Ganji Purnachandra Nagaraju
Pallaval Veera Bramhachari *Editors*

Role of Transcription Factors in Gastrointestinal Malignancies

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The original version of this book was revised. The book was inadvertently published without Abstracts and Keywords, which are now included in all the chapters. An erratum to this book can be found at https://doi.org/10.1007/978-981-10-6728-0_39

Transcription Factors in Gastrointestinal Malignancies

1

Pallaval Veera Bramhachari
and Ganji Purnachandra Nagaraju

Abstract

Gastrointestinal cancer are complex diseases and most lethal amongst all other cancer types. Recent studies have revealed that transcriptional factors are ubiquitously expressed and modulates various physiological processes such as progression and metastasis in Gastrointestinal malignancies. Therefore, targeting these transcriptional factors is an important therapeutic strategy against cancer. In this book, we will discuss some of the important transcriptional factors in depth and further evaluate their role in Gastrointestinal malignancies.

Keywords

Gastrointestinal malignancies · Transcription factors · NF-κB · HIF-1α · AP1 · E2-F1 · STAT-3

1.1 Introduction

Gastrointestinal (GI) malignancies have high mortality rates due to the lack of proper diagnoses in their early stages as they do not cause any symptoms until they have progressed to advanced stages. A high amount of fat content in food intake, lifestyle, genetic risk, consumption of excessive alcohol, and smoking are some of

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the risk factors for cancer [1]. Surgery is the choice of therapy for some patients with GI cancer, but it is not possible if the cancer has metastasized. The therapeutic choice of surgery, however, for completely removing the liver or pancreatic cancer is not feasible as it is highly malignant and spreads very quickly to other parts of the body. All of these cancers show poor responses to either radiation or chemotherapy, so the recurrence of cancer is very high [2], and due to transcriptional reactivation, patients have poor survival rates [3]. Therefore, it is essential to target transcriptional factors in people who have a GI-type cancer. Furthermore, understanding the mechanism of initiation of GI cancers and the role of different agents in regulating the cancer aids in therapeutically curing the cancer at an early stage. Transcriptional inactivation leads to selective death of GI cancer cells, whereas healthy (noncancerous) cells regularly tolerate the loss of transcriptional activity with little significance because of inactivity in regular signaling pathways. For the past two decades, there has been considerable progress in targeting tumorigenic factors and discharging tumor-suppressive transcriptional molecules, as well as therapeutically modulating transcription at the chromatin level.

Attempts at improving the outcome of GI malignancies by incorporating cytotoxic agents such as chemo drugs have been disappointing [1]. These results indicate that the main challenge remains primary resistance of GI malignant cells to cytotoxic chemotherapy in the majority of patients. Therefore, improvement in the outcomes of GI malignancies is dependent on the introduction of agents that can modulate the intrinsic mechanisms of resistance.

Mode of resistance to radio-chemotherapy in GI malignancies includes the activation of NF-κB, the hypoxia-inducible factor (HIF-1α), AP1, E2-F1, and STAT-3. All three of these transcriptional factors control the expression of several oncogenes involved in tumor growth, angiogenesis, and inflammation [2–5]. These transcriptional factors are well-known to be activated in GI malignancies [4]. Therefore, NF-κB, HIF-1α, AP-1, E2-F1, and STAT-3 are rational targets for GI malignancy therapy.

1.2 Significance

Worldwide GI malignancies are the leading cause of most cancer deaths and represent a challenging therapeutic problem. Survival from GI malignancies is particularly poor even when diagnosed during early stages. Patients at the possibility of increasing GI malignancies can be diagnosed based on risk factors (such as taken from familial history). At the current time, these groups of high-risk patients have two options, either close monitoring or surgical resection, which both carry significant risks. Therefore, an effective therapy approach will provide a rational strategy to lower rates of mortality, morbidity, and cost associated with this disease and as such will have a significant impact on this group of high-risk patients. This study will also provide insight into the effects of modulation of inflammatory pathways by selected agents on the development of GI malignancies. These pathways are also central for the development of several other malignancies as well as degenerative diseases; thus, the results of this study may provide a basis for prevention for other

disease types. Therefore, we selected major transcriptional factors and their role in GI malignancies.

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YY1 and KLF4: Their Role in Gastrointestinal Malignancies

2

Himanshu Tillu and Ganji Purnachandra Nagaraju

Abstract

Gastrointestinal (GI) malignancies are among the most serious threats to global health and are among the major causes of morbidity and mortality. KLF4 and YY1 occupy a central niche and can influence the process of oncogenesis of the various tissues of the GI tract in a major way by being closely associated with several cellular processes such as cell proliferation, differentiation, DNA repair, epigenetic modifications, and apoptosis. Although evidence over the years has implicated KLF4 and YY1 in the process of tumorigenesis, significant loopholes still remain. This review is an attempt to evaluate the relative contributions of KLF4 and YY1 to various aspects of GI malignancies.

Keywords

Krüppel-like factor 4 (KLF4) · Yin Yang 1 (YY1) · Gastric cancer (GC) · Colorectal carcinoma (CRC)

2.1 Introduction

The gastrointestinal (GI) tract comprises of the organs esophagus, stomach, small and large intestines, and rectum and other accessory organs playing a crucial role in digestion, namely, the liver, gallbladder, and pancreas. Gastrointestinal cancers

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account for 22% of all malignancies and are an important cause of morbidity and mortality worldwide [36]. The public health burden of GI malignancies is evident by the statistical observation implicating GI cancer as the reason for 12% of all the deaths caused by cancer annually [16, 78]. Tumorigenesis of the GI tract is the result of tumor suppressor gene inactivation and dysregulated proto-oncogene activation of p53 (TP53) [13], anaphase-promoting complex (APC) [23], E-cadherin/CDH1 [1], and p16 [55]. Accumulating evidence over the years has implicated KLF4 [59] and YY1 [7] in the pathogenesis of GI malignancies.

Krüppel-like factors (KLFs) are the transcription factors which are homologous with Krüppel, a transcription factor that is crucial for segmentation during *Drosophila* embryogenesis [40]. KLFs contain three C-terminal C2H2-type zinc fingers, which are able to bind to guanidine-cytidine-rich promoters of several genes [49]. KLFs also are considerably homologous with the specificity protein (SP) family of zinc-finger transcription factors [51]. The 17 KLF family members identified in mammals [17] are crucial for regulation of proliferation, development, differentiation, and apoptosis of cells of mammalian origin. Of the 17 known KLFs, KLF4 plays a critical role in the regulation of cell differentiation, proliferation, and tumorigenesis in the GI epithelium. Clinical and experimental findings have verified tumor suppressor activities of KLF4 in the context of gastric and CRCs.

Yin Yang 1 or YY1 is another GLI-Krüppel transcription factor family member which binds to DNA by virtue of four C2H2-type zinc fingers located at the C-terminus of YY1 [45, 50]. YY1 is important for numerous processes that influence carcinogenesis including cell proliferation, DNA repair, induction of apoptosis, and epithelial-mesenchymal transition. The levels of YY1 have also been found to be altered in numerous cancers [61]. Unlike KLF4, YY1 is required for the induction of carcinogenesis. However, very few studies have elucidated the role of YY1 in GI malignancies.

2.2 Alterations in the KLF4 and YY1 Expression Levels in GI Malignancies

KLF4 or gut-enriched KLF (GKLF) is a transcription factor that shows extensive expression in the GI tract. In mice, KLF4 was found to be expressed primarily in terminally differentiated epithelial cells of the GI tract, especially in the middle to upper epithelium of the crypt [49] in addition to epithelial cells in other organs [14]). In neonatal mice, higher levels of KLF4 have been observed in the colon as compared to the small intestine, the expression at both sites increasing with age [54]. KLF4 expression is essential for terminal differentiation of goblet cells of the murine intestines [20]. In humans, prominent expression of KLF4 is observed at the epithelium surface in normal intestine, with gradual decrease in expression toward the crypt [47, 48, 63]. Colonic bacteria continuously synthesize butyrate by fermentation of dietary fiber [41]. Butyrate has been reported to induce KLF4expression [5, 47, 48].

Independent investigations employing microarray [22], real-time PCR, and immunohistochemistry [20] also have yielded data suggesting reduced expression of

KLF4 in GC. The levels of KLF4 mRNA were found to be significantly reduced in the intestine of the (APC $^{Min/+}$) mice during tumor formation in multiple intestinal neoplasia [11, 54]. Significantly reduced expression of KLF4 was found in the dysplastic epithelium of the colon, such as an adenomatous polyp, and in CRC (CRC) [9, 47, 48]. KLF4 protein has primarily cytoplasmic expression in CRC-derived cells, suggesting that hindered nuclear translocation of KLF4 contributes to carcinogenesis [46]. Patients with familial adenomatous polyposis (FAP) have significantly reduced KLF4 expression in colonic adenomas as compared to adjacent normal mucosa [11, 54]. In addition, lower levels of KLF4 mRNA were observed in sporadic intestinal carcinomas and adenomas compared to normal colonic tissues [47, 48]. Expression of KLF4 is reduced in esophageal cancer [57]. Reduced or lost expression of KLF4 was also observed in esophageal squamous cell carcinomas as compared to the normal esophageal tissue [31]. KLF4 knockdown in the cell line EC9706 of esophageal cancer enhanced cell proliferation and reduced cell adhesion [57].

KLF4 expression was elevated in pancreatic intraepithelial neoplasia, a precursor lesion of pancreatic cancer [39]. However, ectopic KLF4 expression was reported to cause cell cycle arrest of BxPC-3 cells which are derived from pancreatic carcinoma. Furthermore, tumor growth and metastasis were found to be inhibited via the KLF4-p27 (Kip1) promoter interaction in human pancreatic cancer cells inoculated into ectopic and orthotopic mouse models. These findings suggest that increase in KLF4 expression may be an attempt of the transformed cells to halt cell cycle progression to prevent tumor formation [60].

Epigenetic modifications such as promoter hypermethylation and hemizygous deletion reduce KLF4 expression in GI malignancies. Hemizygous deletion of the KLF4 gene was found in SK-GT5 and SNU-16 GC cell lines [58]. Loss of heterozygosity (LOH) at the KLF4 locus has also been observed in some of the CRC specimens and cell lines. In addition to epigenetic modifications, numerous point mutations contained in the open reading frame (ORF) of the KLF4 gene have been reported in CRC (CRC) cell lines leading to flocculated KLF4 protein distribution in the nucleus which in turn is thought to hinder the ability of KLF4 to activate the $p21^{WAF1/Cip1}$ promoter. Hypermethylation of the 5′ untranslated region (5′-UTR) of KLF4 which is found in CRC, GC tissues, and cell lines could lead to the reduced expression of KLF4; blockade of gene hypermethylation was found to reactivate the expression of KLF4 in the cells of human GC [74]. However, epigenetic alterations of KLF4 loci may not be important in all the cases of gastric oncogenesis [8]. Xu et al. have presented evidence in the favor of epigenetic modifications altering KLF4 expression levels. The authors have reported that KLF4 expression could be stimulated in CRC-derived cell lines upon treatment with 5-aza-2-deoxycytidine (5-aza-dC), an agent which induces DNA demethylation (hypomethylation) and gene activation by remodeling [63]. Abrogation of KLF4 expression leads to Sp1 overexpression which may contribute to the development and progression of GC [18]. Zheng et al. reported involvement of microRNAs in KLF4 downregulation in GI malignancies. The microRNA (miR)103 that is associated with increased tumor size, lymph node metastasis, and poor survival was

reported to induce KLF4 downregulation in SGC7901 and BGC823, the cell lines derived from human GC [77].

Murine studies revealed that KLF4 deletion enhanced cellular proliferation and decreased the frequency of pit mucous cells. MUC2, a marker of intestinal goblet cells, which is silent in disease-free gastric tissues, was found to be highly induced in KLF4-deleted cells at the base of antral glands. This finding was replicated in human GC tissue; MUC2 expression was inversely associated with KLF4 expression. Thus, KLF4 appears to be required for antral stem cell homeostasis; the loss of KLF4 expression and induction of MUC2 expression might be crucial diagnostic markers of GC [70].

Several groups have reported changes in expression levels of KLF4 in gastrointestinal cancers. However, there is paucity of data regarding changes in expression levels of YY1. Kaufhold et al. analyzed the expression levels of certain markers of cancer stem cells such as sex-determining region Y-box 2 (SOX2), B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1), and octamer-binding transcription factor 4 (OCT4) in the context of YY1in several types of cancers. Low expression of YY1 was found to be accompanied by low expression of SOX2 and high expression of BMI1and OCT4 in stomach, liver, and pancreatic cancers. On the other hand, CRC was characterized by high-intensity expression of YY1 accompanied by high levels of SOX2 and OCT4 and reduced BMI1 expression [21]. Elevated YY1 expression was reported in nine GC cell lines and in nine out of ten primary gastric adenocarcinoma tissue samples. YY1 was not detectable in three noncancerous gastric tissue samples. YY1 knockdown led to G1 cell cycle arrest via inhibition of Wnt/β-catenin pathway. Ectopic expression of YY1 augmented cell proliferation as seen from in vitro experiments in MKN28, AGS, and NCI-N87 cells and in vivo in nude mice. Significant miR-205 downregulation was observed in cancerous tissue with respect to adjacent healthy tissue in the GI tract. Inhibiting miR-205 activity in vitro significantly augmented the proliferation of AGS and NCi-N87 GC cells. miR-205 was able to suppress YY1 expression by binding to 3′-UTR [66]. Chinnappan et al. have detected six isoforms of YY1 mRNA in mice as well as humans. Higher levels of 7.5 and 2.9 kb isoforms of YY1 mRNA were found in SIIA GC cell line, DLD-1, HT-29, Caco-2 colonic adenocarcinoma cell lines, and BON-I, an endocrine cancer cell line derived from the pancreas, as compared to normal skeletal muscle tissue which has the highest YY1 mRNA expression among normal tissues. YY1 overexpression has been detected in primary colon cancer (CC), especially in poorly differentiated and mucinous tumors. However, surprisingly, lower YY1 expression correlated with poor survival [7].

2.3 Role of KLF4 and YY1 in Cell Cycle and Cell Proliferation

The proliferation of somatic eukaryotic cells is regulated through stringent control of cell cycle progression. The cell cycle has four phases: Gap 1 or G1 phase, where the cell prepares for DNA replication; synthesis or S phase, where actual replication of DNA occurs; G2 phase, where the cell prepares for mitosis; and mitosis or M

phase, when actual cell division occurs. Differentiated, nonproliferating cells are said to lie in the G0 phase. Cyclins and their corresponding cyclin-dependent kinases (CdKs) form cyclin-CdK complexes which act at G1-S, S-G2, and G2-M checkpoints and mediate cell cycle progression. In the event of abnormalities, the inhibitors of cyclin-CdK complexes mediate cell cycle arrest. Aberrant cell cycle regulation is linked to the process of carcinogenesis [44].

KLF4 was initially reported to be essential for enterocyte differentiation and negatively regulate cell proliferation. KLF4 expression was higher in growth-arrested cultured cells [49, 73]. Constitutive KLF4 expression inhibited synthesis of DNA [49]. KLF4 inhibits growth in p53-dependent fashion and is mediated through the interaction of KLF4 with the Sp1-1 binding loci in the p21 $^{WAF1/Cip1}$ promoter, a CdK inhibitor [4, 73]. In addition, KLF4 represses the transcription of numerous cell cycle promoters such as cyclin D1 [47, 48] and ornithine decarboxylase (ODC) [6] and induces G1/S cell cycle arrest [4, 69]. KLF4 promotes the expression of cell cycle inhibitors such as p21, p27, p53, and retinoblastoma and inhibits the proliferation of redox-sensitive vascular smooth muscle cells [33]. In mice with gastric epithelium-specific KLF4 ablation, marked hypertrophy of gastric epithelia was observed, the number of dividing cells in gastric unite was increased by fourfold, and p21$^{WAF1/CIP1}$ mRNA expression in gastric epithelia was reduced by 45% [20]. KLF4 is crucial for p53-mediated cell cycle arrest at G1/S junction after DNA damage in CC HCT116 cells [69]. Yoon et al. also found G2/M arrest in HCT116 cells post γ-irradiation, which was associated with increased KLF4, p53, and p21$^{WAF1/CIP1}$ expression but decreased expression of cyclin B1 through a specific GC-rich element in the cyclin B1 promoter [68]. KLF4 and KLF5 can both upregulate p21$^{waf1/cip1}$ expression in response to UV-induced DNA damage [64]. Thus, in addition to G1/S arrest, KLF4 is also involved in G2/M arrest. Mice with gastric epithelium-specific KLF4 ablation expression display premalignant features such as gastric epithelial hypertrophy as well as altered expression profile of acidic mucins and TFF2/SP-positive cells [20]. The antiproliferative action of KLF4 is also mediated via transcriptional repression of survivin in esophageal squamous cancer cells [71].

YY1 favors S phase entry; the process is regulated by the retinoblastoma protein [38]. A previous genome-wide study has reported prostate stem cell antigen (PSCA) as a susceptibility gene for diffuse-type GC. PSCA protein inhibits the proliferation of GC-derived cell lines. The expression of PSCA is reduced in diffuse-type GC, indicating a tumor-suppressive activity of PSCA. The T allele of the single nucleotide polymorphism rs2294008 (C/T) is associated with GC [43]. The variant with T allele suppresses the transcriptional activity of the −3.2 kb PSCA upstream region. The authors reported that existence of the T allele at rs2294008 created YY1 binding site leading to recruitment of YY1 to the PSCA promoter leading to in vivo repression of PSCA expression which eventually predisposes gastric epithelial cells carcinogenesis [42]. However, there is no information regarding the role of YY1 in cell cycle progression in GI malignancies. A study by Yokoyama et al. has revealed that YY1 indirectly influences cell proliferation via repression of double-negative lymphoid enhancer factor 1 (dnLEF-1) in intestinal cancer cells. LEF-1 is an important mediator of Wnt signaling and is abnormally expressed in CCs. Two promoters

P1 and P2 transcribe LEF-1. TCF-β-catenin complexes activate P1, a Wnt target gene. P2, a second promoter in an intron 2, transcribes dominant negative form of LEF-1 (dnLEF-1). Ectopic expression of dnLEF-1 in CC cells has been reported to slow their rate of proliferation. Yokoyama et al. have identified YY1 as the P2-specific protein essential for the suppression of dnLEF-1. YY1-binding locus at +25 position has been identified in P2 using site-directed mutagenesis and EMSA; chromatin immunoprecipitation assays have confirmed the binding of YY1 to the P2 promoter. The authors have proposed that YY1 plays an important role in evading growth arrest in CC by inhibiting the expression of dnLEF-1 [67]. Investigations of YY1 activity in the context of hepatocellular carcinoma (HCC) also highlighted the oncogenic potential of YY1. The ratio of YY1 to Raf-1 kinase inhibitor protein (RKIP), a potent tumor suppressor, was found to be uniformly inverted in the cancer cells relative to the adjacent normal cells. Upregulation of YY1 in tumor tissue was accompanied by its localization to the nucleus and by increase in the levels of YY1AP, a YY1 coactivator which is usually undetectable in normal liver, and the survivin [34]. YY1 expression was also found to be upregulated in QGY7701and QGY7703 HCC cell lines. YY1 silencing inhibited the proliferation of HCC cells. YY1 was also found to directly downregulate CCAAT/enhancer-binding protein alpha (CEBPA), a factor that is critical for hepatocyte differentiation. CEBPA restoration in HCC cells expressing YY1 was found to induce growth inhibition and cellular differentiation, while CEBPA knockdown in normal hepatocytes promoted cell proliferation [72].

2.4 Role of KLF4 and YY1 in Invasion and Metastasis

Loss in KLF4 expression contributes to metastasis of the gastrointestinal tumors. As compared to normal mucosa, KLF4 was expressed at lower levels in primary gastric tumors and especially in metastasized tumors. Progressive loss in KLF4 expression was observed in American Joint Committee on Cancer stage I to stage IV of GC and was a predictor of poor survival [59]. KLF4 overexpression was found to hinder colony formation, invasion, and migration in vitro. Reduced/lost KLF4 expression was also observed in cell lines HTB103, AGS, N87, SK-GT5, HTB135, TMK1, and SNU-1 derived from GC. The restoration of expression of KLF4 in human GC cells hindered their tumorigenicity and abrogated metastasis [59]. MicroRNAs 103 and 107 (miR-103 and miR-107) inhibit death-associated protein kinase (DAPK) and KLF4 and favor metastasis of CRC. Investigations by Chen et al. have revealed that DAPK and KLF4 downregulation via miR-103/miR-107 resulted in increased cell motility and cell-matrix adhesion and enhanced colonization of CRC cells at the site of metastasis, while the epithelial marker expression and cell-cell adhesion was decreased. The clinical profile miR-103/107^{high}+ DAPK^{low}+ KLF4^{low} favored lymph node and distant metastases in CRC cases and could predict the recurrence of metastasis and poor survival [3]. miR-29a was also reported to mediate KLF4 downregulation. The overexpression of miR-29a and knockdown of KLF4 both promoted MMP2 expression and inhibited the E-cadherin expression. In addition, the extent

of KLF4 expression was negatively correlated with the expression of MMP2 but positively correlated with the expression of E-cadherin. Moreover, clinical studies suggested that both elevated miR-29a expression and reduced KLF4 mRNA levels were significantly associated with metastases and poor outcome in CRC patients [52]. miR-10b also downregulated KLF4 and promoted metastasis of esophageal cancer cell migration and invasion [53]. Leng and group generated spheroidal cells from DLD-1 CC cells in vitro. These spheroidal cells possess the hallmarks of cancer stem cells. The tumorigenic and invasive abilities of spheroidal cells in addition to their capabilities of resisting chemicals were attributed to KLF4 [25]. Ectopic expression of KLF4 in murine HCC cell lines was found to reduce anchorage-independent cell growth in soft agar and cell invasion and migration in in vitro assays. Ectopic KLF4 expression was also found to favor epithelial phenotype and directly downregulate Slug, a crucial epithelial-mesenchymal transition (EMT)-associated protein. Low expression of KLF4 was reported in HCC tumors [29].

Qi et al. also found reduced/lost expression of KLF4 in primary HCC samples, in particular, lymph node metastases, as compared to normal liver tissue. Loss of KLF4 in primary tumor correlated with poor survival. Ectopic KLF4 expression in HCC cells inhibited their migration, invasion, and proliferation in vitro. Nude mice inoculated with KLF4 expressing HCC cells displayed tumors which had reduced growth kinetics and inhibition of metastasis as compared to the mice inoculated with HCC cells not expressing KLF4. KLF4 was found to increase the expression of vitamin D receptor, thereby rendering the cells sensitive to inhibitory effects of vitamin D [26].

Wang and colleagues have recently highlighted the action of YY1 in favoring tumor growth, invasion, and metastasis. Experimental data revealed that the (miR-34) family members miR-34a, miR-34b, and miR-34c target 3′-UTR of YY1 mRNA. miR-34 overexpression led to YY1 downregulation in NUGC-3 cells derived from GC, causing suppression of tumor sphere and colony formation, migration, and invasion. Conversely, silencing of miR-34 family promoted tumorigenesis via YY1 upregulation, in AZ521 and SC-M1 GC cells. YY1-deleted SC-M1 cells formed smaller tumors as relative to the control cells. The expression of pluripotency genes SOX-2, Nanog, CD44, and Oct4 was found to be upregulated by YY1 in SC-M1 cells [56]. YY1 was highly expressed in esophageal squamous cell carcinoma (ESCC) tissues with lymph node metastasis as compared to those without lymph node metastasis. YY1 overexpression also promoted the invasive abilities of the esophageal cancer TE-1 cells. Higher YY1 expression was observed in advanced tumor grades as compared to stage I/II [30].

2.5 Role of KLF4 in Angiogenesis

Angiogenesis or the generation of blood vessels is important for the growth and survival of transformed cells in vivo by virtue of their dependence on blood for oxygen and nutrient supply. Very few reports have characterized the role of either KLF4 or YY1 in mediating angiogenesis. KLF4 was reported to inhibit the

formation of a vascular network through induction of miR-15a in the endothelial and vascular smooth muscle cells [76]. miR-15a has been previously reported to inhibit angiogenesis [65]. Angiogenesis requires Notch signaling. Notch signaling pathway downregulates KLF4 expression in CC cell line HCT116 [75]. Thus, it is conceivable that KLF4 inhibits angiogenesis, although no studies have investigated the contribution of KLF4 toward inhibition of angiogenesis in the context of GI malignancies. De Nigris et al. have reported that YY1 has the ability to enhance vascular endothelial growth factor (VEGF) expression in nude mice inoculated with SAoS osteosarcoma cells, resulting in neoangiogenesis [12]. Silencing of YY1 in HCT116 CC cells which were then transfected into nude mice has also been reported to attenuate angiogenesis [62].

2.6 Role of KLF4 and YY1 in the Induction/Prevention of Apoptosis

Apoptosis or programmed cell death is indispensable for normal cellular turnover. Failure of apoptosis is important for tumor cell survival. KLF4 is known to induce apoptosis in HT29 CC cells [47, 48], but not in RKO [4, 10] and HCT116 CC cells [69]. KLF4 induces the 14-3-3σ protein expression CRC cells upon DNA damage [2, 15]. The 14-3-3σ protein is highly expressed in GC tissue relative to healthy gastric tissue and correlates positively with tumor size, tumor node metastasis, and expression of antiapoptotic Bcl-2 [27]. Other reports have also suggested that 14-3-3σ protein is associated with apoptosis prevention in CRC [37] as well as pancreatic cancer [28, 32]. Nuclear 14-3-3σ expression correlated significantly with lymphatic invasion, depth of invasion, stage, and poor outcome in esophageal cancer [35]. Immunohistochemical analysis of gastric tumors from 96 GC cases revealed KLF4 positivity in 78.1% cases; 15.6% cases showed strong positivity. Strong Fas positivity was associated with a weak KLF4 expression [24]. Human esophageal squamous cancer cell line TE2 obtained from cases with poorly differentiated esophageal squamous cancer typically does not express KLF4 or KLF5. However, TE2 cells with stably transfected KLF4 show reduced viability in response to hydrogen peroxide treatment and have increased anoikis. However, KLF5 but not KLF4 can upregulate the proapoptotic protein Bax in response to ultraviolet (UV)-induced DNA damage [64].

There is a scarcity of data regarding the contribution of YY1 to induction or prevention of apoptosis. YY1 was found to be upregulated in primary CRC tumors as compared to normal adjacent tissue. The proliferation of sh-YY1-stably transfected HCT116 tumor cells in nude mice was significantly slower as relative to vector control mice. Ectopic expression of YY1 was reported to suppress apoptosis and increase proliferation of CC cell lines HCT116 as well as LOVO. YY1 was reported to exert carcinogenic effect by inhibiting the activity of p53 and thus altering the activity of the downstream effectors c-Jun, p15, and caspase cascades. YY1 was also reported to activate Wnt signaling pathway by activating β-catenin, antiapoptotic survivin, and fibroblast growth factor 4. Moreover, high YY1 expression

favored poor overall survival [72]. YY1 knockdown also resulted in apoptosis as seen from increased cleavage of poly(ADP-ribose) polymerase (PARP), in three GC cell lines, namely, MKN28, NCI-N87, and AGS. In primary gastric adenocarcinoma, the nuclear YY1 expression favored poor outcome in early stages and correlated with shorter survival [19].

2.7 Conclusions and Future Perspectives

KLF4 and YY1, the members of same transcription factor family and both binding to guanidine-cytidine-rich DNA, have opposite relevance with respect to GI malignancies. The studies by most of the labs agree that KLF4 is a tumor suppressor (Fig. 2.1) while YY1 favors oncogenesis (Fig. 2.2) in GI malignancies. However, there are significant loopholes in the available literature. Most of the studies have focused on gauging the differential expression profiles of the two transcription factors. Comparatively, there are very few studies aiming at elucidating the molecular mechanism of KLF4 or YY1 by which they participate in development or prevention of cancer. The contribution of KLF4 or YY1 toward angiogenesis of the tumors of the GI tract is a largely unexplored field. The exact mechanism of action by which YY1 helps the transformed cells to evade apoptosis has not yet been described. The investigations aimed at filling these loopholes would further our understanding regarding the exact niche which these transcription factors occupy in the context of GI malignancies. It is a well-established fact that a certain protein may favor oncogenesis in certain cancers, while the same protein may be a tumor suppressor in some others. One of the main reasons for this observation is that the

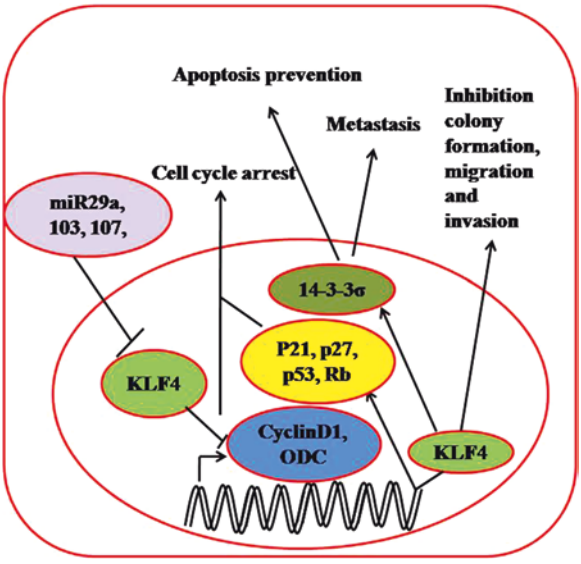

Fig. 2.1 *KLF4* is a tumor suppressor in GI malignancies. *KLF4* induces cell cycle arrest by downregulating *cyclin D1* and ornithine decarboxylase (*ODC*) expression and upregulating the expression cell cycle inhibitors *p21*, *p27*, *p53*, and *Rb*. *KLF4* also contributes to metastasis inhibition. *miR-29a*, *miR-103*, and *miR-107* inhibit *KLF4* activity and favor tumorigenesis

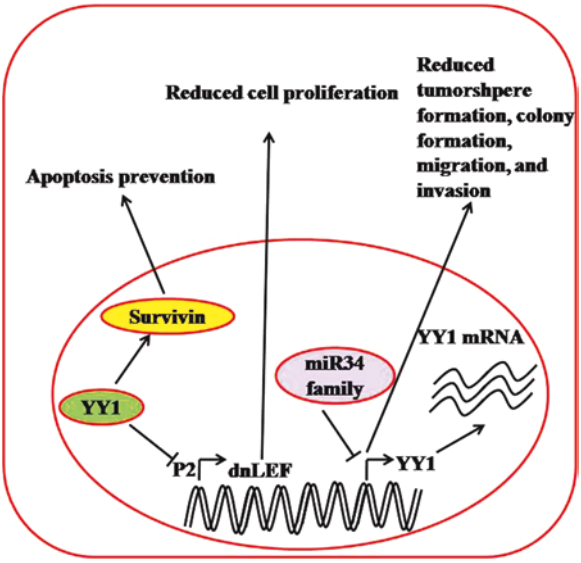

Fig. 2.2 *YY1* is highly expressed in transformed GI tract cells. *YY1* favors cell cycle progression by inhibiting the transcription of double-negative lymphoid enhancer factor 1 (*dnLEF*) isoform which slows down the rate of proliferation. *YY1* also assists tumor cells to evade apoptosis through *survivin*. *miR-34 family* of microRNAs exert their anticancer activity through inhibition of YY1 transcription

proteomes of different tissues are very different which gives rise to differing interactomes. It is the interactome which finally determines whether a particular transcription factor will favor carcinogenesis or not. Thus, the generation of extensive, all-encompassing interactomes will help us to generate a holistic picture of the relative importance of various transcription factors in GI malignancies and allow us to design effective therapies.

Conflict of Interest We declare that we don't have any conflict of interest.

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AP-1: Its Role in Gastrointestinal Malignancies

3

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Abstract

The role of transcription factor AP-1 (activator protein 1) in human physiology is distinct due to its involvement in tissue regeneration in which the metabolism is instigated by the signals which trigger undifferentiated proliferative cells to proceed toward cell differentiation. Consequently the functions of AP-1 may be altered in response to extracellular signals. The studies on gene-knockout mice and AP-1-deficient cell lines propose that AP-1 regulates multiple gene targets and accomplishes accurate physiological functions. There is a significant breakthrough in unveiling the molecular mechanisms and signaling pathways that monitors AP-1 activity. AP-1 functions as a double-edged sword in cancer progression through monitoring gene expression involving cell proliferation, cellular differentiation, cell death, and tumor invasion. AP-1 can be oncogenic and antioncogenic too. The activities of AP-1 in cancer appear to rely on composition of AP-1 dimers and type, stage, and genetic basis of cancer. c-Jun protein, one of the subunits of AP-1 up on activation, is expressed primarily at invasive front in carcinomas leading to the proliferation of malignant cells. Thus, c-Jun mainly has oncogenic functions, while JunB and JunD have antioncogenic effects. AP-1's role is being studied not only in cancers but also in disorders such as psoriasis, asthma, and transplant rejection. AP-1 emerged as drug discovery

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target in recent years. This review is being structured to highlight the role of AP-1 transcription factor in the gastrointestinal malignancy progression.

Keywords
AP-1 transcription factor · c-Jun · c-Fos · Cell proliferation · Cellular differentiation · Apoptosis · Gastrointestinal cancers

3.1 Introduction

Cancer is an out-of-control proliferation of particular cell type originating with an undesirable mutation, which results in aggregation of abnormalities in many classes of genes. The proto-oncogenes accelerate the cell cycle, and the tumor suppressor genes control the cell growth. The signal transduction pathways along with two stress response pathways act as molecular circuits found to be highly conserved in all the vertebrates. The transcription factors are the proteins that participate at the endpoint in signal transduction pathways resulting in alteration of specific genes. Most of the cancer-causing genes participate in these pathways by transferring the exogenous and endogenous signals at the cellular level. Tumor progression can be due to the cross talk between the healthy cells, loss of communication in between cancer-causing genes, abnormal DNA methylation status, hypermutability, and genetic instability. Cancer has multifaceted etiology, a large number of defects in thousands of genes leading to pernicious disease. Transcription factors execute distinct transcription programs. The activator protein 1 (AP-1) may be referred to as a matrix of transcription factors which functions throughout the trajectory of tumor progression. This perspective outlines the current perusal of changes in AP-1 and its role in gastrointestinal malignancies which includes carcinoma of the liver, pancreas, esophagus, stomach, and gallbladder and colorectal cancers.

AP-1 is a transcription factor that has a heterodimer structure made up of protein molecules belonging to families JDP, ATF, c-Fos, and c-Jun. AP-1 monitors diverse cellular processes which include cellular differentiation, cell growth, cell proliferation, and apoptosis or programmed cell death (Fig. 3.1) [1]. It was discovered as TPA-activated transcription factor bound to a cis-regulatory unit of human metallothionein IIa promoter and SV40. The AP-1 binding site was distinguished as the 12-O-tetradecanoylphorbol-13-acetate (TPA) response element (TRE) with 5′-TGA G/C TCA-3′ as consensus sequence [2, 3]. Jun (the subunit of AP-1) and Fos-associated p39 protein were identified as an oncoprotein of avian sarcoma virus and the transcript of cellular Jun gene, respectively. Wagner reported that Fos is a cellular homologue of two viral v-Fos oncogenes (which induces osteosarcoma in rats and mice) [4].

AP-1 is reported to regulate the expression patterns of target genes in reciprocation with external stimuli like stress, cytokines, growth factors, and viral as well as bacterial infections [5]. The AP-1 activity is in turn regulated by means of posttranslational modifications, by its DNA-binding dimer composition and also mainly their

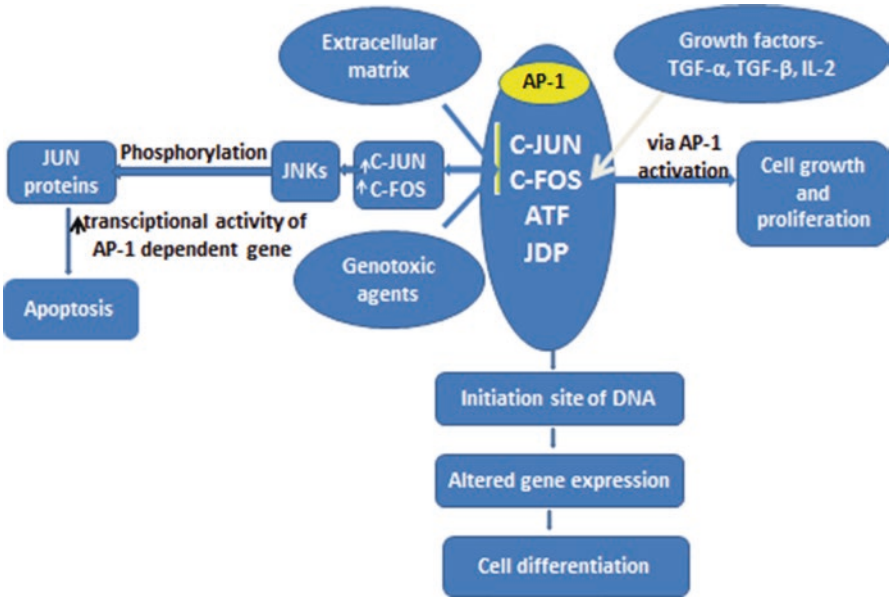

Fig. 3.1 Mechanism of AP-1 transcription factor in cellular process.The growth factors stimulating c-Fos resulting in cell growth and proliferation via AP-1 activation. AP-1 modulating gene expression leading to cell differentiation. Extracellular matrix and genotoxic agents induce AP-1 activity resulting in apoptosis

interaction within different binding partners. Right from the time of discovery, AP-1 is associated with many physiological functions (principally in determination of life span of an organism and in tissue regeneration process) as well as regulatory processes, while some of its novel functions are still under investigation.

The AP-1 subunits, c-Fos and c-Jun, form dimers and play a crucial role in cell growth and cellular proliferation. Hence, the activation and functions of AP-1 are primarily determined by critical patterns of the AP-1 dimers [1]. The AP-1 subunits may bind to the palindromic DNA motif (5′-TGA G/C TCA-3′) in order to modulate the target gene expression, but specificity depends on the composition of dimers of corresponding bZIP subunit [1]. The role of c-Jun is imperative for fibroblast proliferation [6], and both the subunits were expressed above the basal levels in the course of cell division [7]. The expression patterns of c-Fos increase in reciprocation with the introduction of growth factors into the cell, besides strengthening its evocative participation in cell cycle. IL2, TGF-α, and TGF-β-like growth factors were displayed to invigorate c-Fos and reinforce the process of cellular proliferation by activating AP-1 subunits.

Several systems proposed the involvement of AP-1 in cellular differentiation. AP-1 was reported to participate in synchronizing the expression of target genes. The alterations in gene expression at cellular level reflect the DNA synthesis initiation resulting in the generation of differentiated derivatives which in turn leads to cellular differentiation. In a study on chicken embryo fibroblasts (CEF),

it has been proved that when AP-1 is formed by stable heterodimers with c-Jun, the bZIP region of the v-Fos enhances the binding potential of the transcription factor to the target genes through c-Jun; this activation intiates differentiation of CEF [8].

AP-1 and its association with apoptosis is widely identified. Its mechanism is instigated through several genotoxic agents and extracellular matrix proposing their participation in apoptosis [1]. The c-Jun N-terminal kinases (JNKs) are triggered by these stimuli resulting in Jun protein phosphorylation and increased transcriptional activity of genes dependent on AP-1 [1]. Increased range of JNK activity and Jun as well as Fos protein levels was reported in cells where apoptosis has taken place. Few studies reported cells of inactivated c-Jun-ER showing general morphology, whereas c-Jun-ER activated cells are apoptotic [9]. Increased levels of AP-1 lead to the activation of target gene expression. Thus, the regulation of activity of AP-1 is crucial for cell function which is monitored by events such as posttranscriptional and posttranslational events and dimer composition as well as their interaction with respective accessory proteins [10].

AP-1 is known to play a vital role in physiology of the skin along with tissue regeneration. The metabolism is instigated by extracellular signals which set off undifferentiated proliferative cells and undergo cellular differentiation. Thus, the AP-1 subunit activity in response to the extracellular signals can be modified under circumstances when the balance of keratinocyte proliferation as well as differentiation has to be temporally and rapidly altered [11]. Earlier studies reported the involvement of AP-1 in the growth of breast cancer through multiple mechanisms, which include regulation of the genes downstream to E2F and cyclin D1 expression regulation, and their target genes. The AP-1 subunit, c-Jun, was proved to regulate the breast cancer cell growth. The activated c-Jun is known to be mostly expressed at the invasive front in squamous breast cell carcinoma and is said to be prominently linked with breast cell proliferation [12].

3.2 AP-1 and Hepatocellular Carcinoma

The liver secretes bile juice which can break down the fat consumed in food to ease absorption. These fats are being processed along with some proteins which play an important role in clotting of blood. In addition the liver processes alcohol, toxins, poisons, and some medicines to flush them out of the body. The liver malignancy, which is diagnosed in 500,000 cases annually as hepatocellular carcinoma (HCC), is the third most common cause of cancer deaths in the world [13] .The major risk factors of HCC are consumption of alcohol and infections due to hepatitis B and hepatitis C [14]. The incessant intrahepatic inflammation due to infections maintains a balance in the cycle of cell (liver) destruction and regeneration which often ends up in HCC [15, 16]. To examine the molecular mechanisms behind HCC progression in different stages, a wide range of mouse models were developed [17] which imitate the etiology of hepatocellular carcinoma in man. HCC may be due to DNA damage induced in hepatocytes by external chemical carcinogens. When mice

are injected with diethylnitrosamine (DEN), a tumor initiator, they can develop liver cancer [18, 19]. Inflammation also plays a promising role in liver cancer progression [20, 21].

AP-1 transcription factors identified near the receiving ends of numerous signaling pathways are made up of homodimers or heterodimers of leucine zipper (bZIP) protein family [4].The functions of AP-1 protein were established through experimental mice model in which the functional manipulation of bZIP proteins was studied. Johnson and his co-workers suggested that c-Jun disruption in mice model resulted in embryonic lethality during midgestation. The embryos evidenced cardiovascular imperfections and impaired liver development [22]. HCC development was completely inhibited in the DEN liver cancer model (c-Jun knockout in the liver of an adult mice), thus demonstrating the importance or prominence of c-Jun in liver cancer progression [23]. Smeal and co-workers' study has shown that c-Jun coordinates with Ha-Ras during normal cell transformation to cancer cell [24].

Multiple studies demonstrated the role of JDP2 in malignant cell transformation and inhibit AP-1 transcription by interfering with the c-Jun oncogenic properties. JDP2 is implicated in cell differentiation like differentiation of skeletal muscle cell [25] and osteoclasts [26] in stress response to ultraviolet irradiation [27]. JDP2 prevents cellular transformation influenced by Ras in vitro and also in xenografts implanted into SCID mice [28]. In some studies, in mice model with viral insertional mutagenesis, JDP2 was identified as a candidate oncogene in high-throughput screening. Several gene expression studies noticed the increased levels of JDP2 in cancers of the kidney, skeletal muscle, liver, and prostate. Transgenic mice were developed with liver-specific expression of JDP2 and chemically induced cancer hepatocellular model to examine/investigate the involvement of JDP2 increased expression in hepatocellular carcinoma. It was proved that increased JDP2 expression enhanced liver cancer severity.

3.3 AP-1 and Pancreatic Cancer

The pancreas, a flat pear-shaped gland with both exocrine and endocrine function, is located in the abdomen. It is both an exocrine and endocrine gland. The exocrine cells help in digestion, while the endocrine cells of the pancreas help in blood sugar regulation. The lining of the pancreatic duct usually divides more rapidly than the normal cells; thus, there are more chances for an abnormal cell to develop, which divides abnormally and can spread inside the pancreas and then to the nerves and the blood vessels around the pancreas bringing about blockage in the bile duct. Pancreatic cancers mainly spread through the blood as well as through the lymphatic system to other organs of the body.

Signaling pathways as well as their transcription factor targets may be dysregulated in pancreatic ductal adenocarcinoma (PDAC) [29]. The determined activation of the two main transcription factors, nuclear factor-κB (NF-κB) and AP-1, is a characteristic of cancer. Despite the fact that NF-κB was extensively studied [30,

31], very little information is available on AP-1 in PDACs. The AP-1 protein is a dimeric complex (homodimer and heterodimer) composed of Jun (Jun, JunB, and JunD) and Fos (Fos, Fra1, Fra2, and FosB) families, activating transcription factor subfamily (Atf and Creb), and Maf subfamily. Each complex can be functionally defined in determining the certainty of the genes being regulated [32, 8]. AP-1 complexes which bind to palindromic DNA sequences are interpreted as TRE or cARE (cyclic AMP response elements) in promoters as well as enhancers of c-Jun and other target genes [23, 33]. The genetic deletion of c-Jun, JunB, or Fra1 may lead to the embryonic lethality in mice model because of abnormal organogenesis. c-Fos and c-Jun were initially discovered as viral oncoproteins, which are implicated in bone, skin, and liver carcinogenesis. Hezel and co-workers reported that c-Jun is found to be overexpressed in Hodgkin's as well as anaplastic large cell lymphoma and may increase oncogenic Ras-mediated cell transformation [29, 34]. c-Jun and c-Fos might be overexpressed because of an epidermal growth factor receptor-mediated autocrine pathway in PDACs [35–38]. In comparison with c-Fos, FosB, and c-Jun, the AP-1 proteins Fra1, Fra2, JunB, and JunD may have poor transactivation potential and with less or without transforming activity. However, the overexpression of Fra1 and Fra2 in mice induced tumors in different organs which include the pancreas suggesting that they are dimerized with AP-1 proteins which have more potent transactivation domains. JunB and c-Jun have overlapping developmental functions; JunB may be a tumor suppressor which antagonizes the tumorigenic potential of c-Jun [8, 23]. A study reported that Rap1, a Ras antagonist, with the increase in transforming growth factor-β type II receptor expression through a JunB-dependent pathway, reduces the tumorigenicity of pancreatic tumor cells. The regulating ability of c-Jun in cellular proliferation, survival, and cell death may contribute to counteracting its roles in development and tumorigenesis. Thus, the mouse fibroblasts of c-Jun are proliferation defective, and liver regeneration is damaged without c-Jun [39]. c-Jun is considered essential for cell survival in the livers of fetal mice and is also required for apoptosis [40, 41]. c-Jun's ability to induce apoptosis mediators called Fas L and Bim and transcriptionally repress tumor suppressors such as p53 may explain these opposing roles [8]. The activity of AP-1 is not only regulated by dimer composition but also by other mechanisms. Interactions with NF-κB and MPK (mitogen-activated protein kinase) or PI3K (phosphoinositide 3-kinase) signaling pathways [42, 43] may be critical for AP-1 function in pancreatic cancer cells. In comparison with c-Jun, NH2-terminal kinase (JNK) controls c-Jun transcription via phosphorylation of Ser63 and Ser73, and PI3K and its protein kinase mediator Akt may regulate AP-1 at various levels. There are evidences that Akt induces c-Fos and Fra1 expression and also it may suppress the phosphorylation of c-Jun (Thr239) via glycogen synthase kinase-3 (GSK-3) in order to stabilize it [44, 45], [46–49]. Feedback loops may be involved because c-Jun can activate Akt and enhance proliferation and survival in cells through Ras stimulation or suppression of the PI3K antagonist PTEN [50, 51]. Although JNK and AKT may interact through the ASK, POSH, and JIP1 proteins, they are known to use distinct mechanisms to regulate c-Jun. When compared with JNK [52], still there is a controversy regarding the role of Akt in pancreatic cancer cells. It was earlier studied

that AKT and P13K are activated in pancreatic cancer cells because of aberrant PTEN expression as well as insulin receptor substrate-1-mediated signaling [53, 54] . Shun and his co-workers identified the probability of activation in these cells which is regulated by the PI3K signaling pathway. Their inference shows that different AP-1 proteins are expressed in pancreatic cancer cells; c-Jun is imperative for their proliferation and is regulated by Akt signaling through transcriptional activity independently of Thr239 and Ser63/Ser73, the phosphorylation sites regulated by GSK-3 and JNK, respectively.

3.4 AP-1 and Esophageal Cancer

The esophagus or in simple terms the food pipe transfers food from the mouth to the stomach. The cancer can develop throughout the length of esophagus. Glands around esophageal walls produce mucus which helps food to slide down after swallowing. These mucous glands may turn out to be carcinogenic and develop adenocarcinoma of the esophagus, one of the frequent cancers. Other cancer types include squamous cell carcinoma.

Esophageal cancer is known to be one of the most virulent malignancies, ranking eighth in incidence and sixth in mortality rate globally [55]. These neoplasms are particularly incident in China and few other countries in Asia, where esophageal squamous cell carcinoma (ESCC) is the most prevalent [55]. A 5-year overall survival rate was not improved evidently despite the progressed surgical techniques and new therapeutic approaches in the past few decades [56]. Adjuvant chemotherapy for ESCC may reduce postoperative recurrence as well as improve survival [57]. Evidences report that the cancer often acquires resistance through chemotherapy after the nonlethal exposure [56, 58]. Thus an integrated view of chemoresistance can provide a more valuable approach for developing novel therapies for this disease.

ID1 belonging to the helix-loop-helix (HLH) protein family contributes to cancer by counteracting cellular differentiation and stimulating cell proliferation as well as enabling tumor neoangiogenesis [59]. ID1 was known to be overexpressed in multiple human tumor types which include breast, colon, prostate, and esophagus. The overexpression of ID1 is most common in human primary ESCC. ID1 expression directly correlates with tumor invasion and metastasis as well as poor prognosis in esophageal cancer patients [58, 60, 61]. It was known that ID1 was involved in radiotherapy resistance and chemotherapy resistance in human cancers like breast cancer, pancreatic adenocarcinoma, colorectal cancer, lung cancer, and esophageal cancer which becomes a novel potential therapeutic target [62–64]. ID1 is transactivated in the 5-FU therapy, which can provide a resource for the future study directing the molecular mechanisms of chemotherapy in breast cancer patients. In a study of p53 protecting cells from cell cycle arrest caused by arsenic, ID1 is extensively induced by arsenite in p53-proficient cells than p53-deficient cells, which show greater resistance to arsenite-induced apoptosis and mitotic arrest [65]. According to recent study, the competitive binding degeneration and thymidylate synthase expression take place to stimulate chemoresistance among esophageal cancer

patients. The ID1-E2F1-IGF2 regulatory axis has prominent implications for cancer prognosis as well as treatment options. It is indicated that ID1 is upregulated by chemotherapeutic drugs and can be involved in chemoresistance although the mechanisms of ID1 affecting chemoresistance are yet to be investigated.

AP-1 is a menagerie of dimeric basic region-leucine zipper (bZIP) proteins which are identified either as TRE (5′-TGAG/CTCA-3′) or cARE (CRE, 5′-TGACGTCA-3′) [5]. AP-1, a mammalian transcription factor, collectively illustrates a group of functionally as well as structurally related members of Jun protein family and Fos protein family [5]. Previously it was reported that AP-1 is involved in multidrug resistance along with cell survival [66, 67]. Recently researchers demonstrated that aberrantly high levels of ID1 expression in neoplasms are due to induction of transcriptional activity by several proteins that are activated in a constitutive way among cancerous cells and affect the chemoresistance in patients [68, 69]. Identifying the important roles of ID1 and AP-1 in chemoresistance as well as transcriptional regulation between these ID proteins and AP-1 is a challenge to be addressed.

According to earlier studies, ID1 communicated etoposide chemoresistance via inhibiting caspase 3 activity and PARP cleavage, and etoposide-induced apoptosis was promoted via ID1 ablation. Spontaneously c-Jun/c-Fos can bind directly to the ID1 promoter region and activate its transcription in vivo. Ectopic expression of c-Jun/c-Fos enhances ID1 transactivation. Contrarily knockdown of c-Jun/c-Fos prevents ID1 transactivation. Overexpression of ID1 retrieves cells from apoptosis in c-Jun/c-Fos knockdown cells. The expression levels of ID1 are positively correlated with c-Jun/c-Fos in human cancers. More significantly analysis of the gene expression profiles of different cancer types indicated that high expression of ID1 and c-Jun or c-Fos may be associated with poor survival rate among patients. These findings suggest that c-Jun/c-Fos is involved in the chemosensitivity mechanisms and they contribute to the regulation of ID1 with response to chemotherapeutic drugs instigating apoptosis.

AP-1 may transcriptionally regulate ID1 in response to DNA damage thus causing chemoresistance to therapeutic drugs in ESCC cells. ID1 expression may be directly correlated with c-Jun and c-Fos in majority of malignancies. More predominantly, high ID1 and c-Jun/c-Fos expression levels in human neoplasms are significantly correlated with shorter survival rates among cancer patients. In addition they demonstrated the prominence of c-Jun/c-Fos-ID1 signaling pathway in chemoresistance of esophageal cancer cells. This study provides an insight in targetting c-Jun/c-Fos-ID1 for cancer therapeutic strategies. Moreover their results evidence the importance of developing novel anticancer therapies and pathways in understanding the unrevealed mechanisms among ESCC cell studies.

3.5 AP-1 and Gallbladder Cancer

Gallbladder (GB) malignancy is quite a rare tumor of the biliary tract especially in Western societies and Asia-Pacific countries including Korea, Australia, and Japan. In 2011, among 771 Australians, half of the patients were diagnosed with

gallbladder cancer and other half with biliary tract cancer. Majority of patients with these cancers were diagnosed in later stages where the tumor becomes too large to be removed surgically.Only one fourth of gallbladder cancer patients were reported to be eligible for surgery.The survival rate of these patients is very low still. According to the reports, the average 5-year survival rate for these patients is only 18.5%. For the patients who are not eligible for surgery, chemotherapy remains the other treatment option. At present, there are no prescribed chemotherapy regimens for GB cancer that was shown to specifically help patients to survive longer.

In gallbladder patients, tumor necrosis factor-alpha (TNF-α) was identified to play an important role in lymphatic metastasis. Vascular endothelial growth factor-D (VEGF-D) is another factor considered to be associated with lymph node metastasis and lymphangiogenesis. However VEGF-D's role in TNF-α-induced lymphatic metastasis in GB cancers remains unknown. The TNF-α levels are correlated with VEGF-D expression in clinical specimens. According to earlier studies, the effects of TNF-α are due to multiple signaling pathway activations in combination with TNF-α and its receptors (via NF-κB or AP-1 pathway). The two binding sites in VEGF-D promoter core region reveal that the upregulation of VEGF-D is through the AP-1 pathway. TNF-α upregulates the expression of protein as well as promoter activity of VEGF-D via ERK1/2/AP-1 pathway. Additionally TNF-α promotes HDLEC tube formation and lymph node metastasis among GBC patients by upregulating VEGF-D in vivo and in vitro. So considerably it suggests that TNF-α may promote lymphangiogenesis and lymphatic metastasis of GBC via ERK1/2/AP-1/VEGF-D pathway.

The studies by Schafer and Ming suggested that HNF-4α (hepatocyte nuclear factor 4α), COUP-TF1 and COUP-TF2 (chicken ovalbumin upstream promoter transcription factors 1 and 2), and AP-1 bind to VEGF-D promoter, [70]. Multiple transcription studies demonstrated that NF-κB or AP-1, [71] is associated with tumor progression. TF bind and promoter scan were used to determine the potential binding sites of NF-κB or AP-1 in the VEGF-D promoter having three fragments with higher activities. Eventually, it is confirmed that both the AP-1 sites can bind to the VEGF-D promoter and that TNF-α might enhance the combination by site-directed mutagenesis.

3.6 AP-1 and Gastric Cancer

The stomach receives and stores food from the esophagus. Ingested food is passed from the stomach to the small intestine where nutrients are absorbed into the bloodstream. Majority of gastric cancers develop within the cells of mucosa resulting in adenocarcinoma of the stomach. Gastric cancers develop steadily and take several years before the onset of symptoms.

The activity of c-Jun is augmented in several tumor types, but its role in gastric cancer is largely unknown. The aminoterminal phosphorylation of c-Jun by JNKs shows that the phosphorylation-dependent interaction between c-Jun and TCF4 regulates intestinal tumorigenesis by integrating JNK and APC/beta-catenin.These two distinct pathways are activated by Wnt signaling [72]. It was proposed by Wong

and colleagues that a COX-2 inhibitor suppresses AP-1 via JNK in carcinoma of the stomach. Earlier studies reported c-Jun positivity in a large number of gastric cancer patients.

3.7 AP-1 and Colorectal Cancers

Colorectal cancer is sometimes referred to as bowel cancer. The bowel connects the stomach to the anus taken together with the large colon and rectum. The bowel usually develops small growths called polyps which appear like tiny dots near the bowel lining. However, all the polyps are not cancerous.The early detection of polyps in the colon or rectum may reduce the risk of colorectal cancers.

AP-1 functions to regulate gene expression in conjugation with multiple stimuli and is also involved in multiple cellular processes, such as differentiation, proliferation, and apoptosis, like other gastrointestinal cancers [1, 5]. Various genes may encode the monomers of the AP-1 complex. These transcription factors play a crucial role as they are located downstream to many transduction pathways. The hallmark of CSC phenotype is interpreted by several genes; however, NANOG, *POU5F1* (OCT3/4), and SOX2 have prominent roles [73].

Recent experimental studies indicate that c-Jun is salient for the maintenance of self-renewal as well as tumorigenicity of glioma stem-like cells [74] . Another study reports that in colon cancer c-Jun and TCF4 stimulated a subpopulation of colorectal cancer tumor cells to endorse a stem-like phenotype through the *NANOG* promoter [75] . Furthermore c-Fos enables to continue hematopoietic stem cells in the quiescence [76]. Panagiotis Apostolou and colleagues focused at demonstrating the association between the AP-1 complex and the stemness transcription factors. They addressed whether the AP-1 transcription factor is required to activate or suppress NANOG, OCT3/4, and SOX2 transcription factors and also whether it has an effect on apoptosis and cell cycle events.

c-Fos which is a proto-oncogene has a leucine zipper DNA-binding domain. c-Jun is also a proto-oncogene which has got important roles in cellular proliferation and cell death [77]. The AP-1 transcription factor operates downstream of multiple transduction pathways; thus various processes were implicated. Few studies have elaborated that c-Jun and c-Fos may be involved in the stemness pathways. c-Jun has a crucial role in the maintenance of self-renewal as well as tumorigenicity in glioma stem-like cells. In contrast a study has detailed that AP-1 and NF-B induce differentiation of mouse ESCs [66, 74, 78].

Thus there exists an association between AP-1 and stemness. The AP-1 has its contributions in apoptosis, along with that of individual proteins. AP-1 appears to deliver a central role in balancing stemness through monitoring OCT3/4, SOX2, and NANOG. Repression of AP-1 leads to *NANOG* level reduction as well as expression of *SOX2* gene, which in turn may lead to an increase in cells encountering apoptosis. The cells which are unable to conduct stemness undergo apoptosis.

The recent studies evidence that AP-1 could be potentially associated with the stemness phenotypes in colorectal squamous cell carcinomas. The decrease of its expression may lead to changes in expression of the major transcription factors

which are requisites for balancing pluripotency as well as undifferentiation. Additional studies are required to investigate this association further.

3.8 Conclusion

Advances in the field of gene regulation have commenced to discover the transcriptional networks which are operated in the neoplastic cells. These approaches in research offered insights into transcriptional regulatory molecules that can be targeted to rectify irregular gene functions. An accurate survey on signaling networks that are involved in oncogenic transcription factors can provide up-to-date features in transcription that has to be addressed. Advanced, structure-based minute drug molecules with minimum side effects and high selectivity can be generated in the future basing on the transcription factors instrumental in cancer formation. The mechanisms contributing to AP-1 activity regulation and its gene targets whose expression has to be regulated by AP-1 are still under investigation. Few mechanisms were disclosed such as modulation of transcription factor activity by protein phosphorylation and methodology used by cell surface receptors to interface nucleus. Forbye the recognition of critical AP1 gene targets can divulge the activities of AP1. One of the major challenges to be tackled in cancer biology is comprehending the probable mechanisms that confer the actions of protein kinases and transcription factors. The generic and ubiquitous signaling proteins like components of AP1 may be involved in immensely specific biological responses.

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Conflict of Interest None declared.

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Overview of Transcription Factors in Esophagus Cancer

4

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Abstract

Esophageal cancer is one of the most malignant cancer types which rapidly invade into the neighbouring tissues, metastasize to adjacent lymph nodes, reside at distant organs, and develop secondary tumors. Transcription factors (TFs) are frequently deregulated in the pathogenesis of esophagus cancer and are a key class of cancer cell dependencies. Deregulated activation and inactivation of transcription factors in addition to mutations and translocations play central role in tumorigenesis. In normal physiological conditions, TFs are regulated in highly specific manner by upstream transcriptional regulators. However, in cancer, aberrant activation of transcriptional factors guide deregulated expression of numerous genes is coupled with tumor development and progression. This review will summarize about the transcriptional factors involved in poor prognosis of esophagus cancer and the chemotherapeutic drugs targeting transcriptional factors.

Keywords

Esophagus cancer · STAT3 · NF-κB · HIF-1α · Sp1 · E2F1 · KLF4 · YY1

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4.1 Introduction

Esophageal cancer is the sixth most widespread and eighth most common cancer in the world cause malignancy-related deaths [28]. There are two main types of esophagus cancer with diverse malignant behaviors: esophagus squamous cell carcinoma (ESCC) and esophagus adenocarcinoma (EA). Enormous numbers of genetic and epigenetic alterations were found to be concerned in the growth and progression of esophagus cancers [2].

Transcription factors are important regulators for the gene expression pattern in cell and organize copious physiological processes such as self-renewal, development, proliferation, differentiation, survival, and invasion. Aberrant activities of TFs are due to several mechanisms, viz., changes in expression, posttranslational modifications, protein stability, and protein-protein interactions. In addition to mutations and translocations, deregulated activation plus suppression of TFs plays crucial roles in cancer cell proliferation, survival, and metastasis [7]. Depending on tumor stage, conventional treatment and therapies including chemotherapy and radiation therapy are prevailing for treating esophagus cancer. Despite therapeutic approaches, the prognosis of esophageal cancer in patients stays behind underprivileged, with a survival rate 15–34% [4].

The resistance to radiation and chemotherapy contributes to the high recurrence and poor survival rate. Mechanisms of resistance to radio-chemotherapy in esophagus comprise the activation of several transcription factors including NF-κB, HIF-1α, STAT3, E2F1, and specificity protein 1 (Sp1). TFs can be targeted at different levels that comprise inhibition of their interactions with coactivators and corepressors along with other interacting proteins or hindering their binding to DNA [25]. The failure of these therapeutic approaches in efficient treatment of esophageal cancer has attracted the attention toward the therapeutics with the aim of selectively targeting molecular pathways in cancer cells.

4.2 Transcription Factors

4.2.1 STAT3

STATs are signaling proteins, intricate in mediating cell signaling originated by binding of extracellular proteins to ligands [49]. The STAT protein family comprises six members, encoded by different genes: STAT (1–6). Numerous studies have highlighted overexpression of STAT3 in ESCC. In addition to that, STAT proteins perform dual functions: signal transduction in cytoplasm and activation of transcription in nucleus [64].

Several cytokine factors like IL-6 and IL-22 [9] and growth factors like EGFR [1] and FGFR [66] are identified to activate STAT3. The binding for these ligands to the receptor phosphorylates and activates STAT3; phosphorylation of STAT3 is mediated by non-receptor tyrosine kinase JAK2. Upon activation STAT3 dimerizes and translocates to the nucleus where it activates the transcription of genes intricate

in cancer metastasis (VEGF, MMP-2), progression (survivin), and cell cycle regulation (p^{53}, cyclinD1) [61, 64]. Interestingly, the overexpression of STAT3 proteins promotes tumor angiogenesis that intercedes immune evasion and confers resistance to apoptosis by chemotherapeutic agents. STAT3 is an essential mediator of the tumorigenic effects of EGF and TGF in squamous cell carcinoma of the head, neck, and non-small cell lung cancer [15].

The JAK/STAT signaling pathways also plays significant role in several physiological processes. Inhibition of the JAK/STAT signaling pathway may suppress cancer cell growth and support apoptosis in many cancers [21]. Moreover, the JAK/STAT pathways play an important role in upregulation of COX-2, involved in the progression, tumorigenesis, and recurrence of various tumors together with breast, prostate, stomach, and esophagus cancer. Nimesulide, a selective COX-2 inhibitor, downregulates the expression of COX-2 and survivin. In addition to that, nimesulide also upregulates the expression of caspase-3 in ESC cells by inactivation of JAK2/STAT3 pathway [29].

4.2.2 NF-κB

The nuclear factor-κB (NF-κB) pathway is an essential mediator for immune responses in mammals. NF-κB, a ubiquitous transcription factor, influences all six hallmarks of cancer through the transcriptional stimulation of target genes tangled in cancer cell proliferation, angiogenesis, metastasis, tumor promotion, inflammation, and repression of apoptosis [23, 37, 52]. NF-κB in mammals contains five protein forms, namely, RelA (p65), RelB, c-Rel, p50 (NF-κB1), and p52 (NF-κB2). However, these proteins assemble into homo- or heterodimers and form NF-κB. The protein of NF-κB family has a conserved Rel homology domain essential for dimerization and nuclear translocation along with inhibition [19]. NF-κB p50/p65 dimers are usually coupled with inhibitory molecule IκB and sequestered in cytoplasm. NF-κB can be triggered through numerous stimuli which include pro-inflammatory cytokines, viz., TNF-α, IL-1β, and IL-32, bacterial and viral products, and neutral pH [16]. Upon activation of NF-κB, phosphorylation, ubiquitination, and proteolysis of IκB inhibitory proteins by IκB kinase (IKK) complex occur. However, this allows the translocation of NF-κB p50/p65 to the nucleus and induces the transcription of several target genes [53].

Nkx2-8, a novel NK2-related transcription factor, is downregulated in various cancers and elicits an imperative role in development and progression of cancer. Nkx2 restricts the nuclear localization of NF-κB p50/p65 and suppresses the activity of NF-κB via downregulation of AKIP1 and also by binding to the promoter regions of AKIP1. AKIP1 (A-kinase-interacting protein 1) is a binding partner of NF-κB p50/p65 complex. Promoters of angiogenesis (COX-2, VEGF-C) are the downstream targets of NF-κB [26]. Activation of NF-κB is accompanied via stimulation of several factors associated with infiltration and metastasis, such as MMP, vascular endothelial growth factor (VEGF), adhesion molecules (ICAM, VCAM), and E-selectin [48].

Survivin, the smallest member of the IAP (inhibitor of apoptosis proteins) family, affects the expression of NF-κB p65, IKKα, and IKKβ at gene and protein levels in esophagus cancer cells. In Eca109 cancer cells, survivin activates the activity and expression of NF-κB p65 via binding to the IKKβ promoter region and increases transcriptional activity. Interestingly, downregulation of survivin arrested the cell cycle at the G_2/M phase and induced apoptosis in ESCC. However, the upregulated expression of survivin eventually activates NF-κB p65 which is a significant factor in the acquisition and maintenance of the oncogenic features of ESCC [65].

4.2.3 HIF-1α

HIF-1α mediates the adaptive responses to changes in levels of tissue oxygen. The HIF-1α protein is a good intrinsic marker of tumor hypoxia. HIF-1α was upregulated in a group of cancers that include prostate, gastric, breast, and colon cancer. Expression of HIF-1α is stimulated by EGF signals and also by hypoxia [40]. Furthermore, HIF-1α is a heterodimeric protein encompassing constitutionally expressed oxygen-sensitive HIF-1α subunit and oxygen-insensitive HIF-1β subunit. Under hypoxic environment (reduced oxygen levels), HIF-1α is stabilized, dimerizes with HIF-1β, and interacts with coactivator CBP/P300 which then translocates to the nucleus and activates target genes [45, 46]. Notably, in normoxic condition, hydroxylation of asparagine residues in HIF-1α C-terminal transactivation domain (CAD) is mediated by FIH (factor-inhibiting HIF-α). FIH evades the binding of p300/CBP coactivator, rendering HIF-1α transcriptionally inactive [22].

HIF-1α modulates transcription of numerous genes involved in stemness of esophagus cancer cell [68], proliferation (IGFBP-3) [36], invasion/migration, metastasis, and angiogenesis (VEGF, COX-2) [20, 35] under hypoxia. HIF-1α stabilizes p53 protein and contributes to hypoxia-induced p53-dependent apoptosis which is linked with cytochrome c release [14]. Furthermore, HIF-1α regulates the expression of insulin-like growth factor-binding protein (IGFBP)-3 which promotes tumor progression by binding to the HRE elements in promoter region. This leads to augmented transcription of IGFBP-3 and translation in cap-dependent manner under hypoxia conditions [36].

4.2.4 Sp1

Specificity protein 1 (Sp1), a zinc-finger protein belonging to the Sp/Krüppel-like factor (KLF) family, plays a significant role in carcinogenesis and is thought to play an important role in cancer metastasis. The literature witnesses that increased expressions of Sp1 have been reported in esophageal, breast, bladder, lung, and pancreatic cancer cell lines [44, 58]. Sp1-dependent transcription is controlled in response to various stimuli. Sp1 controls a group of genes that play a critical role in cell proliferation and growth (EGFR, HGFR, TGF-α), cell survival and apoptosis resistance (Bcl-2, Bcl-x, and survivin), cell migration and invasion (MMP-2 and

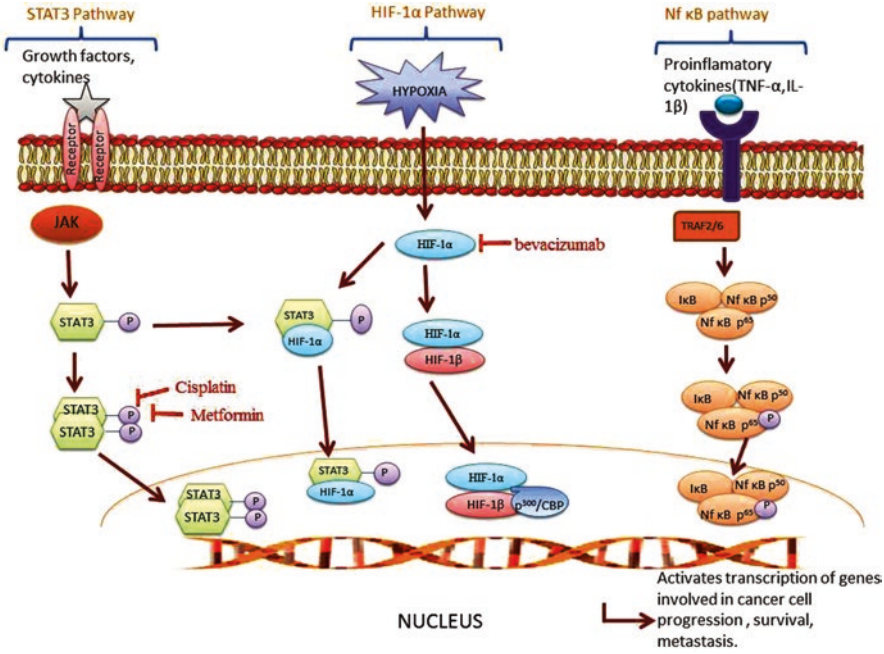

Fig. 4.1 Schematic representation of *STAT3*, *HIF-1α*, and *NF-κB* signaling pathways. In response to various stimuli, transcriptional factors get activated and promote the transcription of genes involved in cancer progression, survival, metastasis, and angiogenesis. Cisplatin, a most commonly used anticancer drug, targets Stat3 proteins and exerts growth inhibition in esophagus cancer cells

urokinase-type plasminogen), and angiogenesis (VEGF) [18]. Sp1 and Ap1 bind to the promoter regions of *VIL2* which encodes ezrin, a membrane cytoskeletal linker protein, critical for various cellular processes which comprise determination of cell polarity, cell adhesion, cell-cell interaction, migration/invasion, and signal transduction [12, 67]. AKIP1 increases the binding affinity of Sp1, NF-κB, and AP-2 to VEGF-C promoters and increases the expression of VEGF-C that promotes angiogenesis/lymph angiogenesis of ESCC cells [27] (Fig. 4.1).

4.2.5 E2F1

E2F1 belongs to the E2F family of transcription factors that are crucial for cell cycle progression. E2F1 controls the proliferation of cells in quiescent stage through G_1/S phase transition by trans-activating genes involved in chromosomal DNA replication, including its own promoter [60]. Zinc-finger protein 282 (ZNF282 also known as HUB1), often upregulated in esophageal squamous cell carcinoma, is an independent adverse prognostic factor. It is notable that depletion of ZNF282 decreases the expression of E2F1 target genes. However this depletion also increased

apoptosis and inhibited cell cycle progression at G_1/S. ZNF282 serves as coactivator of E2F1 and plays an important role in tumor progression in esophagus cancer [63]. Remarkably, E2F1 can stimulate proliferation and apoptosis; therefore, it functions as an oncogene and a tumor suppressor. The tumor suppressor and oncogene functions of E2F1 depend on its protein levels in cells.

E2F1 is inactivated through pocket protein binding of pRb (retinoblastoma). Hypo-phosphorylated pRb protein forms a complex with E2F1 and blocks its activity. CyclinD-CDK4/CDK6 complex phosphorylates pRb during G1 phase and releases E2F1, allowing transcription of genes involved in cell cycle progression [59]. However, inactivation of the pRb pathway may perhaps lead to inappropriate activation of E2F1 and increased cellular proliferation rates and tumorigenesis. As a consequence, inhibition of E2F activity has been proposed as a therapeutic strategy for the treatment of cancer [57]. E2F1 triggers apoptosis, via p53-dependent and p53-independent pathways. Furthermore, E2F1 directs p53-dependent and p53-independent apoptosis through transcriptional activation of ARF gene and p73 (p53 family member), respectively [51].

4.2.6 KLF4

KLF4 (Krüppel-like factor 4), belongs to the Krüppel-like factor family, and is a zinc-finger-type DNA-binding transcription factor expressed highly in different human tissues and implicated in pathogenesis of numerous cancers. Tumor suppressor activity of transcription factor KLF4 has been reported in several cancers such as gastrointestinal cancer, pancreatic cancer [62], colorectal cancer [55], and breast cancer [34]. The literature reports suggest that downregulation/loss of KLF4 promotes tumor invasion, development, and progression in numerous cancers. KLF4 is a crucial regulator of normal cell proliferation and squamous epithelial differentiation [33, 56].

Many studies have successfully demonstrated that KLF4 is controlled directly or indirectly by the transcription factors ZNF750, p63, PKCδ, and lncRNA in squamous epithelia [5, 30, 47]. Tetreault et al. [56] delineated novel pathway for epithelial stratification and differentiation in ESCC. KLF4 controls esophageal epithelial differentiation and stratification via *WNT5A*, a noncanonical Wnt ligand and direct transcriptional target of KLF4. KLF4 upregulates WNT5A and inhibits activity of CDC42, a small GTPase of the Rho family essential for normal differentiation. KLF4 together with WNT5A and CDC42 control β-catenin-dependent Wnt signaling, and play a role in esophageal squamous cell carcinogenesis [56].

4.2.7 YY1

YY1(Yin Yang 1) is a ubiquitous and multifunctional GLI-Krüppel zinc-finger transcription factor that belongs to the polycomb protein family that plays critical roles in cell proliferation, differentiation, cell homeostasis, and tumorigenesis [43].

Nevertheless, YY1 was also reported to upregulate in several types of tumors, which affect the clinical outcome of cancers. Expression of YY1 is high in ESCC tissues with lymph node metastasis and tissues of stages III–IV to stages I–II compared to tissues without lymph node metastasis. YY1 inhibits cell proliferation by increasing the binding potential of P21 to cyclin D1 and CDK4 in ESCC. Interestingly, YY1 also upregulates the expression of heme oxygenase 1, implicated in inhibition of ESCC proliferation [32]. However, this clearly indicates that YY1 has a tumor suppressor effect in ESCC. Luo et al. [31] depicted that YY1 promoted the invasion of ESCC cells, while the YY1 inhibition could retain invasion of EC cells. Notably, several TFs have intricate biological pathways and are involved in ESCC progression. Nevertheless, YY1 is one of the significant transcription factors that can inhibit ESCC proliferation but also promote metastasis. Conversely, the functional role and mechanisms of YY1 in esophageal cancer are still ambiguous and necessitate additional investigation.

4.3 Drugs Targeting Transcriptional Factors

4.3.1 Cisplatin

Cisplatin is a commonly used anticancer compound and most frequently used for esophagus cancer treatment. But the clinical efficiency remains undesirable. Dasatinib had a synergistic effect with cisplatin in growth inhibition of esophagus cancer cell via suppressing PI3K/AKT and STAT3 pathways. Chen et al. [3] reported that disatinib treatment targets downstream proteins of PI3K/AKT and STAT3 pathways and may be an alternative for adjuvant chemotherapy in esophagus cancer. Cisplatin represses the transcriptional activity of NF-κB while activating p53 [8]. Propofol (2, 6-diisopropylphenol, PPF), a general sedative and hypnotic reagent, enhances the cisplatin-induced cell apoptosis cervical cancer cells via EGFR/JAK2/STAT3 pathway. Moreover, the drug propofol was shown to suppress angiogenesis, invasion, and proliferation of cancer cell via ERK-VEGF/MMP-9 signal pathway in esophagus and human colon carcinoma [24]. Esophagus cancer cell lines induced the cell death mechanisms in response to chemotherapeutic drugs 5-fluorouracil (5-FU) and cisplatin [38].

4.3.2 Metformin

Metformin, an antidiabetic drug, employs chemopreventive and antineoplastic effects, and mechanisms responsible for action of metformin seem diverse and differ with cancer type. Metformin-mediated inhibition of cell growth in ESCC tumor cells is achieved by inactivation of STAT3 and Bcl-2 with induction of apoptosis and autophagy. Metformin suppresses the activity of Bcl-2 proto-oncogene, an inhibitor for apoptosis and autophagy [10].

4.3.3 Tolfenamic Acid

Tolfenamic acid (TA) has been used in treatment of rheumatoid arthritis and migraine headaches in humans. TA is also used as a veterinary drug product for treating pain and stress responses in animals. Papineni et al. [39] reported that TA, a nonsteroidal anti-inflammatory drug, can contribute to anticancer activity. TA reduces expression of Sp family proteins and some Sp-dependent genes and proteins such as VEGF, survivin, cyclin D1, and bcl-2.

4.3.4 Ramucirumab

VEGF (vascular endothelial growth factor)-mediated and VEGFR-2 (VEGF receptor-2)-mediated angiogenesis plays a crucial role in the pathogenesis of gastroesophageal cancer. In animal models of gastric adenocarcinoma, VEGFR-2 inhibition was previously depicted to reduce tumor growth and vascularity [17]. Ramucirumab is a human IgG1 monoclonal antibody and VEGFR-2 antagonist that avoids the ligand binding and receptor-mediated pathway activation in endothelial cells. In a recent report, ramucirumab drug depicted prolonged survival in patients with advanced gastric cancer or gastroesophageal junction adenocarcinoma progressing after first-line chemotherapy [11].

4.3.5 Bevacizumab

Bevacizumab, a human monoclonal antibody against VEGF (vascular endothelial growth factor), reduced microvessel density and increased intra-tumor hypoxia, but did not prompt apoptosis. Administration of bevacizumab with topotecan (TPT) triggered prominent antitumor effect, with tumor regression. Topotecan is a topoisomerase I poison that induces DNA damage and cytotoxicity and also potently blocks HIF-1α translation by a DNA damage-independent mechanism [42]. Furthermore, bevacizumab alone increased intra-tumor hypoxia and HIF-1 transcriptional activity and decreased proliferation, but did not prompt apoptosis of cancer cells. In contrast, by adding TPT to bevacizumab, HIF-1 activity and tumor cell proliferation were reduced effectively and apoptosis was induced [41].

4.4 Conclusion and Future Perspectives

Transcriptional factors STAT3, NF-κB, HIF-1α, Sp1 and E2F1 are potent oncogenes that are ubiquitously expressed and control abundant physiological processes in development, progression, and metastasis of cancer. There exist collaboration and cross talk between STAT3 and NF-κB that play a significant role in governing the discourse between the tumor cell and its microenvironment. Transcription factors STAT3 and NF-κB interact with each other and exert tumorigenic effects in cancer types [13]. Notably, in head and neck squamous cell carcinoma (HNSCC),

anomalous expression of the transcription factor NF-κB kindles the expression of STAT3 by an autocrine or paracrine mode that involves the release of IL-6 in an EGFR-independent manner [50]. Moreover, STAT3 interacts with HIF-1α and activates the target genes of HIF-1α, by recruiting the coactivators, CBP (CREB-binding protein), p300, and RNA polymerase II (Pol II) [6]. NF-κB was also a significant regulator for gene transcription of HIF-1α and VEGF. Additionally, NF-κB modulates HIF-1α expression at the level of transcription by binding to the promoter regions [54].

A more and complete understanding of oncogene targets and cross talk between transcriptional factors will help reveal the complex transcriptional networks involved in regulation of cancer progression, metastasis, and prognosis. Interestingly, few chemotherapeutic drugs that are clinically efficient drugs have been reported for treating esophagus cancer. Despite the potential effects of transcription factors, they signify a tremendous approach to combat cancer, and their inhibition not only affects growth and survival of tumor cells but also confers resistance to drugs.

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NF-κB Role and Potential Drug Targets in Gastrointestinal Cancer

5

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Abstract

A multifactorial disease cancer arises due to mutation in the gene encoding particular transcription factor or proteins. Globally cancer is one of the diseases which is responsible for maximum mortality annually. Transcription factor plays an important role in cell physiology, and any alteration in this transcription factor may lead to diseases like cancers. NF-κB is a transcription factor which has immense homeostasis role in cell physiology and in several diseases. NF-κB is actively expressed in many cancers and helps in initiations, cell proliferations, and metastasis of different cancers. The present chapter discusses the role of NF-κB in cancer promotion and different drug targets, targeting NF-κB pathway for the treatment of cancers.

Keywords

NF-κB · Carcinogenesis · Treatment · Noncanonical pathway and canonical pathways

The original version of this chapter was revised. The book was inadvertently published without Abstracts and Keywords, which are now included in all the chapters. An erratum to this chapter can be found at https://doi.org/10.1007/978-981-10-6728-0_39

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5.1 Introduction

Cancer is a multifactorial disease which occurs due to mutations in few genes and leads to abnormal growth of cells. According to the WHO, cancer accounts for a large number of deaths worldwide, and it is the world's leading health problem. Cancers originate from any tissue. The cancer cells lose normal control mechanism and attain a number of characteristic properties like immortality, invading of neighboring tissue, and migrating to remote parts of the body. Continuous growth and multiplication of cancerous cells form tumors which can invade to normal tissues and make them cancerous. However tumors can be cancerous and noncancerous.

In general various cellular developmental signaling pathways like MAPK, PI3K-Akt, and NF-κB take part in controlling regular cell proliferation, motility, cell survival, and cellular metabolic processes. Mutational alteration in signaling molecules or receptors in these pathways plays a significant role in development of cancer [1]. Important molecules responsible for cancer development and progression are transcription factors; these factors play an important role in various cellular gene expressions in different stages of development and cellular metabolic activities. Because of their role in development, intercellular and intracellular signaling, and cell growth, some human diseases are also associated with mutation in the transcription factors. Cancer suits as the best example of the disease which results due to mutation in few transcription factors. Transcription factors are central in many human cancers. Many signaling proteins are often mutated in cancers and change the transcription pattern directly in cancer cells. In general more signaling proteins are mutated in cancer than the transcription factors; finally transcription factors are elected as the strong targets for treatment of cancer because transcription is the final outcome of any cancer prognosis.

Therefore they are of important clinical significance for at least two following reasons:

1. Mutation can be associated with specific transcription factor with specific cancer.
2. They can also act as targets of medication.

There are many inter- and intracellular checkpoints in signal transduction pathways and one or more stress sensing checkpoints. For example, early developmental pathways (Wnt, Sonic Hedgehog pathways, TGF-β receptor pathways, Notch/Delta pathways, cytokine receptor (cytoplasmic tyrosine kinase, JAK/STAT) pathways), mid-developmental pathways (IL-1/toll-like receptor pathways, nuclear factor-kappaB (NF-κB) pathways, nuclear hormone receptor pathways, apoptosis pathways (TNF, Fas L, TNK pathway)), late developmental pathways (receptor guanylate cyclase (FOS, JUN, AP1), GPCR pathways, cadherin pathway, gap junction pathways), and finally stress- or checkpoint-induced pathways (oxidative stress-induced pathway, UV-induced stress response, and checkpoint for DNA damage and replication (p21 and p53) checkpoint pathways) are some of the important cellular physiological pathways. Finally at the end of all these pathways, innumerable transcription

factors participate ultimately as an outcome of signaling. All primary and modified cancer genes also participate in one or other of these pathways; many of the transcription factors are tumor suppressor or oncogenes/proto-oncogenes. Mutations or aberrant regulation of these factors is associated with outcome of different cancers. For example, tumor suppressor APC transcription factor participates in Wnt pathways via β-catenin transduction pathway where alteration in APC can lead to colons cancers. Generally four groups of transcription factors are known to be important in many human cancers. They are NF-κB and AP1 families, STAT family, and steroid receptor family. The present article focuses on NF-κB role in regulating cancer cell proliferation, apoptosis, cell invasiveness, metastasis, tumor angiogenesis, and finally targeting transcription factor NF-κB in cancer therapy for treatment of various cancers.

5.2 NF-κB Gene Family and Proteins

NF-κB is one of the transcription factors which is made up of hetero- or homodimers produced by the NF-κB family members (Fig. 5.1). Nearly 30 years ago, Dr. Ranjan Sen discovered NF-κB in 1986 while studying its interaction with few sequences of immunoglobulin light chain enhancer in B cells. The coincident discovery of three proteins NF-κB, v-Rel, and dorsal had been shown different nucleocytoplasmic subcellular distributions, but soon these proteins have shown to be members of the same family proteins notably NF-κB family proteins [2–5]. From the time of discovery to the present date, the role and importance of NF-κB in regulating cellular processes has been extensively studied. NF-κB is well known for its immune regulatory function, and it is a key pathway in inflammatory responses, but growing evidences suggest that mutation in this factor also has significant consequences of outcome of cancer. NF-κB was found to be involved in regulating expression of the crucial genes which are necessary in certain developmental stages and in diseases like cancer.

NF-κB family proteins are ubiquitous in nature and present in all vertebrate cells. p65 (RelA), p50/p105, RelB, p52, and c-Rel are five NF-κB family proteins which are ubiquitous. These proteins have distinctive structural features like N-terminal

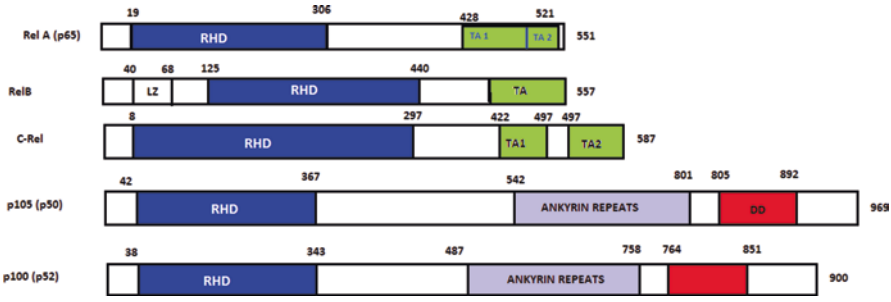


Fig. 5.1 Family member of NF-κB transcription factors

Rel homology domain (RHD) and C-terminal transactivation domain (TAD) [6]. Rel homology domain forms dimers which can bind to DNA and can also bind to its inhibitors. At C-terminal TAD interacts with the transcription machineries which ultimately enhances gene transcription, it is harbored by proteins like p65 (RelA), RelB, and c-Rel of NF-κB family members. In the absence of this TAD, generally the homodimers of either p50 or p52 act as a transcriptional repressors that give a stimulus for the activation of NF-κB [7]. In cell signaling, and in protein sorting, the conserved nuclear localization signal (NLS) sequence is commonly required for translocation of the proteins to nucleus. Similarly the transcription factor NF-κB also requires NLS sequence for translocation to the nucleus. This NLS sequence in NF-κB family proteins is positioned in the center.

5.3 Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-Cell Inhibitor

IκB is the physiological inhibitor of the NF-κB. Binding of IκB to NF-κB in normal quiescent cells leads to the formation of NF-κB-IκB complex. This binding masks the NLS sequence in NF-κB proteins and makes it to be in inactive state as it inhibits nuclear localization. IκB family proteins specially bind to NF-κB RHD domains and inhibit NF-κB proteins by squelching it in the cytoplasm [8]. IκB family proteins are IκBβ, IκBα, IκBε, IκBγ, BCL-3, and p10 and p100 (Fig. 5.2). The IκB kinase (IKK) is a protein that specifically binds to IκB family members and inhibits IκB by causing its degradation. Degradation of IκB is a controlled event and initiated upon phosphorylation of specific residue by activated IKK. This event leads to the dissociation of IκB from NF-κB and its imgrations into nucleus to induce the expression of more than 150 genes which also include

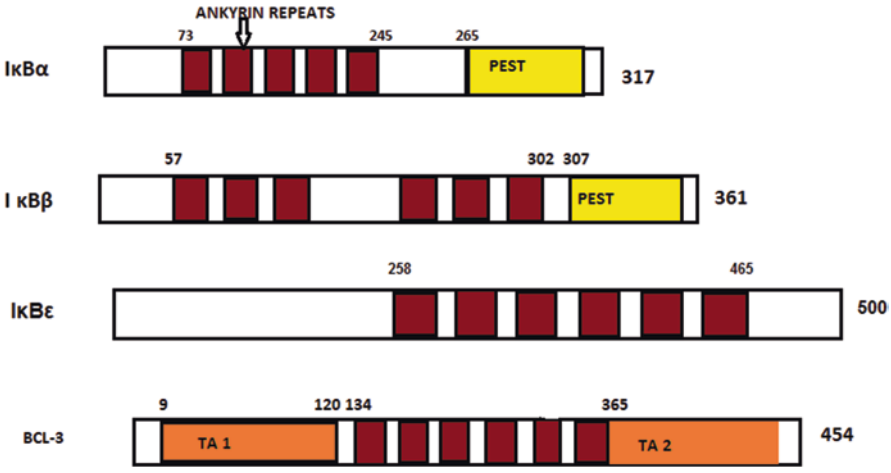


Fig. 5.2 Family members of IκB

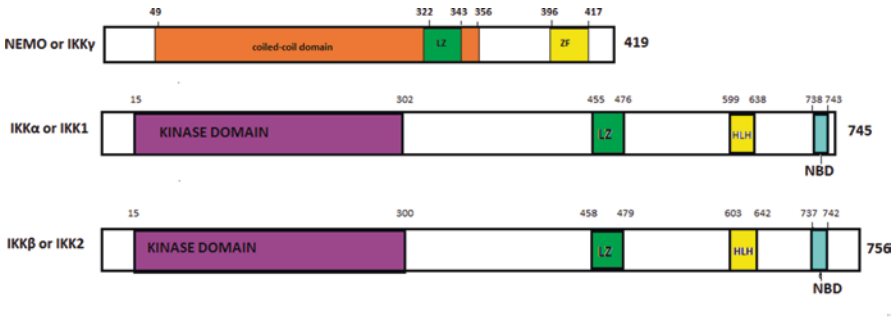


Fig. 5.3 The IκB kinase (IKK) proteins

anti-apoptotic genes. The IKK activity in cells can be purified as a 700 to 900 kDa complex. IKK contains different subunits such as IKK alpha (IKKα), IKK beta (IKκβ), and IKK gamma (IKKγ) or NEMO (NF-κB essential modifier) [9, 10] (Fig. 5.3).

5.4 NF-κB Pathways

NF-κB signaling occurs mainly through the classical pathway which is called as canonical pathway and by the alternative pathway which is also called as noncanonical pathway.

5.5 Classical Pathway or Canonical Pathway

p65, c-Rel, and p50 proteins play an important role and drives the classical pathway.

This pathway has been characterized in many cell types as one of the most established pathways. This pathway also consists of an IKK which consists of complex catalytic kinase subunits like IKKα, IKKβ, and one nonenzymatic regulatory scaffold protein called IKKγ or NEMO. These are known as the essential modulators of NF-κB signaling.

In this pathway after activation of NF-κB, p50 and RelA dimers migrate to nucleus and result with output of increasing the transcription of genes which encodes the chemokines; cytokines; cell adhesion molecules such as ICAM-1, VCAM-1, and ELAM-1; inhibitors of programed cell death; and many other enzymes which produce inflammatory mediator. In the classical pathway, NF-κB/Rel proteins are bound and strongly inhibited by IκB proteins in normal cells. In viral or bacterial infections, stimulatory signals in cells lead to release of various proinflammatory cytokines, LPS (lipopolysaccharides), growth factors, toll-like receptors (TLRs), various cell-induced stress signals, and antigen receptors that can

activate an IKK complex which is made up of both catalytic and regulatory proteins IKKα and IKKβ and NEMO/IKKγ, which phosphorylates IκB proteins which bound to NF-κB to make it inactive and reside in the cytoplasm. The phosphorylation by IKK complex to IκB leads to ubiquitin-mediated proteasomal degradation of IκB and finally generates NF-κB/Rel complexes free from its inhibitor IκB and makes NF-κB/Rel complex partially active. Now this partially activated NF-κB/Rel complex undergoes few posttranslational modifications like phosphorylation, acetylation, and glycosylation and gets completely activated and translocated to the nucleus. Alone or in combination with few other transcription factors like AP-1, E26 transformation-specific (ETS), and signal transduction molecule and transcription activator (STAT), it induces the multiple target gene expression of innate immune genes [11, 12] (Fig. 5.4).

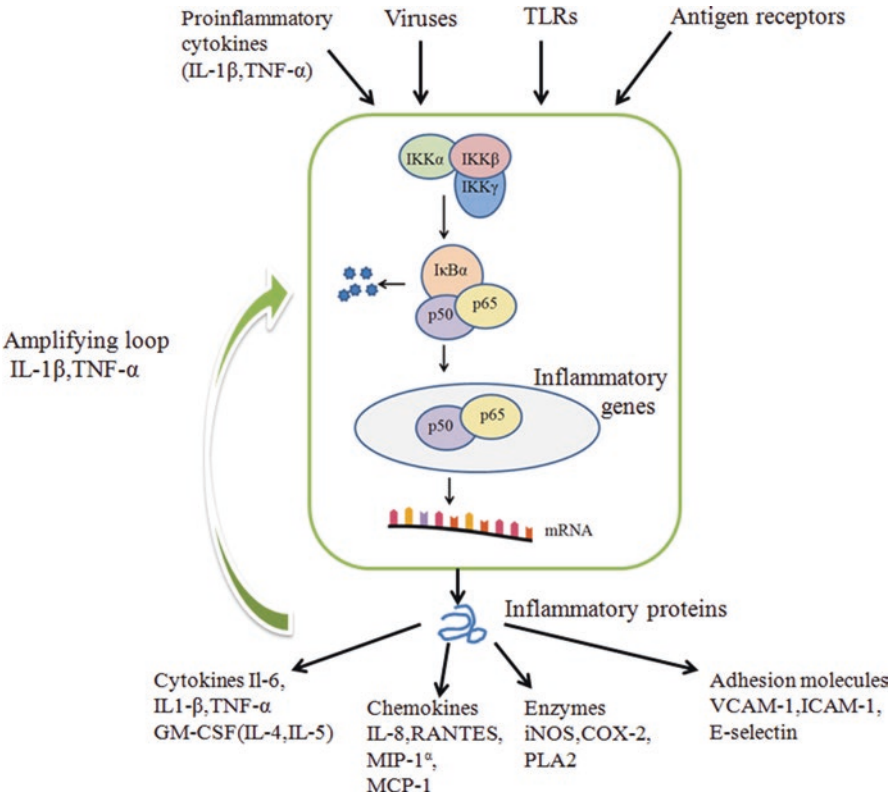


Fig. 5.4 NF-κB pathways (**a**) canonical pathway (**b**) Noncanonical pathway

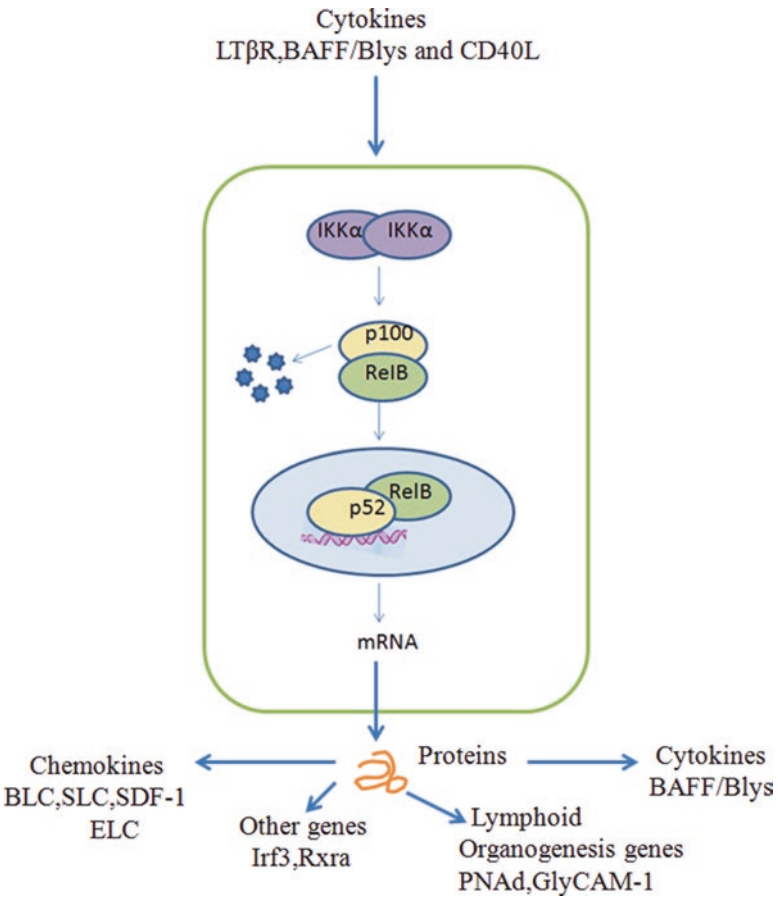


Fig. 5.4 (continued)

5.6 Alternative Pathway or Noncanonical Pathway

The alternative pathway is generally activated by the members like tumor necrosis receptor family such as lymphotoxin-beta (LTβ), CD40, and viral proteins of Epstein-Barr virus (EBV) such as LMP1 and B-cell-activating factor (BAF). In this pathway p52 and RelB NF-κB complexes are the central targets to be activated. p100 is a molecule which is the precursor of p52 and RelB. Inducible processing of p100 generates p52 and a RelB-specific inhibitor. NF-κB-inducing kinase (NIK) plays a central role in this pathway. NIK integrates signals from TNF family receptor and activates the downstream kinases like IKKα to initiate the p100 phosphorylation triggering its processing into p52 and RelB. Phosphorylated form of IκBs is ubiquitinated and subsequently assigned for degradation, or processed in the case of p100, by the proteasome [13]. The untied, NF-κB dimers migrate to the nucleus,

where they bind to specific sequences in the promoter regions of target genes and induce gene expression of target gene (Fig. 5.4b).

Apart from canonical and non-canonical pathways of NF-κB, other pathways had also reported. For example, NF-κB also gets activated by short wavelength of ultraviolet (UV) rays, this involves the IKK casein kinase 2 (CK2)-mediated phosphorylation and calpain-dependent IκB degradation. In this UV-mediated pathway, there is no role of IKK-independent NF-κB activation. In some cases, CK2 activity toward IκB is UV inducible through a mechanism that depends on activation of p38 MAP kinase. Thus, the p38-CK2-NF-κB axis is an important component of the mammalian UV response [14]. NF-κB also gets activated by hydrogen peroxide through the phosphorylation of tyrosine at position Tyr42 of IκB by the involvement of spleen tyrosine kinase (Syk kinases) [15].

5.7 Biological Significance of NF-κB

Due to NF-κB importance and involvement in many physiobiological and pathological processes, NF-κB can be termed as multifunctional transcription factor. Many reports stated that it has an immense role in immune responses as it regulates expression of growth factors involved in immune responses, in cytokine signaling, and also in regulation of both T-cell receptors (TCR), B-cell receptors (BCR), CD40, tumor necrosis factor receptor superfamily member 13C (TNFRSF13C), TLRS, and interleukin-1 (IL-1) receptor family. Genes which are located outside the immune system are also known to be regulated by the NF-κB, and hence it can influence multiple aspects of disease and normal physiology. NF-κB was also known that it has a role in embryonic development and in the development and physiology of tissues including the mammary gland, bone, skin, and central nervous system, tissue homeostasis, and inflammation. While at the molecular and cellular level, NF-κB regulates gene expression, cell apoptosis, and cell proliferation [11, 16, 17].

5.8 NF-κB in Gene Expression

NF-κB plays a very important task in transcription. In transcriptions it acts as a transcription activator or enhancer by binding at the promoter to assist transcription. Most physiological response such as growth, immune response, and inflammation are induced by the expression of NF-κB genes. Previous studies revealed that activation of NF-κB can be done by endotoxins, carcinogens, tumor promoters, stress (pH, hypoxia, heavy metals), apoptosis inducers such as chemotherapeutic agents, gamma radiations, bacterial and viral infections and cytokines like TNF family, IL-1, IL-17 plays a major role in NF-κB activation and can induce cellular transformation, through continous cell proliferation mediated invasion and angiogenesis, metastasis. NF-κB activation can prevents tumorigenesis [18]. The transcriptional suppression of NF-κB is only a probably cell type-specific property as some of this agent-induced NF-κB was clearly transcriptionally active in different cells [19].

5.9 Role of NF-κB in Apoptosis

Programed death of cell is known as apoptosis. This is a physiological process of removal of unnecessary old cells or damaged cells or cells of which DNA damage is not repairable. Apoptosis is crucial for organ development; it creates diverse organ from mass of cells. For apoptosis to start, it needs a variety of induction stimuli. All necessary components are constitutively expressed in cells; as soon as the signal comes cells start suicide program. In different cells Rel/NF-κB factor has shown to have regulatory function for apoptosis pathway. Regulatory function involves activation of pathway or inhibition of pathway. Overall activation or inhibition depends on different conditions as cell type, inducing agents, and damage of cells. This defines anti- or proinflammatory effects of Rel/NF-κB complexes. Few proapoptotic proteins have an effect on Rel/NF-κB signaling pathway, e.g., Bcl-2 and caspases. There are three ways by which NF-κB regulates apoptosis pathway as follows: first is by direct regulation of genes that inhibits or enhances apoptosis, second is by controlling cell cycle, and third is by interaction of NF-κB with cellular proteins whose concentration is vital for cell survival, thereby altering cell survival [20].

As viruses are intracellular pathogens, many viruses found to be interfering with NF-κB metabolism. Viruses may increase the signaling by direct interaction with NF-κB factor. Increased NF-κB may enhance replication by binding with promoter binding site in viral genome or may enhance pathogenicity of viruses. In HIV-1 genome, there are two sites for NF-κB binding in the promoter region. In some conditions binding of NF-κB supports viral replication in cells. NF-κB also protects HIV-1 by inhibiting apoptosis of infected myeloid cells. Apart from HIV-1 virus, other viruses such as hepatitis C virus, and encephalomyocarditis virus infection, NF-κB blocks apoptosis [21]. Many targets of NF-κB like physiological apoptosis inhibitors (cIAP-1, cIAP-2), TNF receptor-associated factors (TRAF1, TRAF2), B-cell lymphoma-extra large (Bcl-XL), X-linked inhibitor of apoptosis (XIAP), manganese superoxide dismutase (MnSOD), and immediately early gene 1 (IEX-1) were shown to have anti-apoptotic properties. Sometimes, NF-κB could be proapoptotic in such scenario; it enhances expression of apoptosis mediators such as death receptor DR5, Fas ligand, PUMA, and Bax [22, 23].

In tumor cells NF-κB found to be constitutively expressed, and it is responsible for enhanced growth or resistance to induced apoptosis.

5.10 Role of NF-κB in Cellular Proliferation

NF-κB family members are shown to regulate cell proliferation on several kinds of cells. Different cytokine secretions including growth factors are under control of NF-κB transcription factor. TNF and many other different interleukins mostly proinflammatory interleukins are under regulation of NF-κB. This leads to either of inflammatory signals or cell proliferation signals; thereby it is involved in

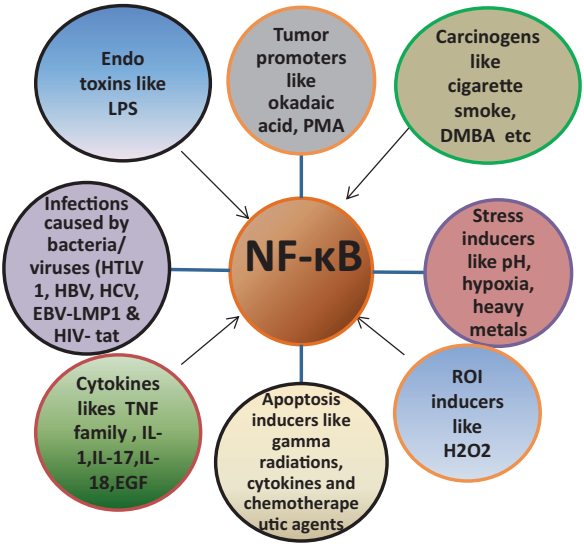
carcinogenesis. NF-κB-mediated increase in TNF or IL-1β and IL-8 is acute myeloid leukemia, Hodgkin's lymphoma, cutaneous T-cell lymphoma, and gliomas. Growth factors signal via NF-κB factors and finally lead to increased signaling which finally helps to the proliferation of cells. In multiple myeloma IL-6 cytokine [24] which involves NF-κB signaling acts as inducer. Epidermal growth factor is one important factor that utilizes NF-κB signaling to increase proliferation of cells [25–28]. In cancers like breast and prostate and in some other cancers, epidermal growth factor receptor is overexpressed. This effect is mediated through activation of NF-κB [29]. Cell cycle regulator genes such as cyclins D1, D2, and D3 and cyclin E and c-myc are also regulated by NF-κB and help in cell cycle progression [18].

5.11 NF-κB in Cancer

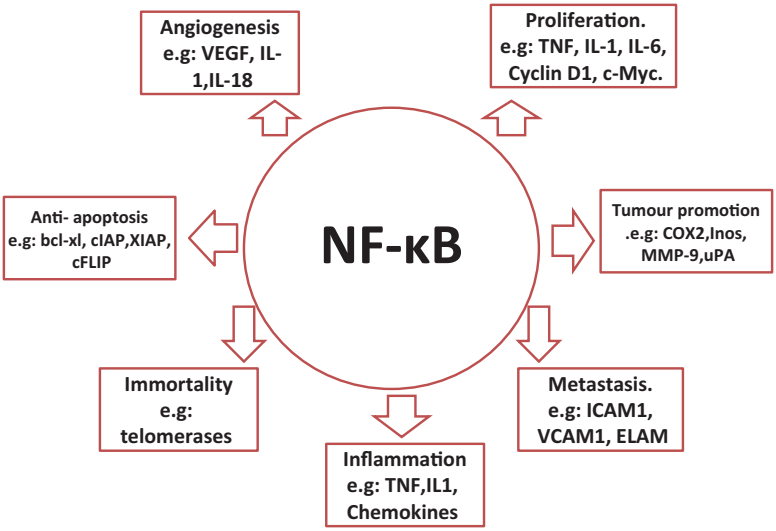
5.11.1 Activation of NF-κB in Cancer

Very common factor involved in tumorigenesis is NF-κB. Several studies on normal and tumor cells and in vivo models proved the NF-κB involvement in cancer development. In 2001 Balkwill and Mantovani showed a role of NF-κB in inflammation and tumorigenesis. NF-κB can be activated by most of the carcinogens and tumor promoters and by inflammatory agents which can finally lead to cancer [30]. Agents like few chemotherapeutic drugs, radiations, and few cytokines generally known to induce apoptosis are also known to activate the NF-κB expression as part of cell auto defense mechanism and mediate the desensitization, chemoresistance, and radioresistance [31]. In some human hematopoietic and solid tumors, chromosomal amplification, overexpression, and rearrangement of gene coding for Rel/NF-κB factors have been identified. Epigenetic alteration or mutations in NF-κB protein lead to continuous expression of NF-κB in a certain type of cancers. In some cancer cell lines, the persistent activity of NF-κB in nucleus was observed due to the mutation of inhibitory IκB subunits. These mutations inactivate the inhibitory IκB subunits or due to constitutive activation of upstream signaling kinases also make NF-κB to be persistent in nucleus. Continuous activity of NF-κB was observed in Hodgkin's lymphoma due to the mutation in the gene of IκBα. Her2/Neu is an oncogene; its overexpression is most commonly observed in the aggressive cancers like breast cancers. Apart from breast cancer, it is also observed in lung and prostate. The overexpression of Her2/Neu is mediated through NF-κB. NF-κB may also be activated by few viral-derived proteins like T-cell leukemia virus (HTLV) Tax proteins, reticuloendothelial (REV-T) retrovirus protein, hepatitis B virus X protein, and EBV latent membrane protein (LMP)-1 and can cause the viral infection-associated cancers [32, 33].

5.11.1.1 Inducers of NF-κB Activation



5.11.1.2 Expression of Numerous Genes by Transcription Factor NF-κB



5.12 NF-κB Role in Inflammation and Cancer

According to the fact sheet data of World Health Organization, cancer is the major cause of the highest number of mortality worldwide. Around 14 million new cases and 8.2 million deaths solely associated with different types of cancers and nearly 15% cancer in world population are exclusively caused by inflammation [34]. Infectious agents are the one which can cause inflammation in specific tissues. There are different ways/mechanisms by which infectious agents can cause the carcinogenesis like by chronic infections (e.g., hepatitis B virus and hepatitis C virus chronic viral infections in case of hepatocellular carcinomas), making normal cells to become transformed cancer cells by insertion of oncogenes and further inhibiting the tumor suppressor genes and finally suppressing the induction of immune responses. Evidences proved that NF-κB plays a major role as like that of other transcription factors like p53, E2F, and sp1 in generalized cellular processes like inflammation, stress response, and cell proliferation apoptosis. In a process of defense mechanism by body's immune system, NF-κB gets activated with an aim of targeting and eliminating the altered and transformed cells in acute infections by recruiting the highly active cytotoxic immune cells against cancer cells. In various stress-induced responses like in inflammatory responses, activation of NF-κB in cancerous tissues usually results in upregulating a variety of pro-tumorigenic functions such as upregulating the anti-apoptotic genes and thereby helping to provide best cell survival signals to develop the progression of cancer and escape of immune defenses mechanism. Cytokines like TNF-α, IL-1, IL-6, and IL-8 that regulate the immune responses and cell adhesion molecules are induced by NF-κB and recruit different immune cells at the site of infection. In innate immune responses, neutrophils release the reactive oxygen species (ROS) to destroy invading pathogen by ROS-mediated DNA; this might cause genetic mutations in host cells which may be responsible for tumor initiations. In gene knockdown experiments carried out by Greten et al. [35] in mouse model, knocking down the IKK gene in enterocytes results in blocking the activity of NF-κB and also resulted into reduction of tumor multiplicity by 80% in dextran sulfate sodium-induced chronic inflammatory colitis cancer in mouse. This states that NF-κB takes part in early development of cancer. Blocking of NF-κB reduced the expression of few other anti-apoptotic gene expressions of Bcl-XL and also the tumor multiplicity to half by reducing cytokine growth factor like IL-6 in myeloid cells. These show that NF-κB interacts with many cellular proteins and promotes the tumor progression. In some other cases, the obstruction of NF-κB into the hepatocyte-specific, IκB expression leads to increased number of liver cell apoptosis and reduced the level of hepatocellular carcinoma. In mouse model the progression of hepatocellular carcinoma and activation of NF-κB are most likely mediated by the cytokines like TNF-α, because the administration of TNF-α antibody surpassed the levels of nuclear RelA immunostaining in liver cell and lowered the hepatocellular carcinoma [36]. In some cancers like mucosa-associated lymphoid tissue-derived lymphoma (MALT), the overexpression of Bcl-10 and MALT resulted in aberrant expression of NF-κB [37]. In some cases the deletion of IKKβ in myeloid cancer showed low expression of certain proinflammatory cytokines and ICAM 1 genes in macrophages and reduced the tumor growth in

myeloid cells without showing an effect on apoptosis [38]. Increased levels of COX2 and MMP were observed in enterocyte-specific deletion of IKK, and myeloid-specific deletion of IKKβ decreases the levels of COX2 and MMP. Thus IKKβ promoted tumor injury growth in myeloid cells through the production of tumor growth, promoting paracrine factors rather than inhibiting the tumor cell apoptosis which shows that IKKβ is involved in inflammatory mediator's production to promote tumor growth. This shows that IKKβ has different approach to initiate tumor growth because in enterocytes tumor are formed by expressing the anti-apoptotic proteins like Bcl-2, whereas in the myeloid cells, tumor imitations and progression are by tumor growth factors.

5.13 Role of NF-κB in Cellular Transformation and Tumor Growth

In cellular transformations and in tumor growth, the role of NF-κB is very much significant as it interacts with apoptotic and anti-apoptotic and with several cell cycle-regulating proteins and genes to initiate cancer cell transformation and tumor growth. In human cancer cells of colon, prostate, fibroblast, and lymphocytes, several oncogenes like HTLV tax genes, PIM, and RAS cause the cell transformation with the help of NF-κB. It also has a positive role in cell transformation with many transcription factors. In case of DNA damage which is caused by cell stress like UV and inflammations or by heavy metals, toxicity, NF-κB comes into the picture and protects from regular cell apoptosis by stimulating the enhanced proliferation of cells to resist the stress. This enhanced cell proliferation leads to the neoplastic transformations and finally initiates the cancer. In this process many anti-apoptotic factors and growth factors are involved like Bcl-XL, survivin, and p21WAF, cyclin D and C-myc which are cell cycle regulators, and cytokines which act as growth factors like IL-6 and IL-1β and certain growth factors such as EGF and HIF-1α which are hypoxia-induced factors expressed highly in tumor cells; all these interact with NF-κB making it active and lead to tumor formation. HIF-1α in myeloid cells interacts with NF-κB and increases the expression HIF-1α and leads to formation of NF-κB and HIF-1α-induced tumors [38–40].

5.14 Role of NF-κB in Tumor Cell Invasiveness and Metastasis

In any cancer processes, cell adhesion, migration, and invasion are the three basic steps involved in tumor metastasis as it is the only process and special property of cancer disease that drives cancerous tissue to occupy and translocate to distant remote tissues in the body making the invaded area cancerous. In cancer cell invasion and metastasis, the role of NF-κB has been investigated in different types of cancers both in vitro and in vivo. Epithelial-mesenchymal transition (EMT) is the process involved in cell invasion and metastasis, and this process in one and some other way is also mediated by NF-κB by interaction of NF-κB with EMT genes. In some aggressive cancers like breast cancers, EMT genes ICAM 1, ELAM-1, and

VCAM-1, twist, MMPs, and serine protease urokinase-type plasminogen activators (uPA) are enhanced by the activation of NF-κB. Bcl-2 activated by NF-κB also promotes the EMT in breast cancer. Sometimes NF-κB has also been shown to have different roles in cell invasion and metastasis. In one (in vitro) study, it was shown that an increased level of TNF enhanced the ability of the tumor cells to adhere strongly with mesothelium, whereas in in vivo it showed opposite results. Increased TNF levels in in vivo showed the enhanced tumor migration and invasion. This is mediated by the NF-κB-dependent induction of some chemokine receptor like CXCR4 and upregulation of monocyte chemokine attractant protein-1 (MCP-1) and IL-8 and ICAM-1 [41–43].

5.15 Role of NF-κB in Angiogenesis of Tumor

Angiogenesis is the development of new blood vessels, a process which is most important for progression of cancer. It helps in tumor invasion and metastases to take cancerous cells to remote tissues by means of newly formed blood vessels. Angiogenesis of the tumors is generally dependent on many signals like proinflammatory cytokine growth factor signals like IL-8, TNF-α, and VEGF that are secreted by macrophages and other inflammatory cells. Continuous activation of NF-κB triggers the autocrine of angiogenesis by angiogenic chemokines in cancerous tissues. Inhibition of this angiogenic chemokine suppresses the tumor growth and angiogenesis and finally cancer progression. Several angiogenic genes are associated via NF-κB and mediate the tumor angiogenesis. For example, in basal cell carcinoma, a gene called stromal cell-derived factor 1 alpha (SDF-1α) is known to boost the tumor angiogenesis by regulating the genes that are associated with angiogenesis via NF-κB. In bone marrow-derived cells, tumor vascularization is much essential for tumor angiogenesis, and IL-8 is mediated in this process. Many cytokines, chemokines, and growth factors are also involved in angiogenesis apart from IL-8, and this process is finally mediated through the NF-κB [44] (Table 5.1).

5.16 Targeting of NF-κB for Cancer Therapy

NF-κB is a transcription factor that plays a central role during tumorigenesis in many cancers types. It has numerous roles in cellular activities like in development, immune system, and in maintaining cellular plasticity and promoting the expression of several genes which controls cellular activities like apoptosis, angiogenesis, and metastasis and in interaction with many other transcription factors, chemokines, and cytokine growth factors and receptors. As it is expressed in many cells, NF-κB family genes and their pathways are the one of best targets for cancer therapy. There are many treatment approaches based on NF-κB like gene silencing mechanisms and chemotherapy targeting the NF-κB genes and proteins [139]. NF-κB is mainly regulated by the processes like methylation, phosphorylation, and ubiquitination-mediated modifications.

From past few years, many investigations had been done on the NF-κB pathways, mainly on the canonical pathways, and opened a door for targeting the

Table 5.1 Constitutive NF-κB activity in human cancer cells and proposed mechanism (Modified from http://www.bu.edu/nf-kb/the-gilmore-lab)

Cancer type	Proposed mechanism	References
Burkitt's lymphoma (EBV)	EBV-encoded latent membrane protein 1 (LMP1) – TRAF2-mediated NF-kappaB activation, AP-1 induction, and JAK3/STAT activation	[45]
Acute nonlymphocytic leukemia	NF-κB continual activity is mediated by the overexpression of WT1 and MDR1	[46]
Breast	Altered IkBα activity	[47]
		[48]
		[49]
Cervix	Altered IkBα activity	[50]
		[51]
		[52]
Ovary	Altered IkBα activity	[53]
		[54]
Vulva	Continuous activation of NF-κB in vulva cancer tissue is by growth inhibitory cytokines	[55]
Prostate	Altered IkBα activity is observed	[56–60]
Kidney	Altered IkBα activity is observed	[61]
Bladder	Altered IkBα activity	[62–64]
Lung	Altered NF-κB p65 nuclear expression leads to continuous activation of NF-κB in lung cancers	[65–69]
Mesothelioma	TNF-α signaling through NF-κB pathway is anti-apoptotic	[70, 71]
Non-small-cell lung	Altered NF-κB and p65 nuclear expression leads to continuous activation of NF-κB in lung cancers	[72–74]
Liver	Defective IkBα activity	[75–78]
Pancreas	Defective IkBα activity	[79, 80]
		[81, 82]
Esophageal/gastric	NF-κB blocks apoptosis and mediates tumor cell proliferation	[83, 84]
		[85]
Laryngeal	By altered levels of NF-κB p65, protein makes NF-κB continuously active	[86, 87]
Stomach	Constitutive activation of NF-κB is likely due to the activation of ubiquitin-proteasome pathway	[88, 89]
Colon	Defective IkBα activity	[90–92]
Thyroid	Defective IkBα activity	[93–95]
Parathyroid	Defective IkBα activity	[96]
Melanoma	Defective IkBα activity	[97]
		[98]
Head and neck	Defective IkBα activity	[99–102]
Endometrial (uterus)	NF-κB and IkB families of genes may be important in endometrial carcinogenesis, by controlling apoptosis and cell proliferation	[103, 104]

(continued)

Table 5.1 (continued)

Cancer type	Proposed mechanism	References
Cylindromatosis	CYLD, a tumor suppressor gene which is mutated in familial cylindromatosis, interacts with NEMO, the regulatory subunit of IKK, and makes NF-κB active continually	[105–107]
Hilar cholangiocarcinoma	Defective IkBα activity	[108]
Oral carcinoma	Altered IkB kinase alpha and Akt kinase activity lead to continual activity of NF-κB	[109, 110]
		[111, 112]
		[113]
Astrocytoma/ glioblastoma	ING4 interaction with p65 (RelA) subunit of nuclear factor NF-κB involved in regulation of brain tumor angiogenesis via transcriptional repression of NF-κB-responsive genes	[114, 115]
Neuroblastoma	Constitutive NF-κB DNA binding activity specifically involving p65 and p50 in neuroblastoma	[116, 117]
Glioblastoma	Defective IkBα activity	[118, 119]
Hodgkin's lymphoma	Constitutive IKK activity; IkBα and IkBε mutations	[120–122]
Acute lymphoblastic leukemia	Constitutive IKK activity; NF-κB1 chromosomal rearrangement	[123, 124]
Acute myelogenous leukemia		[125, 126]
Acute T-cell leukemia (+/−HTLV-1)	Increased IkBα degradation	[127, 128]
Acute nonlymphocytic leukemia		[46]
Chronic lymphocytic leukemia	STAT-3 activates NF-κB in chronic lymphocytic leukemia cells	[129, 130]
		[131]
Multiple myeloma	RelA amino acid substitution	[132]
		[133, 134]
MALT lymphoma	Translocations are involved in few genes which lead to induction of NF-κB pathway	[135]
		[136]
		[137]
Waldenstrom macroglobulinemia	Continuous NF-κB is mediated by different proteasomal and Akt pathways	[138]

molecules involved in these pathways. Much more efforts had also taken for developing therapy against NF-κB factor as well as its characterization. By trying different natural and synthetic compounds and their combinations of NF-κB inhibitors, more than 700 inhibitors are identified. It is an important drug target because of the central role that NF-κB has in many pathologies, besides inflammation and cancers [140]. Best approaches according to known literature are the use of anti-inflammatory drugs, DNA binding inhibitors and inhibitors of nuclear translocation migration of NF-κB, IKK activation, proteasome inhibitors, and targeting genes to inhibit transcription. Degradation of IkB and nuclear targeting of NF-κB and DNA binding is

the main targeted action of NF-κB signaling pathways. So far discovered NF-κB targets are based on these steps as follows.

5.17 Inhibitors of IKK

IKK is one of the major and well-studied molecular targets for NF-κB inhibition as it has a central important role in the activation of NF-κB. IKK inhibition and inhibition of its related kinases had been investigated as good therapeutic targets for the treatments of many inflammatory diseases and in many different types of cancers. SC-514, TPCA-1, IMD-1, bardoxolone methyl, BMM-345541, IKK16, BAY 3264, BAY 11-7085, BAY 11-7082, MLN120B, BMS345541, SC-514, and CHS828 can inhibit IKK by direct interaction with IKK. Inhibition might depend on inhibition ability of kinase or indirectly inhibiting IKK activation [141–145, 156–158].

5.18 Inhibitors Based on Proteasome

Bortezomib is a reversible 26S proteasomal inhibitor which acts as inhibitor of the activity of proteasome which blocks NF-κB pathway by IkB protein degradation. It is one of the first drugs approved by both the European Medicines Agency and FDA for the treatment of multiple myeloma. In many malignant tumors like breast, colon, lung, and bladder and in prostate cancers, bortezomib is tested in combination with other anticancer drugs including DNA damage-inducing drugs and achieved a very better response compared to administration of bortezomib alone. Carfilzomib, RP-171, and NPI-0052 are some of other proteasomal inhibitors [146].

5.19 Inhibitor for Nuclear Translocation and DNA Binding

Translocation of NF-κB into the nucleus and DNA binding is inhibited by this category of drugs. SN50 is one drug that acts in the nucleus by inhibiting both nuclear translocation and DNA binding of NF-κB. Hence NF-κB is retained in the cytoplasm after IkB protein degradation. SN 50 is a peptide 41 amino acids long consisting of P50 NLS sequences that block the machinery of nuclear transport in sensitized cisplastin anticancer activity in ovarian cancer cells [147]. Apart from the IKK inhibitors, proteasome inhibitors, nuclear translocation, and DNA binding inhibitors of NF-κB, some anti-inflammatory drugs are also used in blocking the NF-κB. Sulindac, aspirin, ibuprofen, COX2 inhibitors, and indomethacin which are nonsteroidal anti-inflammatory drugs (NSAIDs) are also used as potential targets of NF-κB. These drugs suppress the inflammatory cell response which in turn suppresses the NF-κB or act by suppressing the NF-κB at key agenesis NF-κB activation pathways. Combination of these agents with other anticancer agents is widely used for chemosensitization [148]. Epigallocatechin gallate, eicosapentaenoic acid, curcumin, and luteolin which are natural anti-inflammatory agents are also able to block the NF-κB [149].

5.20 Gene Therapy Based on Targeting NF-κB

Gene therapy is one of the promising areas for various cancer treatments as genes are the key component of carcinogenesis pathways. One advantage of gene therapy is that it can directly target key components of pathways so it is very sensitive than other approaches.

- Overexpression of IkB SR with viral vector or with plasmid
- RNA interference which specifically eliminates the NF-κB gene expression
- IKKα, IKKβ, and kinase TAK1 are targeted by siRNA for blocking NF-κB expression for treatment of cancer [150–152] (Table 5.2)

Table 5.2 Drug targets of **NF-κB** pathways for cancer therapy

Drugs	Mode of action	Reference
BAY 11-7085	It irreversibly inhibits IkBα phosphorylation, preventing activation of NF-κB by cytokines and lipopolysaccharide	[153]
		[154]
BAY 11-7082	It irreversibly inhibits TNF-α-induced IkBα	[154, 141]
	Phosphorylation and its selective IkBα phosphorylation inhibitor	
MLN120B	Directly binds to IKKβ and inhibits activity of IKKβ kinase and blocks NF-κB activation	[149, 155]
BMS3345541	Directly binds to IKK and inhibits IKK kinase activity and blocks NF-κB activation	[142]
SC-514	Binds and inhibits IKKβ	
CHS828	Directly binds to IKK and inhibits IKK kinase activity and blocks NF-κB activation	[144]
TPCA-1	TPCA-1 is an inhibitor of IKK2 and inhibits NF-κB pathway	[156, 157]
IMD0354	It blocks IkBα phosphorylation in NF-κB by inhibiting IKKβ	[158, 159]
Bardoxolone methyl	It is an IKK inhibitor	[162]
IKK16	Acts on IKKα and IKKβ	[160]
A2D3264	Novel IKK2 inhibitor	[161]
LY2409881	IKKβ inhibitor	[163]
BMS-345541	Inhibits both IKKβ and IKKα	
Bortezomib	Inhibits proteasome action by blocking NF-κB activation during the process of IkB protein degradation	
Carfilzomib	Acts on p50 and p65	
RP-171	Proteasomal inhibitors	[146]
NPI-0052	Proteasomal inhibitors	[146]
SN50	Inhibits the translocation of NF-κB and its binding to DNA	[147]
Sulindac	Selectively acts on IKKβ	[164]
Ibuprofen	It is an anti-inflammatory agent that blocks the constitutive activity of NF-κB	[57]
Aspirin	Inhibits the kinase activity of IKKβ	[148]

5.21 Conclusion

NF-κB is one of the well-studied transcription factors which has diverse roles in regulation of cellular physiology. It is well known to involve in many signal transduction pathways such as cell metabolic pathways, cell growth pathways, and apoptotic pathways and even in several immunological pathways especially innate immune signaling pathways. Besides its role in normal cell physiology, it also plays a critical role in many diseases. One such disease is cancer. As its expression is seen in many cancers, NF-κB is involved in different progression stages of cancer like tumor initiations, cell proliferations, apoptosis, tumor invasion, and metastasis. In this chapter activation of NF-κB and drug targets based on NF-κB signaling have shown in different cancers. Many therapeutic targets are also used against different stages of NF-κB pathways to cure cancers. Approaches like RNA-based gene therapy on NF-κB are very much promising for treatment of cancers.

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HIF-1α: Its Role in Metastasis of Oesophageal Malignancy

6

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Abstract

Hypoxia is a significant factor of the tumor microenvironment. Transcriptional factor HIF-1α serves as a drive for tumor hypoxic microenvironment and triggers gene transcription that intricate in important aspects of cancer biology. Hypoxic effects can be either positive or negative, depending on the context, severity and duration on a tissue. In oesophagus cancer, hypoxia stabilizes the transcription factor HIF-1α and regulates diverse functions such as metastasis, angiogenesis, cell cycle regulation and apoptosis chemo-resistance. miRNAs also play essential roles in the adaptive response of tumors to hypoxia. Therefore, HIF-1α acts as a promising target in advancement of new therapeutics for oesophagus cancer therapy. Recent developments in regulation of HIF-1α and functional involvement in tumor growth, migration, stemness and drugs affecting HIF-1α expression will be discussed in this review.

Keywords

Hypoxia · HIF-1α · Cell proliferation · Metastasis · Angiogenesis · Apoptosis · Growth factors · Cytokines · Stemness · Tumor resistance

6.1 Introduction

Oesophageal cancer (EC) is malignant tumor of upper digestive tract. Oesophageal cancer is extremely lethal disease with an overall 5-year survival in less than 15% and mostly prevalent in developing and underdeveloped countries [49]. Oesophageal cancer (EC) is the eighth most common cancer and the sixth most common cause of

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cancer death worldwide. Oesophageal adenocarcinoma and squamous cell carcinoma are two major types of cancer which typically arises in gastric reflux-associated Barrett's oesophagus, squamous epithelial cell, respectively. Risk factors are associated with chronic smoking and alcohol consumption [2]. More than 90% of oesophageal cancers arise within the stratified squamous epithelial cell (ESCC) [87] and account for oesophageal malignancies in East Asia, including China and Japan. In recent years incidence of oesophageal adenocarcinoma (EAC) is increasing in Western counties [70].

Mammalian cells usually require adequate levels of oxygen to execute aerobic metabolism and energy production to support their biological functions. Tumor expansion increases the diffusion distances from the existing vascular supply. Consequently, the regular normal blood supply into tumor cells is impaired, resulting in a low oxygen level (hypoxia) [48]. In this unique microenvironment of hypoxia, intratumoral cells start to activate the transcriptional factor, HIF-1 (hypoxia-inducible factor-1), which controls transcriptional response of several genes. HIF-1controls the expression of over 100 genes involved in cell survival, proliferation, invasion, tumor metabolism and angiogenesis and stimulates cytokines such as VEGF (vascular endothelial growth factor) [48, 56] and COX-2 (prostaglandin (PG) H synthase-2). However, tumor growth generated hypoxic environment due to insufficient blood supply, stimulates ROS (reactive oxygen species) production and HIF-1 expression [22, 39]. HIF-1α is a good intrinsic marker of tumor hypoxia.

HIF-1α is overexpressed in several cancers like pancreatic cancer, colon cancer, gastric cancer, oesophagus cancer and breast cancer. Furthermore, up-regulation of HIF-1α affects the biological action of tumor cells, recruits the infiltrated lymphocytes and promotes angiogenesis in tumor microenvironments [19, 57, 79]. Increased expression of HIF-1α also activates protein kinase C (PKC) which promotes tumor differentiation and proliferation [6]. HIF-1α facilitates the transcription of several genes encoding for autocrine and angiogenic growth factors including VEGF, TNF-α (tumor necrosis factor-α) and transforming growth factor (TGF)-β1, which consequently benefit the progression of cancer cell metastasis and angiogenesis [20, 104]. Increased expression levels of HIF-1α in both tumor cells and TILs (tumor-infiltrating lymphocytes) were significantly associated with poor survival in ESCC patients [97–99]. Notably, HIF-1α up-regulation is associated with mortality in cervical cancer, ovarian cancer and breast cancer and treatment failure in oesophagus cancer and squamous cell carcinomas. Hypoxia is one of the most prominent conditions that promotes both radio-resistance and chemo-resistance of cancer cells. Resistance to therapy is partly intervened by defect in autophagy or an inherent apoptosis [68].

6.2 Hypoxia Inducible Factor-1α

HIF-1 is a heterodimeric protein composed of a constitutional expressed an oxygen-sensitive HIF-1α subunit and oxygen insensitive HIF-1β subunit. The α-subunit consists of three isoforms, viz. HIF-1α, HIF-2α and HIF-3α, whereas HIF-1β is the

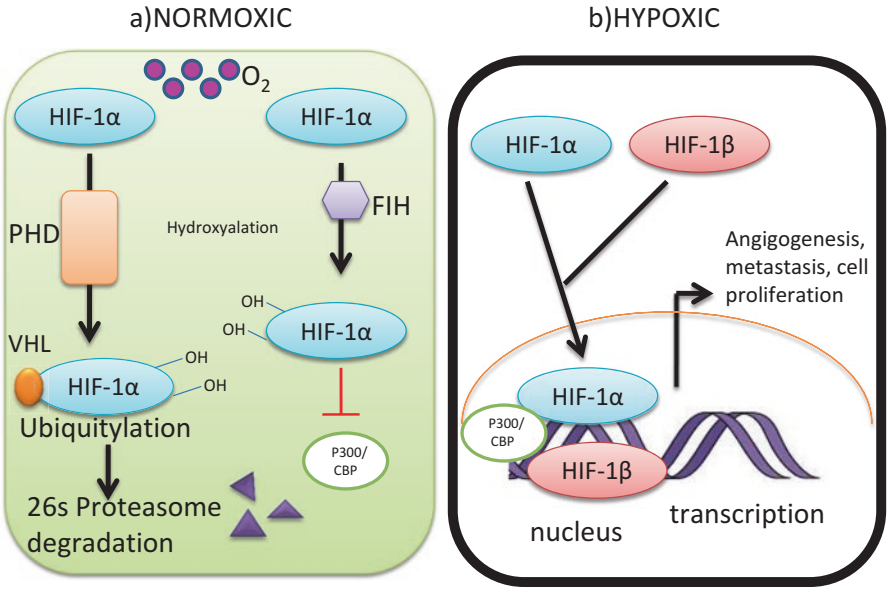

Fig. 6.1 Regulation of HIF-1α under normoxic and hypoxic conditions. (**a**) HIF-1α is hydroxylated by PHD at proline residues. Hydroxylated HIF-1α is polyubiquitinated by tumor suppressor protein VHL and undergoes 26s proteasomal degradation. FIH (factor inhibiting HIF-1α) hydroxylates the asparagine residues of HIF-1α and prevents interaction with p300/CBP rendering HIF-1α transcriptionally inactive. (**b**) Under hypoxic condition, HIF-1α interacts with HIF-1β and translocates to nucleus where it binds to co-activator p300/CBP and activates the transcription of genes by binding to HRE elements

only β-subunit known. HIF-1β subunit is also known as the aryl hydrocarbon nuclear translocator (ARNT). Both HIF-1α and HIF-1β subunits belong to basic-helix-loop-helix (bHLH) PER, ARNT and SIM (PAS) superfamily of eukaryotic transcription factors. DNA binding is mediated by the basic domains and subunit dimerization by the bHLH domains [3, 72, 73].

Under hypoxic conditions, HIF-1α is stabilized and dimerize with HIF-1β. HIF-1 heterodimers then translocate to the nucleus where it binds with cofactors p300/cAMP (response element-binding protein) and forms an active transcription complex (Fig. 6.1). This assembled complex is able to interact with hypoxia response elements (HRE 5′-G/ACGTG-3′) and regulate transcription of various genes [42, 56, 100]. In normoxic condition, hydroxylation of Asparagine residues in C-terminal transactivation domain (CAD) of HIF-1α is mediated by factor inhibiting HIF-α (FIH) to prevent the binding of p300/CBP co-activator, rendering HIF transcriptionally inactive [3, 42]. Nuclear translocation of HIF-1α is achieved by importin-α nuclear transport protein. Binding of importin-α to HIF-1α facilitates transport of HIF-1α into nucleus involves importins 4 and 7 [1].

6.2.1 HIF-1α Stabilization in Mitochondria

In mitochondria under normoxic conditions, prolyl hydroxylases (PHD1, PHD2 and PHD3) hydroxylate ODD (oxygen-dependent degradation domain) domain of HIF-1α at proline and arginine residues (Fig. 6.1). PHDs are dioxygenases that require oxygen, Fe2+ and 2-oxoglutarate as co-substrates and as well as ascorbic acid and iron as cofactors [66]. The von Hippel–Lindau tumor suppressor protein now binds to both hydroxylated HIF-1α and Elongin-C, which in turn recruits Elongin-B, CUL2 and RBX1 and E3 ubiquitin ligase and targets HIF-1α for ubiquitination [32, 63]. Ubiquitination targets HIF-1α for degradation, which can be hindered by proteasome inhibitors. In the absence of oxygen, prolyl hydroxylase cannot alter HIF-1α, and the protein remains stable. Furthermore, reactive oxygen species (ROS) helps in stabilization of HIF-1α and impedes the action of PHD [54]. However, inhibition of cell growth during hypoxic condition leads to formation of more malignant phenotype in tumors by provoking genes involved in angiogenesis and energy metabolism (Fig. 6.2).

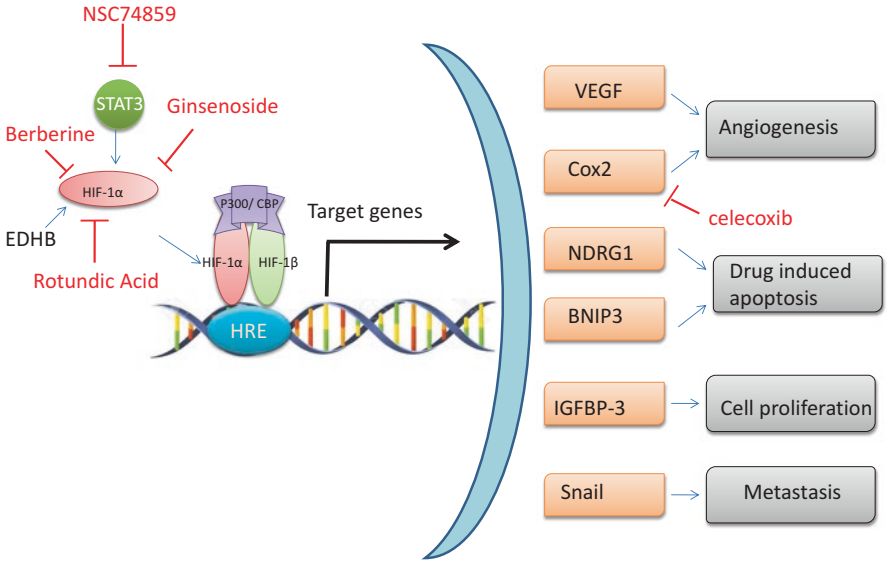

Fig. 6.2 HIF-1α target genes under hypoxia and therapeutic intervention. HIF-1α dimerizes with HIF-1β, and co-activator CBP/p300 binds to HRE regions of DNA and transcribes the genes necessary for ECC progression. STAT3 interacts with HIF-1α and stabilizes under hypoxia condition. Berberine, Ginsenoside and rotundic acid inhibit the expression of HIF-1α

6.2.2 Oxygen-Independent Regulation of HIF-1α

In addition to hypoxia induced activation, growth factors stimulate the HIF-1α protein synthesis via a signal transduction pathway.HIF-1α can be activated by genetic alterations of oncogenic proteins PTEN; VHL appears to depend on the signal transduction pathways that are active in a particular tumor cell [38]. Growth factors, viz. EGFR, IGFR and HER2, mediate activation of HIF-1α through RAS/MAPK and the PI3K-Akt-mTOR signal transduction pathway [43]. This pathway is negatively controlled by the tumor suppressor PTEN, which dephosphorylates downstream targets of the PI3K. Increased levels of PTEN inhibit HIF-1α expression and HIF-1α facilitated gene transcription in prostate cancer and glioma cells [102]. Gain-in-function mutation of oncogene and loss-of-function mutation of tumor suppressor gene promote tumor vascularization and increase glucose uptake and lactate production (Warburg effect). In addition, the notable frequency with which genetic alterations occur in cancer cells is linked with increased HIF-1α expression and tumor progression.

6.3 HIF-1α in Cell Proliferation and Survival

In hypoxic microenvironment, cells may adapt to certain metabolic changes and cellular signalling. Under hypoxia several growth factors and signalling pathways are induced which promote cell proliferation and survival. Growth factors such as cytokines, mitogen-activated protein kinases, insulin-like growth factor-3 (IGF3) and transforming growth factor-α (TGF-α) stimulate HIF-1α expression by binding to their cognate receptors and amplify the HIF-1 activity together with Ras and Myc oncogenes [12, 41]. HIF-1 target gene *IGF2* encodes insulin-like growth factor-2 (IGF2), which promotes tumor cell survival. IGFBP-3 (insulin-like growth factor-binding protein-3), a major carrier protein of IGFs, regulates the cell proliferation in oesophagus squamous cell carcinoma in IGF (or IGF1R)-dependent and IGF-independent manners [84]. The expression of insulin-like growth factor-binding protein (IGFBP)-3 is regulated by HIF-1α but not HIF-2α in ESC cells. HIF-1α binds to the HRE elements in promoter region of IGFBP-3 [60], leading to increased transcription of IGFBP-3, translation in cap-dependent manner under hypoxia conditions.

In addition to cancer cells, tumor microenvironment also contains immune cells (lymphocytes) and stromal cells. The collaborations of diverse cell subsets in tumor microenvironment play a vital role in clinical behaviour of cancer patients. Prospero homeobox 1 (PROX1) is a transcription factor and master regulator of lymphangiogenesis and associated metastasis. PROX1 expression in colon cancer [65], hepatocellular carcinoma [52] and malignant [11] is associated with cancer progression. PROX1 acts as a tumor suppressor in hepatocellular carcinoma [52], neuroblastoma [13], breast cancer [90] and pancreatic cancer [71], suggesting that the oncogenic potential of PROX1 is cancer type-dependent. However, the clinical significance of PROX1 in human solid cancers is controversial. PROX1 regulates lymph node

metastasis via induction of VEGFR3 (vascular endothelial growth factor receptor 3) and FOXC2 (forkhead box protein C2) expression. PROX1 regulates cell proliferation in ESCC patients, is a downstream target of hypoxia-induced gene and NF-κB. PROX1 involves in cancer progression by increasing the protein stability and nuclear accumulation of HIF-1α. High expression of PROX1 reduces expression of epithelial marker E-cadherin and increases lymph node metastases in ESCC patients. PROX1 is independent diagnostic marker in ESCC patients [94]. Hypoxia is a characteristic attribute of tumor environments and up-regulates HIF-1α in lymphocytes which in turn promotes Foxp3 (master regulator of regulatory pathway in development and function of regulatory T cells) expression. Foxp3 mounts the suppressive function of regulatory T_{reg} cells and impairs cytotoxic T cells function. Increased levels of HIF-1α in both TILs and tumor cells were significantly associated with poor survival in ESCC patients [99].

6.4 HIF-1α in Cell Cycle

Cells can respond differently to a wide range of oxygen and also alter homeostatic functions of normal or cancer cells. Hypoxia prompts cell cycle arrest by inactivation of enzymes responsible for nucleotide synthesis, eventually inhibiting DNA replication [18]. Acute and chronic hypoxia induces cell cycle arrest in G_0–G_1 phase and reduces the percentage of cells in G_2/M and S phases. Chronic and acute hypoxia down-regulate the expression of DNA repair genes BRCA1, BRCA2 and RAD51 which account for G_0–G_1 arrest [35]. In hypoxia normal amount of BRCA1 regulate the stability of HIF-1α, possibly by interacting with HIF-1α [33].

MicroRNAs are small (17–25 ntd long), conserved, non-coding single-stranded RNAs that work as post-transcriptional regulators of gene expression. miRNAs play a significant role in cancer development by regulating cell proliferation, invasion and apoptosis. miRNAs have potential dual role in cancer: both as oncogenes and tumor suppressor genes [26]. Up-regulation of several miRNAs has been detected in ESCC such as miR-93, miR-210, miR-106b, miR-192, miR-147and miR-196a. miRNAs such as miR-375, miR-203, miR-145, miR-133a, miR-133b and miR-125b were reported to be down-regulated in ESCC that may exert tumor suppressive functions. [97–99] reported that miR-145, miR-133a and miR-133b suppressed tumor cell growth and invasion via targeting *FSCN1* gene (organize F-actin to parallel bundles). MiR-21 unregulated in several malignancies including ESCC, pancreatic, colon, lung and breast cancers in response to hypoxia and allows cell survival in hypoxic environment. MiR-21 overexpresses and promotes cell proliferation, which inhibit cell cycle arrest at G_2/M phase in lung cancer [103]. Unregulated expression of miR-203 down-regulates mir-21 expression through suppression of small GTPase Ran (downstream target of miR-203) [98]. Down-regulation of miR-21 inhibits cell growth and invasion and induce cell apoptosis by targeting RECK, FASL and TIMP3 genes [92]. MiR-210, a downstream target of HIF-1α, promotes cancer cell proliferation and induces G_0/G_1phase transition of cell cycle by targeting E2F3 and FGFRL1. Increased levels of MiR-210 reduce PLK1 (Polo-like kinase 1)

a crucial regulator of mitosis which facilitate the activation of Cdk1 (cyclin D kinase 1)/cyclin B an essential factor in G_2/M transitions of the normal cell cycle. MiR-210 is elevated in ESCC patients and also serves as a biomarker for cancer diagnosis [47].

Down-regulation of gankyrin (also known as PSMD10 and p28GANK) expression inhibits cell proliferation and accumulation of cells in the G_1/S phase in vitro in ESCC cell lines. Gankyrin is reported as negative regulator for several tumor suppressors, viz. RB and p53. Binding of Gankyrin hyperphosphorylates RB protein through binding of CDK4 (cyclin-dependent kinase 4) and promotes ubiquitination of p53 by MDM2 that ensures degradation by 26 s proteasome. Gankyrin stabilizes E2F1 in relation to the G_1/S transition [16, 64, 101]. Gankyrin regulates the stability of HIF-1α via ubiquitination and degradation in ovarian cancer cells [4].

6.5 Role of HIF in Metastasis

Cancer metastasis is associated with propagation of tumor cells from primary tumor mass to another site (secondary tumor) via blood/lymphatic vessels. Metastasis includes invasion of cancer cells into neighbouring tissues and the growth of secondary tumor in distant organs through angiogenesis. Metastasis of tumor cells occurs via detachment and migration of metastatic cells from the primary tumor invasion from the basement membrane (BM) and ECM into the blood and or lymphatic vessels (intravasation). These metastatic cells adhere to the endothelium of capillaries of the target site (adhesion), and cells invade through the endothelial cell layer and surrounding BM (extravasation). Finally, these cells reside and promote their growth for the formation of secondary tumors at the target organ site [8, 27, 75].

HIF-1α activates the transcription of cell adhesion genes like L1 cell adhesion molecule (L1CAM) [74]. Loss of the epithelial markers β-catenin and E-cadherin and the expression of the mesenchymal markers N-cadherin, vimentin and fibronectin are the molecular hallmarks of EMT [50, 80]. EMT is regulated by various oncogenic signalling pathways that include TGF-β, NF-κB, Wnt and Notch.

6.5.1 HIF-1α in Invasion and Migration

EGFR, IL-6R increase expression of STAT3 (JAK2/STAT3 pathway), hitherto induces migratory phenotype, a fibroblast-like morphology with the up-regulation of mesenchyme-associated N-cadherin and vimentin expression and the nuclear translocation of β-catenin. STAT3 increases stability of HIF-1α protein by interacting with HIF-1α and bind to promoters of HIF-1α target genes. Silencing of STAT3 and HIF-1α under hypoxic conditions shows reduced hypoxia-mediated EMT and increased in E-cadherin expression while reducing vimentin expression. STAT3 controls EMT by targeting transcription factors SLUG, TWIST and SNAIL, which regulates E-cadherin expression [7]. HIF-1α binds to E-box elements of SNAIL and inhibits E-cadherin expression while promoting MMP-2 expression [29].

NSC74859, a STAT3 inhibitor, down-regulates the HIF-1α expression and increases radiosensitivity of ESCC [97]. Transcription factor SOX2 that belongs to the family of SOX (SRY-related high-mobility group box) plays a key role in maintenance of pluripotency of undifferentiated neuronal and embryonic stem cells. SOX2 mediates cell migration/invasion by elevated expression of a zinc-finger protein SLUG (an EMT regulator) that down-regulates E-cadherin expression via STAT3/HIF-α activation [15].

Oncoproteins like p63 and p28GANK are overexpressed in several other types of cancer including hepatocellular carcinoma, lung, colon, gastric and oesophageal cancers and promote invasion, metastasis in ESC cells [14]. p63 belonging to p53 gene family increases the mRNA expression and protein levels of TWIST (oncogene inhibits expression of E-cadherin), vimentin (mesenchymal marker), uPA (serine protease) and SUSD2 (cell-cell and cell-matrix adhesion protein). Knockdown of p63 reduced the levels of β-catenin and c-Myc (cell cycle-regulating genes) and also target genes of β-catenin: cyclin D1 [86], uPA and MMP-7 (genes related to the metastasis and invasion of cancer cells). p63 in part regulates WNT signalling pathway through activation of β-catenin/c-Myc pathway [45].

In addition to growth factors, oncoproteins, expression of chemokine receptors such as CXCR4 (bone marrow-homing receptor) and CCR7 mediates tumor invasion. Fibroblasts secrete chemokines, particularly SDF-1 (stromal cell-derived factor-1, also known as CXCL12) [10]. Interaction between CXCR4 and SDF-1 participates in homing of ECC (oesophagus cancer cells) to bone marrow and lymph nodes, stimulates tumor cell growth, induces angiogenesis, promotes motility, invasiveness, and employs tumor cells to metastatic sites [9, 23, 30]. SDF-1/CXCR4/CXCR7 expression has been reported in both ESCC and EAC, and activity of this axis is coupled with survival as well as tumor invasion and metastasis. CXCR7 is expressed predominantly in ESCC and occasionally in EAC [69, 83]. Under hypoxic conditions, HIF-1α activates the transcription of CXCR4 mRNA in human renal carcinoma and oral squamous cell carcinoma cell lines [28]. CXCR4, a receptor for stromal cell-derived factor-1 (CXCL12/SDF-1α), also binds to MIF and plays a significant role in tumor progression and antitumor immunity. In tumor cells, CXCR4 and MIF were also independent prognostic markers for ESCC [99].

6.5.2 HIF-1α in Angiogenesis

Angiogenesis is involved in formation of new blood vessels, important for tumor growth and metastasis. COX-2 and VEGF play key role in carcinogenesis, angiogenesis and poor overall survival in oesophagus malignancy [67]. HIF-1α controls the expression of genes involved in angiogenesis of ESC cells by binding to HRE promoter regions of VEGF [37], COX-2 [106] and other angiogenic growth factors such as angiopoietin 2 (ANGPT2) and stromal derived factor 1 (SDF1). COX-2 and VEGF serve as independent prognostic markers for poor prognosis in EAC and ESCC [67].

VEGF belongs to the family platelet-derived growth factor and is comprised of six VEGF members (VEGF A-E) and placenta growth factor (PIGF). HIF-1α

activates transcription of VEGF via direct binding to HRE regions, and by recruiting additional transcriptional factors such as P-CREB and P-STAT3, to the promoter. Inhibition of HIF-1α down-regulates the expression of VEGF and increases apoptosis of cancer cells. This supports the role of HIF-1α in tumor progression and angiogenesis and is a potential target for cancer therapy. VEGF-D and HIF-1α act as independent survival predictors and therapeutic targets in ESCC. VEGF expression was associated with a high micro-vessel density [1, 88].

Cyclooxygenase is a rate-limiting enzyme in biosynthesis of prostaglandin from arachidonic acid. COX-1 and COX-2, two isoforms of COX genes, have been reported. COX-1 is constitutively expressed in many tissues, whereas COX-2 expression is induced by inflammation or by a variety of stimuli, such as cytokines, mitogens and various growth factors [34, 76]. HIF-1α transcriptionally regulates the expression of COX-2 by binding to HRE elements in COX-2 promoter region and increases theCOX-2 expression. Prostaglandin E synthase (PTGES) a functional downstream of COX-2 in synthesizes prostaglandin E2 (PGE2) from prostaglandin H_2 (PGH_2), is synthesized from arachidonic acid catalyzed by COX-2. Pro-inflammatory stimuli such as IL-1β (interleukin-1β) and lipopolysaccharide also activate COX-2-PTGES axis [31]. PTGES is up-regulated in gastrointestinal cancers [89], colon cancer [59] and hepatocellular carcinoma [85]. Overexpression of PTGES boosts PGE_2 levels and leads to vascularization. In ESCC PTGES overexpressed at both mRNA and protein levels, expression of mRNA is regulated by HIF-dependent transcriptional activation; PTGES protein is stabilized upon reoxygenation [44].

Apurinic/apyrimidinic endonuclease-1 (APE-1) is a multifunctional protein that interacts with HIF-1α and STAT3 and activates hypoxia-induced expression of VEGF. APE-1 is a crucial enzyme responsible for DNA base excision repair. APE-1 stimulates the expression of COX-2 via NF-κB activation by stimulating the DNA-binding activity of NF-κB [58]. Additionally, the MCP-1 (monocyte chemoattractant protein-1) receptor and CC-chemokine receptor 2 (CCR2) are reported to influence on angiogenesis and VEGF production. MCP-1 plays a key role in tumor vascularity of oesophageal cancer [62]. The non-steroidal anti-inflammatory drug (NSAID) celecoxib, a selective COX-2 inhibitor [95], also repressed APE-1 expression through reduction of IkBα phosphorylation [58].

6.6 HIF-1α in Apoptosis

In contrast to necrosis, apoptosis is an energy-dependent process which occurs in the presence of ATP. Cells under environmental stress can endure apoptosis. Transcription factor p53 controls cell cycle inhibition, apoptosis and blood vessel formation. p53 protein, an important regulator of apoptosis after DNA damage, induces the Bax and Bak proteins. These proteins control the release of cytochrome C from the mitochondria, thereby initiating apoptosis cascade (Apaf-1 and caspase-9 activity) [21, 24]. In early-stage oesophageal cancer, the combined expression of HIF-1α and anti-apoptotic protein BCL-2 is significantly associated with treatment failure [40].

HIF-1α stabilizes p53 and contributes to hypoxia-induced p53-dependent apoptosis. The interaction p53 a (tumor suppressor protein) and HIF-1α depends on the microenvironment. Under severe prolonged hypoxia, dephosphorylated HIF-1α is induced and stabilizes p53. In case of mild hypoxic conditions, HIF-1α is phosphorylated and transactivates various genes, including the angiogenic factor VEGF [36, 82]. In addition to HIF-1α, p53 is stabilized by the ATR and also by decreased expression of MDM2 or by phosphorylation of MDMX E3 ubiquitin ligases. p53 induces apoptosis through intrinsic pathway. Hypoxia-induced p53-dependent apoptosis occurs through inhibition of AKT signalling via *PHLDA3* and *INPP5D* genes. AKT inhibition may increase response to radiotherapy in p53-deficient tumors [46].

TRAF6 (tumor necrosis factor receptor-associated factor 6), a signal transduction molecule, belongs to both the tumor necrosis factor receptor (TNFR) superfamily and the interleukin-1 superfamily. TRAF6 is involved in regulation of IL-1R/TLR family signalling pathway to activate NF-κB. Independent of oxygen TRAF6 regulates the expression of HIF-1α and enhances HIF-1α activity in a proteasome-dependent manner [81]. TRAF6 regulates caspase-8-dependent apoptosis and activating NF-κB. siRNA-mediated silencing of TRAF6 reduced proliferation and enhanced apoptosis of EC109 cells, and levels of NF-κB were simultaneously reduced [55].

Drug-induced ESC cell death is induced by mitochondrial apoptotic pathway. Apoptosis is mediated by BCL family of proteins such as Bax which increase permeability of outer mitochondrial membrane through loss of mitochondrial membrane potential that enhances the release of cytochrome C and Caspase cascade such as caspase-3 and caspase-9. Bax suppress the expression of anti-apoptotic factor Bcl-2. Protocatechuic acid ethyl ester ethyl-3,4-dihydroxybenzoate (EDHB) is an antioxidant and common food additive found in the testa of peanut seeds. The prolyl hydroxylase inhibitor EDHB stabilizes HIF-1α protein by inhibiting proteasomal degradation and increases HIF-1α protein expression, impacting HIF-1α-mediated downstream gene expression. EDHB promotes the expression of NDRG1 (N-myc downstream-regulated gene-1) and BNIP3 a pro-apoptotic protein and promotes apoptosis. HIF-1α induces the expression of NDRG1 and BNIP3 by binding to HRE elements. EDHB also promotes caspase-dependent apoptosis [25].

6.7 HIF-1α in Stemness

Notch, Wnt/β-catenin and Hedgehog pathways play a significant role in proliferation, differentiation and self-renewal of stem cells. These pathways have been associated in the regulation of EC CSCs and are potential therapeutic targets. Although not reported in EC, hypoxia targets Notch, Wnt/β-catenin, Hedgehog, PI3K/mTOR and unfolded protein response (UPR) pathways to regulate EMT and CSC stemness and is stimulated by a number of oncogenes or loss of tumor suppressor genes [91]. WNT10A, an activator of the Wnt/β-catenin pathway, was highly expressed in ESCC tissue which associated with poor outcome. WNT10a-expressing cells showed

enhancement for $CD44^+/CD24^-$ cells, increased self-renewal and invasive and metastatic potential [53]. Inhibition of the PI3K/mTOR pathway or a hypoxic environment leads to activation of autophagy and may also be of attention in EC. Cell surface marker for potentially identifying EC CSCs are ABCG2, $CD44^+/CD24^-$, $CD44^+/ICAM1^+$, $CD44^+/CD133^+$, CD90 or Thy-1. In EC, cancers stem cells which express these markers that might be of prognostic or predictive value [91].

6.8 HIF-1α in Tumor Resistance

Hypoxic condition of tumors is identified to be associated with resistance to radiation, photodynamic therapy and chemotherapy (CRT) in ESCC cancer [78]. Hypoxic environment with the more malignant tumor phenotypes increased metastatic potential, invasiveness and poor survival. Radiation up-regulates expression of HIF-1α via enhancement in oxidative stress and increases the availability of glucose and oxygen in oesophageal cancer cells. Therefore, HIF-1α increases VEGF expression, which guards vascular endothelial cells against cytotoxic effects of radiation. After radiation therapy, recombinant human endostatin could enhance the radiosensitivity of ESCC via the down-regulation of VEGF and HIF-1α expression [105]. Berberine [93] and Ginsenoside Rg3 [17] enhance radiosensitivity of oesophageal squamous cancer cell lines under hypoxic conditions by targeting HIF-1α and VEGF expression. Under hypoxic condition, HIF-1α induces the expression of MDR-1 in gastric cancers [51] and colon cancer [5]. The direct role of HIF-1α in the regulation of MDR gene expression is not discovered in ESCC so far. Metronomic chemotherapy is an effective treatment option described in most cancer types, due to its very low reported toxicity, modest efficacy, low cost and ease of administration. Chemotherapeutic drugs administrations at a lower dose than the MTD (maximal tolerated dose) without prolonged drug-free intervals refer to metronomic chemotherapy. Capecitabine is the most common drug used in metronomic therapy; other drugs include cyclophosphamide and paclitaxel. In oesophagus cancer, metronomic chemotherapy is under clinical trials, and it can be considered to be a therapeutic option [61].

6.9 Conclusion and Future Directions

Oesophageal cancers are one of the least studied cancers and are highly aggressive in nature with poor survival rate. Its mortality rate ranks sixth among all cancers. The impact of hypoxia in most of the cellular metabolic pathways is still not clearly established. Nonetheless, hypoxia drives malignant progression in oesophagus cancers, resulting in poorer survival through increased metastatic potential and resistance to therapy. Hence it is warranted to understand how hypoxia alters cellular metabolism in order to target these pathways, thereby killing malignant cells. High levels of MIB-1 and NF-κB and low levels of HER2 and ER (oestrogen receptor) were good prognostic factors following definitive curative

chemoradiation therapy for ESCC [77]. No efficient or specific HIF-1α inhibitors/drugs have completed clinical trials for the treatment of ESCC. Since HIF-1α has a significant role in oesophagus cancer, targeting hypoxia may become an effective approach in preventing or reducing metastasis and resistance to therapies in oesophagus cancer.

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A Potential Role of Hypoxia-Inducible Factor-1 (HIF-1) in Esophageal Cancer

7

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Abstract

Hypoxia-inducible factor-1 (HIF-1) is a regulatory protein, mainly responsible for maintaining oxygen homeostasis in response to reduced oxygen concentration in cells and tissues. The protein is a heterodimer which consists of subunit HIF-α and HIF-β. It is an important transcription factor involved in the transcriptional regulation of many genes related to embryonic development, metabolism, cell proliferation, angiogenesis, metastasis, and response to radiation therapy, making it an important regulator in most cancer therapies. Esophageal cancer (EC) is not a well-studied and a poorly understood cancer. It is highly aggressive in nature with poor survival rate. Its mortality rate ranks sixth among all cancers. Overexpression studies of HIF-1 in correlation with other target gene expressions revealed its role in both upregulation and downregulation of certain molecules in particular cancer types. Considering all parameters, HIF-1 inhibitor could present a potential approach to cancer therapy. This chapter summarizes the potential roles of HIF-1α in cell cycle, proliferation, apoptosis, and metastasis and future perspectives in targeting esophageal cancer for developing novel anticancer therapies.

Keywords

Squamous cell carcinoma · Adenocarcinoma · Hypoxia

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7.1 Introduction

Hypoxia-inducible factor-1 (HIF-1) as the name suggests is a key regulator of oxygen homeostasis in cells and tissues in case of lower oxygen concentration. It is an important transcription factor involved in the transcriptional regulation of many genes related to embryonic development, metabolism, cell proliferation, angiogenesis, metastasis, and response to radiation therapy, making it an important regulator in most cancer therapies [1]. In this chapter, we can study and understand the potential roles of HIF-1α in cell proliferation, cell cycle, metastasis, and apoptosis and future perspectives in targeting esophageal cancer for developing novel anticancer therapies.

HIFs are made up of heterodimers which contain one α-subunit of 120 kDa (HIF-α) and one 94 kDa β-subunit (HIF-β). HIF falls in the basic helix-loop-helix (bHLH) transcription factors of PAS (PER-ARNT-SIM) family. HIF-α has three isoforms that are named as HIF-1α, HIF-2α, and HIF-3α. HIF-1α is the extensively studied one of all the three isoforms. These isoforms are formed due to alternative splicing, and these subunits are encoded by discrete loci on gene. HIF-1α is discovered after the recognition of sequence 5′-RCGTG-3′, a hypoxia-response element (HRE) present in 3′ enhancer of the erythropoietin gene (EPO): it induces transcription process in response to hypoxic condition [2, 3]. This transcriptional factor targets more than 60 genes where HIF-1α binds to cis-acting elements present in the promoter region. HIF-1α binds to DNA in heterodimeric form constituting oxygen-dependent subunit-α (HIF-α) and an oxygen-independent, constitutively expressed subunit-β (HIF-β). The HIF-1α subunit has four distinct domains from N- to C-terminal of the protein: a DNA-binding and dimerization domain or bHLH domain, followed by a dimerization and target gene specificity domain or PAS domain and an oxygen-dependent degradation domain or ODD domain which utilizes ubiquitin-proteasome pathway for degradation [4]. Two transactivation domains or TAD are present in the C-terminal end [5] (Fig. 7.1).

The HIF-2α and HIF-3α also contain bHLH-PAS domain and ODD motifs [6], can dimerize with ARNT/HIF-1β like HIF-1α, and bind with hypoxia-response elements (HREs) in vitro. Multiple splice variants are formed by HIF-3α, in that the best-studied one is an inhibitory domain PAS protein (IPAS). IPAS is formed by truncated HIF-3α protein which does not have a transactivation domain. IPAS is a dominant negative; it binds to HIF-1α and prevents to construct HIF-1α/ARNT hetero complex. In vivo function of HIF-3α in the hypoxia-induced gene regulation is not understood properly [7].

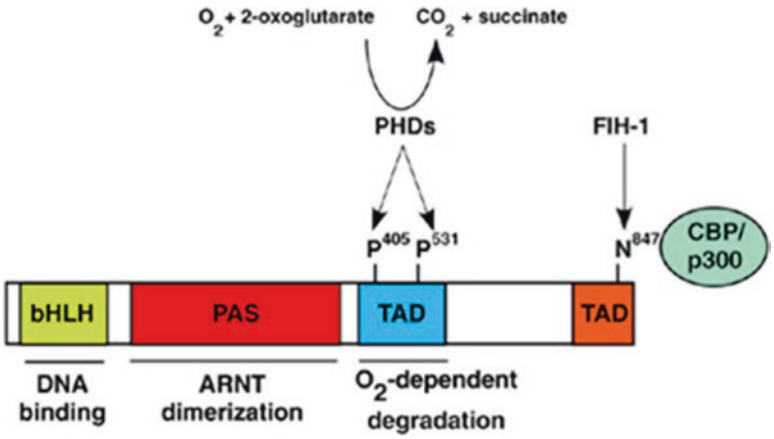

Fig. 7.1 Schematic of HIF-1α*:* the HIF-1α subunit has four distinct domains from N- to C-terminal of the protein: a DNA-binding and dimerization domain (bHLH), followed by a dimerization and target gene specificity domain or PAS domain and an oxygen-dependent degradation domain (ODD).Two transactivation domains (TAD) at C-terminal end

7.2 Regulation and Stability of HIF

DNA-binding and transcriptional activities are mainly reliant on the levels of HIF-1α [8]. Expression of HIF-1α in hypoxic microenvironments is a critical step and occurs through increased mRNA expression, protein stabilization, and nuclear localization. When oxygen level in the cells is poor and insufficient, proteasomal degradation gets ceased, thereby stabilizing the HIF-1α protein, and is accumulated by different post-translational modifications, i.e., acetylation, phosphorylation, and hydroxylation.

In case of normal oxygen tension, HIF-1α remains low because of its continuous proteasomal degradation by von Hippel-Lindau (VHL) tumor suppressor via pVHL-mediated ubiquitin-proteasome pathway [9]. pVHL provides a substrate binding site for ubiquitylation of HIF-1α by E3 ubiquitin ligase complex. HIF-1α/E3, ubiquitin-proteasomal degradation is mediated and depends on oxygen concentration. The process of degradation is very rapid during normoxia and the half-life is predicted lower than 5 min. In state of cell's normal oxygen condition, HIF degradation is maintained by proteins containing-prolyl-4-hydroxylase domain (PHD). It mediates the binding of pVHL with HIF-1α by hydroxylation of conserved proline (402 and 564) and the acetylation of lysine residue in the ODD of HIF-α ([10, 11]. The PHD enzymes show oxygen-dependent hydroxylation activity. There are three non-heme-containing PHD (iron-dependent) enzymes that help progress the hydroxylation of HIF-1α. When the levels of oxygen decrease, hydroxylation of proline also decreases and the interaction of VHL with HIF is dissociated. Inactivation of VHL leads to increased target expression and stabilization of HIF irrespective of oxygen concentrations in the cells [12].

Hypoxia conditions increase the transcription level of HIF-1 resulting in enhanced expression. In poor oxygen concentration, the HIF-1α subunit translocated to the nucleus where HIF-1α heterodimerizes with ARNT/HIF-β and binds to hypoxia-response elements (HREs) located on the regulatory regions of HIF target genes. Now, HIF-1α subunit turns stable by interacting with its co-activators such as p300/CBP and increases its transcriptional ability. Also, this HIF-p300/CBP interaction is oxygen concentration dependent and regulated by factor inhibiting HIF-1 (FIH-1). FIH-1 belongs to the superfamily of 2-oxoglutarate and Fe^{+2}-dependent oxygenase. In normal concentrations of oxygen, FIH prevents binding of p300/CBP by hydroxylation of asparagine residues present in HIF-α C-terminal transactivation domain (CTAD) [13], so that the HIF-1α/HIF-β heterodimer cannot bind to HREs. When oxygen concentration goes low (hypoxic conditions), disruption occurs in asparaginyl hydroxylation leading to increased interaction of HIF-1α and CBP/p300. In this way, both stabilization of HIFα and activation of CTAD are requisites to activation of transcriptional activity of HIF [14] and are regulated by FIH hydroxylation. Thus for a number of hypoxia-inducible gene, HIF-1α is a master regulator under hypoxic conditions.

Recently it has been observed that under hypoxia, mitochondrial reactive oxygen species (ROS) play a critical role to regulate the HIF protein levels. The hypoxic condition in mitochondria leads to inhibition of electron transport chain due to genetic and chemical changes and produces ROS resulting in decreased stability of HIF-1α [15–17].

7.3 Esophageal Cancer and Its Risk Factors

Esophageal cancer (EC) is not a well-studied and a poorly understood cancer. It is highly aggressive in nature with poor survival rate. Its mortality rate ranks sixth among all cancers. Esophageal cancer commonly develops inside the esophagus lining and progress outward. Esophageal cancer is not easily identified, but, as the tumor progresses, it can block the esophagus passage, which makes swallowing of food and liquid difficult and painful. Squamous cell carcinoma and adenocarcinoma are the most common types of esophageal cancers.

Squamous cell carcinoma is the most common type and presents the 95% of all esophageal cancers worldwide. A thin layer of flat squamous cells normally makes the lining of the esophagus and can develop squamous cell carcinoma at any portion, but it is most common in the middle portion.

The second most common type of esophageal cancer is adenocarcinoma. The incidences for adenocarcinoma have become almost equal as squamous cell carcinoma. Adenocarcinoma develops in glandular tissues found in the lower part of the esophagus near the opening of the stomach as a symptom of Barrett's esophagus. It is a precancerous stage; the chronic acid reflux or gastroesophageal reflux disease (GERD) induces the transformation in squamous cells present in the lower esophagus into glandular cells. Patients having Barrett's esophagus are usually 30–40 times more susceptible toward adenocarcinoma of the esophagus than the general population.

7.4 Risk Factors

Though the exact cause to develop esophageal cancer is not known, genetic factors are found to play a crucial role. General risk factors include alcohol consumption, smoking, a habit of drinking hot tea, and poor oral health, and red meat consumption, inadequate consumption of fresh fruit and vegetables, and low socio-economic condition have been linked with high risk of esophageal squamous cell carcinoma. GERD also might create an environment wherein the cells are transformed with altered genetic and metabolic functions which may further develop in cancer.

7.5 Cell Proliferation and HIF-1

In hypoxic microenvironment, cells may adapt to certain metabolic changes and cellular signaling. There are several growth factors and signaling pathways which are induced during hypoxia to stimulate proliferation cell and its growth. Several cell growth inducers like growth factors, such as cytokines, mitogen-activated protein kinases, insulin-like growth factor-3 (IGF3), and transforming growth factor-α (TGF-α), can stimulate HIF-1 expression by binding to their corresponding receptors and increase the HIF activity along with Ras and Myc oncogenes [18, 19]. Cytokines and growth factors can activate MAP kinase and PI3K signaling pathways, which not only to promote proliferation of cells but also contributes to increase the HIF-1 activity. This increased activity of HIF-1 improves transcriptional activity of HIF-1 target genes which helps cell proliferation [20]. When HIF-1α is phosphorylated by mitogen-activated protein kinases such as p42/p44, transcription activity HIF-1α target genes are increased which further helps in cell proliferation. Generally HIFs are known to play a devoted role in both hypoxia and normoxia by regulating the expression of the genes involved in glucose metabolism like glycolysis, lactate and pyruvate metabolism, and glucose transport. Studies with transformed cell lines showed the regulatory role of HIF-1 in the regulation of mitochondrial respiration process. In *von Hippel-Lindau tumor suppressor (VHL)* gene-deficient renal cancer cells (RCC), HIF-1 also regulates mitochondrial oxygen consumption in negative manner which shows that HIF plays a key role in cancer cell metabolic processes [21].Hypoxia signaling pathways may also be affected by cross talk from other cellular metabolisms. Apart from HIF-1, other genes such as nitric oxide synthase and heme oxygenase-1 can also be induced during hypoxic conditions producing nitric oxide (NO) and carbon monoxide(CO). Generally NO and CO can bind directly to HIF-1 or indirectly through c GMP or guanylate cyclase-dependent protein kinases [22]. Binding of NO and CO to HIF-1 inhibits the genes induced by hypoxia by regulating the dimerization or DNA-binding activity of HIF-1 showing any effect on HIF-1 protein levels [23]. This shows that both NO and CO can downregulate HIF-1 activity. In few cancers like human oral squamous cell carcinoma, when the levels of nitric oxide synthase and its isoforms are increased, the protein stability of HIF-1 is highly improved. Mutations in genes such as p53, PTEN, and pVHL which are tumor suppressor and in few oncogenes also lead to induction and amplification of the HIF system [24].

7.6 Cell Cycle and HIF-1

To maintain the cell viability, the processes of cell growth and cell cycle arrest are two important phenomenons. In response to a wide range of oxygen concentrations, cells can respond differentially through the changes in both their metabolic processes and growth kinetics. Hypoxic conditions cause cell cycle arrest by inactivating nucleotide biosynthesis enzymes which leads to inhibition of DNA replication and alterations in cell proliferation and finally causes the programmed cell death (apoptosis). Hypoxia-induced apoptotic pathways were triggered by any mutations in the p53. HIF-1 regulates the activity of both p53 and p21. Overexpression of HIF-1 leads to the aberrations in p53 gene which leads its accumulations in tumors. This p53 in turn induces the Bax and Bak genes and proteins which cause the cytochrome C-mediated apoptosis ([25, 26, 27]). Also, mutations in cell cycle-regulating genes also play a key role in the progression of carcinogenesis, and some of them may act as prognostic factors in esophageal cancer. Altered expressions of p53, p16, pRB, and cyclin D1 proteins were observed in esophageal cancers.

7.7 Metastasis and HIF

Metastasis is one of the critical steps in progressions of tumor, and it is one of key causes of difficulty in treatments resulting in deaths caused by human cancers. HIF controls the expression of some factors that have both anti-metastatic and metastatic factors in cell adhesion, invasion, migration, and angiogenesis. HIF regulates cell adhesion proteins like E-cadherin and vascular endothelial growth factor (VEGF) during angiogenesis [28]. In human esophageal squamous cell carcinoma, VEGF-C expression was correlated with the tumor invasion, tumor stage, venous invasion, lymphatic invasion, and lymph node metastasis [29]. Transfection and inhibitory experiments have confirmed the importance of VEGF in angiogenesis and tumor growth. HIF-1 may be a potential target for angiogenic therapies to treat esophageal squamous cell carcinoma. HIF has a great potential to directly regulate the over activation of pro-angiogenic and metastatic factors such as VEGF and its receptors (FLT-1 and FLK-1), PDGF-B, PAI-1, and angiopoietins ANG-1 and ANG-2. Kitadai et al. showed that VEGF-C expression in esophageal squamous cell carcinoma was highly associated with tumor development and invasion, venous invasion, lymphatic and lymph node invasion, and metastasis. Inhibitory experiments of VEGF proved the importance of VEGF in angiogenesis and metastasis. HIF-1 is an important angiogenic drug target in esophageal squamous cell carcinoma [29].

7.8 Hypoxia-Induced Apoptosis

Hypoxia-induced apoptosis is a general process in solid tumors. This is essential for the cells to remain untransformed. Depending on the available oxygen concentration and nutrients, cells may choose to survive by adapting to the hypoxic

environment or undergoing apoptosis. Cells, which are adapted to repeated hypoxic conditions, can easily gain resistance to hypoxia-induced apoptosis and transform into aggressive tumor phenotypes with low or no response to the treatment. HIF-1 plays a crucial role in balancing cell proliferation and apoptosis, thereby activating intrinsic or extrinsic apoptotic pathways and preventing accumulation of cells with hypoxia-induced mutations. HIF-1α can induce the genes involved in apoptosis such as p53 and Bcl-2 family members. HIF-1 can cause apoptosis by two different mechanisms: first, by increasing the stability of the products of the p53 gene, and, second, by activating pro-apoptotic BNIP3 and NIX proteins in the peri-necrotic regions of tumors. As earlier mentioned, in hypoxic stress or during DNA damage, p53 induces apoptosis through apoptotic protein Bax. This can be achieved by direct binding of HIF-1α to MDM2 which is p53 ubiquitin ligase in both in vitro and in-vivo [30, 31].

7.9 Diagnosis of Esophageal Cancer

Screening of esophageal cancer-suspected cases appears to provide an early assessment of responsiveness to preoperative chemotherapy. It can aid to determine the extent or the stage of cancer. Further tests can help in the treatment and recovery of the patients. It includes esophagram, endoscopy, biopsy CT scan, and PET scan. Esophagram is done by letting the patient swallow liquid containing barium to coat the inner walls of the esophagus, followed by X-ray imaging. In Esophagram it is easy to identify the early formation of cancer. Endoscopy includes upper endoscopy, endoscopic ultrasound, bronchoscopy, thoracoscopy, laparoscopy, etc.; this helps in identifying the spread of the tumor to nearby lymph nodes, the trachea, lungs, the stomach, and other body parts. The extent of spread of a tumor to lymph nodes and other nearby organs can be assessed by computed tomography (CT) scans. Positron emission tomography (PET) scanning helps in the assessment of the presence of distant metastatic lesions. It provides accurate information regarding the spread of the tumor, which is helpful in further radiation therapy. A biopsy is most often done after endoscopy. It involves the removal of the small piece of tissue from an abnormal region. It shows the abnormal pattern of tissue or cells seen under the microscope.

7.10 Treatment Strategies

Initial treatment of esophageal cancer depends on various factors including patient's age, stage of cancer, and overall health of the patient. Surgical endoscopic resection alone primarily offers a potential cure. Effective palliation can be achieved with surgical resection in combination with chemotherapy, radiation therapy, stents, and photodynamic therapy. Preoperative chemotherapy and radiotherapy offer survival benefits in early cancer treatments. In some cases, preoperative chemoradiotherapy provides limited survival benefits, but surgical resection along with

chemoradiotherapy may limit the reoccurrence of esophageal cancer and metastasis which may provide a better survival outcome. Clinical trials in the usage of anti-angiogenic factors, stem cell therapy, and gene therapy including esophageal-specific gene silencing methods are in progress.

7.11 Conclusion

HIF-1 plays a prominent role in inducing directly or indirectly many target genes involved in physiological functions of a cell. The study on the regulation of HIF-1 is very essential in the treatment of specific cancers. Tumor cells are more hypoxic and express higher level of HIF-1α as compared to normal cells, making it a potential molecule for therapeutic approach. Overexpression of HIF-1α observed in biopsies of brain, ovarian, cervical, breast, oropharyngeal and esophageal cancer is correlated with treatment failure and mortality. Overexpression studies of HIF-1 in correlation with other target gene expressions revealed its role in both upregulation and downregulation of certain molecules in particular cancer types. Considering all parameters, HIF-1 inhibitor could present a promising approach to cancer therapy. Extensive research and understanding of signaling mechanisms and cross talks are to be considered in treating esophageal cancer cell.

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Genistein and Its Role in Regulation of AP-1 in Colorectal Cancer

8

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Abstract

Colorectal cancer (CRC) is a prominent source of cancer-related deaths across the world. AP-1 is involved in CRC growth and metastasis. AP-1 is a transcription factor that regulates many oncogenic transduction pathways. In the current chapter, we discuss the importance of AP-1 on CRC growth and metastasis. Additionally, we discuss the mechanism of genistein, a tyrosine kinase inhibitor, and its effect on CRC treatment.

Keywords

Genistein · Colorectal cancer (CRC) · Activator protein-1 (AP-1)

8.1 Introduction

The third most leading cause of cancer-associated fatalities is CRC in developed countries [7]. Higher incidences of CRC have been seen in developing countries, most likely due to changes in the environment and dietary habits. CRC patients with initial stages of the disease have more than 90% of 5-year survival rate. On the other hand, patients with advanced stages of metastatic CRC have a survival rate of less than 20% [8]. Therefore, the advancement of tumor metastasis is a key determinant of patient prognosis and survival. The development of such therapies and drugs that

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could effectively inhibit CRC metastasis could serve as an important discovery in cancer treatment. Incidences of CRC are highly associated with the country and region. For instance, Asians, who are known to historically consume a traditional diet that is rich in soy, tend to have lower rates of clinical CRC incidences in general [9]. On the other hand, Asians, who migrate to Western countries and adopt the Western diet, tend to have higher incidences of CRC [10].

CRC progression involves multiple stages of genetic variation of cancer promoter and suppressor genes. These variations lead to deregulation of important molecular pathways [11]. Alteration of the anti-oncogene, adenomatous polyposis coli (APC), results in the deregulation of the APC/β-catenin/T-cell factor 4 (Tcf-4) signaling, which is observed in the initial stages of a large number of CRC patients [12]. Several investigations have identified the potential downstream target proteins of the APC/β-catenin/Tcf-4 signaling cascade, which are known to be very significant in regulating the transformation of non-malignant colon and rectal cells into malignant colorectal epithelium. These genes belong to the activator protein-1 (AP-1) transcription factor family, namely, fra-1 and c-jun [13]. Several investigations reveal that diet and lifestyle influence the occurrence of CRC and the importance of soy (genistein) in controlling such transcription factors.

8.2 Genistein

Genistein is an isoflavone, a type of phytoestrogen that is abundantly found in soy. As a protein tyrosine kinase inhibitor, genistein is identified as an inhibitor of angiogenesis, cell proliferation, apoptosis, and uncontrolled tumor growth by regulating the vital components of signal transduction cascades like Akt [1], EGFR [2], and MAPK [3], as well as important transcription factors like AP-1. Akt is an important downstream controller of phosphatidylinositide-3-kinase (PI3K). Abnormal expression of Akt is associated with the progression and advancement of various human malignancies, including CRC [4]. Earlier investigations have revealed that genistein is responsible in inhibiting CRC cell migration and reducing Akt phosphorylation, signifying that Akt pathway suppression could be a key regulator associated with the anti-cancerous properties of genistein in colorectal malignancies [5]. Moreover, additional tumor-associated factors, like EGFR [6], have been known to be involved in the tumor progression and are negatively regulated by genistein in CRC cell lines. Further, genistein also sensitizes tumor cell lines toward chemotherapy.

8.3 Activator Protein-1

The transcription factor AP-1 is a dimeric complex, which can form different combinations of homo- and heterodimers. AP-1 comprises of dimers of the Fos (FosB, c-Fos, Fra-1, Fra-2) and Jun (c-Jun, JunB, JunD) subfamilies, which harbors a standard leucine zipper (bZIP) domain and is capable of forming duplexes between themselves as well as with other bZIP genes. The capability of forming complexes

allows AP-1 transcription factors to aim a larger variation of DNA-binding domains and modulate oncogenes that engage in cellular cycle and inflammation, such as cyclin D1, p53, etc. and COX-2, respectively. AP-1 is involved in basal protein expression and also in the initial cellular response toward a variety of pathological and biological stimuli comprising of oncogenic signals. Activation of AP-1 is induced by the cis-elements in promoter regions of AP-1-encoding proteins, succeeded by a spontaneous phosphorylation of AP-1 genes essentially via mitogen-stimulated protein kinase (MAPK) signaling. Growth factors trigger pro-inflammatory cytokines, extracellular signal regulated kinase (ERK), as well as genotoxic stress-mediated p38 MAPKs and JNKs, whereas oncogenes such as Src and Ras stimulate the ERK or JNK signaling cascade [14].

In CRC patients, AP-1 aids in regulating transcriptional stimulation of redox-modulating enzymes in regions with solid tumors [15]. Investigations have revealed that AP-1 activity is positively associated with the expressions of VEGF, EGFR, and COX-2, which are its downstream targets [16] (Fig. 8.1). Elevated

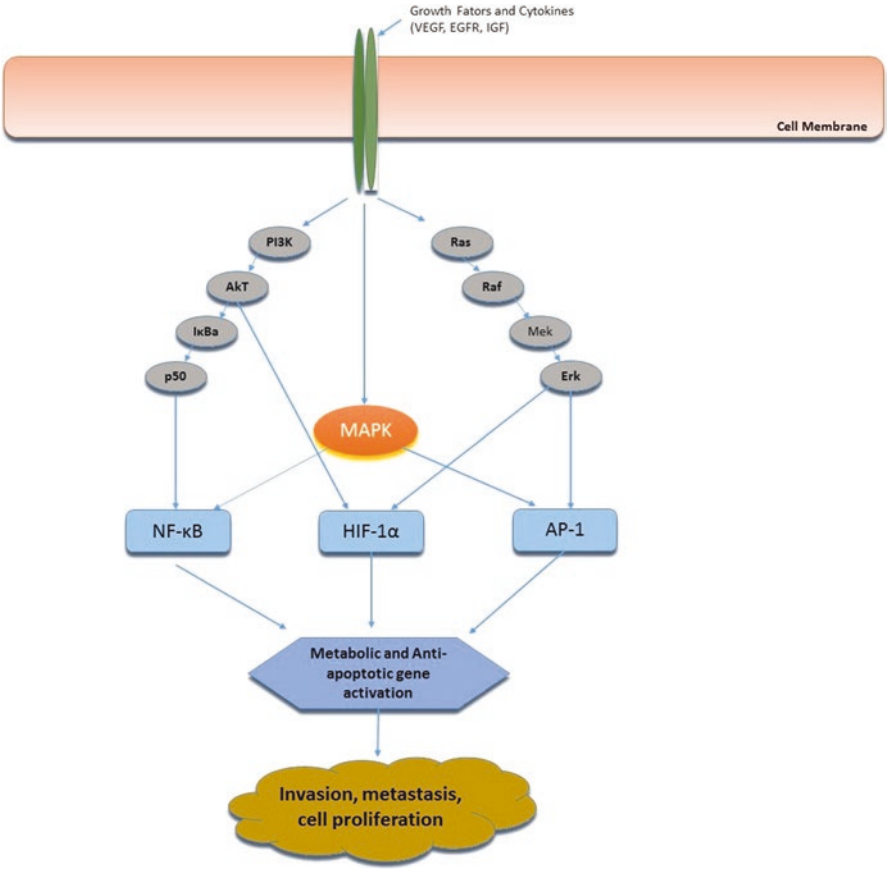

Fig. 8.1 Role of transcription factors in regulation of growth and metastasis

concentrations of bile acids increase the expression levels of AP-1 via ERK and protein kinase C (PKC) pathway and leads to COX-2 activation, which regulates invasion, anti-apoptosis, and motility [17]. A series of pathways in CRC cell lines that are regulated via AP-1 activity include the stimulation of Wnt/β-catenin signaling pathway that regulates the c-jun and fra-1 proteins [13], as well as the Ras-GTPases signaling pathways. Gain-of-function alterations in the K-ras protein activates the ERK and JNK signaling cascade, initiates AP-1 transcription factor, and is even known to play a critical role in the progression of CRC [18]. AP-1 acts like a homeostasis switch that controls cell cycle. AP-1 is capable of upregulating cyclin D1 and also acts as an anti-apoptotic factor through negative regulation of p53 [19] in addition to stimulation of the anti-apoptotic Bcl proteins. Furthermore, AP-1 can also motivate cell death via inducing Fas ligand and therefore initiate apoptosis depending upon the stimulus. In CRC cell lines, AP-1 facilitates an anti-apoptotic reaction to the hypoxic situations that are often discovered in the solid tumor microenvironment and therefore aids in the resistance toward radio- and chemotherapy [19]. Besides, AP-1 regulates the activity levels of VEGF and MMPs of malignant cells [17].

AP-1 activates epithelial-to-mesenchymal transition (EMT) signaling pathways, in addition to the EGFR and mTOR cascades, revealing that AP-1 is an important target to regulate EMT as well as CRC progression. Various inhibitors that target these pathways and upstream EMT signaling, which is elevated by receptor and non-receptor tyrosine kinases like Src, EGFR, integrins/focal adhesion kinase (FAK), VEGF-R, IGF-R, and G-protein-coupled receptors (GPCR), are undergoing preclinical and clinical investigations for CRC treatment [20]. The invention of without adverse side effects could also offer further options in developing novel treatment strategies to cure CRC.

8.4 Mechanism

Genistein has been known to exert anti-proliferative properties via targeting NF-κB as well as AKT pathways. Genistein has been linked with the initiation of cell death through modulating the AKT/GSK3β/FOXO3a/androgen receptor signaling pathway [21]. Moreover, stimulation of cell death via genistein has been observed in CRC cell lines through induction of the BAX and p21WAF1 pro-apoptotic activity [22]. In vitro studies have revealed that genistein acts in conjunction with chemotherapy to inhibit cancer cell progression and metastasis. CRC cell lines reveal enhanced apoptosis upon addition of genistein to fluoropyrimidine 5-FU [23]. Genistein is also known to sensitize different types of cancer cell lines toward apoptosis once added to the chemotherapeutic drugs such as gemcitabine, cisplatin, docetaxel, and doxorubicin [24]. Genistein acts simultaneously with oxaliplatin in order to inhibit cell amplification in PC cell lines [25] and decreases the size of PC tumor in mice. These investigations suggest that genistein might improve response rates in CRC patients as well since oxaliplatin and 5-FU are the standard chemotherapy drugs that are utilized in CRC treatment regimen.

Genistein exhibits several regulatory mechanisms such as inhibiting Wnt pathways, inhibiting NF-κB pathways, inhibiting EGFR pathways, as well as influencing other signaling pathways that are operative in CRC and signaling via estrogen receptor. Due to its extremely low toxicity, genistein might be therapeutically valuable for preventing primary CRC in patients with elevated risk or even as a secondary CRC preventive factor in order to lessen the risk of deterioration following therapeutic surgical resection as well as adjuvant analysis. This methodology is reinforced by epidemiologic results, which show that prolonged exposure to soy and its derivatives decreases the occurrence of CRC.

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Role and Regulation of Transcriptional Factors in Gastric Cancer

9

Nageswara Rao Reddy Neelapu

Abstract

Gastric cancer is the second major cancer diagnosed worldwide. Cytokines, chemokines, metalloproteinases, prostaglandins, and reactive oxygen nitrogen species induce, amplify, and sustain inflammation (in the lining of the stomach) in the host. Signalling pathways like β-catenin, NF-κB, etc. bring about changes in the genetic material leading to diversification of genetic material. Genetic diversification in oncogenes and tumour suppressor genes leads to gastric cancer. Transcriptional factors' role in apoptosis, cell cycle, cell proliferation, and metastasis (adhesion, invasion, migration, angiogenesis) is well known and established. The role and regulation of transcriptional factors in relation to gastric cancer was reviewed.

Keywords

Apoptosis · Cell cycle · Cell proliferation · Gastric cancer · Metastasis · Transcription factors

9.1 Introduction

Helicobacter pylori in a coordinated cascade manner colonizes the host. Neelapu et al. [81] reviewed the mechanism of pathogenesis in *H. pylori*. *H. pylori* adapt to the harsh environment (acidic) initiating colonization in the stomach with the help of chemoreceptor TlpB and enzyme urease. The pH of the stomach is perceived by

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H. pylori and moves towards the less acidic region of the stomach with the help of chemoreceptor TlpB [25]. Urease produced by *H. pylori* neutralizes its periplasm and cytoplasm [134]. The gastric mucosal barrier (GMB) prevents the passage of the *H. pylori* and further colonization [4]. Pathogen uses toxin VacA, cytokines, and gastrin to weaken GMB by slackening/disrupting the mucous layer or modifying the glycoproteins in mucous [81]. Once the GMB is weakened, adhesion molecules are used by *H. pylori* to attach to the lining of the stomach [39, 71, 76, 93]. Further, pathogen institutes gastritis in the host by injecting toxins like CagA, peptidoglycan, and VacA via T4SS system. CagA is known to alter host cell expression; brings about elongation of the cell, loss of cell proliferation, and cell polarity; decreases acid secretion; and degrades cell–cell junctions [140]. Cytokines, chemokines, metalloproteinases, prostaglandin E2, and reactive oxygen nitrogen species induce, amplify, and sustain the inflammation in the host [81]. Sustained inflammation activates G cells, and the hormone gastrin is secreted, "in turn stimulating loads of acid damaging duodenum leading to a condition known as ulcers" [109]. DNA of the *H. pylori* genome is damaged as it is unprotected from acidic environment, peristaltic movement, harmful effects of oxidative stress, and phagocytes [86]. For successful infection, *H. pylori* uses the acquired DNA repair mechanism [74]. The changes in the host cell result in double-stranded breaks (DBS) and sometimes defective mitotic checkpoints (DMC). Directly or indirectly β-catenin and NF-κB signalling pathways induce DBS and DMC and deregulate HR pathway of DSB repair and enzymes of DNA repair heading towards chromosomal instability (CI) and microsatellite instability (MSI) [12]. Genetic material of the host is randomly diversified due to MSI and CI; subsequently oncogenes are turned on, and tumour suppressor genes are turned off leading to gastric cancer. Several studies have identified tumour suppressor genes like ASPP2, GKN1, GKN2, p53, TFF1, TFF2, TFF3, and RUNX3 [81].

9.2 Genetic Alterations, Epigenetic Alterations, and Gastric Cancer

Genetic alterations (in p53, KRAS, PIK3CA, ARID1A, MLL3, C-MET, ERBB4, CD44) and epigenetic alterations (due to methylation of CpG islands, microRNAs, non-coding RNAs, nucleosome positioning posttranslational modifications of histones) are involved in gastric cancer [99]. Genetic alterations such as mutations or amplifications and epigenetic alterations are responsible for acquiring oncogenes function and forfeiture of tumour suppressor genes function. Epigenetic alterations directly or indirectly affect transcription mechanism in gastric cancer. Evidences are increasing in favour of epigenetic alterations leading to either transcriptional activation or repression, and epigenetic alterations are now considered as an important hallmark of cancer cells. Epigenetic alterations were known to effect apoptosis, cell adherence and cell cycle, cell growth and differentiation, DNA repair, invasion and migration, transcriptional regulation, Ras pathway, retinoic acid pathway, STAT pathway, Wnt pathway, and others in gastric cancer till date [99].

9.3 Role of Transcriptional Factors in Cell Proliferation

Cell proliferation is described as "the balance between cell divisions and cell loss through cell death or differentiation…". The cell is said to be proliferative, "…if an increase in the number of cells…" was witnessed in any tissue as in case of tumours. Increased cell proliferation was observed in several cancers like breast, gastric, colon, stomach, etc. Transcription factors like BTF3, GATA6, GKN1, NF-κB, Sp1, and STAT3 play a significant role in proliferation of gastric cancer cell (Table 9.1, Figs. 9.1 and 9.2).

9.3.1 NF-κB, Cell Proliferation, and Gastric Cancer

Peptidoglycan of *H. pylori* enters the host cell and stimulates intracellular pathogen receptor Nod1, to signal and activate transcription factor NF-κB. Keates et al. [51] observed activated NF-κB in epithelial cells of gastric biopsy that were infected with *H. pylori*. NF-κB is a dimer with a motif related to the nucleotide sequence of the κB site (REL homology domain- RHD). There are two classes of REL homology domains – REL proteins and NF-κB. REL proteins (A, B, C) contain RHD at amino-terminal and transcription-modulating domain at their carboxy terminal. RHD at amino-terminal is responsible for DNA binding and dimerization. NF-κB (β1 and β2) also contain RHD at the amino-terminal and ANKYRIN REPEATS at their carboxy terminal [23]. NF-κB is essential to stimulate innate and adaptive immune responses against pathogens [81]. Cancer and chronic inflammation are due to constitutive and sustained expression of NF-κB [33]. Constitutively activated NF-κB transcription helps in proliferation of cancer cell, prevents apoptosis, and increases tumour's angiogenic and metastatic potential [48].

NF-κB is activated in two ways; the first regulatory pathway is turned on as result of microbial infections, proinflammatory cytokines, and viral infections activating I B kinase (IKK) complex. Two serines which are conserved, present in the I B

Table 9.1 Transcriptional factors involved in regulation of cell proliferation, cell cycle, metastasis, and apoptosis

S. No	Transcription factor	Cell proliferation	Cell cycle	Metastasis				Apoptosis
				Adhesion	Invasion	Migration	Angiogenesis	
1	NF-κB	Yes	Yes	Yes	Yes	Yes	Yes	Yes
2	STAT3	Yes	Yes	No	No	No	Yes	Yes
3	BTF-3	Yes	Yes	Yes	No	No	No	Yes
4	Sp1	Yes	No	No	Yes	No	Yes	Yes
5	GKN1	Yes	No	No	No	No	No	Yes
6	GATA	Yes	Yes	No	Yes	Yes	No	Yes
7	FOXM1	No	Yes	No	Yes	Yes	Yes	Yes
8	RUNX3	No	No	No	No	No	No	Yes
9	SOX2	No	Yes	No	No	No	No	Yes

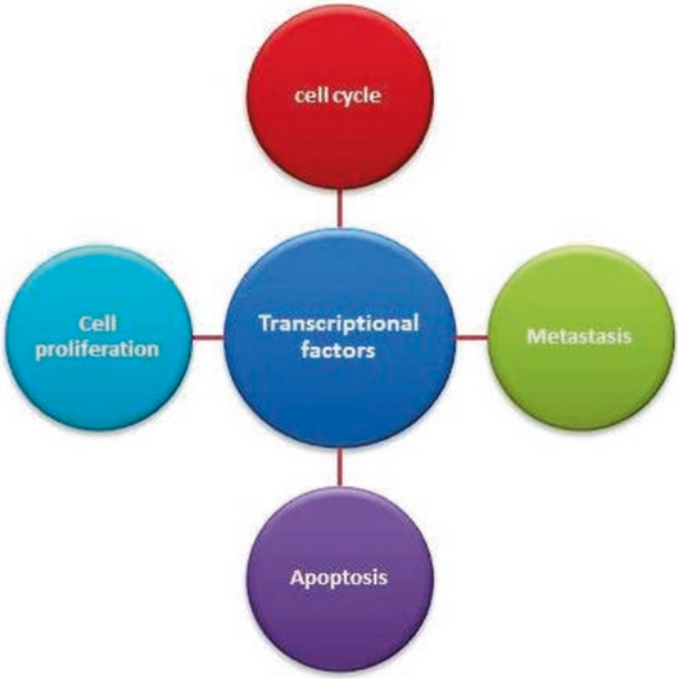

Fig. 9.1 Transcriptional factors regulating various stages of gastric cancer

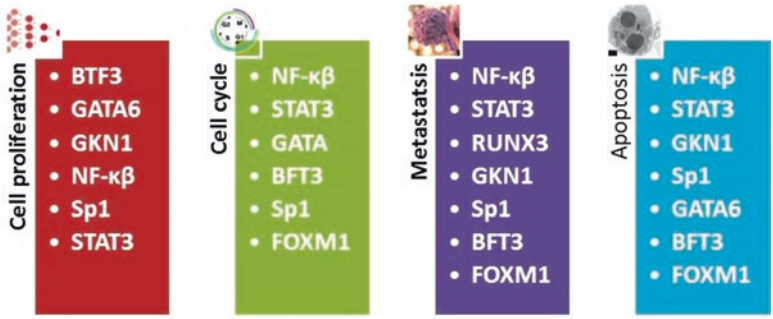

Fig. 9.2 Transcriptional factors involved in regulation of cell proliferation, cell cycle, metastasis, and apoptosis

N-terminal regulatory domain, are phosphorylated by IKK targeting I B for ubiquitin-dependent degradation translocating the released NF-κB dimers to the nucleus [24, 47, 62]. The second pathway activates NF-κB2, by dimerizing with RELB5, where members of tumour necrosis factor (TNF) cytokine family trigger this process. TNF selectively activates IKK and NIK, inducing phosphorylation-dependent

proteolytic removal of the I B like C-terminal domain of NF-κB2. This allows RELB–p52 dimers to translocate to the nucleus [110, 115]. NF-κB is phosphorylated in the nucleus to transcriptionally activate several genes with broad functional categories contributing to tumorigenesis. Immunoregulatory and inflammatory genes, antiapoptotic genes, cell proliferation genes, and genes that encode negative regulators of NF-κB are the genes activated transcriptionally [48]. NF-κB activates genes such as interleukin (IL)-2, granulocyte–macrophage colony-stimulating factor (GMCSF), and CD40 ligand (CD40L)-stimulating proliferation of cells [48].

Li et al. [64] demonstrated constitutive activation of transcriptional factor NF-κB leading to proliferation of gastric cell lines. Inhibitory activity of pyrrolidine dithiocarbamate (PDTC) on NF-κB was measured by MTT method. MTT assay measured inhibition of NF-κB by pyrrolidine dithiocarbamate (PDTC) and proliferation of gastric cancer cell lines [64]. PDTC was able to inhibit the activity of NF-κB and proliferation of gastric cell lines. Ishikawa et al. [41] knocked out NF-κB1/p100 in mice to constitutively express NF-κB2 and established that NF-κB can stimulate gastric epithelium proliferation. NF-κB signalling must be turned off appropriately to prevent sustained and harmful inflammatory responses. The cell employs several strategies at multiple levels for termination of NF-κB signalling. Downregulation of NF-κB signalling leads to loss or overpowered expression of cylindromatosis (CYLD) and synthesis of 1kβα. Degradation of NF-κB and direct ubiquitination are the other mechanisms which turn off NF-κB [141].

9.3.2 STAT3, Cell Proliferation, and Gastric Cancer

Signal transducers and activators of transcription (STATs) are a family of transcription factors. STAT3 is a regulator of cell proliferation in gastric cancer. In gastric cancer, IL-26 activates STAT3 signalling pathway. The activated STAT3 pathway induces upregulation of Bcl-2, Bcl-XL, and c-Myc expression, which lead to proliferation of cell [145]. Inhibitors of STAT3 suppress cell proliferation in gastric cancer [45].

9.3.3 BTF3, Cell Proliferation, and Gastric Cancer

Basic transcription factor 3 (BTF3) is an evolutionarily conserved 27-kD protein [46, 69, 96, 149]. BFT3 involvement in proliferation of human gastric cancer cells is known [69]. BTF3 is expressed in two isoforms, BTF3a and BTF3b [90]. Isoform BTF3a is transcriptionally active, whereas isoform BTF3b is transcriptionally inactive [90]. BTF3 is a transcriptional initiation factor that interacts with proximal promoter elements and forms a complex with RNA polymerases [6, 148]. Overexpression of BTF3 gene was reported in colorectal cancer, glioblastomas gastric cancer, and hepatocellular carcinomas [18, 69, 83, 105, 132]. BFT3 and BFT3a mRNAs were increased by 1.3 and 4.6 folds, respectively, when compared to the normal tissues in the pancreatic ductal adenocarcinoma. BFT3 expression was downregulated using small interfering RNA (siRNA) [91, 119]. Liu et al. [69]

reported BFT3 mRNA and protein levels in gastric tumours and normal samples and also downregulated BTF3 expressions using siRNA-BTF3 in gastric tumour cells. Liu et al. [69] also detected that BTF3 expression is connected with enhanced cell proliferation, and its silencing decreased proliferation of gastric cancer cells.

9.3.4 Sp1, Cell Proliferation, and Gastric Cancer

Sp multigene family with sequence-specific DNA-binding proteins transcribes many genes having GC boxes in their promoter [120]. Sp1, Sp2, Sp3, and Sp4 are the transcriptional factors of Sp multigene family [11, 15, 30, 32, 59, 72, 124]. Sp1 is an essential transcription factor contributing to gastric cancer. Sp1 is involved in signal transduction pathways linked to cancer [60]. Investigations reported rise in Sp1 mRNA and binding activity. Inhibition of cell growth when Sp1 is interfered further corroborates its role in gastric cancer [8]. Sp1 was directly correlated with proliferation of cell and cancer [28]. The interrelation between levels of Sp1 expressed, development of gastric cancer, and patient survival was observed [129]. Wang et al. [129] reported distinct expression of Sp1 in gastric cancer cell lines when compared with normal gastric tissue and proposed that abnormally activated Sp1 directly contributes to gastric cancer development and progression.

9.3.5 GKN1, Cell Proliferation, and Gastric Cancer

Gastrokine 1 (GKN1), a novel tissue-specific secretory protein, is present under the apical plasma membrane. GKN1 is of 6 kb in length, located on chromosome 2p13, and contains six exons. Several mammalian species expressed GKN1 in gastric mucosa cells [73]. GKN1 was known for maintaining the gastric mucosa integrity and mediating repair after injury. Oien et al. [84] measured the expression of GKN1 in normal and in gastric tissues to establish transcriptional silencing of GKN1 gene in gastric cancer. Oien et al. [84] reported abundant mRNA in the normal human stomach, whereas GKN1 was absent in gastric adenocarcinomas, gastro-oesophageal adenocarcinoma cell line, and other normal and tumour gastrointestinal tissues.

H. pylori-positive chronic gastritis patients reported low levels of GKN1 protein [103]. Nardone et al. [79, 80] analysed 28 gastric cancer patients and demonstrated downregulation or the complete absence of the protein. Reduced colony formation in MKN-28 gastric carcinoma cells was reported when transfected with GKN1 [112]. GKN1 increased apoptosis and reduced proliferation of gastric cancer cells [104]. These data suggest that GKN1 may function as gastric tumour suppressor gene [17].

Yoon et al. [143] with the objective of investigating the mechanism of silencing GKN1 gene analysed 81 gastric carcinoma and 40 gastric adenoma samples. Studies identified hyper-methylation of GKN1 gene promoter in two tumours and decreased number of GKN1 levels in gastric cancer. This study provided insights on the epigenetic mechanisms that could contribute to silencing of GKN1 gene. Overall,

studies suggest that hyper-methylation of GKN1 gene promoter silences GKN1 gene reducing the levels of GKN1 and thus demonstrates the possibility of tumour suppressor activity of GKN1 operating downstream of the pathways.

9.3.6 GATA6, Cell Proliferation, and Gastric Cancer

Transcription factors of the GATA family share conserved zinc fingers (C_2H_2 type) that mediate DNA binding and protein interactions. GATA factors bind to A/TGATAA/G and control activation or repression of transcription leading to carcinogenesis. It was established that loss/expression/silencing of GATA factors was responsible for breast, colorectal, lung, and gastric cancer. Studies identified different types of factors like GATA 1, 2, 3, 4, 5, and 6. Silencing (GATA 4 and GATA 5 genes) and loss (GATA6) were observed in colorectal and gastric cancer. Sulahian et al. [118] studied the transcriptional regulation of GATA6 in gastric cancer cell lines. Chromatin immunoprecipitation and sequencing (ChIP-seq) was used to identify GATA6-bound genes in primary gastric cancers. Analysis of genes suggests that GATA6 directly regulates 75 genes that control cell replication; and cell proliferation was promoted by 41 of these genes. This study suggests that GATA6 directly regulates genes promoting cell proliferation.

9.4 Role of Transcriptional Factors in Cell Cycle

Cell cycle involves a series of events leading to duplication (DNA), and cell division produces two daughter cells. The cell cycle is divided into three phases: interphase, mitotic (M) phase, and cytokinesis. The following are the checkpoints G_0, G_1, G_2, S, and M for the cell cycle. G_1, S, and G_2 checkpoints of interphase control transitions from G_1 to S phase, S phase to G_2, and G_2 to M phase, respectively. M checkpoint controls the transition from M to G_0 phase, whereas G_0 checkpoint controls the transition from G_0 to G_1 phase. Several studies have identified cell cycle regulators, which control the transition from one phase to another. Transcription factors like NF-κB, STAT3, GATA, BFT3, Sp1, and FOXM1 play a vital role in regulating cell cycle of gastric cancer cells (Table 9.1, Figs. 9.2 and 9.3). Deregulation at checkpoint controls may lead to tumour formation.

9.4.1 NF-κB, Cell Cycle, and Gastric Cancer

NF-κB master transcriptional regulator has a role in cell cycle regulation. Receptor activator of NF-κB ligand (RANKL), a member of TNF family, binds to the receptor activator of NF-κB (RANK) activating IKK and NIK, inducing phosphorylation-dependent proteolytic removal of the I B like C-terminal domain of NF-κB2. Freeing of NF-κB2 translocates it to the nucleus activating cyclin D1 expression, leading to cell cycle progression [48]. κB site present in promoter of cyclin D_1 induces

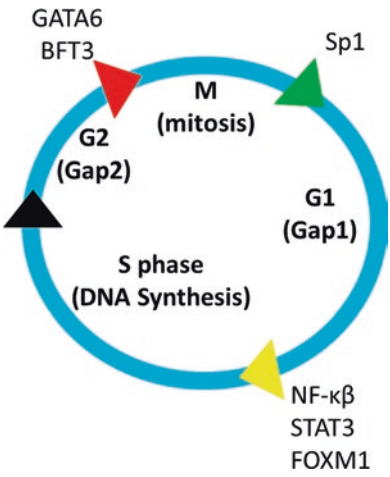

Fig. 9.3 Transcriptional factors regulating various transition stages of cell cycle

proliferation. Several other studies have identified cell cycle regulators like D_1, D_2, and G_1 which were associated with NF-κB. Transition from G_1 to S phase is stimulated by D_1, D_2, and G_1 which are activated by NF-κB ([5, 29, 34]; Fig. 9.3).

9.4.2 STAT3, Cell Cycle, and Gastric Cancer

Cell cycle is controlled by STAT3 in gastric cancer, and data in favour of cell cycle transition by STAT3 was well proven [113, 138]. Shirogane et al. [113] studied the synergistic roles of Pim-1 and c-Myc in STAT3-mediated cell cycle progression. The study established that two STAT3 genes, c-myc and pim of JAK/STAT signalling pathway, mediate cell cycle transition from G_1 to S (Fig. 9.3; [113]).

9.4.3 GATA, Cell Cycle, and Gastric Cancer

GATA6 of the GATA family controls transcription leading to carcinogenesis. Sulahian et al. [118] investigated the regulation of transcription using ChIP-seq to identify GATA6-bound genes in primary gastric cancers. Studies demonstrated that M-phase of the cell cycle is controlled by GATA6 [118]. Decrease in levels of GATA6/protein controlled the expression of the genes downstream, and the cells in G_2 and M phases are arrested (Fig. 9.3). This research comprehends the involvement of GATA6 in regulating cell cycle at G_2 and M phases.

9.4.4 Sp1, Cell Cycle, and Gastric Cancer

Progression of cell cycle and growth of cell are inhibited by Sp1. SiRNA and decoy oligonucleotides of Sp1 supressed progression of cell cycle and growth of cell [1, 40]. In the cell cycle protein, Sp1 is prevailing in the G_1 phase and directly

correlates with cell proliferation ([28]; Fig. 9.3). Overexpression of Sp1-responsive genes, ODC, and cyclin D_1 augment the proliferation. The results of study indicate that Sp1 contributes to the progression of gastric cancer.

9.4.5 BFT3, Cell Cycle, and Gastric Cancer

BFT3 expression is correlated with reduced cell cycle regulation of gastric cancer cells. Human Protein Atlas reported high levels of BTF3 protein in gastric cancer [125]. Liu et al. [69] examined samples of gastric tumours, healthy cell lines of gastric tumour for the mRNA, and protein patterns of BFT3 and also studied the silencing of BFT3 expression using SiRNA BFT3. Liu et al. [69] reported that BFT3 was upregulated in gastric tumour samples when compared with normal samples. At the same time, the expression levels of BFT3 were similar in all gastric cancer cell lines. Liu et al. [69] also reported that there is a connection between G_1, G_2/M, and S phases regulation and expression of BFT3. The shift in the phases from G_1 to the G_2/M and S in the cell cycle was witnessed when expression of BFT3 was silenced (Fig. 9.3). This study clearly indicates that there is a close association between regulation of cell cycle and BFT3 expression.

9.4.6 FOXM1, Cell Cycle, and Gastric Cancer

Forkhead proteins contain a motif helix-turn-helix (loops in appearance of butterfly or winged helix DNA-binding domain) acting as a transcription factor. This family contains proteins from Forkhead box protein (FOX)A-FOXC, and FOXM1 is one of the members of the family with 100 amino acids [135]. FOXM1 promotes tumour development in several cancers along with overexpression in gastric cancer [68, 85, 142]. Li et al. [66] studied the relationship between expression of transcription factor FOXM1 and gastric cancer. Li et al. [66] reported overexpression of FOXM1 in samples of gastric cancer when compared with normal samples. Ras-MAPK and hedgehog signalling pathway activate FOXM1 promoting transition from G_1 to S phase (Fig. 9.3). The genes such as Cdc25B, CDK1, and p27KIP promote the progression to mitosis [13, 131]. These results suggest that FOXM1 plays an important role in the progression of human gastric cancer.

9.5 Role of Transcriptional Factors in Metastasis

Metastasis is one of the "hallmarks of cancer" where cancer is spread from the place of genesis to the other sites. Cancer cells spread through the vessels and circulate in the bloodstream to establish themselves in multiple new sites. Cancer cells to metastasize shall have the ability to adhere, invade tissue, migrate, and form new blood vessels (angiogenesis). Hanahan and Weinberg [31] proposed the essentials of tumorigenesis, "…altered cell physiology; self-sufficiency in growth signals;

Fig. 9.4 Various stages of metastasis

insensitivity to growth inhibition; evasion of apoptosis; immortalization; sustained angiogenesis; tissue invasion and metastasis…". Transcription factors like NF-κB, STAT3, RUNX3, GKN1, Sp1, BFT3, and FOXM1 play an important role in metastasis of gastric cancer cells (Figs. 9.2 and 9.4).

9.5.1 NF-κB, Metastasis, and Gastric Cancer

NF-κB has the metastatic potential to increase tumour's angiogenesis. NF-κB's activity directly or indirectly was involved in cell migration, extracellular matrix destruction, angiogenesis, and tumour invasion. Increased NF-κB activity deregulates production of chemokines inducing migration of the cell. Koch et al. [54] revealed that NF-κB deregulates the production of chemokine IL8 and promotes angiogenesis. Proteolytic enzymes matrix metalloproteinases (MMPs) were known to promote tumour invasion. κB sites were identified in promoters of genes that encode MMPs. NF-κB activation was also reported to contribute to extracellular matrix destruction by cancer cells [3, 121, 128]. NF-κB may act as a TF, inducing MMPs promoting destruction of extracellular matrix and tumour invasion. Huang et al. [37] reported that the activation of NF-κB induces vascular endothelial growth

factor (VEGF) and IL8 expression thus stimulating angiogenesis. Thus, studies on NF-κB provided information that NF-κB has the metastatic potential and is involved in cell migration, extracellular matrix destruction, angiogenesis, and tumour invasion.

9.5.2 STAT3, Metastasis, and Gastric Cancer

STAT3 is associated with evasion of immune system and angiogenesis of tumours. Immune evasion and angiogenesis are the properties representing the metastatic potential of a gastric tumour. Immunosurveillance detects and eliminates cancer cells; however, cancer cells have the capability to evade immune system. Several roles of STAT3 like inhibition of cytokine expression, negative regulation CXCL10 expression, promotion of NF-κB, and IL-6/GP130/JAK pathways were reported in immune evasion. Wang et al. [130] hypothesized that STAT3 signalling regulates innate and adaptive immune responses in tumour cells. Activation of STAT3 inhibits cytokine expression leading to decrease in MHC expression. This subsequently decreases presentation of antigen and T-cell activation [130]. Saudemont et al. [107] reported that STAT3 negatively controls CXCL10 expression significantly enhancing natural killer cells that can kill cancer cells. STAT3 promotes NF-κB and IL-6/GP130/JAK pathways preventing antitumour immune responses of T helper 1 [146]. Therefore, STAT3 regulates immune responses and helps in immune evasion.

Angiogenesis includes "degradation of the vascular basement membrane, vascular epithelial cell proliferation and migration, and new vessel reformation and resolution…" [44]. There exists a relationship between STAT3, metalloproteinase 2 (MMP-2), vascular basement membrane, basic fibroblast growth factor (bFGF2), and VEGF A. Vascular basement membrane, bFGF2, and VEGF A are important for proliferation of vascular endothelial cells. Inhibition of STAT3 downregulates MMP-2 which in turn degrades vascular basement membrane, basic fibroblast growth factor (bFGF2), and VEGF A. So, suppression of STAT3 inhibits angiogenesis and thus prevents tumour formation.

9.5.3 RUNX3, Metastasis, and Gastric Cancer

RUNX family (mammalian runt-related genes) code for proteins with DNA-binding function. Members like RUNX1/AML1, RUNX2, and RUNX3 belong to the family RUNX genes [42, 88]. Hyper-methylation of the CpG islands in the promoter region can lead to loss of RUNX3 gene. RUNX3 promoter was hypermethylated in different cancers, including gastric cancers [63]. In gastric cancer methylation of RUNX3 gene was observed in chronic gastritis (8%), intestinal metaplasia (28%), and gastric adenomas (27%). RUNX3 knockout mice exhibited hyperplasia, reduced apoptosis, and reduced sensitivity to TGFβ1. These reports suggest that epigenetic gene silencing of RUNX3 operates downstream as the signalling pathways demonstrating tumour suppressor activity [53, 106]. *H. pylori*

infection induces nitric oxide production resulting in methylation of the RUNX3 promoter and may downregulate RUNX3 gene with an epigenetic mechanism [50]. Hsu et al. [35] reported that damage of RUNX3 expression correlates with the metastatic spread of gastric cancer [35].

9.5.4 GKN1, Metastasis, and Gastric Cancer

Epithelial–mesenchymal transition (EMT) is a critical pathophysiological state observed during tumour genesis and progression. According to Huang et al. [36], "…activation of EMT promotes cancer cell stemness, initiation, invasion, metastasis, and repression of E-cadherin allowing tumour cells to disseminate and spread throughout the body …". Pathogens like *H. pylori*, stress, and hypoxia activate EMT thereby inducing and aggravating gastric cancer. Overexpression of transcription factors downstream of signalling pathways like TGF-β, Wnt/β-catenin, Notch, etc. and microRNAs trigger microenvironmental, membrane, and intracellular cues modulating EMT.

GKN1 known as gastric tumour suppressor gene is having specific role in metastasis of gastric cancer. Yoon et al. [144] reported that GKN1 inhibits EMT in GKN1-transfected AGS cells, whereas EMT was observed in AGS cells enabling mesenchymal cells to migrate, invade, and resist apoptosis. Transfected GKN1 and recombinant GKN1-treated AGS cells revealed decreased levels of reactive oxygen species (ROS), phosphatidylinositol 3-kinase (PI3K)/Akt pathway proteins, EMT-related proteins (cytoplasmic and nuclear b-catenin, slug, snail, fibronectin, and vimentin), and re-expression of E-cadherin. This study suggests that GKN1 effects the progression of cancers by inhibiting EMT. Xing et al. [137] reported that GKN1 inhibits cell growth by activating p16/Rb and p21waf pathways and also showed that Ras/Raf/MEK/ERK signalling is activated when cells of gastric cancer and xenograft nude mouse model are treated with GKN1. Therefore, GKN1 has a specific role in metastasis of gastric cancer.

9.5.5 Sp1, Metastasis, and Gastric Cancer

Sp1 regulates several aspects of metastasis like invasion and angiogenesis. Shi et al. [111] showed that VEGF is expressed constitutively due to Sp1 activation. Wang et al. [129] revealed that multiple genes leading to development of tumour and progression of cancer are regulated by transcription factor Sp1. Several studies established the fact that Sp1 controls "promoters of multiple growth-regulated genes…" for cell growth. Sp1 regulates promoters of epidermal growth factor receptor (FGFR1) [16, 89, 92, 147], hamster dihydrofolate reductase [43, 82], insulin-like growth factor-binding protein 2 [58], insulin-like growth factor receptor 1 [16, 89, 92, 147], ornithine decarboxylase [56], rep3a [108], serum response factor [117], thymidine kinase [116], and VEGF [75, 111] for cell growth. Sp1 also regulates multiple aspects of tumour angiogenesis like vessel formation by the expression of VEGF and basic

fibroblast growth factor [10, 20, 22, 70, 78, 111]. Further, Sp1 is involved in tumour invasion and metastasis by upregulating matrix metalloproteinase-2 [97, 98] and urokinase-type plasminogen activator [38]. These studies clearly suggest that Sp1 regulates tumour cell survival, growth, and angiogenesis.

9.5.6 BFT3, Metastasis, and Gastric Cancer

Transcription factor BFT3 involved in development and progression of gastric cancer regulates cell adhesion in gastric cancer cells. The association between downregulation of the BTF3 expression and cell adhesion was reported. Liu et al. [69] studied both upregulation and downregulation of BTF3 expression in samples of gastric tumours, normal tissue, and cell lines of gastric cancer. The expression of BFT3 is downregulated and is correlated with adhesion of cells in gastric tumours/cancers and also reported change in cell adhesion [69]. The plausible mechanism of action could be that heparanase 2, an enzyme involved in cell adhesion, is reduced when BTF3 expression is downregulated [91, 119]. This study clearly indicates that there is a close relationship between BFT3 expression and adhesion of gastric cancer cells.

9.5.7 FOXM1, Metastasis, and Gastric Cancer

FOXM1 is correlated with angiogenesis, growth, and metastasis of gastric cancer [65]. Okada et al. [85] showed that there is no relationship between expression of FOXM1 and any of the clinical pathological parameters (tumour size, depth of invasion, lymph node metastasis, proliferation activity of the tumour cells). Li et al. [66] studied the influence of FOXM1 expression levels on tumour size (>5 cm), depth of invasion (pT – T3 and T4), and pTNM (stage III–IV) and further confirmed that there is no significant relationship between FOXM1 expression, survival of patients, stage T1-2, I–II, and smaller tumour size.

FOXM1 induces angiogenesis by controlling expression of VEGF gene. Li et al. [65] studied the correlation between expression of VEGF gene and microvessel density (MVD) and established that there exists a correlation with expression of VEGF gene and MVD. Cancer cells showed increased invasive and migratory abilities when FOXM1 was overexpressed [9]. The above studies clearly show the metastatic properties like invasion, migration, and angiogenesis are correlated with the expression of FOXM1.

9.6 Role of Transcriptional Factors in Apoptosis

Apoptosis or programmed cell death (PCD) takes place predominantly in multicellular organisms where altered biochemical events lead to changes in morphology of cell and death; “Blebbing, cell shrinkage, chromatin condensation, chromosomal DNA fragmentation, nuclear fragmentation, and global mRNA decay” are the

changes [26]. NF-κB, STAT3, GKN1, Sp1, GATA6, BFT3, and FOXM1 are the TFs known to control apoptosis (Table 9.1, Fig. 9.2).

9.6.1 NF-κB, Apoptosis, and Gastric Cancer

Production of proinflammatory cytokines, infection, and activation of oncogenic constitutively activate the expression of NF-κB. Constitutively activated NF-κB prevents apoptosis. NF-κB is an inhibitor of PCD and transcriptionally activates anti-apoptotic factors [2, 67, 126, 127]. Cellular inhibitors of apoptosis (cIAPs), caspase 8/FADD (FAS-associated death domain) like IL1-converting enzyme (FLICE) inhibitory protein (cFLIP), and members of the BCL2 family (such as A1/BFL1 and BCLXL) are the antiapoptotic factors that are induced by NF-κB [49]. NF-κB upon inhibition of PCD prevents the death of cells, thereby increasing the cells with chromosomal rearrangements or DNA damage. So, "prevention of apoptosis increases the pool of genetically altered cells, which will eventually give rise to transformed progeny…". In general, genetically altered cells are rejected by checkpoint controls [133].

9.6.2 STAT3, Apoptosis, and Gastric Cancer

STAT's role was studied well in development of cancer and apoptosis. STAT3's role in increasing the repression of apoptosis was established [14, 19, 21, 45]. Fukuda et al. [21] studied and showed that STAT3 is engaged in anti-apoptosis. STAT3 induces upregulation of Bcl-XL to repress apoptosis [21]. Inhibitors of STAT3 also promote apoptosis in gastric cancer [45]. Kanai et al. [45] studied the role of inhibitors on STAT3 in gastric cancer and demonstrated that inhibition of STAT3 promoted apoptosis in gastric cancer. Thus, these studies provide comprehensive information on STAT3 in gastric cancer development and apoptosis.

9.6.3 GKN1, Apoptosis, and Gastric Cancer

Several studies have established that GKN1 controls proliferation in cells of gastric cancer by increasing apoptosis [77, 104]. Moss et al. [77] attempted to study the influence of GKN1 and GKN2 expression in tumour and gastric cancers. Moss et al. [77] related that individuals with a lower expression of the GKN1 and GKN2 proteins have risk of gastric diseases. Decrease in proliferation of cell lines – AGS gastric cancer by GKN1 when compared to other cell lines, HEK 293, human lung epidermoid carcinoma cell line (H1355), and non-gastric cancer cells – was observed in MTT assay. This study suggests that GKN1 and GKN2 act like a modulator of apoptotic signals controlling proliferation of cells in gastric cancer. Further, GKN1 overexpression in cells of gastric cancer was studied [104]. Overexpression of GKN1 increased apoptosis in cells of gastric cancer when compared to control

cells [104]. These findings suggest that GKN1 and GKN2 have a role in apoptosis, and overexpression of GKN1 can increase apoptosis.

9.6.4 Sp1, Apoptosis and Gastric Cancer

Gastric cancer progression is dependent on how cells show resistance to apoptosis. Sp1 is known to control the promoters of apoptosis-related genes like Bcl-2, Bcl-x [27, 101], survivin [61], and TGF-ß [52, 136]. Thus, apoptosis is regulated by transcriptional factor Sp1 [7, 57, 95, 102].

9.6.5 GATA6, Apoptosis and Gastric Cancer

GATA6 of GATA family controls transcription leading to carcinogenesis. Sulahian et al. [118] studied regulation of GATA6 at the level of transcription using ChIP-seq to identify GATA6-bound genes in primary gastric cancers and observed that GATA6 controlled apoptosis. Sulahian et al. [118] also observed that GATA6 binds DNA and directly regulates dependent genes implicated in cell death.

9.6.6 BFT3, Apoptosis and Gastric Cancer

Transcription factor BFT3 is overexpressed in cells of gastric cancer. The relationship with reference to downregulation of BFT3 and apoptosis rates in gastric cancer was reported. Liu et al. [69] investigated the relationship between BTF3 expression, apoptosis, and gastric cancer. Overexpression of BTF3 in gastric cancer samples was observed when compared to normal cells [69]. Downregulation of BFT3 expression using SiRNA in cells of gastric cancer increased apoptotic rates significantly. This study clearly establishes relationship between BTF3, apoptosis, and gastric cancer.

9.6.7 FOXM1, Apoptosis and Gastric Cancer

The association between FOXM1 expression, apoptosis, and gastric cancer was well studied. Overexpression of FOXM1 mediates inhibition of apoptosis [66]. The probable mechanisms which work against induced apoptosis are "…upregulation of MDR1 (multi-drug resistant protein 1); a P-Glycoprotein; CIAP (inhibitors of apoptosis) family members including survivin; and the altered microtubule dynamics" [87]. Li et al. [66] investigated the expression of FOXM1 in regulating the dynamics of microtubules during mitosis in tumour cells. Li et al. [66] reported that overexpression of FOXM1 mediates the alteration of microtubule dynamics and prevents induced apoptosis in gastric cancer cell lines.

9.7 Other Transcriptional Factors

Several other transcriptional factors were also studied and reported in relation to cell proliferation, cell cycle, metastasis (adhesion, invasion, migration, angiogenesis), and apoptosis. Studies on SRY-related high-mobility group (HMG)-box protein- 2 (SOX2), hepatocyte nuclear factor 4 alphas P1(HNF4aP1), CDX2, etc. reported association with gastric cancer. SOX2 is a member of the HMG-domain DNA-binding protein family and is highly expressed in upper gastrointestinal tract [100], regulating transcription and chromatin architecture [94, 123]. SOX2 inhibits growth by preventing the apoptosis and cell cycle. CDX2 is a mammalian homeobox gene involved in identity and polarity of a cell. CDX2 transcriptionally controls specific genes of intestine [114]. CDX2 is expressed in the colonic epithelium and intestinal epithelium of the stomach [123], whereas half of gastric cancer cells express CDX2. Hepatocyte nuclear factor 4 alphas (HNF4a) is necessary for development of the liver and metabolism of fat [122]. Mutations in HNF4a cause maturity onset diabetes of the young-1 (MODY-1). Splicing of HNF4a by alternative promoter (P1 and P2) is responsible for different isoforms and also depends on the organ. In the colon and small intestine, P2-driven HNF4a (HNF4aP2) and P1-driven HNF4a (HNF4aP1) are expressed. HNF4aP1 is expressed in about half of gastric cancers, whereas in the normal stomach, HNF4aP2 is expressed [55, 122].

Xu et al. [139] constructed the transcriptional network to screen the TFs driving the gastric cancer progression. The analysis identified 70 differentially expressed transcriptional factors in a transcriptionally regulatory network. The top ten transcriptional factors that regulate the downstream genes were ARID3A, BRCA1, EHF, FEV, FOXC1, FOXD1. FOXL1, GATA3, SOX10, and ZNF263.

9.8 Conclusion

Transcriptional factors regulate cell proliferation, cell cycle, metastasis (adhesion, invasion, migration, angiogenesis), and apoptosis of gastric cancer cells. Proliferation in gastric cancer cells is regulated by transcription factors like ARID3A, BRCA1, BTF3, CDX2, EHF, FEV, FOXC1, FOXD1, FOXL1, GATA3, GATA6, GKN1, HNF4aP1, NF-κB, Sp1, SOX2, SOX10, STAT3, and ZNF263. BFT3, FOXM1, GATA, NF-κB, Sp1, and STAT3 are the TFs which control the cycle of cells in gastric cancer. Metastasis of the cell in gastric cancer is regulated by TFs like BFT3, FOXM1, GKN1, NF-κB, RUNX3, Sp1, and STAT3. BFT3, FOXM1, GKN1, GATA6, NF-κB, Sp1, and STAT3 are the TFs known to control apoptosis.

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Role of Hypoxia-Inducible Factor (HIF) in the Initiation of Cancer and Its Therapeutic Inhibitors

10

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Abstract

The inadequate oxygen (O2) supply to a large extent alters the cellular microenvironment and results in hypoxia or even anoxia. Hypoxia-inducible factor (HIF) facilitates the cellular response to hypoxia. HIF, a heterodimer composed of two subunits, the subunit α and subunit β, is involved in several signaling pathways which involves both survival and death pathways, their activation and regulation. HIF is believed to be the best molecular target in the treatment of cancer, and also numerous inhibitors for HIF-1α are available today. This chapter explains the HIF-1α role in cancer and its therapeutic applications that potentially target HIF pathway.

Keywords

Cancer · Hypoxia · Hypoxia-inducible factor (HIF) · HIF-1α inhibitors · Angiogenesis · Small molecule inhibitors

10.1 Introduction

Constant supply of O2 is required for all the cells to carry out oxidative phosphorylation in the mitochondria for the generation of ATP by oxidative phosphorylation. Under normal regularized conditions, with the normal supply of oxygen, the cells

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divide in an orderly way and are replaced with new cells when they die, are worn out, or are damaged. In contrast, in an inadequate supply of oxygen, the lack of oxygen leads the cells to enter into abnormal and stressful conditions, where the regulated cell division becomes irregular and thereby activating several mechanisms in the process to sustain their viability. The inadequate supply of oxygen (O2) in large extent alters the cellular microenvironment and results in hypoxia or even anoxia leading to the cellular transformation [56]. Under the hypoxic conditions, the transformed cells divide rapidly and result in the formation of tumors by crowding out the normal cells. In such condition, the energy requirement and production are the most important aspects to understand the differences between the proliferating and nonproliferating cells [91]. The heterogenous cells in a complex structure of tumor are undergoing different stresses, e.g., low oxygen levels in the interior, so often the core of a tumor is necrotic [32, 60, 71, 94].

Under the hypoxic conditions, due to nonavailability of oxygen, tumor cells generate energy by non-oxidative breakdown of glucose, followed by fermentation of lactic acid in cytosol [25, 28, 32, 36, 47, 91]. In such conditions, hypoxia plays a major role at different stages of cancer (initiation, accumulation, angiogenesis, and metastasis) by initiating the changes in the microenvironment, altering the oncogenic genes and normal metabolism, and in the development of new blood vessels, thereby inducing the metastasis. The cellular response to hypoxia is mainly mediated by the HIF. HIF is found in mammalian cells grown under hypoxic condition. It is stimulated in response to intrahumoral hypoxia leading to genetic alterations by activating the oncogenes and inactivating the tumor suppressor genes. HIF plays an important role in adapting the cancer cells to low oxygen condition by triggering the transcription of over 100 target genes that regulate the tumor survival and progression [122–125].

10.2 HIF Structure

Hypoxia-inducible factor (HIF) is a heterodimer composed of two subunits, the subunit α and subunit β. The HIF-1α subunit is oxygen sensitive and is a cytoplasmic protein. It is degraded by the ubiquitin–proteasome system continuously in well-oxygenated cells. The HIF-1β subunit is also known as aryl hydrocarbon receptor nuclear translocator (ARNT), a nuclear protein, independent to oxygen tension and a heterodimeric partner of aryl hydrocarbon receptor (AhR). HIF-1β is constitutively expressed to levels within the nucleus that remain relatively constant and binds to AhR and facilitates its translocation. These two subunits (α and β) belong to the family of basic helix-loop-helix (bHLH) and PER-ARNT-single-minded protein (SIM) (PAS) transcription factors. The characteristic feature of these family proteins is that they have recognizable domains and can regulate their own transcription. Among all the family members, the PAS domain was the only domain that is conserved. The N-terminal region of this PAS domain is essential to mediate DNA binding and interaction with HIF-1β subunit [118].

The subunit α has three different isoforms, HIF-1α, HIF-2α, and HIF-3α. Analogs of α subunits of HIF-1α and HIF-2α are more comprehensively studied and were compared to HIF-3α. HIF-3α is less analyzed when compared with the other HIF-α homologs. The inhibitory PAS domain protein (IPAS), a spliced variant of HIF-3α discovery, led practical information about HIF-3α. It functions as dominant-negative regulator of hypoxia-inducible gene expression and does not show any intrinsic transactivation activity as compared to the COOH-terminal transactivation domain (C-TAD) of HIF-1α and HIF-2α [111, 148].

The analogs of HIF-1α and HIF-2α share high percentage sequence identity (48%) and can heterodimerize with HIF-1β subunit. These two analogs when heterodimerized with HIF-1β subunit have distinct tissue-specific expression. The ubiquitously expressed HIF-1α is constantly expressed and degraded in presence of induced hypoxic conditions. However, HIF-2α distribution is restricted to specific tissue origins like vascular endothelial cells, the kidney, catecholamine-producing cells, renal interstitial fibroblasts, and some glomerular cells [95].

HIF-1α in its C-terminal has two transactivation regions: the N-terminal transactivation region or N-TAD (AA 531–575) and the C-terminal transactivation region or C-TAD (AA 786–826) (Fig. 10.1). HIF-1α transcriptional activity is mostly dependent upon these two domains. Under hypoxia conditions the transcription of HIF-1α is modulated by C-TAD whereas stabilization by N-TAD. The requirement of C-TAD or N-TAD for different gene sets regulation is completely dependent on oxygen tension. N-TAD, also known as an oxygen-dependent degradation domain (ODDD), is responsible for stabilizing HIF-1α against degradation as hydroxylation of conserved prolyl residues resides in this region. This domain is also important in mediating oxygen regulation stability. Prolyl-4-hydroxylases (PHDs), 2-oxoglutarate-dependent oxygenase superfamily enzymes, mediate this hydroxylation and promote the subunit degradation [77].

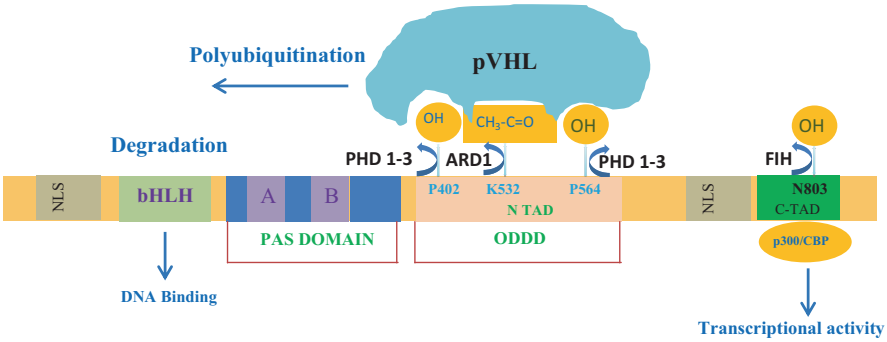

Fig. 10.1 Oxygen-dependent regulation of HIF-1α activity (This figure is adapted from [95, 121] with modifications)

HIF-1α hydroxylation does not occur in hypoxic conditions. In this condition α subunit along with other cofactors acts as transcription factor and thereby migrates to nucleus and dimerizes with β subunit and initiates its transcriptional program [18]. The resultant active protein that is HIF-1 is a messenger which is translocated to the nucleus to induce transcriptional responses to hypoxia [171].

The active HIF-1 protein activates transcription of target genes by adhering to specific hypoxic response elements (HRE) which comprises A/GCGTG consensus motif. Similarly HIF-2 and HIF-3 are resultant active heterodimers of HIF-2α or HIF-3α with ARNT [119]. The presence of two nuclear localization signals in bHLH domain (17–33 amino acids) and COOH-terminal regulatory domain (718–721 AA) results in translocation of HIF-1α into nucleus [110].

The interaction of C-TAD with coactivators CBP/p300 results in the change in transcription of HIF-1α under hypoxia. This interaction is governed by the CH1 region of p300/CBP and also improved by SRC-1, and synergistic effect was observed at limited concentrations. Phosphorylation of p300 by the MAPK pathway increases the HIF-1 α/p300 complex formation and thereby increases the transcriptional activity of HIF-1. Upon blocking of HIF-1α/p300 CH1 interaction, HIF-1 transactivation is inactivated as the p300-CH1 interacting protein and p35srj (for serine–glycine-rich junction) bind to p300/CBP. C-TAD interaction with p300/CBP does not occur in normal conditions. This is due to oxygen-dependent hydroxylation of N803 residue in the carboxyl-terminal transactivation domain (CAD) of HIF-1α by factor-inhibiting HIF (FIH-1), a 2-oxoglutarate-dependent dioxygenase enzyme [77]. It prevents the interaction of HIF-1α with transcriptional coactivators, p300 and CBP (cAMP response element-binding protein). Small redox protein thioredoxin-1(Trx-1) under both normoxic and hypoxic conditions has been reported to enhance the binding of CBP/p300 to the C-TAD of HIF-1α. This leads to the expression of HIF-1α and its downstream target VEGF and improved angiogenesis [39]. Transactivation of HIF-1 by Ref-1 leads decrease of a cysteine residue in the C-TAD of HIF-1α. But, the useful status of this cysteine residue and the consequence of CBP/p300 remains doubtful [59, 77].

The PHD enzymes (prolyl hydroxylase-domain protein) hydroxylate the proline 402 and 564 residues that are present in LXXLAP amino acid motif of ODDD of HIF-1α subunit under normal oxygen conditions. This allows modified HIF-1α at prolyl sites to bind to the von Hippel–Lindau (VHL) tumor suppressor protein. Only modified HIF-1α is able to bind to the VHL protein whose binding may also be promoted by acetylation of K532 residue by the arrest-defective-1(ARD1) acetyltransferase [42]. This VHL protein is a recognition component of an E3 ubiquitin-protein ligase. This ligase finally targets the HIF-1α for proteasomal degradation by 26S proteasome. OS-9 is another factor that impacts on the degradation of HIF-1α. OS-9 interacts HIF-1α directly, and the prolyl hydroxylases PHD2 and PHD3 and forms a ternary complex. This complex formation stabilizes the interaction between HIF-1α and PHDs, thus helping HIF-1α hydroxylation and pVHL-mediated ubiquitination, and finally leads to degradation of HIF-1α [34].

The HIF-1 activity depends on the regulation of its subunits (α and β) at several levels including transcription, translation, ubiquitin-mediated protein breakdown, and nuclear translocation. The loss of this activity decreases the vascularization, tumor growth, and energy metabolism. HIF-1, by employing transcriptional coactivators, controls the expression of many genes. The HIF-1 expression directly regulates the tumor growth. The overexpression of HIF-1 promotes the tumor growth by increasing HIF-1 transcription factor activity. The protein products play important roles in the severe and long-lasting adaptation to hypoxia, including angiogenesis, erythropoiesis, and pH regulation glycolysis. Pulse-chase studies of MCF-7 breast cancer cells stimulated with heregulin increase HIF-1α synthesis but do not activate transactivation-region function that was stopped by rapamycin in PC-3 prostate cancer cells. In another study when Rat-1 fibroblasts and breast cancer cells (MCF7) were overexpressed with BNIP3 (BCL2/adenovirus E1B 19 kDa interacting protein 3) and NIX (BNIP3 homolog) at the transcriptional level, it induced apoptosis. The cell death induced by BNIP3 is mediated by binding of BNIP3 to anti-apoptotic proteins Bcl-2 and Bcl-xL and inhibiting those proteins. This hypoxia-induced apoptosis may be HIF-1α dependent because BNIP3 promoter contains HRE [46].

10.2.1 Glucose Metabolism

The glycolytic rates in normal cells when compared to cancerous cells are very high even in the presence of oxygen, and energy required for cancerous cells is generated by glycolysis followed by fermentation of lactic acid in cytosol rather than oxidation of pyruvate in mitochondria, also defined as “aerobic glycolysis” [25, 28, 32, 36, 47, 91].

The aerobic glycolysis is an important pathway by which cells in the body could generate energy using glucose as main fuel source, whereas glutamine becomes the secondary fuel source for carcinogenic cells [91]. Glucose, the primary fuel source after entering the cell, is metabolized to pyruvate by a multistep set of reactions called glycolysis [32]. In typical normal cells, this pyruvate undergoes oxidative phosphorylation (OXPHOS) in mitochondria through Krebs cycle (TCA cycle) to generate energy (ATP) in order to meet the energy demands of the cell; however if oxygen levels are low, pyruvate is converted into lactate in cytoplasm through the action of lactate dehydrogenase (LDH) enzyme [28, 44]. In glycolysis one glucose molecule is broken down into two molecules of pyruvate thus generating two ATPs by consuming NAD^+, whereas in OXPHOS one glucose molecule produces 30 ATPs by oxidation of NADH and FADH2, clearly stating that OXPHOS is more efficient than glycolysis [36, 139]. The main difference between cancer and normal cells dwells here. In cancer cells the pyruvate is converted into lactate even when an ample amount of oxygen is available [28]. For creation of new biomass such as nucleotides, lipids, amino acids, and nonessential amino acids, cancer cells require more nitrogen. The excess glucose that is generated is deviated to produce

nucleotides through pentose phosphate stunt (PPS) [32]. In multiple steps, PPS pathway by the action of malic enzyme generates NADPH reducing equivalents to produce more pyruvate. These NADPH reducing equivalents are required to produce acetyl CoA from citrate through the action of ATP-citrate lyase (ACL) in cytosol [25]. This production leads to synthesizes of fatty acids that are required for membrane production. Glutamine an essential metabolite acts as an intermediate in the bloodstream to transport reduced nitrogen and is also required for cell growth. This metabolite is utilized by tumor cells as secondary energy source because it plays a crucial role in uptake of essential amino acids and can replenish the TCA cycle by supplying carbon, and also through the action of malic enzyme, it can produce more pyruvate [24]. More NADPH in PPS pathway is produced by transactivation of TP-53-induced glycolysis and apoptosis regulator (TIGAR) by p53 oncogene. PI3K/Akt and Ras are activated through RTKs by stimulation of growth factor. RTK signaling to C-Myc activates many genes that are involved in lactate production and glycolysis [25, 28, 32, 47, 91].

The sequence initiation of angiogenesis and glycolysis in differentiating cells is arbitrated partly by triggering HIF-1. HIF-1 target genes are mainly the genes that are intricate in the glucose uptake and glycolysis. HIF-1 controls expressions of phosphoglycerate kinase 1, aldolase A, and pyruvate kinase M in the glycolytic pathway, as well as expression of the glucose transporters (GLUT1 and GLUT3), which facilitate uptake of glucose by the cells [62]. It also induces adaptive responses to ensure that the cells should have sufficient energy levels and thus allowing their survival in a hostile environment [77, 140].

10.3 HIF-Associated Pathways

Although HIF-1α transcription is constant, the mRNA translation and transactivation activity of HIF-1α are induced by associated pathways and cell surface receptors of tyrosine kinases and G protein-coupled receptors. In pseudohypoxia circumstances, HIF-1α subunits are stabilized by a variety of oxygen-independent signaling and cellular stress events. In hypoxia condition, in response to growth factor stimulation, the HIF-1α levels increase in a specific manner. If hypoxia is associated with decreased degradation of HIF-1α, growth factors, cytokines, and other signaling molecules stimulate synthesis of HIF-1α through stimulation of the phosphatidylinositol 3- kinase (PI3K) or mitogen-activated protein kinase (MAPK) pathways [98].

Activation of phosphatidylinositol-4, 5-bisphosphate-3-kinase (PI3K)/AKT pathway has been shown to upregulate the HIF-1α protein translation. Under non-hypoxic conditions, due to extremely short half-life, HIF-1α protein expression is particularly sensitive to changes in the rate of synthesis. In the phosphatidylinositol-3-kinase (PI3K) pathway, binding of a growth factor (e.g., insulin-like growth factor 1, IGF-1) to its cognate tyrosine kinase receptor activates PI3K by phosphorylation and stimulates the downstream serine/threonine kinase Akt (protein kinase B). This

stimulation subsequently phosphorylates mammalian target of rapamycin (mTOR), providing a link between the microenvironment and HIF signaling [118, 120]. mTOR increases protein translation and mediates its action by phosphorylation of the mRNA cap-binding protein eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP1). mTOR provide a potential mechanism for increasing HIF-1a levels under normoxic conditions by disrupting the integrity of 4E-BP1, which is essential for inhibiting cap-dependent mRNA translation. In hypoxic conditions mTOR may increase HIF-1α levels by the mechanism in which it occurs independently without eIF4E. Alternatively, mTOR induces protein translation by phosphorylation of p70 S6 kinase (S6K) which promotes ribosomal protein S6 phosphorylation, a substrate. This pathway is upset by a tumor suppressor protein (PTEN) which backs the phosphorylation of PI3K products.

In MAPK pathway, certain growth factors are involved in activation of RAS; this activation in turn stimulates RAS/RAF/MEK/ERK kinase cascade and induces HIF-1α transactivation-domain function. Growth factors activate the mitogen-activated protein kinase (MAPK) to phosphorylate MAPK (extracellular signal-regulated kinase, ERK). Activated ERK is then capable of phosphorylating p70S6K1, 4E-BP1, S6K, and MAP kinase interacting kinase (MNK) [107, 161]. MNK can also phosphorylate eIF-4E directly that activates the translation initiator factor together with mTOR by inhibiting the 4E-binding protein (4E-BP). These signaling events result an increased rate of HIF-1α protein synthesis through its effects on eIF4E. ERK and p70S6K1 are essential factors that are required for HIF-1α mRNA translation. ERK regulates HIF-1α synthesis and also plays a pivotal role in its transcriptional activation. ERK phosphorylates the coactivator CBP/p300, hence increasing HIF-1α/p300 complex formation, and thus stimulates its transcriptional activation function (Fig. 10.2) [7, 26, 67, 70, 97].

The von Hippel–Lindau protein (pVHL) pathway along with p53, a tumor suppressor gene which induces apoptosis by regulating proteins such as Bax, regulates the levels of HIF-1α. In environmental stress or DNA damage, p21 mediates p53 to cause growth arrest (Fig. 10.2). The murine double minute 2 (Mdm2) ubiquitin-protein ligase mediates ubiquitination and proteasomal degradation of HIF-1α. Direct binding of the p53 tumor suppressor gene to the ODD domain of HIF-1α causes the ubiquitination and degradation [46]. It is evident that absence of p53 tumor suppressor gene in certain types of tumor cells enhances HIF-1α levels. In hypoxic tumors, mutations in tumor suppressor genes cancel the Mdm2-mediated degradation of HIF-1α. It was studied that Hsp90 inhibitors such as geldanamycin (GA) could nullify HIF-1α levels even in cell lines lacking von Hippel–Lindau protein (pVHL) regardless of the availability of oxygen. Mutation of prolyl residues (p^{402} and p^{564}) in HIF-1α does not protect HIF-1α from geldanamycin (GA)-induced degradation, suggesting that Hsp90 degradation involves a novel E3 ubiquitin ligase [46, 131, 140].

Redox (reduction-oxidation)-dependent processes displays a vital role in the control of HIF-1α. Some studies have shown that generation of ROS can start both MEK/ERK and PI3K/Akt signaling pathways. This activation leads to enhanced HIF-1α expression in human cancers such as ovarian, prostate, and breast cancer

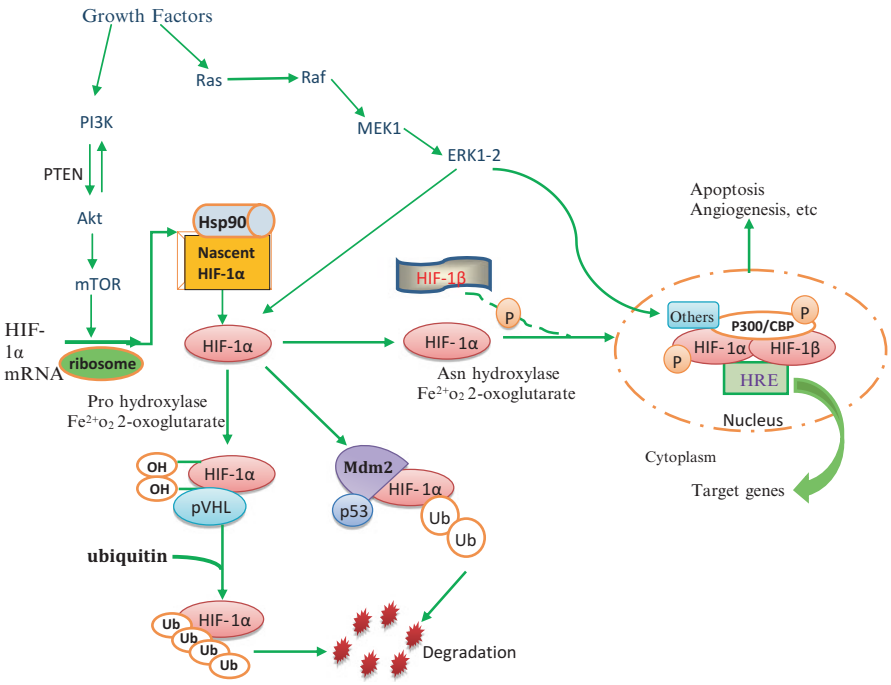

Fig. 10.2 Regulation of HIF-1α activity at different levels (This figure is adapted with some modifications from [95, 153])

[34, 171]. Breast carcinoma is characterized by persistent ROS generation. In human prostate cancer cells, carcinogens such as vanadium and arsenate were shown to elevate ROS and induce HIF-1α and VEGF expression through p70S6K1 activation. In human ovarian cancer cells, it is shown that p70S6K1 activation is stimulated by elevated epidermal growth factor (EGF) and its receptor (EGFR) which triggers H_2O_2 production.

Under hypoxia, mitochondrial ROS and intracellular secondary messengers such as CaM (calcium binding protein) levels increase and stimulate the accumulation of HIF-1α. CaM targets proteins (CaM kinase II, calcineurin, and actin) involved in the stimulation of transcriptional activity of HIF-1α expression. Thus, the inhibition of Ca2þ/CaM by a CaM-dominant mutant, Ca2þ/CaM antagonist such as HBC, or Ca2þ chelator downregulates the transcriptional activity of HIF-1, and subsequently angiogenesis is suppressed. The ROS levels in mitochondria increase through transfer of electrons from ubisemiquinone to molecular oxygen at the Q_0 site of complex III electron transport chain (ETC). HIF-1α activation is modulated by inhibiting its hydroxylation by the prolyl and asparaginyl hydroxylases. Mitochondrial ROS also induces signaling components of HIF-1α (ERK and p38 MAP kinase pathways) under hypoxic conditions. The activated ERK2 phosphorylates HIF-1α and increases its transcriptional activity [34, 42, 110].

10.3.1 HIF and Cell Cycle

Under hypoxia, there are different adaptive responses to lessen oxygen and nutrients for hypoxia-/hypoglycemia-regulated genes, which are involved in the cell cycle regulation. These genes are either HIF-1α dependent (p53, p21, Bcl-2) or HIF-1α independent (p27, GADD153). Hypoxia causes a HIF-1-dependent escalation in the expression of the cyclin-dependent kinase (CDK) inhibitors p21Cip1 and p27Kip-1and hypophosphorylation of retinoblastoma protein (Rb). Decreased activity of CDK complexes and hypophosphorylation of retinoblastoma protein regulate the cell cycle progression in response to hypoxia. HIF-1α activation may serve as a primary gatekeeper at the G1/S transition through at least two distinct mechanisms – the action of CKIs and another by cyclin E regulation. HIF-1α regulates cyclin E, not the cyclin A protein levels, but both may bind CDK2 and control its kinase activity dependent upon phase of cell cycle [15, 42, 43].

10.3.2 HIF and Cancer

HIFα is expressed in various types of cancers that include colorectal, liver, gastric, pancreatic, renal, gastrointestinal (IBD), esophagus, and many others. But mechanism and the factors that regulate the HIF1α expression remains poorly understood in cancer. Several studies demonstrated the associated mechanisms that activate the HIFα and their upstream or downstream factors. In this context, we explore recent updates on the impact of HIFα in different types of cancers.

HIF-1α and HIF-2α play a significant role and have overlapping and distant functions in inflammatory bowel disease (IBD) [154, 158]. IBD, a chronic inflammatory disease of the intestine, is characterized by repeated mucosa wounding and losing of intestinal epithelial barrier functions. It comprises two distinct pathological entities, ulcerative colitis (UC) and Crohn's disease (CD) [157, 158]. Immunohistochemical and immunostaining studies of surgical specimens from patients with IBD revealed higher vascular density in diseased tissue than in normal tissue [40].

Studies revealed that HIF was essential for restoration and intestinal barrier integrity [63]. Mouse models and cell studies demonstrated distinct functions for HIF-1α and HIF-2α and regulate diverse sets of genes to modulate the epithelial barrier [41, 92, 132, 154]. Regulation of HIF-1α and HIF-2α by different subset of genes also promotes disruption of intestinal tight junctions and increased barrier permeability. HIF-1α is a critical transcriptional factor in intestinal epithelial cells and is beneficial in regulating the epithelial barrier following inflammation. HIF-1α activation in intestinal epithelial cells decreases proinflammatory cytokines. Two mouse models of colon cancer, a sporadic and a colitis-associated colon cancer model, were assessed and proved that activation of HIF-1α in intestinal epithelial cells did not result in spontaneous tumor formation. HIF-2α activates several proinflammatory mediators and is important in wounding response, whereas its activation increases inflammation [157, 158, 168].

Pharmacologic inhibition of prolyl hydroxylases (PHDs) primes more vigorous activation of HIF-1α rather than HIF-2α. PHD inhibitors activate HIF-1α and HIF-2α in pulsatile manner and protect acute colitis in murine models. DMOG, a pan-hydroxylase inhibitor, activates the HIF pathway by mimicking hypoxia through the inhibition of hydroxylase activity, leading to stabilization and transactivation of HIF-1α [22]. AKB-4924, a HIF-1-specific prolyl hydroxylase inhibitor (PHDi), enhances innate immunity by robustly activating HIF-1α [61]. FG-4497, a novel PHD inhibitor, provides a protective adaptation in murine TNBS colitis [114].

HIF-1α is a critical protein in the development of colorectal cancer (CRC) [84]. Various studies have reported the role of HIF-1α in angiogenesis and tumor progression via regulation of VEGF in human colorectal carcinoma [75]. In colon cancer HIF isoforms have different cellular functions. In human colon cancer tissues, expression of HIF-1 α and, to a lesser extent, HIF-2 α was linked to upregulation of VEGF and tumor angiogenesis [52]. Overexpression of HIF-1α was found in the tissue of stage III and stage IV lymph nodes and liver metastases [13]. HIF-1α expression was strongly observed in the epithelium around the necrosis region of tumor compared to normal mucosa suggesting a significant correlation of HIF-1α expression along with CXCR4, VEGF, and microvessel density. Immunohistochemical studies of tumor cells in colon cancer cases by Wu et al. [152] also indicated that HIF-1*α* expression correlates with tumor TNM stage, lymph node status, tumor invasion, and distant metastases. JMJD2B upregulates hypoxia-inducible genes involved in cancer cell proliferation, apoptosis, cell cycle arrest, and invasion through specifically demethylating the H3K9me3 on their promoters. Study by Fu et al. [33] suggested a significant role of JMJD2B in CRC tumorigenesis and progression in HIF-1α-dependent manner under hypoxia. Activation of HIF-1α results in increasing transcription of STAT-3 and HSP90 in the CRC cell lines. This interaction between HIF-1α and STAT-3 in the CRC cell lines is dependent on the presence of an active HSP90 [35]. HSP90 in HCC cells regulated the levels of HIF-1α by inhibiting the ubiquitination and proteasomal degradation of HIF-1α. Further studies also analyzed a positive correlation between HSP90 and HIF-1α, with statistical significance, showing they may exert a synergistic effect on the occurrence, development, invasion, and metastasis of colorectal cancer [88, 155]. The results by Zhang et al. [167] and Zhang et al. [169] suggest that HIF-1α enhances EMT and cancer metastasis by binding to ZEB1 promoter in CRC and proposed a novel molecular mechanism for HIF-1α-inducing epithelial–mesenchymal transition (EMT) and cancer metastasis. LRG1 plays a crucial role in the progression of CRC by regulating HIF-1α expression thereby inducing VEGF-A expression and EMT markers of E-cadherin, VDR, N-cadherin, α-SMA, vimentin, and Twist1. In human CRC cells, HIF-1α under hypoxia induces B-cell CLL/lymphoma 9 protein (BCL-9) expression, an important underlying mechanism for increased BCL-9 expression [135].

In esophageal squamous cell carcinoma, HIF-1α expression levels significantly correlates with the expression of VEGF protein and with initial response to concurrent CRT. HIF-1α expression strongly apparent within nuclei and/or cytoplasm of

tumor cells and its expression are also found to be different in two separate tumor microenvironments: SCCs and ACs of the esophagus cancer proposing a different mechanism for HIF-1α expression in esophagus cancer [45, 96, 106].

Under hypoxic conditions, ERK1/2 phosphorylates and activates HIF-1α in pancreatic cancer cells. This activation contributes the $ABCG_2$ expression by inducing binding of HIF-1α to target promoter region for transcription [51]. Recent findings in pancreatic cancer patients indicated that HIF-2α induces cell migration, invasion in vitro, and regulated E-cadherin and MMPs protein expression; these are vital to epithelial–mesenchymal transition (EMT). It is regulated by binding of Twist2 protein to E-cadherin promoter; this indicates HIF-2α may act as an effective therapeutic target for prevention of pancreatic cancer [159].

HIF-1α is an important mediator and also acts as potential target for treatment of gastric cancer. The overexpression of HIF-1α in human gastric cancer proves the fact of it being a potential target. While regulating VEGF expression in cancer cells, it also plays a major role in the formation of complex proangiogenic microenvironment in tumors, and thereby affecting vessel morphology and vessel function. The in vitro studies in metastatic human gastric cancer cells evidenced that HIF-1α was not required for cellular proliferation. The inactivation of the HIF-1α activity by 2ME significantly reduced migratory, invasive, and adhesive features of gastric cancer cells. Inhibition of its function has proven the antitumor efficacy in rodent models and angiogenesis. In human gastric cancer cells, inhibition of HIF-1α activity by transfection with a construct expressing a dominant-negative mutant version of HIF-1α (pHIF-1αDN) that dimerizes with HIF-1β to form HIF-1 complexes that cannot activate transcription leads to impaired gastric tumor growth, angiogenesis, and vessel maturation [115, 131]. HIF-1α also regulates transcription factors (NF-κB1, BRCA1, STAT3, STAT1) and their corresponding network genes (MMP1, TIMP1, TLR2, FCGR3A, IRF1, FAS, and TFF3) that were associated with hypoxia, inflammation, and immune disorder in gastric cancer [145]. In the recent study, it is revealed a novel mechanism in three GC cell lines, 44As3, 58As9, and MKN45, and the integrity of mitochondrial autophagy (mitophagy) might determine the aggressiveness of cancer via the mitochondrial ROS (mtROS)/HIF-1α interplay under hypoxic conditions [127]. Relative mRNA expression of miR-421 (microRNAs), a crucial factor in carcinogenesis, was found to be upregulated by HIF-1α in gastric cancer tumor tissues [38]. Low expression of microRNA-186 (miR-186) facilitates aerobic glycolysis and suppresses cell proliferation induced by HIF-1α in gastric cancer cell lines. The in vivo xenograft tumor studies demonstrate that the miR-186/HIF-1α axis has an antioncogenic role in gastric cancer [86]. The in vitro and in vivo results revealed that dextran sulfate (DS) may reduce tumor metastasis through inhibition of HIF-1α and ITGβ1 expression in gastric cancer cells [156]. In hypoxic gastric cancer cells, angiopoietin-like protein 4 (ANGPTL4), a hypoxia-inducible gene expression, is independent of HIF-1α [73]. Expression of HSP60 or HIF2α serves as predictive marker for diagnosis of gastric cancer. In gastric cancer cells, HSP60 or HIF2α inhibition induce apoptosis and suppresses cell mobility by negative relation of MEK/ERK signaling [138].

10.3.3 HIF Pathway Inhibitors

Research is currently focused to target HIF involved pathways, and several drugs have been developed by considering the fundamental role of HIF and the analogs in the activation of various pathways involved in tumor progression in several cancers. Based on the mechanism of action, HIF inhibitors can be divided into the agents that modulate HIF1α *(1)* mRNA expression, *(2)* protein translation, *(3)* protein degradation, *(4)* DNA binding, and *(5)* transcriptional activity. The inhibitors representing each group are depicted in Fig. 10.3 and discussed below and listed in Table 10.1.

In diverse human cancer cell lines, the elevation of HIF-1α protein is by PI3K/Akt/mTOR signaling pathway. Various compounds for inhibiting PI3K/Akt/mTOR signaling pathway are under the exploitation stage, and few compounds are in clinical trials. Inhibitors *wortmannin*, *LY294002*, *GDC-0941*, and *PI-103* specifically inhibit PI3 kinase in dose-dependent manner [105]. FDA-approved drugs like *rapamycin* and its chemical derivatives (*temsirolimus* and *everolimus*) have more potency to target mTOR and inhibit the protein translation of HIF-1α at cellular levels [113].

Glyceollins, a set of phytoalexins present in soybean, potentially inhibit the HIF-1α synthesis and decrease stability by blocking the PI3K/AKT/mTOR pathway and interaction of Hsp90 with HIF-1α [81].

TSL-1, an agent in aqueous extracts of *Toona sinensis* (TS) leaves, which induces apoptosis via mitochondria-dependent pathway. TSL-1 stops cell division in G0/G1 phase via the decrease in cyclin D1, cyclin-dependent kinases (CDK2 and CDK4), and induced p53 expression. TSL-1 suppresses progression of cell cycle and motility through phosphorylation inhibition of JAK2/stat3, Akt, MEK/ERK, and mTOR. TSL-1 also inhibits p21, HIF-2α, c-*Myc*, VEGF, and MMP9 expressions and its anti-migration activity [19].

EZN-2968, an antisense oligodeoxynucleotide that precisely targets HIF-1α. A trial with administered EZN-2968 in patients with advanced solid tumors observed modulation of HIF-1α mRNA, protein, and its target genes [55]. In MCF-7 xenografts, *aminoflavone*, a potential therapeutic target for several human diseases, inhibited HIF-1α protein accumulation and expression of target genes [137].

GL 331, a topoisomerase II inhibitor, suppresses tumor-induced angiogenesis. In CL1-5 cells treated with GL331 downregulates HIF-1alpha expression through transcriptional repression. It also exerts cytotoxic effects on the glioma cells [16, 20].

Camptothecins (CPTs) analogs, topotecan and irinotecan, are active in different human tumors and shown significant anticancer activity against various tumors by inhibiting DNA topoisomerase I. *Topotecan* is the approved agent using in the treatment of lung cancer [37]. *Irinotecan* is a cytotoxic drug used for the patients suffering with colorectal cancer (CRC) in advanced stage. *SN-38* (10-hydroxy-7-ethyl-camptothecin) is the active metabolite of irinotecan prevents re-ligation of single-stranded DNA breaks induced during the DNA synthesis [37, 90]. These agents have shown the antitumor activity in xenograft model by inhibiting HIF-1α

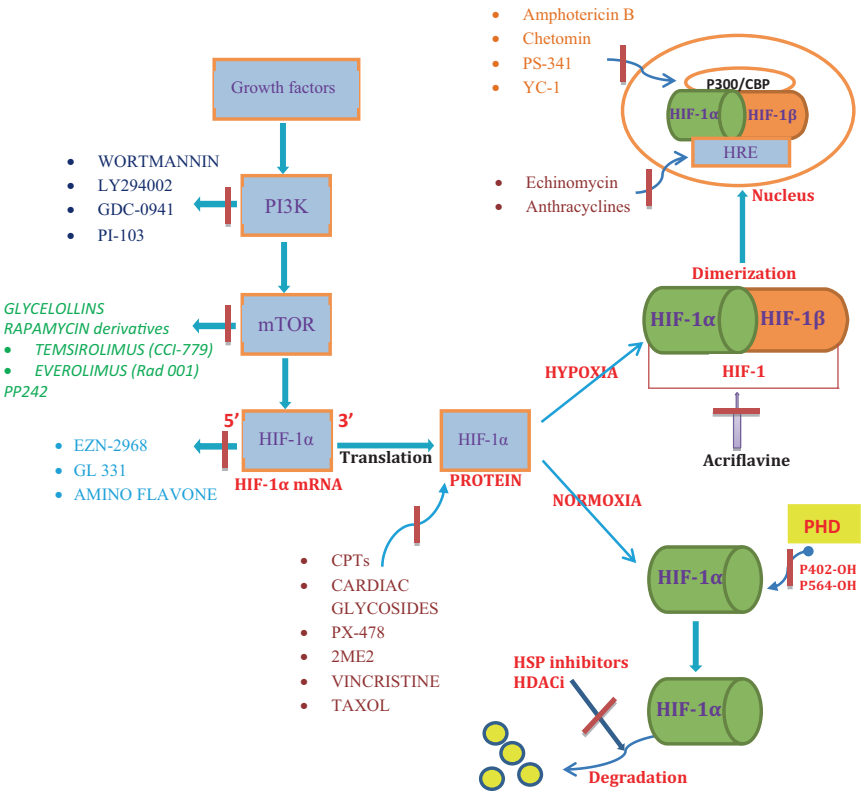

Fig. 10.3 Inhibitors that modulate different HIF-1α pathways

Table 10.1 Classification of HIF-1α pathway inhibitors and their molecular targets

Inhibitory mechanism	Target	Compound
PI3/AKT/mTOR inhibitors	PI3K	Wortmannin
		LY294002
		GDC-0941
		PI-103
	AKT/mTOR, Hsp90	Glyceollins
		Toona sinensis (TSL-1)
	mTOR	Rapamycin derivatives • Temsirolimus (CCI-779) Everolimus (Rad 001) PP242
mRNA expression	HIF-1α mRNA	EZN-2968
		GL 331
		Amino flavone
Protein translation	Topoisomerase I (top-1) inhibitor/ HIF-1α accumulation inhibitor	Camptothecins (CPTs) • Topotecan (NSC-609699) • (PEG-SN 38) • SN-38 Irinotecan

(continued)

Table 10.1 (continued)

Inhibitory mechanism	Target	Compound
	Topoisomerase II inhibitor	GL-331
	HIF-1α/mTOR-independent mechanism	Cardiac glycosides • Digoxin • Ouabain • Proscillaridin Strophanthidin glycoside
	HIF-1α/pVHL and p53-independent mechanism	PX-478
	Disrupts tumor interphase microtubules	2ME2 (2-methoxy-estradiaol) derivatives • ENMD-1198 • ENMD-1200 • ENMD-1237 Vincristine
		Taxol
HIF-1α degradation	HSP90 inhibitors	Geldanamycin derivatives • 17-AAG • 17-AG • 17-DMAG Radicicol derivatives • KF58333 • Apigenin IPI-504 (retaspimycin)
		Y-632
	FIH	YC-1
	Farnesyl transferase inhibitor	SCH66336
	Histone deacetylase inhibitors (HDACi)	Sirtuin1 (SIRT1)
		FK228 (romidepsin)
		Trichostatin A (TSA)
		LW6
		LAQ824
		LBH589
	TRX-1 signaling (thioredoxin-1)	PX-12
		Pleurotin
		AJM 290
		AW 464
HIF-α/HIF-1β dimerization inhibitors	HIF-1α/2α PAS B-domain	Acriflavine
	HIF-2α PAS B-domain	PT-2385
Transcriptional activity	p300 recruitment	Chetomin
		Bortezomib (PS-341)
	FIH interaction and p300 recruitment	Amphotericin B
	Hsp70	Triptolide
	Histone acetylation with repression of p300	FM19G11
DNA binding	HRE	Echinomycin
		Anthracyclines derivatives Doxorubicin (DXR) Daunorubicin (DNR)

accumulation. Clinical trials of these compounds are under progress to provide evidence as anticancer activity agents.

Cardiac glycosides, a group of natural products used in cardiac congestion and cardiac arrhythmias treatment. Recent studies suggested that cardiac glycosides have potential characteristic properties for the treatment of cancer [100]. Cardiac glycosides also inhibit cancer cell proliferation at nanomolar concentrations [117]. For example, *strophanthidin glycoside*, an organic solvent extract from *Crossosoma bigelovii*, showed the HIF-1α translation inhibitory effect [68].

Digoxin, a cardiac glycoside extracted from the foxglove plant, having antitumor activity against many cancers including lung, colon, prostate, and ovary. It shows activity through Erk and stress response pathways [30]. It exerts antitumor properties through antiproliferative and apoptosis mechanisms in HepG2 cell line cultured with different concentrations of digoxin [133]. Digoxin when treated also has shown to prolong tumor latency and hampers tumor xenograft growth in mice. It also inhibits HIF-1α expression and its target genes *VEGF*, *GLUT1*, *HK1*, and *HK2* [166]. Digitoxin in H1975 cells showed a significant cytotoxic effect by causing G2 phase arrest and suppressed microtubule polymerization through decreasing α-tubulin [170].

Ouabain is another cardiac glycoside used as novel anticancer HIF-1α antagonist. It can regulate HIF-1α translation and affects neither HIF-1α mRNA levels nor protein degradation. Studies revealed that inhibitory effect of ouabain on HIF-1α protein synthesis is by eIF4E rather than mTORC1, eIF2α signaling, or Na(+)/K(+)-ATPase inhibition. Mechanistically, ouabain straightly binds to eIF4E and disrupts association between IF4E/eIF4G complex rather than eIF4E/mRNA complex both in vitro and in vivo, finally suppressing the intracellular CAP-dependent translation [14].

Proscillaridin A exerts its cytotoxic activity by targeting both topoisomerase I and II enzymes simultaneously. In human fibroblasts it elevates intracellular Ca2+ concentration, activates caspase-3, and induces apoptosis relatively at high concentration. It exerts the antiproliferative and apoptotic activity at nanolevel drug concentrations (30 and 100 nM) [10, 151].

PX-478 (*S*-2-amino-3-[4′-*N*,*N*,-bis(chloroethyl)amino] phenyl propionic acid *N*-oxide dihydrochloride) decreases Hif-1α levels in both in vitro and in vivo by suppressing mRNA and blocking translation. PX-478 inhibitory mechanism is independent of pVHL or p53. This drug inhibits HIF-1α levels and transactivation in a variety of cancer cell lines including HT-29, PC-3, DU-145, MCF-7, Caki-1, and Panc-1. The effect of PX-478 is limited to hypoxia, as baseline levels of vascular endothelial growth factor is not altered under normoxic conditions [69, 149]. A recent study showed that PX-478 significantly decreased or inhibited extra skeletal bone formation by inhibition of Hif1α. This finding indicates that Hif-1α represents a promising target to prevent and treat pathologic extra skeletal bone or heterotopic ossification (HO) [2].

2-Methoxyestradiol (2ME2) is a natural estrogen metabolite having antiangiogenic, antiproliferative, and pro-apoptotic drug activities. It culminates induction of apoptosis by diverse cellular effects including microtubule disruption, commencement of signal transduction pathways, and generation of reactive oxygen species

[102]. $2ME_2$ targets apoptosis in rapidly proliferating cells of both the tumor cell and endothelial cell compartments and inhibiting blood vessel formation. The ability of $2ME_2$ to inhibit metastatic spread in several models adds to its therapeutic value for cancer treatment at various stages of the disease. Many genes regulating cell death and repression of growth/survival machinery were also induced transiently in multiple myeloma (MM) cells. Cells under normoxia and hypoxia conditions when exposed to 2-ME reduced mRNA expression of HIF-1α and HIF-2α were observed [4, 8]. 2ME2 significantly induced apoptosis in HIF-1α overexpressed AML cells by suppressing the expression HIF-1α. In vivo 2ME2 has been shown to downregulate HIF-1α target genes, such as for VEGF, phosphoglycerate kinase, glucose transporter-1, GLUT1, and HO-1 [8, 172]. In clinical trials the 2ME2 was noticed to target both tumor cells and neovasculature in preclinical models. The report of first Phase I trials of 2-methoxyestradiol, alone and in combination with docetaxel, was well tolerated in patients with metastatic breast cancer (MBC) [23, 53]. 2ME2 analogs (*ENMD-1198*, *ENMD-1200*, and *ENMD-1237*) with superior properties have been identified [76, 109, 128].

Few compounds like *Taxol* and *vincristine* also inhibit protein translation of HIF-1α by disrupting tumor interphase microtubules. Taxol induces static magnetic field (SMF) effect on microtubules to cause abnormal mitotic spindles that delay cell exit from mitosis [93]. Vincristine clinical trials in adults have demonstrated clinical activity without dose-limiting neurotoxicity. The safety, tolerability, and activity of vincristine might be reasons for FDA approval for adults with relapsed acute lymphoblastic leukemia [126].

Hsp90 antagonists induce degradation of HIF-1α proteins because binding of HSP90 to HIF-1α promotes HIF-1α activity [95]. Heat shock protein 90 is a 90-kDa ATPase-dependent molecular chaperone which is a ubiquitously expressed and highly conserved. The expression of Hsp90 in cancer cells is generally higher than that in normal cells. The Hsp90 proteins include a wide variety of signal-transducing proteins that regulate cell growth and differentiation; these are like protein kinases and steroid hormone receptors [101]. Hsp90 inhibitors may be organ-specific and should be carefully monitored, and they have some effects on cell adhesion-associated molecules. Hsp90 has long been regarded as an emerging drug target for a wide spectrum of cancers. Heat shock protein inhibitors are a diverse group of agents which have been verified to have pro-apoptotic effects on malignant cells [3, 129]. The high sensitivity of the inhibitor in cancer cells is proposed due to the formation of the Hsp90–cochaperone–client super complex that is highly unstable and possesses high ATPase activity [89]. Initial development of hsp90 inhibitors, *geldanamycin* and *17-AAG* (17-N-allylamino-17-demethoxygeldanamycin), showed nearly 100-fold higher binding affinity in cancer cells than in normal cells. The effect is restricted by hepatotoxicity and need for solvent carrying agents. On the other hand, *retaspimycin*, or *IPI-504*, a derivative of geldanamycin and 17-AAG, is highly soluble in water and has shown promising activity in gastrointestinal stromal tumor in Phase I/II trials [28]. Currently, Phase I/II trials are underway in the evaluation of dosing schedules and activity for IPI-504 in breast cancer [49, 146].

Y-632, a novel pyrimidine derivative, Hsp90 function suppressed through induced thiol oxidation and disruption of Hsp90–Hsp70/Hsp90 organizing protein complex. This further induces inhibition of cell adhesion, G_0/G_1 cell cycle arrest, and apoptosis [147].

17-DMAG (17-dimethylaminoethylamino-17-demethoxygeldanamycin), another geldanamycin derivative of the HSP90 inhibitor, stalled the viability of human lung cancer cell lines via reduced expression of client proteins, including the proto-oncogene RAF-1. 17-DMAG treatment in human SCLC cell line SBC-5 inhibited the formation of metastatic sites in both liver and bone [134].

KF58333, a novel oxime derivative of radicicol, binds to Hsp90 and destabilizes its associated signaling molecules. KF58333, without altering the HIF-lα mRNA expression, resulted in significant downregulation of HIF-1α under hypoxic conditions. KF58333 also inhibited tumor angiogenesis and vascular endothelial growth factor (VEGF) secretion in a dose dependently [74].

Apigenin a naturally occurring flavonoid exhibits antiproliferative and antiangiogenic activities. Apigenin inhibits VEGF expression via degradation of HIF-1α and interferes with the function of Hsp90 in endothelial cells of human umbilical artery. In pancreatic cancer cells, it inhibits HIF-1α, GLUT-1, and VEGF mRNA and protein expression in both normoxic and hypoxic conditions [99, 108]. It inhibits the growth of UV-induced skin cancer and thyroid cancer cells by activating AMP-activated protein kinase (AMPK), leading to suppression of basal mTOR activity. This suppression of mTOR activity inhibits cell proliferation and arrests the cell cycle at G2/M phase. Apigenin is shown to reduce CDK4 and cyclins D1 and A, but not the cyclin E, CDK2, and CDK6 protein expression. Its growth inhibitory effects are mediated by targeting signal transduction pathways and emerging as a promising anticancer agent [11, 163].

YC-1 [3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole], a HIF-1 inhibitor, acts by reducing HIF-1α expression [50, 104]. YC-1 inhibits HIF-1α expression via the FIH-dependent CAD inactivation as well as protein downregulation [83]. YC-1 suppresses the hypoxic responses by posttranslationally inhibiting HIF-1α accumulation and exhibits novel antiangiogenic anticancer agent properties [21, 160].

SCH66336, a small molecule farnesyl protein transferase inhibitor that shares a common tricyclic nucleus and competes with peptide/protein substrates for binding to farnesyl protein transferase [87]. It also inhibits the interaction between HIF-1α and Hsp90 to inhibit VEGF production in NSCLC and HNSCC cells [48].

Under hypoxia, histone deacetylase (HDAC) inhibitor enhances p53 and von Hippel–Lindau expression and thereby stimulates angiogenesis. This stimulation leads to downregulation of HIF-1α and VEGF thus promoting HIF-1α degradation [65]. Stress-responsive genetic regulator, sirtuin 1 (Sirt1) gene expression, increases in a HIF-dependent manner, and loss of HIF signaling affects Sirt1 deacetylase activity during hypoxia [17]. SIRT1 downregulation was due to decreased NAD levels; this allowed the acetylation and HIF-1α activation. SIRT1 deacetylase and the HIF-1α transcription factor act as redox and oxygen sensors, respectively, whereas hypoxic HIF-1α stabilization requires SIRT1 activation [85]. Sirt1 regulates HIF-1α and HIF-2α by deacetylating Lys674 of HIF-1α and HIF-1α K674 and

HIF-2α K741 by PCAF and CBP, respectively. HIF-1α deacetylation blocks the recruitment of p300 to HIF-1α. This blockade consequently inactivates HIF-1α; represses HIF-1 target genes including VEGF, GLUT1, and MMP2; and finally promotes cancer cell invasion [58, 165].

Trichostatin A (TSA), an antifungal antibiotic showing histone deacetylase (HDAC) activity. In vitro and in vivo studies in human breast cancer and squamous cell carcinoma cell lines assessed the antitumor efficacy and toxicity of TSA [141]. It induced caspase-dependent or caspase-independent apoptosis according to cell types. In gastric cancer cells, TSA increased TRAIL-induced apoptosis [82]. In HSC-3 cells, TSA enhanced the Bim protein expression levels by dephosphorylating ERK1/2 pathway. In Ca9.22 cells TSA damaged MMP and increased cytosolic apoptosis-inducing factor (AIF) [54].

LW6, a small compound, inhibits the HIF-1α accumulation. LW6 degrade HIF-1α via VHL expression, with modifications of P402A and P564A, at hydroxylation sites in the oxygen-dependent degradation domain (ODDD), without affecting the activity of prolyl hydroxylase (PHD) [78]. A recent data revealed that angiogenesis suppression through LW6 inhibited HIF-1α stability via direct binding with calcineurin B homologous protein 1 (CHP1) [64].

LAQ824 and *LBH589*, the inhibitors of histone deacetylase (HDACi) and established cancer therapeutic agents. Both engage in the intrinsic apoptotic cascade which does not require p53. Mitochondrial damage is the key event for LAQ824 and LBH589 to mediate tumor cell death [31].

Thioredoxin-1 (Trx-1), a redox protein usually overexpressed in many human tumors. It increases aerobic and hypoxia-induced HIF-1α protein in the cells and leads to expression of HIF-regulated genes. Trx-1 controls multiple aspects of cell growth and survival [57].

PX-12 (1-methylpropyl 2-imidazolyl disulfide), an irreversible inhibitor of Trx-1. This is currently under clinical development [5, 112]. PX-12 decreases plasma VEGF levels and contributes to the antitumor activity [6]. PX-12 acts independently and increases nuclear Nrf2; this one interacts with PMF-1 to increase SSAT1 expression, and further SSAT1 binds to HIF-1α and RACK1, finally resulting in oxygen-independent HIF-1 ubiquitination and degradation [66].

Pleurotin, a growth inhibitory and antitumor agent shown to decrease HIF-1α protein levels, HIF-1-*trans*-activating activity, VEGF formation, inducible nitric oxide synthase, and the expression of downstream target genes [150].

AJM290 and *AW464* (quinols), two novel anticancer drugs that inhibit Trx-1 function and also inhibit HIF-1α CAD transcription activity and DNA binding. In contrast to other Trx inhibitors, these agents also inhibit HIF degradation [57].

Small molecules can inhibit HIF-1 dimerization and potentially inhibit the tumor growth and vascularization.

Acriflavine antagonizes HIF upon binding to the HIF-α PAS-B domain. It directly binds to HIF-1alpha and HIF-2alpha and suppresses dimerization of HIF-1 and transcriptional activity. It also induces cell death under hypoxic conditions and reduced the expression of the HIF-1 target genes *VEGF, PTGS2*, and *EDN1* [12, 80].

PT2385, HIF-2α inhibitor allosterically binds to PAS-B domain of HIF-2α, thereby preventing HIF-2α dimerization with ARNT (aryl hydrocarbon receptor nuclear translocator, HIF-1β). This results in decreased transcription and expression of HIF-2α downstream target genes, many of which regulate tumor cell growth and survival. Blocking HIF-2α reduces the proliferation of HIF-2α-expressing tumor cells. PT2385 is currently under evaluation in Phase I clinical trials for the treatment of clear cell renal carcinoma [144].

In hypoxic conditions, HIF-1*α* is translocated into nucleus, heterodimerizes with HIF-1*β*, and binds to hypoxia response element (HRE) DNA sequence. *Chetomin*, a metabolite complex, produced by several fungi of the genus *Chaetomium*, disrupts the ability of tumors to adapt to hypoxia by blocking the HIF pathway and reduces hypoxia-dependent transcription. Chetomin targets transcriptional coactivator p300 by disrupting its CH1 domain and impairs the interaction of between HIF-1α and p300 [130, 142].

Bortezomib, the first proteasomal inhibitor (PI) and also confirmed antitumor activity-containing agent in clinical setting. Bortezomib attenuates the transcriptional activity and impairs tumor growth only of HIF-1, and not HIF-2. Bortezomib inhibits HIF-1α protein expression at the translational level under both normoxic and hypoxic conditions and its nuclear targeting through inhibition of PI3K/Akt/mTOR and MAPK pathways, respectively, by dephosphorylation of phospho-Akt, phospho-p70S6 K, and phospho-S6RP [1, 9].

Amphotericin B (AmB), an agent that interferes the HIF-1α expression through CAD-FIH. AmB represses the C-terminal transactivation domain (CAD) of HIF-1α, a target site of the factor-inhibiting HIF-1 (FIH). CAD-FIH interaction inhibits the recruitment of p300 through CAD of HIF-1α [162].

Triptolide possesses anticancer, antiangiogenesis, and drug-resistance activities. Triptolide suppresses HIF-1α through c-Myc-dependent mechanism. Triptolide treatment in SKOV-3 cells resulted in loss of function of HIF-1α protein transcriptional activity and reduced mRNA levels of its target genes [29, 173].

FM19G11, an agent that inhibits HIF-alpha protein expression and suppresses target genes of two alpha subunits in several tumor cell lines. FM19G11 reduces overall histone acetylation with significant p300 repression and behaves as a target gene of HIF2alpha at nanomolar range of FM19G11 inhibiting transcriptional and translational expression of Oct4, Sox2, Nanog, etc. [103].

Echinomycin (NSC-13502), a small molecule that binds in a sequence-specific manner in the DNA and shows dual effect on HIF-1 activity under normoxic and hypoxic conditions. It inhibits binding of HIF-1α and HIF-1β proteins to a HRE sequence. It suppresses cell growth and induces apoptosis with decreased mRNA expression of HIF1 targets, glucose transporter-1 (GLUT1), and B-cell CLL/lymphoma-2 (BCL2). This agent has failed as anticancer agent due to its dual effect [72, 143, 164].

Anthracycline and its chemical derivatives (doxorubicin (DXR) and daunorubicin (DNR)) are the topoisomerase inhibitor family that suppresses hypoxia-inducible factor-1 (HIF-1) transcriptional activity by obstructing its binding to DNA. These

agents are using widely in the prevention of tumors [116]. Doxorubicin (DXR) weakens the transcriptional activity of the HIF by inhibiting the binding of the HIF heterodimer to the consensus – RCGTG – enhancer element and downregulated HIF target lysyl oxidase (LOX) family members [136]. Anthracyclines also inhibit the endogenous HIF-1 target gene expression. In hypoxic cells the VEGF and GLUT1 mRNA levels were significantly decreased by DNR, and DXR, in a dose-dependent manner [79].

10.3.4 Future Approaches

The thrust is continuously inundated in identifying the novel metastasis-associated oncogenes and tumor suppressor genes. Several therapeutic approaches that target HIF and its associated factors in tumor progression are emerging continuously. Further studies are needed for answering how the cells sense hypoxia and how HIF-1α activation occurred along with other signaling pathways. In recent studies, researchers have focused on the determination of the pathways (pro-survival and apoptosis) activated in response to hypoxia in cancer cells, and further it is needed to analyze the hypoxia-response gene expression patterns to the levels such as apoptosis, angiogenesis, and metastasis in human cancer cells through microarray analysis and other high-throughput technologies.

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Activation of STAT3 in Gastric Cancer Development

11

Kishore Kumar Jella

Abstract

The transcription factor STAT3 important for regulating factors that involve in the modulation of gene expression. The factors such as growth factors and cytokines regulate homeostasis of both epithelial and stromal cells. Deregulation of STAT3 activation is important in the initiation of transformation of various cancers that are epithelial in origin. This chapter mainly focuses on STAT3 activation in gastric cancer and its progression with the activation of cytokines; it also discusses how STAT3 associated with progression of gastric cancer. The studies have shown the association of deregulated JAK/STAT in the development of solid cancers especially in gastric cancer. Therapeutic use of STAT3 may prevent the development of gastric cancer. Inhibitors of STAT3 are emerging as a potent drug in treatment options. STAT3 inhibitors are under evaluation in various clinical trials, showing promising results for the treatment.

Keywords

Gastric cancer · STAT3 · JAK/STAT · *H. pylori* · CagA · TLR · EGFR

Abbreviations

ADAM-17	ADAM metallopeptidase domain 17
AP-1	Activator protein 1
Bcl	B cell lymphoma
Cag PAI	Cytotoxic-associated gene pathogenicity island
CagA	Cytotoxic-associated gene A

The original version of this chapter was revised. The book was inadvertently published without Abstracts and Keywords, which are now included in all the chapters. An erratum to this chapter can be found at https://doi.org/10.1007/978-981-10-6728-0_39

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DC	Dendritic cells
DCF	5-Flurouracil
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
EPISA	Mutation of tyrosine to serine
EPIYA	c Terminal Glu-Pro-Ile-Tyr-Ala
ERK	Extracellular signal-regulated kinase
FOLFRI	5-Fluorouracil, leucovorin, and irinotecan
gp	Glycoprotein
H. pylori	*Helicobacter pylori*
HB-EGF	Heparin-binding epidermal growth factor
HER2	Human epidermal growth factor receptor-2
HIF-1	Hypoxia-inducible factor 1-alpha
IEC	Intestinal epithelial cells
IFN	Interferon
IL	Interleukin
IRAK1	Interleukin 1 receptor associated kinase
JAK	Janus kinase
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteases
MPL	Myeloproliferative leukemia
MyD88	Myeloid differentiation primary response gene 88
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OS	Overall survival
PIAS	Inhibitor of activated STATS
PTPs	Protein tyrosine phosphatase
REG	Member of regenerate family
SHP2	*SHATTERPROOF*2
SOCS	Inhibitor of cytokine signaling
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator protein
T_H	T helper cells
TLR2	Toll-like receptors
TNM	Tumor node metastasis
Tregs	The regulatory T cells
VEGF	Vascular endothelial growth factor

11.1 Introduction

Gastric cancer is considered as the third most common cancer deaths in the world. It remains very difficult to cure especially in Western countries, due to its nature and advancement. Especially in the United States, stomach malignancy is considered as the 15th most common type of cancer [1]. In the year 2017, according to the American Cancer Society, nearly 28,000 cases of stomach cancer have been reported, and its more prevalent in males compared with females (17,750 in men

and 10,250 in women) [1]. The median age for the diagnosis is considered as 69 years; out of every ten patients around six patients would be with an age of 65 or older. According to the reports of the World Health Organization in 2012, gastric cancer accounted for 723,000 deaths around the globe. The disease is more prevalent in Asia and some parts of South America and lowest in North America. Record death rates were reported in the countries like the former Soviet Union, Chile, Japan, and South America [1].

Transcription factors play a vital role in the regulation of various cell functions such as activation, repression, and alterations of gene expression that are important for cell growth, development, and differentiation process. Activation of various transcription factors has been found to be associated with acquisition of resistance to gastric cancer treatment. The transcription factors that play a vital role in activation of oncogenes and tumor progression include NF-κB, HIF-1, AP-1, and STAT3. The protein STAT3 is hyperactivated in a wide variety of cancers; specifically it is well shown in head and neck cancer [2]. In gastric cancer, STAT3 protein is hyperactivated via a number of pathways that are associated with poor treatment and metastatic potential [3, 4]. The signaling of STAT3 is considered as central for the propagation cancer stem cells, which is important in cancer metastasis [3, 4]. Both cancer stem cells and hypermalignant cancer cells are metastatic, and they are highly tumorigenic; STAT3 inhibitors have shown an intended results in reducing the growth of cancer [4]; these inhibitors are considered as the most effective. The transcription factor STAT3 activates via a number of routes, and hence there are a number of strategies that are being employed to block these pathways.

11.2 STAT Proteins

STAT proteins are considered as a family of transcription factors [5]. It was first identified in the year 1994 [5, 6]. There are seven STAT proteins, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. These proteins are responsible for the generation of signals from growth factors or cytokines and helps in migration of signals from the plasma membrane to the cell nucleus for its activation [5]. The transcription factors are involved to regulate cell proliferation, differentiation, apoptosis, angiogenesis, and production of inflammatory responses [7]. Non-phosphorylated, inactivated STAT proteins are mainly present in cytoplasm, and they are phosphorylated/activated via cytokines through their receptors. The proteins such as STAT2, STAT4, and STAT6 play a major role in development of T cells and signaling process involved interferon gamma. STAT3 and STAT5 are often involved in the development of cancer progression among humans [5].

STAT proteins share common features in their structure, and the size of STAT proteins ranges from 750 to 847 amino acids (90–155 kDa). Each protein is comprised of N-terminal region for dimerization and C-terminal region for transcription activation through serine residues, a coiled domain for interaction with other proteins, DNA-binding domain to determine the selectivity of STAT proteins and a linker domain, and a src domain for both binding and dimerization [5]. Among all

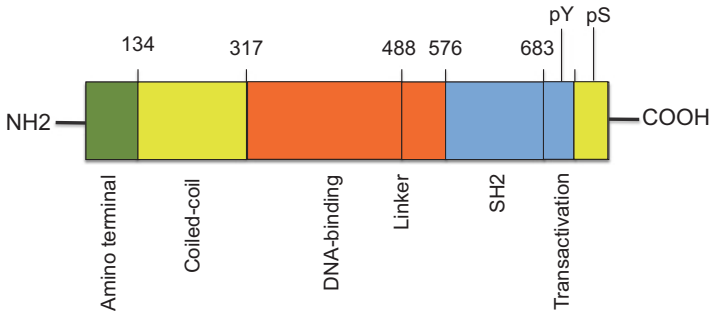

Fig. 11.1 Structure of STAT3 protein

STAT proteins, there is Y located approximately at aminoacid position 700 near SH2 domain. Phosphorylation of Y domain is crucial for its dimerization, function, and nuclear localization that help in DNA binding [8]. STAT1, STAT3, and STAT5 are phosphorylated at its C-terminal end, which is critical for its transcriptional activation (Y727 in STAT3); phosphorylation at this site has both stimulatory and inhibitory effects on gene expression [5, 8, 9]. STAT3 protein is located on chromosome 17q21.31 [9] and its structure is depicted in Fig. 11.1.

11.2.1 STAT3 Signaling Pathway

The STAT3 signaling pathway can be activated via various number of ways. JAKs are activated upon binding to cytokine receptors (e.g., IL 6), via cross-phosphorylation pathway. Figure 11.2 represents the overview of STAT3 signaling pathway. JAK/STAT pathway involved a wide range of cellular and physiological process that includes cellular proliferation, immune responses, renewal of stem cells, and normal homeostasis [6, 10–12]. Deregulation of JAK/STAT pathway involves in a wide range of pathophysiological conditions of many human cancer and immune-related disorders [13].

The signaling cascade is conserved from slime molds to human, across the phyla [60]. This cascade was originally defined as signaling mediated by IFNα, IL-6, and IFN-γ. Along with these signals, attachment of immunomodulator to their receptors results in the activation and dimerization of specific receptors and further results in the phosphorylation of various kinases [14]. The receptors are activated upon phosphorylation by the activation of JAKs and act as a docking site for SH2 domain [15]. STAT protein, upon phosphorylation, translocates to the nucleus and acts a transcription factor, which in turn controls the expression of targeted genes in downstream process. This cascade is mainly regulated at various levels of signaling by three classes of protein molecules which are PTP, PIAS, and SOCS [16]. PTPs involve in the dephosphorylation of JAK, STAT, or its receptors and inactivate them; PIAS molecules inhibit the signaling process and prevent the binding of activated STAT dimers to their downstream targets [17]. SOCS molecules interfere with the recruitment of STAT to its receptors [16]. In mammalians, four members are

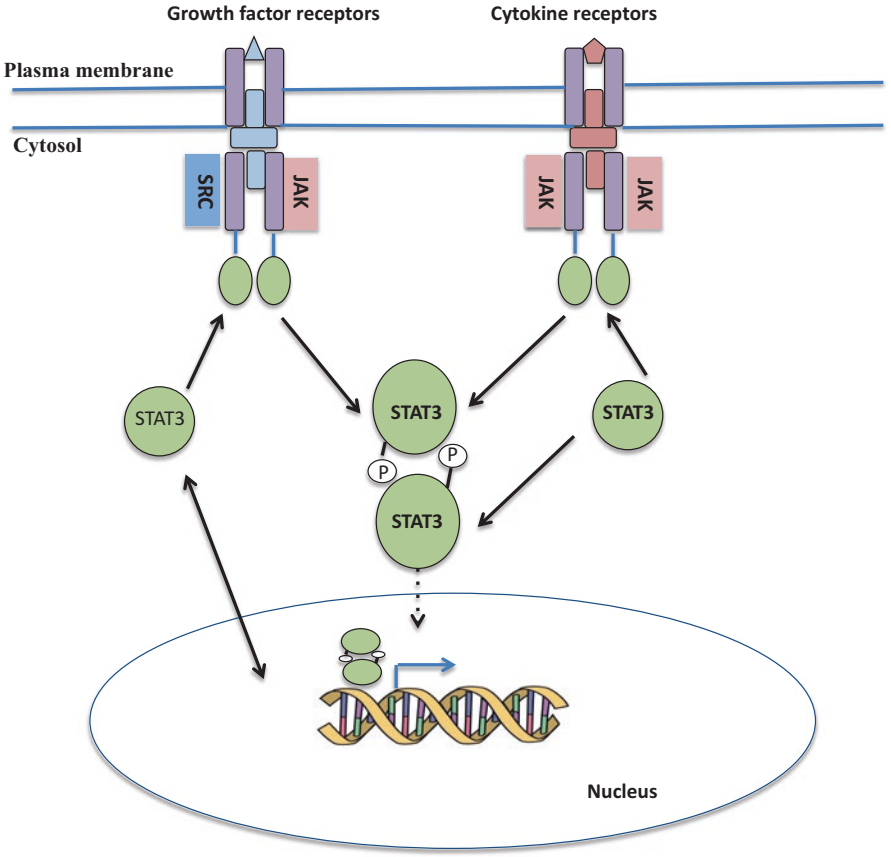

Fig. 11.2 Depicts JAK/STAT signaling pathway

included in JAK family, and they are JAK1, JAK2, JAK3, and TYK2. JAK family members, JAK1 to JAK3, are expressed ubiquitously, and JAK3 is expressed only in hematopoietic cells and involves in the development and regulation of hematopoietic cells [18]. The STAT family involves in various cellular processes, and it includes seven different members from STAT1 to STAT6, whereas STAT5 has both STAT5a and STAT5b [6]. STAT proteins are involved in transcription of various molecules that are involved in proliferation, differentiation, inflammation, and apoptosis. In recent years, JAK/STAT has been found to associate with stability of heterochromatin and epigenetic regulation such as DNA methylation and remodeling of chromatin [19].

Many scientists observed the continuous activation of both JAK and STAT signaling pathways in various cancers. It may be due to increased expression of cytokine and its receptors or decreased expression of its negative regulators [20]. In myeloproliferative disorders, activation of somatic mutations such as JAK2 or MPL

(encodes thrombopoietin receptors) was observed leading to persistent activation of both STAT3 and STAT5 [21, 22]. In pathogenesis of hepatocellular carcinoma, deletion of *gp*130 results in the continuous activation of STAT3 even without the presence of its ligand [69]. In various epithelial cancers, there is a continuous upregulation of JAK2/STAT3 signaling pathways with increase in STAT3 signaling resulting in tumorigenesis of various cancers [23]. The dephosphorylation of STAT3 by tumor suppressor PTP delta was found to frequently mutate in various cancers such as head and neck cancers, human glioblastoma, and lung cancer [24]. Deregulation of JAK/STAT signaling pathway results in tumor initiation and progression in hematopoietic malignancies and other solid cancers [21–23, 25]. STAT3 was found to increase antiapoptotic proteins such as Bcl-2 family proteins and prevent cells to undergo apoptosis [24]. STAT3 mediates mitogenic activity within the cells, resulting in pro-survival pathway via surviving [24, 26]. Other studies revealed that STAT3 controls epithelial-to-mesenchymal transition regulators through metastasis [27]. The protein STAT3 promotes the initiation of various blood vessels by increasing the levels of HIF-1α and VEGF [28–30]. STAT3 facilitates tumor progression through aberrant expression of STAT3 via cell motility and invasion [31]. JAK/STAT pathway regulates several immunomodulatory molecules. An aberrant activation of STAT3 molecules regulates cancer cell proliferation and cell survival, suggesting the role of STAT3 in inflammation and angiogenesis modulating tumor microenvironment of cancer [28].

Several in vitro and in vivo studies have implicated the deregulation of JAK/STAT signaling pathway resulting in various hematological malignancies and solid cancers. The aberrant activation of this pathway has contributed to the propagation of gastric cancer [32, 33]. Inhibition of STAT3 protein resulted in the decrease of antiapoptotic protein levels such as survivin, decreasing cell survival in gastric cancers [34]. In 86 cases of gastric cancer patients, the studies revealed the activation of STAT3 with increase in angiogenesis factors such as microvessel density and the expression of VEGF. The studies on univariate survival analysis revealed the aberrant expression of STAT3 protein that plays a role in angiogenesis of gastric cancer and its progression [30]. To study the expression of IL-6 inducible protein, REG Iα in tissues of gastric cancers suggested the role of STAT3 in inflammation process. Both REG Iα and phospho-STAT3 expression predict the role in tumorigenesis in gastric cancer.

Kim et al. analyzed 100 gastric adenocarcinoma tissues collected after gastrectomy to identify the STAT3 expression through immunohistochemical staining [35]. It was identified that the expression of STAT3 found to be associated with survival and TNM imaging; this STAT3 expression can act as a biomarker for the treatment of gastric cancer. STAT3, SOCSI, phospho-STAT3, and other clinicopathological factors were evaluated by Deng et al. in 53 gastric cancer patient tissue samples with overall survival rate. Both phospho-STAT3 and lymph node metastasis are considered as independent predictors of OS in the patients of gastric cancer, revealed by both univariate and multivariate analysis, and the expression of STAT3 correlates with lymph node metastasis [3]. Nearly 106 gastric cancer tissue samples were analyzed by immunohistochemistry to identify the expression of SOCS3 among

patients; they revealed that SOCS3 acts as a good biomarker to identify lymph node metastasis [36].

Extensive studies on STAT3 revealed its role in gastric cancer-mediated inflammation. Bollrath et al. reported in colitis-associated cancer model that gain and loss of function of STAT3 revealed the link associated between inflammation and gastric cancer. Tumor progression was found to associate with IEC progression and survival mediation through cell cycle progression [37]. In STAT3-deficient mice, progression of intraepithelial lesions to tubular tumors that are in advanced stage confirmed the involvement of STAT3 in tumorigenesis [38]. STAT3 activation that was found to be observed in more aggressive gastric tumors could be due to either introduction of SOCS3-binding-deficient mutation or ablation of SOCS3 [39, 40]. Together these results have revealed valuable details about inflammation driven by STAT3 in gastric cancer [40].

11.3 Inflammation of *H. pylori* and Regulators of STAT3

H. pylori is a stomach bacteria that is mainly associated with the development of gastric adenocarcinoma. Major advances have been made to study the underlying mechanism of *H. pylori* to evade mucosa of host immunity. The pro-inflammatory cytokines that are produced by both immune cells and epithelial cells help in maintaining the chronic inflammatory condition resulting in gastric cancer. Widely discussed the STAT3 role in various organ systems raises a question of STAT3 activity in the immune response to *H. pylori* infection, which may mediate oncogenic effects of *H. pylori*.

Both gastric and immune compartments are influenced by functional effects of STAT3 [2, 41–43] and controlled by specific intracellular signaling events. In pharmacotherapy, STAT3 has been recognized as a potential target for the treatment of gastric cancer as a strategy to prevent the pathology of gastritis, metaplasia, and adenocarcinoma, respectively. H. pylori infections are caused by both immune and mucosal perspectives, and STAT3 is considered as a potential target for therapy. Jackson et al. reported that the hyperactivation of STAT3 is dependent on gastritis tumorigenesis, and they identified the functional role of STAT3 in the early stages of gastric cancer development [44]. Infections of mucosal surface a pit epithelial cell are identified with nuclear localization of phosphorylated STAT3 and its engagement with active gene promoters [44].

11.3.1 Toll-Like Receptors (TLR) and STAT3 Activation

Toll-like receptors play a role in recognizing and responding to microbial pathogens (pathogen-associated molecular pattern) and host danger signals (damage-associated molecular patterns) and facilitate the process of inflammation [45]. TLRs are specifically located on immune cells, especially on dendritic cells and macrophages [46]. The antigens of *H. pylori* such as LPS, flagellin A, and unmethylated CpG DNA

activate TLR2, TLR4, TLR5, TLR8, and TLR9 [47]. *H. pylori* responses to both TLR2 and TLR4 were best studied in both immunocytes and in the stomach; and the outcomes mainly depend on the type of cell and microenvironment-dependent alterations in response with well-defined antigens. In non-gastric tissues, both TLR4 and TLR9 involved in STAT3-dependent activation [48]. But there is no evidence that the same mechanism will occur in gastric epithelial cells. STAT3 was found to increase the gene expression of TLR2 in IL-11/STAT3 dependent in in vivo models; removal of TLR2 blocks the growth of tumor without any inflammation [49]. IL-6/STAT3 regulates the activation of spleen TLR4 and directly involves in inflammation [50]. Altogether, the data suggests that differential cytokine/STAT3 inductions are important for the activity of both TLR2 and TLR4. Together these data suggests that *H. pylori* infection influences the outcome of pathogenicity in stomach.

11.3.2 Regulation of STAT3 in *H. pylori* Tolerance

STAT3 plays important roles in the production of inflammatory cytokines. Emerging evidence from various mouse models reveals that *H. pylori* has evolved toward tolerance at immunity [51, 52]. Inflammatory responses raised against *H. pylori* infections by Tregs resulting in the release of cytokines that involve in the suppression of pro-inflammatory T-cell responses [53]. The transcription factor, FOCP3, forms a complex and regulates the expression of IL-10 in Tregs [54]. IL-19 involves in regulating STAT3, which is important for the suppression mediated by Tregs of pro-inflammatory T_H 17 cells; STAT3 deletion resulted in suppressive function or IL-10Rα but not IL-6Rα [55] in Tregs. DCs upon reprogramming of its phenotype with *H. pylori* infection induce favorable Treg responses [56, 57]. There is less number of studies to show the functional responses of STAT3 protein [58, 59]. This provided a strong evidence that IL-10-mediated signaling of STAT3 proteins involves impairment and tolerization of human DCs maturation [58]. In immune cells, STAT3 plays an important role in gastric epithelial cells that facilitates the persistence of infection caused by *H. pylori*. CagA signals in the activation of IL-11/gp130/STAT3 mediated by tyrosine phosphorylation with the help of C-type lectin [60].

11.3.3 STAT3 and Epidermal Growth Factor Signaling

EGF family members act as ligands for receptor tyrosine kinases such as ERBB1-4. It includes the family members of various factors such as EGF, HB-EGF, transforming growth factor-alpha amphiregulin, betacellulin, and epiregulin that can induce the dimerization of both ERBB1/ERBB2 and ERBB1 heterodimers to begin signaling cascades. There are several signaling networks in EGFR that are interconnected including the pathways such as PI3K/Akt, ERK1/2, STAT, and PLCγ [61]. Ligands such as STAT1, STAT3, and STAT5 utilizes ERBB1 homodimers to activate signaling pathways [62]. ERBB1 receptor-mediated STAT activation is compulsory for STAT3 activation without the involvement of JAK [62, 63].

Ligands of EGF family are involved in the pathophysiology of diverse activities ranging from cell proliferation, apoptosis, differentiation, and cell motility [44]. EGFR is considered as the first receptor that provides a link between various cancers and receptor overexpression; the hyperactivity of these receptors is demonstrated in a wide range of cancers such as the stomach, pancreatic, colorectal, head and neck, breast, lung, and brain. A wide variety of inhibitors are available to inhibit EGFR signaling pathways [61].

11.3.3.1 Ligands of EGF and *H. pylori*

Protein and mRNA expression of EGFR and its ligands are found to be increased in human stomachs infected with *H. pylori*. There is a direct correlation between the increased infection with an increased expression of transcription factors such as amphiregulin and HB-EGF; this has a direct correlation with the proliferation index of gastric carcinoma [64, 65]. The expression of both EGF and EGFR are increased in gastritis and gastric cancer patients infected with *H. pylori* but not in *H. pylori*-negative gastritis [66]. In gastric cancer cell lines, the infection of *H. pylori* caused increased expression of HB-EGF and EGFR [5]. Increased activity of HB-EGF has been directly linked to EMT through increased expression of EMT transcriptome [67]. Infection with *H. pylori* for a longer duration of time results in the increased expression of EGFR resulted with the inhibition of endocytosis [47].

The activation of EGFR in *H. pylori*-infected epithelial cells of gastric carcinoma results in increased survival rate with the decrease in apoptosis rate and with an increased expression of polyamine oxidase [68, 69]; it further enhances the expression of genes that are involved in early growth responses [70]. Modification of EGFR signaling is required for the development of gastric organoids through stem cells derived from pluripotent cells [71]. All the mentioned activities are important in progressing pathologic conditions of cancer induced by *H. pylori* infections.

11.3.4 STAT3 and Cytotoxin-Associated Antigen A (CagA)

Among various numbers of bacterial factors that involve in the pathogenesis of H. *pylori*, a very well-understood mechanism of STAT3 signaling is CagA. Over the last decade, much importance has grown in the biology of H. *pylori* compared with CagA. It is a principal cytotoxin of *H. pylori* with a molecular weight of 120–145 KDa, encoded by virulence gen *CagA*. The *CagA* is located in the region of cag pathogenicity island; this region contains genomic DNA of 40 kb with 27–31 virulence genes; few genes encode key components of bacterial type IV secretion system that involves in the delivery of CagA into epithelial cells of gastritis [72, 73]. *H. pylori* strains exhibit greater virulence with CagA positive, and they carry a high risk of gastric adenocarcinoma [74]. In mouse transgenic experiments, overexpression of CagA resulted in the uniform overgrowth of gastric epithelium and late onset of focal tumorigenesis, without altering significant changes to gastritis or atrophy [75]. CagA involves in autonomous deregulation of gastric epithelial cell

homeostasis; other factors such as secondary somatic mutations or inflammatory cytokines are necessary for oncogenesis. CagA along with other immortalizing agents involves in mediation of oncogenic transformation of primary gastric epithelial cells [76].

Translocation of CagA from epithelial cells of gastritis through type IV secretion system of bacteria [77]. Upon internalization, CagA localizes to the internal surface of the plasma membrane, and the tyrosine is phosphorylated at a specific location of EPIYA repeat motifs by kinases of src and c-Abl [78, 79]. On translocation CagA has been shown to interact with other intercellular signaling cascades that are associated with cell motility and growth [60, 78, 80–83]. The recent literature identifies CagA as the most widely discussed intracellular target of Src-homology protein tyrosine phosphatase SHP2 [72]. Activation of SHP2 results in binding and phosphorylation of CagA resulting in an inappropriate signaling via SHP2-(Ras)-ERK (MAP-kinase) cascade mechanism with deregulated cell polarity of epithelial cells and its increased motility, hummingbird phenotype [60, 80–84]. Knockdown of SHP2 restricts CagA-dependent ERK activation and induction of hummingbird phenotype [84]. Altogether, SHP2 plays an important role of CagA function in gastritis in epithelial cells.

The connection between SHP2 and gastric gp130 signaling is well explained [85] with the regulation of gp130/JAK/STAT3 pathway activation for the maintenance of gastric mucosal homeostasis [42, 85]. Lee et al. reported that the transfection of CagA triggers the activation and phosphorylation of STAT3 in gastric epithelial cells [60]. The phosphorylation of EPIYA motif is considered as a major determinant of CagA-mediated signaling in phosphorylation stage [60, 78, 80–83, 86, 87]. The phosphorylation of tyrosine residue within EPIYA of CagA is necessary for the activation of STAT3; it was revealed by EPISA [59]. Other groups reported that activation of STAT3 occurs independent of tyrosine phosphorylation of CagA [87] or through unphosphorylation of CagA [88].

The other groups performed a detailed investigation of STAT3 activation through CagA dependence in non-gastritis group, laryngeal carcinoma-derived HEp-2 cells [87]. The other group assessed the role of STAT3 response in gastric AGS cells after infection with the different *H. pylori* strains that lacks phosphorylation-defective CagA mutant that lacks EPIYA motif [88]. The studies based on gastric cell lines using an inducible transgene that carries either mutant in EPIYA tyrosine residue or wild-type CagA has been replaced with EPISA [60]. The following approach helped in abolishing EPIYA tyrosine phosphorylation without affecting cell membrane tethering of CagA [89].

11.4 Therapeutic Options for Gastric Cancer

Based on the size and location of initial tumor, either total surgical resection or subtotal gastrectomy will be performed. Both radiotherapy and chemotherapy are considered to be major treatment options for the treatment of cancer. The contributions of nontargeted effect for the development of secondary cancer are far less clear

Table 11.1 Targeted agents for the treatment of gastric carcinoma

Target	Agent	Treatment prospects
Trastuzumab	HER2	FDA approved
Ramucirumab	VEGFR	FDA approved
Sorafenib	VEGF, PDGF	Ongoing phase II and III clinical trails
Marimastat	MMPs	Ongoing phase II and III clinical trails
Erlotinib	EGFR	Ongoing phase II clinical trails
Foretinib	c-Met, KDR VEGFR2	Ongoing clinical trials
Bevacizumab	VEGF	Treatment given individually [103]
Pertuzumab	HER2	Ongoing phase III clinical trials
Sunitinib	VEGF, PDGF, KIT, FLT-3, RET	Promising outcomes [104–106]
Bortezomib	NF-κB	Promising outcomes [107]
Rilotumumab	c-MET	Promising outcomes [108]
Figitumumab	IGFR-IR	Ongoing phase I clinical trials

[87–90]. In patients, chemotherapeutic interventions will be revealed in the adjuvant, neoadjuvant, and metastatic settings. The trial, SWOG 9008, is evaluated by postoperative chemoradiation after removing gastric tumors. Patients were closely monitored under observation versus 5-FU/leucovorin, and the radiation dose was delivered at 45 Gy. After 10 years, follow-up revealed that there is a dramatic improvement in overall survival of the treatment arm; generally, the postoperative chemotherapy is not well tolerated in patients undergoing treatment [98]. The MAGIC trial was evaluated with the treatment of epirubicin, 5-FU, and cisplatin in patients suffering from adenocarcinoma, and they found that chemotherapy had a very good overall survival compared with surgery alone [99]. In patients with metastatic carcinoma, any combinations of DCF, FOLFRI, and DCF are considered as best supportive care [100–102].

Several therapies have been studied till now, but only two therapeutic treatments have been approved in the United States based on positive clinical trials. Characteristics of various molecular-targeted agents in gastric cancer therapy are given in Table 11.1. HER2 inhibitors are considered as the best targeted therapy for several cancers. The monoclonal antibody, trastuzumab, targets HER2 and inhibits its signaling pathways [109]. In phase III international study (ToGA trial), trastuzumab was evaluated in combination with cisplatin plus 5-FU or capecitabine. Patients were classified based on HER2 overexpression. Treatment with trastuzumab and chemotherapy has improved overall survival rate compared with chemotherapy alone [110]. A monoclonal antibody, ramucirumab, given against vascular endothelial growth factor receptors 2 demonstrated survival benefits in patients with advanced gastric cancer [111, 112]. Ramucirumab is the first Food and Drug Administration-approved biologic therapy used as a single agent [112]. Ohtsu et al. [113] demonstrated that monoclonal antibody against VEGFA and bevacizumab in combination with cisplatin and capecitabine that was administered in AVAGAST trial demonstrated improved overall response rate and progression-free survival, but overall survival was not improved.

11.5 Conclusion

Among transcription factors, STAT3 plays a pivotal role in gastric mucosal function. STAT3 activation by hyperphosphorylation constitutively results in increased gene expression that contributes to pre-neoplastic progression in the stomach. The progression leads to cell proliferation, angiogenesis, and inflammation and inhibits cell death. The aberrant expression of STAT3 has made promising biomarkers in gastric cancer patients. Several cell culture and mouse model studies confirmed of STAT3 in precancerous pathological conditions of the stomach, which confirms STAT3 as a diagnostic biomarker for early detection in patients suffering with gastric cancer. The blockage of STAT3 with effective antagonists will be effective in spreading metastasis to secondary sites, a common situation for effective treatment of gastric cancer.

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Role of STAT3 in Gastric Cancer Initiation, Development, and Progression

12

Aleem Basha Pinjari and Abdul Razak Meerza

Abstract

Gastric cancer (GC), a leading cancer that occupies the second position in terms of morbidity and mortality, occupies the fourth place of all cancers in terms of manifestation. Annual gastric cancer manifestations are reaching several millions of deaths along with millions of new cases. Gastric cancer which is not detected in the early stages has very poor prognosis, and the 5-year survival rate is only around 20%. Gastric cancer development includes numerous alterations at genome level leading to changes in the expression of quite a lot of genes involved in several physiological processes. Even though a number of factors showed their role in advancement of GC, a link between STAT3 and the risk of GC has become apparent in current years. Signal transducers and activators of transcriptions (STATs) which are predominantly known for their role as transcription factors are implicated in controlling numerous physiological processes such as cell propagation, differentiation, apoptosis, and angiogenesis by controlling the expression of critical genes in the pathway. Abnormal activation of STAT3 plays a key role in inflammation and transformation in numerous cancers including gastric cancer (GC). Earlier, STAT3 has never been considered as a target, and hence there is no FDA-approved STAT3 inhibitor till now. Recent advances in drug discovery and cancer biology now focused on STAT3 globally for treating different types of cancers. The present chapter summarizes the recent literature and gives an idea about involvement of STAT3 in gastric cancer initiation and progression.

Keywords

STAT3 · Inflammation · Signal transduction · Gastric cancer

The original version of this chapter was revised. The book was inadvertently published without Abstracts and Keywords, which are now included in all the chapters. An erratum to this chapter can be found at https://doi.org/10.1007/978-981-10-6728-0_39

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12.1 Introduction to STATs

In 1994, STATs, which were firstly recognized, constitute a group of proteins which are involved in transcription and control of several physiological processes such as cell division, differentiation, survival, apoptosis, angiogenesis, immune modulation, and inflammatory functions. Mainly STAT family of transcription factors contains seven members which are encoded by STATs 1, 2, 3, 4, 5A, 5B, and 6 genes [60, 74]. The twin roles of STATs show their uniqueness where firstly they communicate from the cytoplasmic membrane to the nucleus and, secondly, they control the expression of some critical genes involved in physiological processes at the gene level [23, 32, 70]. Initially, STATs were identified as part of interferon (IFN) signaling complex [42, 49]. Based on their functional role, STAT proteins are classified into two groups [69, 73]. STATs 2, 4, and 6 form the first set which are actively involved in IFN-γ signaling and T-cell development, while the second set involves STATs 1, 3, and 5 having a significant role in mammary gland development, IFN-γ signaling, and embryogenesis. Each family member of STATs has specific functions, which include regulation of antiviral host response [21, 35], regulation of T-cell development [39–41, 52, 54], stimulation of cell proliferation, and survival to apoptosis [7, 28, 56, 71]. Most of the cellular functions of STATs are elucidated by gene knockout studies in animal models. STAT proteins 1 and 2 elicit a remarkable role in the control of several cellular and physiological processes which is mediated through interferons. STAT1 knockout or deletion studies in mice have shown their inability to respond to interferons and thereby become susceptible to several microbial infections [26]. The most important member of STAT proteins is STAT3, which controls the expression of central genes involved in many physiological processes at DNA level in response to several signaling molecules. In response to cytokine IL-6, STAT3 which gets activated was firstly identified as acute phase response factor [2, 96]. Upon stimulation with cytokines, STAT3 undergoes activation through phosphorylation in the cytoplasm by Janus kinases (JAK), and the active phosphorylated STAT3 moves to the nucleus to control the transcription of several important genes by binding to specific DNA sequences [33]. Within 15 min STAT3 undergoes maximum phosphorylation to exhibit its activity and phosphorylation ceases in 60 min [73]. Activation of STAT3 by several signaling molecules follows two modes in which the first mode is through Tyrosine 705 phosphorylation (cytokines, growth factors, and interferons follow this mode) and the second mode is through Serine 727 phosphorylation [68] (mitogen-activated protein kinases (MAPK) [79] and non-receptor tyrosine kinases follow this mode). Animal model studies clearly showed that the deletion of STAT3 in homozygous condition is embryonically lethal, indicating a crucial role in early stages of embryonic development [75]. From the previous reports, it was clear that STAT3 activation is essential to maintain embryonic stem cells (ESCs) in undifferentiated state though STAT3 is enough for embryonic stem cell (ESC) regeneration [51]. TH17 helper T cells, which are known to exhibit a remarkable role in several autoimmune diseases, also require STAT3 for their differentiation [91]. STAT4 is the predominant STAT protein present in macrophages, activated monocytes, and dendritic cells which perform immunological

functions. Similar to STAT3, STAT4 also gets activated through phosphorylation in response to cytokines specifically IL-12, is translocated to the nucleus, and regulates the transcription of genes involved in inflammation [84, 86, 89]. In mice, it was clearly shown that impairments in IL-12-induced proliferation of activated T cells may result due to the deletion of STAT4 [40, 78]. Even though they have identical names, STAT5A and STAT5B have independent genes coded by different chromosomes, and their knockouts exhibit different phenotypes. The studies related to STAT5A knockout revealed its importance in the development of the mammary gland and lactogenesis in adult mice, whereas STAT5B knockout studies discovered its role in sexual dimorphism of body growth rates and gene expression in the liver [83]. Even if several cytokines activate both STAT5 isoforms, some of them show specificity toward either STAT5A or STAT5B [59]. STAT6, another important associate of the STAT group, exhibits a lead role in the maintenance of equilibrium between immune protection and allergic reactions [89]. One of the important functions of STAT6 is M2 macrophage polarization. Upon stimulation with cytokines specifically IL-4 and/or IL-13, STAT6 displays its transcription activator activity and regulates the transcription of several genes depending upon the cell type [43, 85]. The best examples include the enhancement of transcription of IgE chain and CD23 genes in B cells and transcription of gata3 and crth2 genes involved in Th2 differentiation in T cells [40, 65, 76]. With the involvement of other transcription factors, STAT6 also suppresses the expression of genes besides its original activation function [85]. The loss of IL-4 response in STAT6 knockout mice acts as driving force for Th1 differentiation, whereas loss of IL-12 response shows impaired Th1 differentiation in STAT4 null mice.

12.2 Structural Characteristics of STAT Proteins

Analysis of the STAT group proteins revealed their structural details as they contain seven conserved domains which include both structural and functional domains [13, 59], namely, N-terminal domain, coiled-coil domain, DNA-binding domain, linker domain, SH2 domain, phosphotyrosyl tail segment (Y domain), and C-terminal transactivation domain. The N-terminal domain encompassing about 125 amino acid residues exhibits a remarkable role in the formation of dimers. This domain also plays a dual role by maintaining the STATs in an off conformation and recruiting STAT dimers at the receptor. Coiled-coil domain is one of the important domains (amino acid residues 136–317) containing four α helices. This domain is rich in charged amino acids and has potential to interact with other proteins. The documentary evidence shows that this domain not only is essential in establishing protein–protein interactions with many regulatory proteins [44] but also performs a central role in translocating STAT proteins in and out of the nucleus [55, 60]. The other most important and essential domain is DNA binding (amino acids 320–480) by virtue of which STAT proteins exhibit their remarkable function, transcriptional regulation of genes. Through this conserved domain STAT proteins bind to diverse palindromic sequences and the GAS sequence [22]. In between DNA-binding and

SH2 domains, there exists a highly conserved domain known as the linker domain (463–566 amino acids), which acts as a chief contact point in the formation of STAT-determined transcription complex. Another domain which is thought to be well conserved is Src homology2 (SH2) domain (amino acid residues 575–680). The SH2 domain binds specifically to phosphotyrosine and acts as a phosphorylation-dependent knob to control receptor recognition and binding to DNA. Thus SH2 domain exhibits a straight link with the cell surface receptor activation and transcriptional gene regulation. The crucial step in STAT activation is the tyrosine phosphorylation and the conserved tyrosine residue that is exposed to outer surface in tyrosine activation motif is very much essential for phosphorylation by JAKs [50]. The minimum conserved domain in STAT proteins is C-terminal transcriptional activation domain (TAD) which acts as the docking site for several coactivators and also known to affect the stability of protein [44, 59].

12.3 Activation of STAT3

STAT3 is a well-known transcription factor which gets stimulated by a bunch of cytokines including growth factors, interferons, hormones, and interleukins. Almost all types of growth factor receptors can stimulate the STAT3 activation in response to signaling molecules. Stimulation with growth factors or cytokines through receptor binding promotes their homo- or heterodimerization, thereby switching on intrinsic receptor tyrosine kinases to stimulate tyrosine phosphorylation of STAT3. Receptors lacking tyrosine kinase activity intrinsically get activated by other tyrosine kinases that are bound to receptors such as JAK and SRC. Receptor-associated tyrosine kinases subsequently phosphorylate the tyrosine residues present in the receptor, and these phosphorylated sites serve as docking sites for the binding of proteins containing SH2 domain, for instance, STAT3. STAT3 phosphorylation at Tyr 705 by JAK converts inactive form of STAT3 to active form. Once STAT3 gets activated by phosphorylation, it undergoes dimerization with another STAT3 protein by establishing a connection between phosphorylated tyrosines at position 705 of one monomer with the SH2 domain of the other. Dimerized STAT3 then moves from the cytoplasm to the nucleus and performs its function of transcriptional regulation by binding to specific regions in DNA. The transcriptional activity of STAT3 is regulated by MAPK or mTOR pathways via phosphorylation of Ser727 present in the transactivation domain. Phosphorylation of STAT3 Tyr 705 converts inactive form to active form, whereas to attain complete activation, it requires one more phosphorylation specifically at Ser 727 [11, 92]. Besides these two phosphorylation sites, STAT3 is also regulated by acetylation of Lys 685 which stabilizes STAT3 dimers [94]. Along with phosphorylation, STAT3 also requires association of coactivators such as CBP/p300, c-Jun, APE1/Ref-1, MCM5, Nmi [97], and NCOA/SRC1a [29, 30] for its transcriptional activity. STAT3 regulates wide range of transcription factors predominantly c-Fos and HIF-1α which exhibit broad functions. STAT3 upregulates c-Fos and HIF-1α by directly binding to their promoters. Increased levels of c-Fos and HIF-1α regulate their downstream target genes

involved in various physiological processes related to inflammation, invasion, cell metabolism, angiogenesis, and apoptosis [46, 61, 63, 88, 90]. These three transcription factors STAT3, c-Fos, and HIF-1α alone can stimulate the initiation and progression of tumors. In addition, STAT3 activates the expression of many genes which are related to physiological processes such as cell proliferation, apoptosis, and angiogenesis. STAT3 enhances antiapoptotic gene transcription, for instance, Bcl-2, Bcl-xL, and survivin, and genes related to cell cycle progression such as p21WAF1/CIP2, cyclins, CDC25A, APE1/Ref-1, c-Myc, and Pim1 and also activates genes such as VEGF involved in angiogenesis. In fact, most of the STAT3 target genes play a key role in controlling various physiological processes which include cell proliferation, transformation, and metastasis.

12.4 Abnormal Activation of STAT3

Current literature recommends that continuous activation of STAT proteins plays a significant role development of tumors associated with different malignancies and it is also responsible for poor prognosis [14]. Recent study conducted by Xiong et al. with gastric cancer (GC) samples revealed the importance of phosphorylation of STAT3 in terms of survival rate. Patients having tumors with phosphorylated STAT3 have relatively shorter life compared to those with non-phosphorylated protein. Not only in gastric cancer, pSTAT3 was also associated with shorter overall survival in other cancers which include colorectal carcinoma, B-cell lymphoma, cervical cancer [77], and head and neck squamous cell carcinoma [47, 62]. Pathways that are responsible for continuous activation of STAT3 include loss of negative regulation, excessive stimulation, positive feedback, and somatic mutations of STAT3. Different types of mechanisms which lead to abnormal activation of STAT3 are shown in Table 12.1. The predominant negative feedback regulators of STAT3 are suppressor of cytokine signaling (SOCS) and protein tyrosine phosphatases (PTPs). The SOCS (suppressor of cytokine signaling) family of proteins acts as competitors to STAT3 to bind to JAKs and prevent their interaction with kinase domain of JAKs and thereby stop their signaling [87]. As SOCS proteins are involved in the maintenance of homeostasis of physiological processes by inhibiting the hyperactivation of important signaling molecules, their expression is under tight control. Accordingly, the JAK/STAT3 pathway that is activated with respect to cytokine stimulation enhances the transcription of SOCS3, and this protein functions as a negative feedback regulator to prevent its own overstimulation. Protein tyrosine phosphatases (PTPs) belong to a large group of proteins involved in counteracting the effects of protein tyrosine kinases. These phosphatases maintain the phosphorylation status in the cell. Several phosphatases mediate their action in the same manner to that of SOCS proteins, by regulating JAK/STAT3 signaling pathway. PTPs inhibit the JAK/STAT3 signaling by dephosphorylating the tyrosine residues that are required for interaction of JAKs and STAT3 [5]. SOCS proteins and PTPs which act equally as negative feedback regulators are repressed or silenced in many types of cancers

Table 12.1 Mechanisms involved in aberrant activation of STAT3

Proteins/ cytokines	Type of action	Mechanism	Expression	Reference
SOCS (Suppressor of Cytokine Signaling)	Negative feedback regulation	Acts as competitors to STAT3 to bind to JAKs and prevent their signaling	Repressed or silenced in many cancer	Ward et al. (2009)
PTPs (Protein Tyrosine Phosphatases)	Negative feedback regulation	Inhibits JAK/STAT3 signaling by dephosphorylating the tyrosine residues required for interaction of JAKs and STAT3.	Repressed or silenced in many cancer	Alonso et al. (2004)
IL-6, IL-10, IL-11, IL-21, IL-23, oncostatin	Direct stimulation	Persistent activation of STAT3 through continuous stimulation	Over expressed in many cancers	Yu et al. (2009)
v-src	Positive feedback regulation	stimulates NF-κB which in turn stimulates IL-6 which brings constant activation of STAT3	Over expressed in many cancers	Iliopoulos et al. (2010)
Pyruvate kinase M2 (PKM2)	Positive feedback regulation	Activates STAT3 by phosphorylating Y705	Over expressed in many cancers	Luo et al. (2011)

leading to abberant activation of STAT3. Tumor microenvironment is more important in the persistent activation of STAT3 protein as several surrounding cells release signaling molecules into the environment. Predominant cytokines that are involved in persistent activation of STAT3 belong to interleukin family members along with leukemia inhibitory factor and oncostatin [93]. The activated STAT3 in return stimulates the transcription of those cytokines that are responsible for its activation; hence a round of continuity occurs in between STAT3 activation and cytokine production which often exists in tumors (Yu and Jove 2004; [93]). Several previous reports have shown the contribution of IL-6 to prolong STAT3 activation in cancer cells [19].The transcription of several oncogenic protein tyrosine kinases (PTKs) also results into constitutive activation of STAT3. One such oncogenic member is Src, which is overstimulated in different cancer types. Src is overstimulated by Y416 phosphorylation and Y527 dephosphorylation. Many cancers revealed that the dephosphorylation of Y527 results due to the action of tyrosine phosphatase such as PTP1B, deletion of Y527, or Y527F mutation [6, 27]. Src activation further brings STAT3 activation which in turn regulates the expression of several genes which are very necessary for v-src-mediated cellular transformation [10, 15, 81]. Constitutive activation of STAT3 also results from positive feedback loops. Several mechanisms utilize IL-6/STAT3 signaling pathway which is more prevalent in many cancer types for activating STAT3 in a positive feedback loop. Recent studies conducted on MCF-10A, STAT3 activated by v-src increases the expression of two miRNAs which in turn stimulates NF-κB

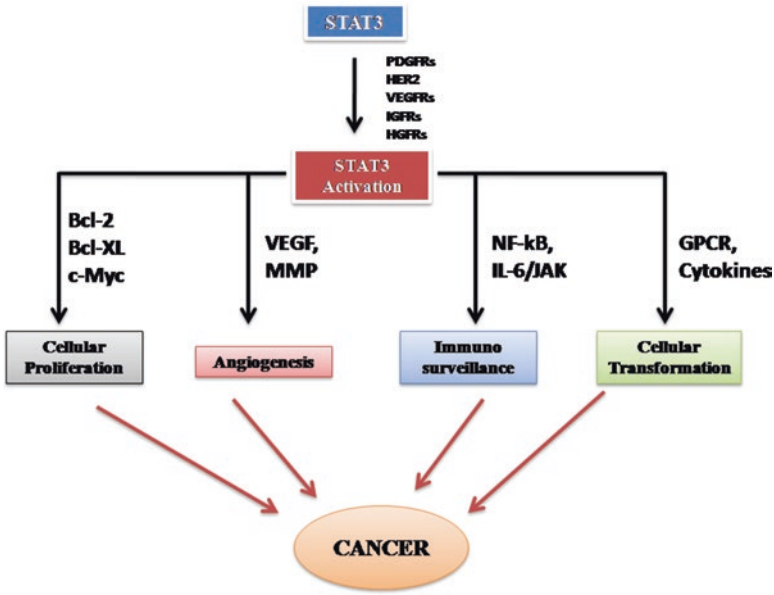

Fig. 12.1 Different mechanisms of STAT3 activation leading to cancer

that inhibits the transcription of two tumor suppressor genes PTEN and CYLD. In addition, the stimulation of IL-6 by NF-κB leads to constant activation of STAT3. In another study conducted in cancer cells and tumor microenvironment, it was shown that G-protein-coupled receptor overexpression in response to transcription factor STAT3 promotes persistent activation of STAT3 by IL-6 production and tyrosine kinase activity of JAK2. Activation of STAT3 through Y705 phosphorylation by pyruvate kinase M2 (PKM2) is another example for positive feedback. Activated STAT3 then promotes the transcription of HIF-1α which in turn enhances PKM2 expression. One more example for positive feedback comes from STAT3 and COX-2 which was established in *Helicobacter pylori*-associated gastric cancer. Various mechanisms of STAT3 activation are shown in Fig. 12.1.

12.5 STAT3 and Gastric Cancer

In malignancy, the second foremost cancer responsible for high mortality is gastric cancer (GC) [3, 9]. Even though there is a rapid development with respect to diagnosis and cure, the actual mechanisms that are responsible for gastric cancer development are still unclear. The aggressive behavior of gastric cancer results due to accumulation of mutations resulting in oncogene activation, tumor suppressor gene suppression, and their aberrant downstream signaling pathways concerned with many aspects of cancer biology [20, 67]. Gastric cancer which results due to

accumulation of damages at gene level involves oncogene activation and suppression of tumor suppressor genes. Some of the oncogenes that specifically altered in gastric cancer include epidermal growth factor receptor (EGFR), human epidermal growth factor receptor (HER2), K-ras, and COX-2. Hyperactivation of these genes may be responsible for the abnormalities associated with gastric cancer and also for its poor prognosis [25, 45]. Many molecular biological factors serve as predictors of clinical importance and explore novel targets in gastric cancer patients. One of the STAT family proteins, STAT3, which controls many physiological processes such as cell proliferation, apoptosis, survival, and inflammation [33], is aberrantly expressed under pathological conditions such as human cancers [15]. The major mechanism of STAT3 activation is tyrosine −705 phosphorylation. Once the STAT3 gets phosphorylated, it undergoes dimerization and then translocates into the nucleus followed by its binding to specific DNA sequences for transcription. Subsequently STAT3 proteins are inactivated by dephosphorylation of tyrosine residue and return to the cytoplasm [69]. STAT3 is often found to be hyperactivated in many cancer cell lines and tumors which include breast [64], prostate [53], renal [31], head and neck, colorectal [82], cervical [77] ovary [8], lung [4], and gastric (Xiong et al. 2012) cancers. Continuous activation of STAT3 has been shown to encourage several physiological processes such as growth, survival, angiogenesis, and suppression of antitumor responses in tumor cells (Yu and Jove 2004; [37]). Persistent activation of STAT3 observed in gastric cancer cell lines is responsible for progression of gastric cancer [36, 38]. The mechanism(s) essential for STAT3 activation in gastric cancer are not clear. Several molecules have been identified in gastric cancer which induces progression of gastric cancer through STAT3 signaling pathway.

In a recent report, it was shown that mitochondrial GRIM-19 (gene associated with retinoid interferon-induced mortality 19) has a role in gastric cancer tumorigenesis. The loss or suppression of GRIM-19 as observed in gastric cancer and chronic acute gastritis (CAG) results in the activation of STAT3 leading to gastric tumor initiation and progression. Reduction or loss of the function of GRIM-19 is correlated by way of advanced stages in gastric cancer along with infections of *H. pylori* with short overall survival. GRIM-19 expression studies in gastric cancer cell lines have shown the suppression of tumor formation which revealed the inhibition of STAT3 activation and its downstream target genes by GRIM-19. On the other hand, knockdown studies of GRIM-19 have led to STAT3 activation in an abnormal manner [34]. Recent report revealed Fas signaling involvement in the development of gastric cancer metastasis happens through the stimulation of STAT3/Fascin signaling pathway. Fas signaling is a very well-known mechanism to stimulate apoptosis by activating several caspases [95]. In some cancers, Fas signaling promotes apoptosis, while in some cancers, it promotes tumor cell proliferation, migration, and invasion [12, 57, 66, 80]. The downstream signaling of Fas has resulted in the upregulation of Fascin through STAT3 signaling pathway [24, 58, 72]. Fascin that plays a key role in tumor metastasis is overexpressed in many cancers, along with gastric cancer [1, 48]. Several studies have revealed that *Helicobacter pylori* CagA positive is more detrimental that CagA negative with respect to gastric cancer [17,

18]. In *H. pylori* infection, STAT3 activity is enhanced leading to gastric cancer progression [16, 36]. These results confirm STAT3 activation in gastric epithelial cells with *H. pylori* infection.

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Role of Sp1 Transcriptional Factor in Gastrointestinal Carcinogenesis

13

A. M. V. N. Prathyusha, Renuka Nawadkar, and Pallaval Veera Bramhachari

Abstract

Sp1 protein binds to GC/GT-rich promoter elements through zinc finger motifs present at their C-terminal domains and regulates expression of multiple genes in normal tissues and tumors. Sp1 protein plays a critical role in the growth and metastasis of gastrointestinal cancers by regulating expression of cell cycle genes and VEGF. However, Sp1 is involved much in growth-related signal transduction pathways, and its overexpression has both positive and negative effects on proliferation of cells. In addition to growth control, Sp1 is intricate in apoptosis and angiogenesis; therefore, Sp1 is involved in several aspects of tumorigenesis. Consistent with a role of Sp1 in cancer, it interacts with oncogenes and tumor suppressors and alters their expression. Effects of changes in Sp1 factor are context-dependent and are paradoxical. Sp1 proteins have been recognized as an essential cancer drug target.

Keywords

Sp/KLF transcriptional factors · Signal transduction · Tumorigenesis · Apoptosis and angiogenesis

The original version of this chapter was revised. The book was inadvertently published without Abstracts and Keywords, which are now included in all the chapters. An erratum to this chapter can be found at https://doi.org/10.1007/978-981-10-6728-0_39

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13.1 Introduction

Gastrointestinal (GI) cancers are malignant tumors of the GI tract and accessory organs of digestion. GI cancers include carcinomas rising in the oral cavity, esophagus, stomach, liver, gallbladder, pancreas, small intestine, large intestine, rectum, and anus. GI cancer represents about 30% of all tumor incidences and is responsible for approximately 40% of tumor-related mortality worldwide. Gastric cancer remains the fourth most commonly detected malignant cancer in the world and ranks the third most cause of cancer-related deaths in 2008. Although the occurrence of gastric cancer (GC) is decreasing worldwide, but it is still higher to a certain extent in Eastern Asia, particularly in China and Japan. Gastrointestinal cancers are highly aggressive tumors, existing in a locally advanced stage with a poor prognosis and survival. Colorectal cancer (CRC) is the third most common cancers globally and accounts for 1.2 million fresh cases and 600,000 deaths per year [30]. The biological and clinical outcome of gastric carcinoma is associated with mutations of various oncogenes, tumor suppressor genes, and aberrations of growth factors and their receptors. These mutations and abnormalities affect the downstream signal transduction pathways that intricate the cell growth and differentiation. Precisely, these perturbations confer a noteworthy survival and growth advantage to gastric cancer cells [39, 42].

Sp1 is a key transcription factor that plays a vital role in regulating various aspects of tumor. Abnormal expression and activation of Sp1 contribute for gastric cancer progression and development. Sp1 was first recognized as a promoter-specific binding factor that is important for transcription of the SV40 major immediate-early (IE) gene. Interestingly, the Sp1 specifically binds at GC-rich sites with the help of three Cys_2His_2 (C_2H_2) domains of zinc finger motif [32]. The Sp/KLF family comprises of 20 transcription factors, Sp1–5, several Krüppel-like factors (KLF1–14) and KKLF Fig. 13.1. Firstly, Sp1 was considered as a general transcription factor, required for transcription of "housekeeping genes," due to its involvement in cell proliferation, metabolism, growth, and cell death [3]. It is notable that there are several housekeeping genes involved in cancer progression and Sp1 is one of them. Approximately about 12,000 Sp1 binding sites were identified from human genome. This review details about the role of Sp1 in transcription of diversified genes involved in apoptosis, cell division, and cell metabolism.

13.1.1 Sp-Transcription Factor Family Member Structure

Sp family has different transcriptional properties and can modulate the activity and gene expression of its own family (Sp1–6 and KLF1–14). All SP family members are made up of three zinc finger motifs at C-terminus and glutamine (Sp)/ proline/ Serine-rich domains at N-terminus [4]. It is noteworthy that Sp factors have specificity toward GC boxes and CACCC boxes to which KLF eventually bind. Broadly SP factors are categorized into two groups based on similarity in domain organization: Sp1–4 and Sp5–6. Domain organizations of first group Sp1–4 are similar, but Sp5–6

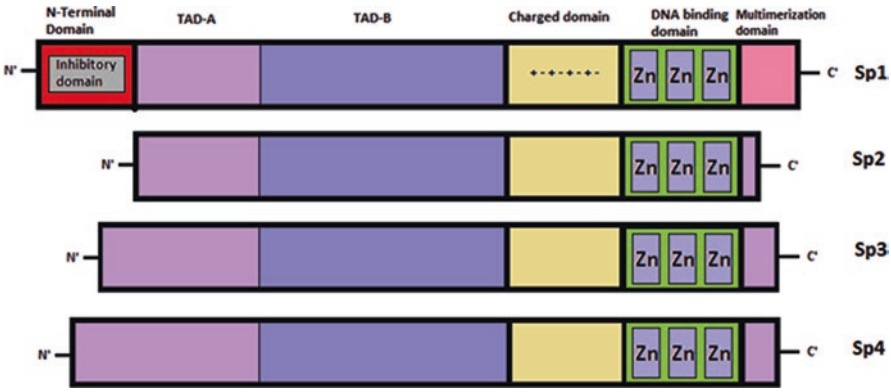

Fig. 13.1 Sp1 transcription factor family. Structural representation of human Sp family proteins. *Sp1*, *Sp2*, *Sp3*, and *Sp4* are aligned based on transactivation domain, and DNA binding (Cys_2His_2 light green) transactivation domains are made up of serine- and threonine-rich domains; glutamine-rich domains are adjacent to it. Charged domain in purple promotes DNA binding domain to bind to DNA. Only Sp1 in Sp family has inhibitory in red and multimerization domain in pink

factors are similar to Krüppel-like factors. N-terminal region have transactivation domains, rich in serine and threonine which most of the times are found near to glutamine-rich regions in Sp (1–4) [16]. Sp1 has multimerization domain, to carry out super-activation of promoter having multiple adjacent Sp sites [10] Fig. 13.1.

13.2 Regulation of Transcription Factor Sp1

Sp1 protein is highly modified by variety of posttranslational modifications, viz., *O*-linked glycosylation, phosphorylation, SUMOylation, ubiquitylation, and acetylation. The 785 amino acid Sp1 protein includes 164 threonine and serine residues, which signifies highly phosphorylated and *O*-glycosylated attribute of Sp1. Nevertheless, posttranslational modifications of Sp1 affect activity and stability, by modulating proteasomal degradation. Various posttranscriptional modifications were revealed in vitro; contrastingly, its associations with specific functions in cells are unclear. Some of these modifications are detailed below with the context of controlling Sp1 stability and transcriptional activity [3].

13.2.1 Posttranslational Regulations

Notably 164 serine and threonine residues in Sp1 explain the first regulatory mechanism of phosphorylation and glycosylation. Several kinases such Ataxia Telangiectasia (ATM), JNK1, ERK 1, CDK2 and PI3K, ATR kinase, and DNA-dependent protein kinase show their action directly or indirectly by interacting with other proteins on Sp1 for posttranslational modifications. Single Sp1 molecule can

be modified in several ways in a single cell which changes stability of Sp1 and its functions in a number of different ways. Such modification and changes in structure of Sp1 make it to interact with numerous proteins and other transcription factors. This phenomenon differentially regulates transcription of several numbers of genes. Several modifications discovered in vitro yet have to be studied in context of cell functions [3].

In gastric cancer, glycosylation is an essential posttranslational modification of proteins and one of the causes of cancer. Fucosylation is one of the most common types of glycosylation in carcinogenesis carried out by fucosyltransferases (FUTs). FUT4 is one of the key enzymes to catalyze the fucosylation, and its elevated expression is stated in diverse types of cancers such as breast, colon, pancreatic, and gastric cancers [43]. Recent reports stated that gastric cancer cell proliferation is stimulated by cytotoxin of *H. pylori* with FUT4 upregulation in cells. Transcription factors HSF1 and Sp1 monitor expression of FUT4. The fucosylated blood group of antigens allow *H. pylori* colonization on gastric epithelial cells which leads to gastric cancer with alteration of specific FUT expression.

13.2.2 Sp1 Protein Stability

Sp1 interacts with a number of binding partners and modulates expression of several genes. Sp1 is important to regulate either at expression level or stability in cell to control subsequent genes. Ubiquitination of Sp1 protein leads to degradation, which cleaves at N-terminus of amino acids [13, 31]. Sp1 cleavage may occur via interaction of proteasome with Skp-cyclin-F (SCF) box ubiquitin ligase complex [40]. β-TCRP of proteasome also interacts with Sp1. Although direct interacting partners have yet to be identified, proteasomal degradation may be regulated by ERK and GSK3-β by phosphorylating Sp1 at 728,732 Ser or Thr residues respectively. SUMOylation to Sp1 occurs at lysine (16), but this needs modification of first seven amino acids. SUMOylation causes destabilization of Sp1 via ubiquitination through ring finger protein 4 (RNF4); this reaction is catalyzed by SUMOdependent ubiquitin ligase. Ubiquitination-independent binding of RNF4 to Sp1 changes the interaction between RNF 4 from its targets and depicts the alterations in Sp1 functions. Nonetheless, the phosphorylation at 238 residue also inhibits Sp1 interaction with RFN4 and helps in maintaining stability. But the role for phosphorylation at 739 threonine is yet unclear. More investigations are warranted to know the developments of phosphorylation on SUMOylation and ubiquitination and stability of Sp1 attained after posttranslational modifications.

13.3 Sp1: Protein Interactions

Sp1 protein has been reported to interact with large number of proteins. At the same time various in vitro studies, evidenced that Sp1 interacts with multiple proteins. As we know, it forms tetramer and then it interacts with promoter and enhancer

simultaneously; it makes the study of this protein in vivo difficult to distinguish specific binding partners of Sp1 from a large protein complex. Large basal transcriptional factors, other transcriptional factors, DNA repair machinery, and cell cycle regulators are found to be associated with Sp1.

13.4 Sp1 in Carcinogenesis

Sp1 expression and dependent transcription are tightly regulated in various developmental stages. Sp1 control several genes involved in many diseases. One well-known example is cancer [8, 37]. Sp1 is one of the important transcription factors found to be upregulated and associated with poor prognosis in cancers which include breast, gastric, pancreatic brain, and lung cancers. Several studies reported that Sp1 can act as target for cancer treatment. Although feasibility of targeting Sp1 is in controversy as it is present in normal cells also, its inhibition can affect cell division of normal cells. It is noteworthy that the inhibitor of Sp1 is yet unidentified. Additionally, it is essential to understand the functions, associations, and binding patterns of Sp1 to completely emphasize its role and inhibition in cancer [17, 29]. Hanahan and Weinberg stated the following four key cancer hallmarks viz., abberrant metabolic pathways, escaping from immune system, genome instability, and inflammation, resistance to death, angiogenesis induction, escaping from the growth suppressors, cancer metastasis and invasion, cellular energetic deregulation, and resistance to death and overexpression of proliferation signals, respectively [14, 15]. Interestingly, Sp1 is involved in regulation of genes that directly or indirectly regulates all eight hallmarks of cancer.

13.5 Role of Sp1 in Proliferative and Survival Signals

In contrast with normal cells, cancer cells maintain their cellular proliferation by escaping need for growth factors. Four growth factors and its receptors regulate cell division and cell cycle; out of four growth factors, epidermal growth factor (EGF), fibroblast growth factor (FGF), nerve factors, and their receptors mediate through Sp1. Insulin-like growth factor 1 receptor (IGF1R) is antiapoptotic in nature; increased expression of IGF1R is associated with cancer [9]. Commonly Sp1 and its receptor are overexpressed in many types of cancer such as breast, prostate, colon, and lung cancers. Sp1 affects proliferation of cancer cells, adhesion, migration, and functions critical for cancer cell survival. Therefore, Sp1 is stated as a potential drug target for cancer therapy [27, 46]. Breast cancer-associated factor 1 (BRAC1) repairs double-stranded breaks in DNA, and if DNA is damaged beyond repair, then it destroys cell. It associates with Sp1 and with other factors and consequently suppresses IGF1R transcription [22, 23]. Furthermore, Sp1 binds to BRCA1 directly and represses the transcription of IGFIR Fig. 13.2.

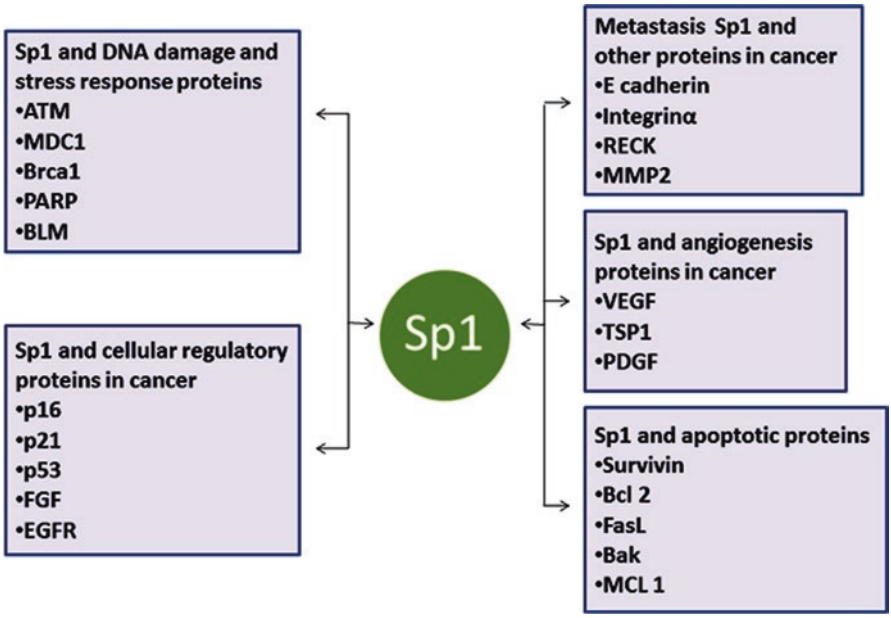

Fig. 13.2 Cancer causing genes regulated by *Sp1*

13.6 Role of Sp1 in Metastasis

EMT is generally associated with loss of cell adhesion molecules. Epithelial mesenchymal transition (EMT) generally falls in three basic categories. EMT engages in secondary epithelial or endothelial cell transitioning to tissue fibroblast in the process called as fibrogenesis. EMT is also involved in primitive epithelial cell transitioning to mesenchymal cells during embryological body plan or in early development. EMT also has role in transition of epithelial carcinoma cells in primary modules to metastatic phenotypes.

MTA2 is vital for activity of NuRD complex and Twist complex which is involved in inhibition of E-cadherin gene transcription. MTA2 protein could alter gastric cancer cell morphology via disrupting F-actin structure. NuRD complex, with MTA, regulates expression of genes involved in cytoskeleton remolding, necessary for cell motility and invasion. Alteration in expression of CD24 and MYLK might be a reason for MTA2 involvement in cytoskeleton. Overexpression of Sp1 regulates the transcriptional activity of MTA2 promoter and might partially contribute to gastric cancer [47]. Both ZEB2 and Sp1 associate with mesenchymal gene activation during EMT in cancer cells. Transcription factor ZEB2 is generally involved in EMT via repressing the E-cadherin transcription and upregulating integrin α5 expression to induce invasion. During EMT in human cancer cells, ZEB2 associates with Sp1 and activates integrin α5, vimentin, and cadherin-11 expression [24, 25].

Although transcription of Sp1 is tightly regulated under normal conditions, overexpression of Sp1 targets many downstream factors, those that promote cell proliferation and oncogenes. In TGF-β mediated induction of epithelial mesenchymal transition (EMT); Sp1 associates with Smad complex to express EMT-associated marker genes in pancreatic cancer. Inhibition or knockdown of Sp1 has key role in decreasing tumor formation growth and metastasis [28, 38]. E2F1 transcription factor modulates the expression of matrix metalloproteases, involved in degradation of extracellular matrix proteins (MMP). E2F1 modulates expression of Sp1 which in turn modulates MMP9 expression in small lung cell cancer [21]. Additionally, *TIAM*2 (neuron-specific protein) and Sp1 bind directly to GC box of *TIAM*2S promoter and function as oncogenes in hepatic cancer tumorigenesis [45].

13.6.1 Adhesion

Tumor metastasis involves invasion into neighboring tissue, intravasion, and persistence in circulation, extravagation, and colonization of distant organs. In order to metastasize, the tumor cells must alter their adhesion molecules to detach from the primary tumor mass and translocate to distant sites for growth of metastatic lesions. Activated leukocyte cell adhesion molecule (ALCAM) of immunoglobulin (IgG) super family is implicated in inflammation, tumor progression, and hematopoietic stem cell differentiation. A recent report stated that Sp1 binds to GC boxes in promoter region of ALCAM. Overexpression of Sp1 significantly increases basal promoter activity [33].

13.6.2 Angiogenesis

Increased cell division and metabolism require additional nutrients and growth factors for tumor microenvironment to fulfill demand through new vessel formation. Angiogenesis is controlled by proangiogenic molecules such as vascular endothelial growth factor (VEGF) and angiopoietin-2 in microenvironment. Cancer cells induce angiogenesis by several mechanisms; hypoxia and acidosis are the major causes for activation of VEGF. Loss of function or activation of tumor suppressor genes is associated with VEGF overexpression [6]. Promoter region of VEGF has a number of different transcription factor binding sites, viz., activator protein (AP)-1, AP-2, early growth response-1 (Egr-1), Sp1, and hypoxia-inducible factor-1 (HIF-1). Interestingly, Sp1and matrix metalloproteases (MMP) play an imperative role in angiogenesis which in turn promotes aggressiveness of human pancreatic adenocarcinoma [41]. MMP-2 involved in remodulation of basement membrane which helps in sprouting of vessels. During this process, the matrix-bound angiogenic factors get released which further helps in generation of new blood vessel. In gastric cancer, MMP-2 expression was depicted to be dependent on Sp1 transcription factor. Furthermore, the MMP-2 levels were elevated in different other cancer types as melanoma, lung cancer, and breast cancer [6].

13.7 Role of Sp1 in Cellular Immortality

Activation of telomerase is essential for cells to attain immortality. Telomerase activity in human cells is tightly regulated by human telomerase reverse transcriptase (hTERT) which in turn is regulated by HDAC. *h*TERT gene is repressed by binding of HDAC which could be the major, universal transcriptional repression of *h*TERT. HMGA2, important in telomere maintenance, is reported to be upregulated in a number of human cancers and promote tumorigenesis. Sp1 interacts with HMGA2 and interferes with HDAC2 recruitment to the *hTERT* proximal promoter and enhances histone H3-K9 acetylation, thereby stimulating *hTERT* expression and telomerase activity. Sp1 interferes with HDAC binding on *h*TERT and relieves the HDAC-mediated repression of the *h*TERT promoter [20]. In contrast to normal cells, tumor cell possesses ability of unlimited replication. Tumor cells overcome antiapoptotic cellular barrier and become cancerous. For unlimited cellular proliferation, tumor cell requires multiple factors, such as CDK inhibitor p16, p53, and telomerase enzyme [5]. Sp1 has a role in transcription of p53, p16, as well as telomerase genes. Sp1 mutation or modulation evades cancer cell through several mechanisms by employing tumor suppressor and *h*Tert genes.

13.8 Sp1 Role in Regulation of EGFR

Epidermal growth factor receptor (EPGR) belongs to receptor tyrosine kinase family. EGFR overexpression is associated with aggressive tumor nature and resistance to chemotherapy. Sp1 is translational regulator of EGFR; it inhibits EGFR gene transcription [12, 18, 19]. Epidermal growth factor receptor gene transcription is inhibited, when Sp1 interacts with promyelocytic leukemia protein (PML). DNA binding domain of Sp1 and PML's dimerization domain interact in different manner. This makes Sp1 to bind the promoter region of EGFR and inhibits its gene transcription [34]. Sp1 is targeted to nuclear bodies by the association of PML proteins [26].

13.9 Encoding Tumor Suppressor Activity

In normal conditions, growth factor and nutrients are absent in environment, and cells remain quiescent. Additionally, damaged DNA prevents cell transition into mitotic phase. Sp1 and Sp family members regulate several stress signals generated in cancer cells (Figs. 13.1 and 13.2).

13.10 Role of Sp1 in Immune Evasion of Tumor

Tumor cells need to evade from immune system. In order to attain resistance to immune system, tumor cells should overcome intrinsic and extrinsic signals for apoptosis and senescence. Sp1 is reported to be involved in regulation of several

anti- and proapoptotic signal, viz., BCl-2 [11], TRAIL and TNF-α, X-linked inhibitor of apoptosis (XIAP), E3 ubiquitin protein ligase, cellular FLICE-like inhibitory protein (FLIP), BCL-2 antagonist (BAK), and Fas ligand (FasL) [1, 7].

13.11 Sp1 and Cancer Chemotherapy

In gastric cancers, Sp1 regulate several genes involved in vital processes ranging from cell cycle, cell differentiation, and apoptosis. Sp1 is one of the negative prognostic factors for survival of patients with gastric cancer. Survival of patients after surgical tumor resection seems to correlate with Sp1 expression [39]. Poor survival in patients with resected gastric, pancreatic cancers occurs due to increased expression of Sp1 and VEGF [44]. Bevacizumab, a neutralizing antibody against VEGF, suppresses angiogenesis and abrogates gastric cancer growth. Bevacizumab block function of VEGF while activating its expression through a positive feedback. Mithramycin A suppresses Sp1 expression in tumors in vitro. Bevacizumab plus mithramycin has synergistic effects in tumor suppression, consistent with suppression of the Sp1 expression. This corroborates that alteration of Sp1 signaling has significant and potential clinical effects for the treatment of pancreatic and gastric cancer in mice [17, 35, 36]. Ginsenoside Rg3, active component in ginseng, has antitumorigenic, proapoptotic properties and antimetastatic significance to treat gastric cancer. Rg3 triggered the activation of caspases 3, 8, and 9 and PARP which considerably increases the expression of proapoptotic proteins. Furthermore, Rg3 repressed FUT4 expression through downregulation of HSF1 and Sp1 upregulation. Hence, Rg3 therapy might be an effective approach in gastric cancer treatment. Moreover Sp1 and HSF1 might serve as potential diagnostic and therapeutic targets for gastric cancer [2].

13.12 Conclusion and Future Directions

This review establishes the highly regulated role of Sp1 transcription factor in regulation and expression of a numerous genes that promote all the "hallmarks of cancer." The role of Sp1 in gastric cancer is not well established. Sp1 activates and suppresses the expression of several oncogenes and tumor suppressors, as well as genes involved in cellular functions, viz., proliferation, differentiation, DNA damage response, apoptosis, senescence, and angiogenesis. Sp1 has a significant role in cancer and important downstream signals transmitter for growth factor in normal cells; further studies on functions of Sp1 are essential for understanding its complete role in tumorigenesis. Targeting Sp1 may become an effective approach in preventing or reducing metastasis and resistance to therapies in gastrointestinal cancer (Table 13.1).

Table 13.1 Sp1 protein interactions

Transcription factors	Factors involved in cellular regulation	DNA repair proteins	Tumor suppressor proteins	Nuclear receptor proteins
TBP	p53	ATM	Brca1	ERα
TAF4	c Jun	Nos1	MDM2	SMRT
TAF7	c MYC	Brca1	VHL	PPARγ

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Curcumin: Its Role in Regulation of HIF-1α in Gastric Cancer

14

Tapan K. Barik and Surya N. Swain

Abstract

Gastric cancer is a malignancy and is the fourth most common cause of cancer globally. An oxygen-deficient microenvironment is a common state of gastric cancer, associated with tumor invasion and metastasis. Under hypoxic conditions, cancer cells start their own adaptive pathways to have a facilitated oxygen and energy supply for survival. In this condition, hypoxia-inducible factors (HIF) such as HIF-1α have a vital role particularly in cellular response to hypoxia and stimulate the major hallmark processes of tumor development, for example, angiogenesis, glucose transport, and metabolism by activating the transcriptional factors of various targeted gene involved in tumor development. Curcumin, a major phytochemical extracted from roots of *Curcuma longa*, inhibits proliferation of many types of solid cancer cells. Earlier, curcumin shows strong therapeutic potential against gastric cancer cell lines by downregulating the levels of the oncogene c-Myc expression and thereby hindering the expression of the targeted gene. Curcumin also exhibits antitumor effects under hypoxic conditions by significantly decreasing levels of hypoxia-induced HIF-1α protein, in cancer cells. Furthermore, curcumin eliminates cell proliferation, migration, and invasiveness generated by a hypoxic microenvironment and associated with HIF-1α accumulation.

Keywords

Gastric cancer · Curcumin · HIF-1α

The original version of this chapter was revised. The book was inadvertently published without Abstracts and Keywords, which are now included in all the chapters. An erratum to this chapter can be found at https://doi.org/10.1007/978-981-10-6728-0_39

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14.1 Introduction

Curcumin ($C_{21}H_{20}O_6$), commonly called as diferuloylmethane, is a natural lipid soluble and major biological active compound extracted from the rhizomes of turmeric plant, *Curcuma longa*. It is a golden-yellow hued flavor ordinarily utilized as a part of Indian subcontinent, for human services and safeguarding of nourishments. This nontoxic natural compound has numerous therapeutic activities for various diseases such as wound, inflammation, hepatitis, hemorrhoids, and liver disorders. Also, its several biological activities are therapeutically beneficial for the treatment of cancer. Curcumin additionally exhibits anti-inflammatory activity by suppressing the enactment of one of the inducible transcription factor that controls the expression of host genes involved in aggravation [3]. Since endless provocative conditions unequivocally favor the development of cancer, curcumin can possibly be a decision preventive agent for the treatment of cancer. Unlike most chemotherapeutic agent, curcumin has the potency to reduce the signs of cancer development, i.e., oncogene enactment [29], cancer cell multiplication [28], apoptosis evasion [12], metastasis [8], limitless replicative potential, and some emerging trademarks such as activator protein 1, B-cell lymphoma 2, JAK/STAT, and steroid receptors, respectively [26]. Along these lines, curcumin can possibly defeat chemoresistance and can hit different intracellular focuses without causing any reactions; these properties render it a promising contender for potential utilization in the treatment of cancer.

Cancer frequently depicted as disease of the genome since it obtains through the accumulation of DNA mutations and genome unsteadiness. Additionally, transformation of proto-oncogenes has been distinguished in several tumors [23]. Curcumin has been accounted for to suppressively affect the oncogenes and dysregulate their downstream action, which is an underlying stride in tumorigenesis. In spite of the fact that curcumin is ineffectively consumed after ingestion, numerous reviews have proposed that even in low concentration, curcumin indicates against proliferative effect which causes cell cycle arrest.

The enhancement of gastric (including gastroesophageal junction) cancer is a multifactorial process emerging through the perplexing interaction of natural and hereditary factors over a patient's lifetime [1, 14]. It is a common malignancy and is the fourth most normal and second driving reason of cancer demise in the world [10]. Albeit the incidence is declining due to amended pabulum, victuals preservation, better aversion, earlier diagnosis, and treatment, the disease still carries a poor prognosis. Surgical resection with adjuvant chemotherapy stayed as the main potential curative treatment choice for gastric cancer. In spite of resection, the disease requests never-ending consideration and research with respect to prevention, early detection, and novel therapeutic options.

14.2 Chemistry of Curcumin

Natural products have always been a venue for the search of new drugs or leads. These natural compounds have increased impressive enthusiasm for their potential as treatment and preventive operators for human diseases. Curcumin is a polyphenolic

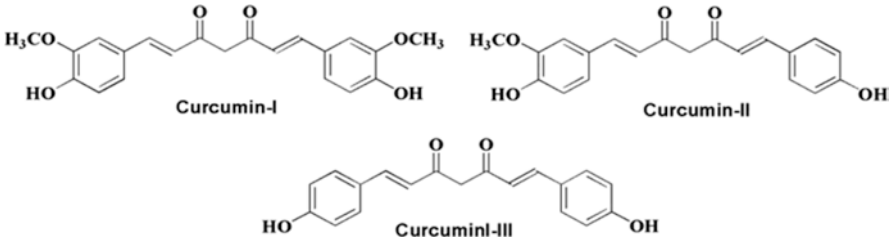

Fig. 14.1 Curcumin structure. *Curcumin I* 1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione, *curcumin II* 1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)hepta-1,6-diene-3,5-dione, *curcumin III* 1,7-bis(4-hydroxyphenyl)hepta-1,6-diene-3,5-dione

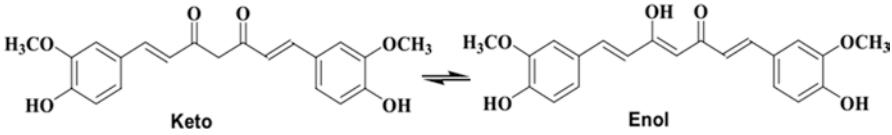

Fig. 14.2 The keto-enol tautomeric forms of curcumin

and major biological active compound extracted from the rhizomes of turmeric plant, *Curcuma longa*, and has been utilized broadly in customary solution since old circumstances as a household remedy against various diseases, including hepatic disorders, cough, sinusitis, rheumatism, and biliary disorders[4, 5]. Recently a large number of studies have shown that curcumin has a surprising array of antioxidant, antitumor, anti-inflammatory, anticancer, and other desirable medicinal properties. Curcumin, isolated from *Curcuma longa*, was once accepted to be a solitary segment yet was later found to have three closely related species, known by the name of "curcuminoids," which embody curcumin (curcumin I), demethoxycurcumin (curcumin II), and bisdemethoxycurcumin (curcumin III) (Fig. 14.1). These three types of curcumin are contrast by a methoxy group apended to the phenolic rings.

Natural curcumin extracted from turmeric usually contains curcumin I (77%) as the significant component, while curcumins II and III constitute approximately 17% and 3%, respectively. Notwithstanding the way that curcumin I, curcumin II, and curcumin III differ in their synthetic structures similarly as to methoxy substitution, they indicate by and large remarkable antioxidant, antitumor, and anti-inflammatory activities. To date there has been no proficient review that evidently interfaces the physiochemical and molecular properties of the three curcuminoids with their biological activities. Curcumin forms a ruddy brown salt with alkali and forms solvent with ethanol, acetic acid, and chloroform. It has two tautomeric forms, i.e., keto and enol (Fig. 14.2). The enol form is more vigorously stable in solution [16].

Curcumin has molecular weight of 368.37 with a melting point temperature of about 180–186 °C and shows a spectrophotometric maximum absorption (λmax) at 450 nm in methanol [22]. The curcumin molecule is one of a kind in its physiological

effects, having an incredible number of molecular focuses than whatever other particles so far are detailed. Curcumin has been accounted for to have different natural activities. Such an assortment of activities might be the consequence of its receptive α, β-unsaturated β-diketone moiety, which can covalently bind proteins [2]. Different normally occurring bioactive compounds have exhibited some auxiliary resemblance to the curcumin molecule; they incorporate ferulic acid, cinnamic acid, caffeic acid, chlorogenic acid, dibenzoylmethane, cassumuin, and yakuchinone [4, 5].

14.3 Anticancer Effects of Curcumin

Cancer is a heterogeneous group of diseases characterized by uncontrolled growth of the cells, distinguished by metastasis into the vital organs of the body through invasion and angiogenesis. Curcumin obstructs the transformation, proliferation, and invasion of tumor cells. Curcumin stifles the development of few tumor cell lines, including drug-resistant lines [21]. Curcumin in like manner stifles the activation of few transcription factors that are involved in carcinogenesis [2]. Some of the transcription factors, such as nuclear factor kappa B (NF-κB), activator protein 1 (AP-1), signal transducer, and activator of transcription proteins (STAT3, STAT5), and some cellular protein-coding genes, such as early growth response protein 1 (Egr-1), peroxisome proliferator-associated receptor gamma (PPAR-γ), β-catenin, and Nrf-2, are intimately involved in the cellular pathway causing tumorigenesis. Curcumin inhibits the activation of both NF-κB and AP-1 and their regulated gene products involved in tumor development. Also, curcumin downregulates the expression of Egr-1, PPAR-γ, β-catenin, and Nrf-2, thereby inhibiting tumor growth. Expression of cyclin D1, a component subunit of cyclin-dependent kinases 4 (Cdk4) and 6 (Cdk6) which are rate-limiting components in progression of cells through the cell cycle, is suppressed by curcumin [21]. Curcumin likewise initiates apoptosis in tumor cells by activating caspase-8, which promotes cleavage of BID, which in turn is responsible for release of mitochondrial cytochrome C, thereby activating caspase-9 and caspase-3, which promote the activation of poly ADP-ribose polymerase (PARP) and apoptosis of tumor cells. Curcumin has the potency to downregulate expression of genes responsible for cell multiplication, cell invasion, metastasis, angiogenesis, and resistance to chemotherapy [2]. Additionally, it also downregulates the expression of Bcl-2, Bcl-XL, cyclooxygenase-2 (COX-2), matrix metalloproteinase (MMP)-9, tumor necrosis factor (TNF), cyclin D1, and the adhesion molecules [27]. Curcumin binds to over 30 proteins and targets transcription factors, growth factors, cytokines, enzymes, and genes regulating cell proliferation and death pathways involved in cancer (Fig. 14.3).

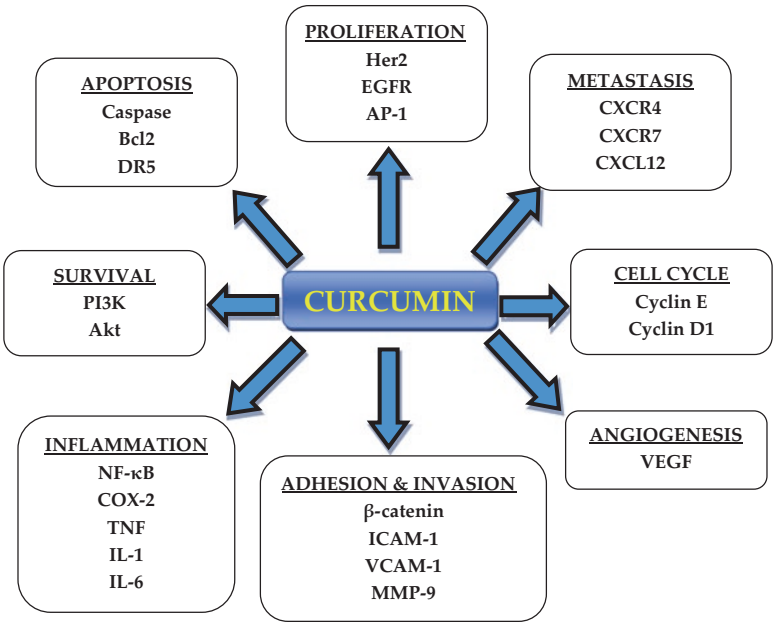

Fig. 14.3 Various molecular targets of curcumin in cancer cell

14.4 Curcumin and Gastric Cancer

Curcumin has been appeared to hinder the development of almost all types of cancer cells, including gastric cancer. Subsequently, there has been a vast collection of in vitro work exploring the restorative viability of curcumin in gastric cancer. Curcumin has potency to overcome the apoptosis resistance of cancer cells by regulating both intrinsic (mitochondrial) and extrinsic (death receptor) pathways. The expression levels of anti-apoptotic Bcl-2 and Bcl-XL, causing cytochrome c release, caspase-3 activation, and PARP cleavage are suppressed by curcumin by promoting apoptosis in human gastric cancer cell lines [6]. Curcumin upregulates the mitochondrial Bax protein expression by involving p53, which acts as a transcription factor, recommending the intrinsic pathway as a leading pathway of curcumin-incited apoptosis in tumor cell. The upregulation of expression of p53 by curcumin induces apoptosis in cancer cells at G2 phase of cell cycle. Curcumin additionally invigorates the extrinsic pathway through activation of caspase-8 by triggering the death activators such as TNF-α and Fas ligand. Curcumin enhances the levels of Fas and caspase-8, thereby inducing apoptosis in cancer cells. Curcumin repress the multiplication of gastric cancer cells by inducing apoptosis by facilitating the collapse of mitochondrial membrane potential (MMP) which was believed to initiate the mitochondria-induced apoptotic pathway. It hinders the opening of the ATP-sensitive potassium channel opening, in this manner, causing apoptosis in gastric

cancer cells [18]. The Wnt/β-catenin signaling pathway is important for the initiation, progression, and prognosis of human cancers. The β-catenin protein is multifunctional and plays a vital role in the canonical Wnt/β-catenin pathway [11], and it is activated by Wnt signaling. Previous reports proposed that the silencing of β-catenin hindered cell growth in different malignancies, indicating that β-catenin is indispensable for cancer growth. Curcumin inhibits Wnt/β-catenin signaling and diminished expression of Wnt target genes in human gastric cell lines, by reducing the levels of low-density lipoprotein receptor-related protein 6 (LRP6) and LRP6-phosphorylation, thereby inhibited cancer cell growth [34]. The development of P-glycoprotein subordinate multidrug resistance in gastric carcinoma is a noteworthy hindrance for effective chemotherapy [7]. Curcumin tweaks the expression of function of P-glycoprotein by lowering the levels and reversing the multidrug resistance capability through caspase-3 activation in gastric carcinoma cell lines [30]. Fluorouracil (5-FU) has been utilized as the standard of care to cutting-edge gastric cancer and has been found to increase general survival by 6% and lessen the risk of mortality by 18% [15]. However, lethal drug resistance to fluorouracil has created an impediment for the treatment of gastric cancer. Curcumin downregulates the NFκB-signaling pathway; a standout among the most critical survival-signaling cascade involved in drug resistance in tumor cells, subsequently, turns around the drug-resistance mechanism to fluorouracil [13].

14.5 HIF-1α: Therapeutic Target in Cancer

Oncogenesis is governed by genetic and epigenetic events that co-opt to malignant progression. Oxygen supply is one of the rate-limiting microenvironmental factors. As all mammalian cells rely on legitimate oxygen hemostasis so as to execute their metabolism and energy generation, cancer cells are no exemption. Hypoxia is the condition in which the partial oxygen pressure has dropped to levels that are no longer sufficient to sustain normal cellular function. In growing tumors, significant areas loose access to supporting blood vessels due to inefficient formation of the tumor vasculature. In these regions, oxygen delivery is insufficient to meet the oxygen demand, and the tumor suffers from hypoxic stress. The most very much described mechanism by which tumor cells adjust to a hypoxic environment is the enactment of the hypoxia-inducible transcription factor, HIF-1. The cellular adaptation to hypoxia is mainly constitutes by hypoxia-induced factor 1 (HIF-1).

HIF-1 is a heterodimeric transcription factor, made out of two subunits, the HIF-1α and HIF-1β subunits [31]. HIF-1β is otherwise called as aryl hydrocarbon nuclear translocator (ARNT). HIF-1α is an oxygen-sensitive subunit, and its expression is induced under hypoxic conditions and has two other isoforms, HIF-2α and HIF-3α [9]. The HIF-1 belongs to basic-helix-loop-helix-PAS (bHLH-PAS) protein family. Four groups of HIF-1α target genes that are particularly relevant to cancer progression are (1) angiogenic factors (LEP, VEGF, NOS, ADM), (2) genes involved in glucose metabolism (HK1, HK2, GLUT1, GLUT3), (3) survival factors (ADM,

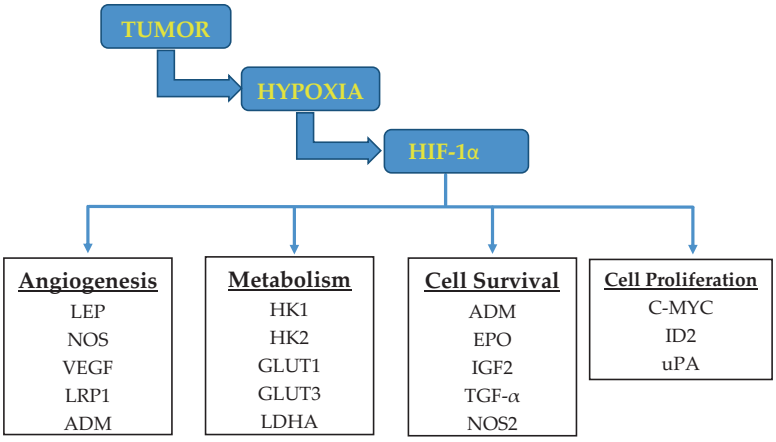

Fig. 14.4 HIF-1α regulatory genes and their impact on tumor progression. *LEP* leptin, *NOS* nitric oxide synthase, *VEGF* vascular endothelial growth factor, *LRP1* LDL-receptor-related protein 1, *ADM* adrenomedullin, *TGF-β3* transforming growth factor-β3, *EPO* erythropoietin, *HK1* hexokinase 1, *HK2* hexokinase 2, *GLUT1* glucose transporter 1, *GLUT3* glucose transporter 3, *LDHA* lactatedehydrogenase, *IGF2* insulin-like growth factor 2, *TGF-α* transforming growth factor α, *C-MYC* myelocytomatosis virus oncogene cellular homolog, *ID2* DNA-binding protein inhibitor, *uPA* urokinase plasminogen activator

EPO, IGF2, TGF-α), and (4) factors involved in invasion and metastasis (C-MYC, ID2, uPA) (Fig. 14.4) [17].

HIF-1α overexpression is related to increased mortality in patients with various tumors, including gastric malignancy [19, 25]. This association depends on the regulation of genes that assumes a fundamental part in the hallmark processes of cancer. HIF-1α targeting anticancer agents can be divided into different groups according to their mechanisms of action:

- Inhibition of HIF-1α protein translation
- Inhibition of HIF-1α protein by promoting its proteasomal degradation
- Inhibition of HIF-1 DNA-binding capacity
- Inhibition of HIF-1 transcriptional activity

HIF-1α regulates both transcription factors and chromatin modifiers to incite metastasis in an EMT-subordinate or EMT-autonomous manner. What's more, different targets controlled by HIF-1α that intervene other biological effects such as metabolism may likewise contribute to metastasis [20]. In gastric cancer, both HIF-1α and HIF-2α play the possible role in the progression of invasiveness and metastasis during hypoxia by involving the JNK signal pathway. Also, the expression levels of both HIF-1α and HIF-2α were significantly very high in metastatic gastric cancer in comparison to nonmetastatic gastric cancer [32].

14.6 Curcumin: HIF-1α Crosstalk

Under hypoxic stress, cells start their own adaptive pathway in response to lower oxygen and imbalanced energy status to continuously grow and survive. HIF-1α is a well-known adaptive pathway-regulating molecule in hypoxic condition, which stimulates the transcription of several genes involved in angiogenesis, metastasis, glucose transport, and apoptosis inhibition. For this reason, protracted exposure of cancer cells to the hypoxic conditions leads to resistance to chemotherapies and tumor malignancy. Aberrant expression of vascular endothelial growth factor (VEGF), a transcription factor, is one of the key regulators in hypoxia-induced angiogenesis. Curcumin inhibits the expression of VEGF through NFκB regulation. Both HIF-1α and NFκB are interdependent in regulating the expression of each other. Curcumin also excreted α1-inhibitory effects in hypoxic condition, resulting downregulation of HIF-1α and HIF-1β (ARNT) through degradation of transcriptional activity. In other ways, HIF-1α protein stability may be initiated indirectly by p53 levels elevated by curcumin. p53 interacts with HIF-1α and limits hypoxia-induced expression of HIF-1α by promoting its ubiquitin-proteasome degradation [24]. Taking everything into account, curcumin downregulates HIF-1α protein levels and activity and prompts the hindrance of VEGF gene expression. Moreover, curcumin adequately hinders the hypoxia-stimulated angiogenesis of tumor cells.

14.7 Conclusion

Given its promising viability in an assortment of variety of diseases including cancer, curcumin has garnered much consideration in research. Be that as it may, the low bioavailability including poor retention, rapid metabolism, and limited tissue appropriation, remain the real worries in the headway of curcumin as a drug. These advantages of curcumin make ready for future research on multidimensional therapeutic approaches to deal with gastric cancer including combinatorial strategies incorporating standard chemotherapies as well as natural compounds, offering the guarantee of conquering imperviousness to chemotherapy and enhancing chemoresistant tolerant results. Curcumin can stifle transformation, proliferation, and metastasis of tumors by regulation of specific molecules involved in cancer progression. Curcumin overwhelms proliferation and prompts apoptosis of human cancer cells in a concentration subordinate manner by hindering the Wnt signaling pathway [33]. The anticancer effect of curcumin was observed to be in part intervened by repressing HIF-1α stabilization in tumor cells, without influencing HIF-1α transcription. These outcomes recommend potential for the treatment of malignancy by altering the upregulation of HIF-1α observed. Finally, these findings on curcumin's role in gastric cancer have the potential to be extended to other types of cancer, ultimately contributing to the eradication of cancer.

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Transcription Factors and Colorectal Cancer: An Overview

15

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Abstract

Colorectal cancer (CRC) is one of the leading causes of cancer deaths worldwide among various cancer malignancies. The drugs and targeted therapies that target various intracellular signaling pathways have improved the progression free survival of CRC patients, but they suffer with therapeutic resistance. Dysregulation or mutations in several oncogenic transcriptional factors such as c-MYC, nuclear factor κB (NFκB), NF-E2-related factor 2 (Nrf2), signal transducer and activator of transcription-3 (STAT-3) and p^{53}, were reported to be associated with CRC. Understanding the transcription factors involved in various CRC pathogenesis will be useful in designing novel therapeutic strategies specifically targeting the dysregulated transcription factors. This chapter emphasizes the role of major transcription factors and their dysregulation in CRC.

Keywords

Colorectal cancer · Transcription factors · Dysregulation

15.1 Introduction

Colorectal cancer (CRC) is one of the most general and most serious malignancies. Worldwide, CRC is the second in women (614,000 cases, 9.2% of the total) and the third most common cancer in men (746,000 cases, 10.0% of the total). The

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incidence of CRC shows wide geographical variation across the world, with almost 55% of the cases occurring in economically developed countries [38]. According to the Centers for Disease Control and Prevention (CDC), around 14.1 million new cancer cases were diagnosed, and 8.2 million people died worldwide in the year 2012. In 2013 colorectal cancer was 10% of all cancers diagnosed, i.e., 1.4 million approximately. Mortality is lower (694,000 deaths, 8.5% of the total) with more deaths (52%) in developing countries, reflecting a poorer survival in these regions. By 2025, 19.3 million new cancer cases are expected to be detected (http://www.cdc.gov/uscs).

Worldwide, CRC is the second most in women and the third most common neoplasia in men [59]. The lifetime risk of developing CRCs is about 1 in 23 (4.4%) for women and 1 in 21 (4.7%) for men, which indicates that risk of developing CRCs is slightly higher in men than in women [112]. Both environmental and genetic risk factors play a crucial role in CRC development. A number of other factors such as diet, obesity or weight, physical exercise, smoking, alcohol consumption, age, chronic intestinal inflammation, and family history also affect person's risk for developing CRC. Generally, CRC consists of colitis-associated cancer (CAC) and sporadic (noninflammatory adenomatous). CAC is the subtype of CRC that is associated with inflammatory bowel disease (IBDs) including ulcerative colitis (UC) and Crohn's disease (CD). Moreover, patients with IBD including ulcerative colitis (UC) and Crohn's disease (CD) show an increased risk of CRC. Both CAC and sporadic CRC are associated with dysplastic cancer sequence and multiple mutations for carcinoma development. However, CAC differs from sporadic CRC; chronic inflammation precedes CAC development, but inflammation does not initiate tumorigenesis in the case of sporadic CRC, but rather chronic inflammation follows tumor development [69, 120].

Nearly 20% of the patients with IBD develop CAC [44]. These statistics are forbidding for the reason that cancer is a well-studied malignancy, which has preneoplastic lesions, slow development, and known risk factors that can be diagnosed and treated. Additionally, the cautioning symptoms and signs related to CRC are often found in the advanced stages, which significantly reduce the chances of using curative therapy. Hence, there is a need to introduce measures for screening and a better management of patients with CRC. The colon, rectal, and colorectal cancers are considered together as CRC in this chapter, unless stated otherwise.

15.2 Colorectal Cancer Genetics

The origin of CRC is not acknowledged specifically, but a few features become visible to amplify the risk of extending the malignancy. Around 10% of CRC patients have a true inherited predisposition to CRC, and the causative genetic event was identified in most of them.

Nearly 25% of diagnosed CRC cases have family history of CRC (familial CRC) [4].

Members of families with certain unusual hereditary circumstances such as juvenile polyposis syndrome (JPS), Gardner syndrome, familial adenomatous polyposis (FAP), Lynch syndrome, attenuated familial adenomatous polyposis (AFAP), Peutz-Jeghers syndrome (PJS) Muir-Torre syndrome, MYH-associated polyposis (MAP), and Turcot syndrome have a considerably high chances of developing CRC. Family members of women with endometrial cancer (uterine) may also have a higher risk of developing CRC compared to others. However, sporadic cases of CRC with no family history or genetic predisposition are found in nearly 90% of the CRC cases [37]. Both familial and sporadic CRC are genetically driven malignancies, but they differ from each other in that familial CRC is driven by germline mutations, whereas sporadic CRC is caused by alternations in DNA structure (mutations) or function (epigenetics) [92].

15.3 Molecular Mechanisms

CRC is highly heterogeneous malignancy with a number of key epigenetic and genetic alterations which lead to malignant transformation [3]. Major molecular pathways that have been identified to play a role in CRC include CpG island methylation pathway, microsatellite instability (MSI) pathway, and chromosomal instability (CIN) pathway. Chromosomal instability (CIN) is the most common pathway leading to CRC, which is characterized by mutations in oncogenes or specific tumor suppressor genes. MSI and CIMP pathways are characterized and distinguished by dysfunction of DNA mismatch repair genes and hypermethylation of CpG islands, respectively [58, 92].

15.4 Transcription Factors and CRC

The clinical and biological behavior of CRC is affected by multiple pathways regulating cellular processes such as proliferation, differentiation, migration, apoptosis, DNA replication, and DNA repair. These pathways are controlled and exercised by transcription factors (TFs) that ultimately regulate gene expression resulting tumor initiation and development. TFs are the DNA-binding proteins, which initiate and regulate expression of gene by controlling the activity of RNA polymerase in a gene-dependent manner. TFs do so by using their DNA-binding domain (e.g., basic helix-loop-helix, zinc finger, homeodomain) and transactivation domain (usually nine amino acids long), which interacts with various cofactors or transcription coregulators to either activate or repress the target genes. In addition, some TFs also possess a third domain called a signal-sensing domain (SSD), which senses external signals or signaling molecules. The human genome encodes nearly 2000 different TFs, many of which show cell-specific expression to regulate gene expression [12, 99, 139].

A very high percentage of oncogenes and tumor suppressor genes encode TFs. The TF expression or activity is tightly controlled in normal tissue conditions to

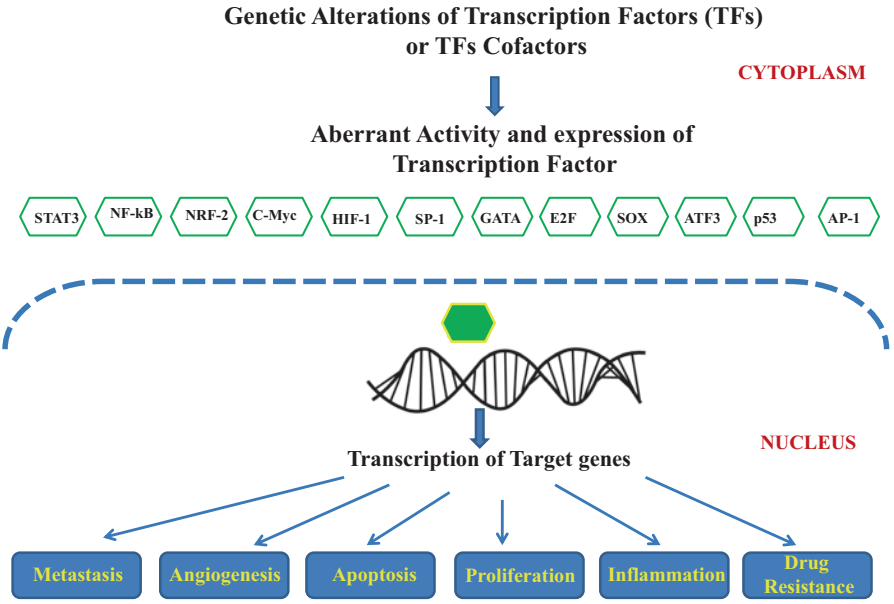

Fig. 15.1 Transcription factor (TF) and colorectal cancer (CRC). TFs expression or activity is triggered by multiple genetic alterations such as amplifications, deletions, insertions, and point mutations that result in gain- or loss-of-function, rearrangement via chromosomal translocations. Deregulation of TFs expression or alteration of TFs functionality leads to activation of intracellular signaling cascade, resulting in nuclear translocation of TFs and transcriptional activation of target genes involved in tumorigenesis-related cellular processes such as proliferation, angiogenesis, metastasis, and apoptosis

maintain tissue homoeostasis. Therefore, when the TF expression is altered or functionality is deregulated, TFs become oncogenic which in turn leads to dysregulation of genes implicated in tumorigenesis-related cellular processes including cell proliferation, apoptosis, angiogenesis, and metastasis. The TF deregulation could be due to multiple genetic alterations including amplifications, deletions, insertions, point mutations, and rearrangement via chromosomal translocations (e.g., TP53 and MYC). Studies also showed that alterations of TF cofactors (e.g., p300/CBP, SWI/SNF and mediator) are also major mechanism that contributes to the tumorigenesis. Furthermore, as TFs are the downstream effectors of many signaling pathways, alteration of TF functions by upstream oncogenic signal transduction cascades leads to tumorigenesis [75, 86, 131] (Fig. 15.1).

Current targeted therapies developed to date have been targeted against cell surface receptors (EGFR or VEGF) or kinase inhibitors targeting kinases including intracellular protein tyrosine kinase, serine/threonine kinases, and receptor tyrosine kinases (RTKs). Though these strategies have made major progress, their efficacy is restricted because of development of drug resistance due to mutation of the target gene or downstream molecules and overexpression of the target gene or parallel signaling pathways activation, thus demanding new therapies or strategies [53].

Conventionally, targeting transcription factors was generally considered too difficult. However, recent technological advances have made TFs as realistic and attractive drug targets. Recent preclinical studies of targeting TFs in animal models and in cancerous cell lines showed promising results. Normal cells often tolerate the disruption or targeting oncogenic transcription factors and/or reactivation of tumor suppressor genes with minimal toxicity because of overlapping cellular signaling pathways. Conversely, as many cancerous cells are dependent on oncogenic TFs, inhibition of their activity leads to selective killing of tumor cells [41, 141, 24]. An improved understanding of dysregulated TFs affecting tumor malignancies may direct to have better ability to design novel therapeutic strategies and predict clinical outcome. In the following sections, we discuss about the major transcription factors dysregulated in CRC.

15.4.1 STAT3

Signal transducer and activator of transcription 3 (STAT3) is a cytoplasmic transcription TF that regulates expression of genes related to many cellular processes including cell survival, growth, apoptosis, and differentiation. Mammalian STAT family consists of seven members (STAT1-4, STAT5α, STAT5 β, and STAT6) encoded by separate genes but shared similarity in structure. STAT3 appears to be more commonly transcribed than the other identified family members [25]. STAT3 is activated through the binding of extracellular signaling molecules such as cytokines (IL-6, IL-10, and IL-11) or growth factors (vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF)) to cell surface receptors via Janus kinase (JAK), focal adhesion kinase (FAK), and Src. Upon activation through phosphorylation, STAT3 forms a homodimer or heterodimer and translocates to the nucleus where it transcribes target genes involved in cell cycle (c-MYC and cyclin D1), angiogenesis (VEGF and IL-8), invasion/migration (MMP-2 and MMP-9), and anti-apoptosis (Bcl-xL, Bcl-2, and survivin). STAT3 also plays a key role in regulation of genes connected with anti-apoptosis, invasion/migration, and angiogenesis [39, 70].

Transitory levels of activated STAT3 are maintained in normal cells. However, clinically, in 70% of solid and hematological tumors, STAT3 is shown to be either constitutively active or overexpressed. Further, the level of activated STAT3 in CRC adenomas (18%) is much lower than that in adenocarcinomas (72%). STAT3 was shown to be a key regulator of tumor initiation and progression in CRC which regulates tumor-related proliferation, survival, inflammation, and angiogenesis and thus results in carcinogenesis and cancer progression [70, 82]. In clinical cases of CRC, STAT3 activation is negatively correlated with clinical efficacy and is associated with the prognosis of CRC. Constitutive activation of STAT3 is frequently high in dedifferentiated CRC cells and infiltrating lymphocytes, than in normal colon epithelium [21, 73]. Further, in vitro and in vivo studies had shown that the inhibition of STAT3 sensitizes CRC cells to chemoradiotherapy [114].

15.4.2 Nrf2

NF-E2-related factor 2 (Nrf2)/nuclear factor-erythroid 2-related factor 2 (Nrf2) is a leucine zipper-containing antioxidative transcription factor that plays a vital role in controlling of battery of genes implicated in multiple pathways including cell survival, proliferation, invasion, inflammation, and oxidative and electrophilic stress. The most important role of Nrf2 is activating the antioxidant responsive element (ARE)-mediated antioxidative response. Expression of Nrf2 is suppressed under unstressed conditions but markedly induced under oxidative stress. Under unstressed conditions, Cul3-dependent E3 ubiquitin ligase complex with cytosolic Kelch-like ECH-associated protein 1 (Keap1)-nuclear factor (Keap1) as substrate adaptor negatively regulates Nrf2 through polyubiquitination and subsequent degradation by 26s proteasome. Upon exposure to reactive oxygen species (ROS), toxicants, electrophilic molecules, and carcinogens, Keap1 sequestered Nrf2 is released and transactivates the expression of cryoprotective and antioxidative genes in the nucleus by forming the active complex with other regulatory proteins such as musculoaponeurotic fibrosarcoma (Mafs) [42, 60]. Nrf2 binds to the antioxidant responsive element (ARE) in promoter regions of target genes involved in antioxidants and xenobiotic metabolism. The Nrf2-ARE target genes include antioxidant genes, glutathione S-transferase (GST), NAD(P)H: quinone oxidoreductase 1 (NQO1), phase II enzymes such as heme oxygenase-1 (HO-1), and glutathione peroxidases. Apart from Keap1 inhibition of Nrf2, stress-induced upregulation of Nrf2 results from enhanced translation of Nrf2, mediated by functional IRES elements (internal ribosomal entry site) present within the 5′ untranslated region of the Nrf2 mRNA [78].

Nrf2 TF plays an intricate role in the cell, and its expression is strongly affected by various external agents or mechanisms. Any interruption of Nrf2 normal expression, either downregulation or overexpression, may encourage CRC initiation and progression. Basal-level expression of Nrf2 reduces the risk of CRC by exerting cytoprotective effect and by activating antioxidant target genes. Nevertheless, the loss of Nrf2 activity compromises the protection against the oxidizing agents and thus increases the occurrence of CRC [20]. On the other hand, many studies have reported that the risk of CRC increases with overexpression or constitutive expression of Nrf2 and its target genes, and the increased risk arises from Nrf2-induced colonic inflammation, increased cell proliferation, inhibition of apoptosis, and resistance to chemotherapy [54]. Increased chemoresistance to the chemotherapeutic agents due to Nrf2 overexpression is mediated by the activity of demethylases and methyltransferases. This overexpression can occur due to either constitutive mutation in the Nrf2 gene or in Keap1 repressor gene. Chronic inflammation is a major contributing factor for the development of CRC, and several recent studies have shown that Nrf2 is associated with chronic inflammation leading to CRC [91, 105]. Thus, Nrf2 can function both as tumor suppressor and also as an oncogene, and hence, targeting the Nrf2 represents a promising alternative approach for the treatment of both oxidative stress-induced CRC and colorectal inflammatory diseases [87].

15.4.3 NF-κB

Nuclear factor κB (NF-κB) proinflammatory TFs are key regulators of inflammation, innate immune responses, and cell survival. Mammalian NF-κB family is composed of five members: NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB, and c-Rel (Rel). All the members of NF-κB TF family contains a structurally conserved unique amino-terminal RH domain (Rel-homology domain), which is required for inhibitor binding, nuclear localization, homo- and heterodimerization, and DNA binding. Further, RelA, RelB, and c-Rel contain an additional transactivation domain that promotes transcription of target genes, while p50 and p52 do not. NF-κB subunits assemble by dimerization of two of the five subunits: p65 (RelA), c-Rel, RelB, p50/NF-κB1, and p52/NF-κB2 [102, 129].

NF-κB signaling in mammalian cells usually operates through two pathways: the canonical (classical) and the noncanonical (nonclassical) pathway. Noncanonical pathway is triggered by binding of tumor necrosis factor receptor family members such as BAFF, CD40, receptor-activated NF-κB ligand (RANKL), and lymphotoxin-B, and this pathway plays an important role in controlling the development and function of secondary lymphoid organs [115]. Canonical pathway is activated by various signals, including proinflammatory cytokines (TNF-α), growth factors (HGF), pathogen-associated molecular patterns (LPS), stress-inducing stimuli such as radiation and oxidative stress, and various other drugs [48]. These stimuli activate membrane-bound receptors, including the TNF receptor superfamily (TNFRSF) and interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR) superfamily as well as TCR or intracellular mediators, culminating in the activation of the inhibitor of kappa B (IκB) kinase (IKK) complex, composed of the scaffold protein NEMO (IKKγ) and two IKK subunits (IKKα and IKKβ).

Without stimulation (under nonactivated conditions), most of the members of NF-κB family are sequestered in the cytoplasm by specific inhibitory proteins, IκBs. Upon activation, NF-κB is released or freed from IκBα through kappa β (IκB) kinase (IKK) complex, composed of the scaffold protein NEMO (IKKγ) and two IKK subunits (IKKα and IKKβ) (IKK)-mediated phosphorylation and proteasomal degradation of the IκB inhibitory proteins. The freed NF-κB subunits form either homo- or heterodimers and translocate to the nucleus and trigger the expression of target genes, including anti-apoptotic factors, proinflammatory cytokines, and proliferation factors [30, 102].

In addition to NF-κB roles in the inflammatory signaling, the NF-κB pathway has been extensively tied to CRC. NF-κB is reported to be constitutively active in various cancer cells, cell lines, xenograft animal models, or clinical sites. NF-κB is found to be constitutively activated approximately in 40% of human CRCs and 67% of CRC cell lines [81, 103, 125]. The NF-κB pathway plays a key role in the progression of colitis-associated cancers (CAC) because of persistent STAT3 activation and sphingosine-1-phosphate (S1P)-mediated upregulation of IL-6 production [80]. Recent studies depicted that constitutively activated NF-κB promotes carcinogenesis by stimulating proliferation of malignant CRC cells, angiogenesis regulation, apoptosis inhibition, promotion of tumor invasion, and metastasis [100, 102, 103].

The process of epithelial mesenchymal transmission (EMT) is important for carcinogenesis, invasion, and metastasis. The EMT driving TF snail (promotes lymph node metastasis) and twist (enhances EMT) is co-regulated by NF-κB and HIF with potential clinical significance in CRC [22]. Moreover, increased expression levels of twist and NF-κB are correlated with tumor metastasis to the lymph nodes [106]. Another important feature of constitutive activation of NF-κB imparts resistance to chemotherapy. Tumor cells with constitutive NF-κB activation are highly resistant to anticancer drugs, and the inhibition of NF-κB activity in those cells has increased tumor sensitivity to inhibitors or drugs. As NF-κB activation contributes to the CRC tumorigenesis, the inhibition of NF-κB activation may be useful in antitumor therapy.

15.4.4 P53

P53 is a tumor suppressor protein encoded by the human TP53 gene. P53 plays a key role in cell cycle and senescence or apoptosis in response to various intrinsic and extrinsic cellular stress signals such as DNA damage by gamma or UV radiations, oxidative free radicals, hypoxia, depurination of DNA, etc. P53 protein has many mechanisms of anticancer functions such as inhibition of angiogenesis, activation of DNA repair, inducing growth arrest, or initiating apoptosis. The human p53 protein contains 393 amino acids and has four functional domains: basic domain or DNA-binding domain (DBD), tetramerization domain (TD), transactivation domain (TAD), and proline-rich domain [62].

During the normal conditions, p53 level is maintained at low level through continuous ubiquitination and degradation process by the E3 ubiquitin ligase MdM2, which functions as negative regulator of p53. The stress signals disrupt or inhibit the Mdm2-p53 interaction and activate p53 through series of posttranslational modifications such as phosphorylation and acetylation of Mdm2 by c-Abl and ATM kinases. Once activated, the activated p53 protein binds target DNA sequence and triggers the expression of diverse target genes (including p21), which results a cell cycle arrest to allow either cellular repair or apoptosis. In addition, activated p53 also interacts with several other proteins (PML bodies (promyelocytic leukemia bodies), Werner helicase) for its transcriptional activity to selectively modulate target genes. In healthy humans, wild-type p53 induces the expression of MDM2; in contrary mutant p53 can't induce MDM2 and results in the accumulation of p53 at very high concentrations [47].

The p53 protein is most commonly altered protein in all types of human cancers and is frequently mutated or inactivated in CRC. Dysregulation of p53 is one of the most common events for the CRC development, progression, and tumor metastasis [79]. Inactivation of the p53 tumor suppressor is a key event in CRC and correlates with the transition from benign adenoma to malignant carcinoma. The *p53* gene is mutated in about 40–50% of sporadic CRC [117]. The mutations mainly occur in DNA-binding domain (DBD) (Exons 5–8) followed by tetramerization domain (TD), majorly in a few hotspot codons, including 175, 245, 248, 273, and 282, consisting of GC>AT transitions which are predominantly located at the CpG

dinucleotide. Loss of heterozygosity (LOH) of the short arm of 17p chromosome is also found in aggressive phenotype CRC tumors. Further, the frequency of occurrence of LOH of the 17p chromosome and p53 mutation was more in carcinomas than in early adenomas in both non-familial and familial adenomatous polyposis patients. Loss of p53 function via allelic loss or mutation also contributes to the chemoresistance and leads to poorer prognosis [96]. Abnormal pattern of p53 expression is closely associated with disease outcome of sporadic CRC. In CRC, it has been shown that accumulation of mutations in a sequential manner in APC, K-Ras, and p53 genes is closely associated with the progression of CRC [79]. The inactive p53 mutations were observed more often in advanced stages of tumor and were negatively associated with patient survival [55].

15.4.5 Hypoxia-Inducible Factor (HIF)- 1

Hypoxia-inducible factor-1 (HIF-1) is an oxygen-sensitive TF induced during hypoxia (low oxygen tension) and plays a key role in cell proliferation/survival, angiogenesis, and glucose and iron metabolism. HIF-1is heterodimeric TF consisting of an oxygen-regulated HIF-1α subunit (or its human paralogs HIF-2α and HIF-3α) and constitutively expressed/oxygen-independent HIF-1β subunit (also called as aryl hydrocarbon receptor nuclear translocator; ARNT). The HIF-1α and β subunits contain basic helix-loop-helix motifs that bind DNA and cause dimerization of HIF subunits. HIF α subunit has an additional oxygen-dependent degradation (ODD) domain apart from Per-ARNT-Sim (PAS) domain [22].

The activity and stability of HIF-α subunit are regulated at posttranslational level by phosphorylation, ubiquitination, hydroxylation, and acetylation. Under normoxic conditions, ODD domain is hydroxylated by non-heme, Fe2+ and 2-oxoglutarate (2OG)-dependent dioxygenase enzyme proline-hydroxylase-2 (PHD-2). Subsequently, the hydroxylated HIF-α is recognized by the von Hippel-Lindau (pVHL) E3-ubiquitin ligase complex rendering HIF-α Lys48-linkedpoly-ubiquitination and subsequent HIF-α subunit degradation via ubiquitin-proteasome pathway. Further, FIH (factor inhibiting HIF) provides another layer of regulation by blocking HIF binding with its coactivators p300/cAMP response element-binding (CREB) protein (CBP) through hydroxylation of asparaginyl residue in the transactivation domain of HIF-α subunit [93, 122].

In hypoxia (oxygen deprivation conditions), the HIF-α subunit becomes stable due to diminished PHD activity then translocates into the nucleus, and dimerizes with the HIF-1β subunit. The HIF- α and β dimer then binds to the hypoxia responsive elements (HRE) along with its coactivators and regulates transcription of downstream target genes implicated in a large variety of processes [89]. In hypoxic conditions, HSP90 and STAT3 interact with HIF1α transactivation domain and PAF domain, respectively, and stabilize HIF-α [63, 67]. Further, an intimate bidirectional crosstalk between HIF and NF-κB has been demonstrated under inflammatory conditions to regulate the common targets against activating stimuli [8, 22, 123].

Elevated levels of HIF-1α and HIF-2α have been found in CRC [16, 98] due to either hypoxia or upregulation of signal transduction pathways or loss of the von Hippel-Lindau (VHL) expression. Immunohistochemical studies have shown that HIF-1α is detected in both adenomas and colorectal adenocarcinomas, and moreover HIF-1α is frequently expressed in adenocarcinomas compared to adenomas [43, 57]. Expression of HIF-1α is also frequently correlated with disease stages [72]. In contrast to HIF2α (EPAS1), HIF1α regulates numerous genes essential for development of CRC. Though both the isoforms are induced by hypoxia, HIF-1α and HIF-2α have divergent and specific roles in colon cancer [56].

Hypoxic microenvironment created in tumors supports tumor growth, invasion, metastasis, and angiogenesis in CRC. HIF1α plays an essential role in mediating these effects of hypoxia. HIF1α regulates expression of battery of genes involved in CRC survival (GLUT-1) [90], tumor vascularization (COX2) [64], angiogenesis (VEGF) [16], invasion and metastasis (cathepsin D, matrix metalloproteinase 2, urokinase plasminogen activator receptor (uPAR), fibronectin 1, keratins, vimentin, and transforming growth factor α) [72], chemo-/drug and radiation resistance (MDR-1/P-gp) [29, 89], and hypoxia survival (Nur77) [6, 119, 133].

15.4.6 Specificity Protein-1 (SP1)

Specificity protein-1 is a zinc finger TF that belongs to SP/KLF (Krüppel-like factor) family of TFs that binds to GC-rich *Sp1*-binding sites located on promoter regions of several genes having diversified roles in cell growth, differentiation, apoptosis, DNA damage, and immune responses. Sp1 is 785 amino acids long protein encoded by *sp1* human gene located at the 12q13.1 locus. Sp1 plays a key role in cell proliferation, angiogenesis, metastasis, and apoptosis. There are 12,000 Sp1-binding sites in the human genome, and Sp1 serves as activator as well as repressor based on the promoter to which it binds and also the coregulators or interacting partners with which it interacts [26].

Sp1 comprises of four distinct domains (A, B, C, and D), of which A and B domains rich in glutamine serve as transactivating domains (TAD) which directly interact with components of the transcription machinery, i.e., TBP (TATA-binding protein) and TAF4 (TBP-associated factor 4). Domain C of sp1 is a highly charged group of 69 residues comprising 12 negative and 6 positive charges. Domain D lacks its own transactivation and is essential for synergistic transactivation by Sp1. Three Cys2-His2 type zinc finger motifs located between domain C and D of Sp1 serve as its DNA-binding domain (DBD). Each of three Sp1 zinc finger motifs possesses its own preference for specific DNA sequence, and all of them are required for binding to the *Sp1*-binding site. The N-terminus of Sp1 consists of small inhibitory domain (IB) at the extreme N-terminus end, which regulates the domains A and B functions by directly interacting with corepressors such as SMRT (silencing mediator of retinoid and thyroid receptor), NCoR (nuclear hormone receptor corepressor), and BCoR (BCL-6 interacting corepressor) [7, 132].

Sp1 was the founding member of the Sp TF family, which consists of two broad groups: Sp1–4 group and the Sp5–9 group. Members of Sp1–Sp4 group have similar domain organization having N-terminal transactivation domains, which are absent in Sp5–Sp9 group. Sp1, Sp3, and Sp4 have two TADs, whereas SP2 possesses only one TAD. The activity of Sp1 is compensated by other members of the family due to homologous sequences among members of sp. [10]. The transcriptional activity of Sp1 is regulated by posttranslational changes such as phosphorylation, glycosylation, acetylation, and proteolytic processing as well as by interactions with tumor suppressors and oncogenes [18]. Sp1 binds to consensus GC box 5′-(G/T)GGGCGG (G/A) (G/A) (C/T)-3′ through its C2H2-type zinc fingers and recruits other proteins and general transcription factors, coregulators associated with transcriptional complex. A promoter can have either single or multiple Sp1-binding sites. Sp1 acts as a transactivator and binds directly to other Sp1 molecules and forms homooligomers. Sp1 transactivates simply via a single or synergistically via two or more Sp1-binding sites without cooperative DNA binding or super activates the Sp1-mediated transcription through interaction with DNA-bound Sp1 molecule. Sp1 also interacts with other cellular factors such as the cell cycle regulators, ATP-dependent remodelers, chromatin modifiers, factors participating DNA repair, and other transcription factors to modulate expression of specific target genes [7, 26, 132].

Sp1 is an important transcriptional regulator that plays a significant role in CRC initiation and metastasis [7]. Recent studies have shown that Sp1 and its other family members are highly expressed in human CRC stem cells and CRC cell lines and tissues [1, 45]. Further, higher levels of Sp1 protein have been observed in colon cancer patients [127, 128]. The role of Sp1 in CRC is described in detail with respect to cell proliferation, angiogenesis, metastasis, and apoptosis. Sp1 also contributes to the resistance to chemo- and radiotherapy in CRC apart from NF-B and hypoxia-inducible factor (HIF-1). Recent study has shown that transcriptional enhancer activator domain 1 (TEAD1) overexpression increases the CRC cell proliferation through enhancing the Sp1 expression levels [143].

15.4.7 GATA

GATA transcription factors are zinc finger motif containing TFs, which play an essential role regulation of the differentiation and organogenesis during vertebrate development. Based on the tissue expression and phylogenetic analysis, the six TFs in vertebrates are classified into two subgroups: GATA1/GATA2/GATA3 and GATA4/GATA5/GATA6. The members of subgroups GATA1/GATA2/GATA3 are implicated in differentiation of ectoderm and mesoderm, whereas GATA4/GATA5/GATA6 are involved in development and differentiation of mesoderm-derived tissues (embryonic stem cells, cardiovascular embryogenesis) and endoderm. All the members of GATA TFs contains two highly conserved DNA-binding zinc finger domains,Cys-X_2-C-X_{17}-Cys-X_2-Cys (ZNI and ZNII), which typically bind to the (A/T)GATA(A/G) elements and also mediate interactions with other proteins [76,

146]. GATA TFs are not conventional tumor suppressors; however their loss leads to malignant transformation.

Impaired function through mutations, reduced expression, or overexpression of GATA transcription factors has been associated with several cancers including CRC. In addition, many factors that associate with these TFs are proto-oncoproteins and function in part by modulating the GATA proteins activities. GATA2 expression levels are correlated with the worse patient survival outcomes in CRC patients with KRAS mutations and associated poor prognosis and disease recurrence in CRC [19, 137]. GATA4 and GATA5 exhibit tumor suppressive properties in human CRC, and these genes are frequently transcriptionally silenced by hypermethylation [2, 50]. Overexpression of GATA6 is found in CRC cells, and in vitro studies on human colon cancer cells have shown that GATA6 regulates the suppression of 15-lipoxygenase (LOX)-1 (15-LOX-1) [111]. Altered GATA6 expression promotes CRC tumorigenesis and tumor invasion by regulating urokinase plasminogen activator genes (uPA) [11] and also regenerating gene 4 (REG4) expressions [68]. Further it was shown that aberrant GATA6 expression in CRC was associated with poor prognosis and liver metastasis [109]. Pradhan et al. [97] have shown a possible link of GATA1 with CRC using system biology approach.

15.4.8 E2F

The E2F family of TFs controls several cellular functions related to cell cycle, DNA damage, and apoptosis and regulates many tumor suppressors. Mammalian E2F family consists of eight proteins, of which E2F1, E2F2, and E2F3a act as activators and E2F3b, E2F4–E2F8 serve as repressors. E2F7 and E2F8 are newly identified members of E2F family and show modest homology with other established E2F family members. The E2F3 gene encodes longer E2F3a and a shorter E2F3b protein products, of which E2F3b lacks the cyclin A binding domain [121]. Traditional E2F family members (E2F1–6) contain several evolutionally conserved domains, including N-terminal DNA-binding domain, dimerization domain with marked box important for dimerization and DNA bending, acidic amino acid-enriched transactivation domain with pocket protein-binding region, and a tumor suppressor protein association domain. C-terminal transactivation domain is absent in E2F6 factor. E2F1–E2F3 have an additional cyclin A binding domain with an adjacent nuclear localization signal (NLS). NLS assists in modulating E2F activity in cell cycle-dependent manner through regular nucleocytoplasmic movement of E2F TF. E2F4 and E2F5 are additionally tagged with nuclear export signal (NES) and require differentiation regulated transcription factor proteins (DP) for their nuclear translocation. E2F1–E2F6 TFs form heterodimers with one of their partner proteins (DP1 or DP2 or DP3) to form functional TF complex which can bind to their target genes [88]. These proteins bind preferentially to retinoblastoma protein pRB or the associated pocket proteins p107 and p130 in a cell cycle-dependent manner and can regulate cell proliferation as well as p53-dependent/independent apoptosis [95, 135].

E2F TF family members are downstream targets of the pRb-CDKs pathway. Free E2F proteins are transcriptional activators, whereas binding of pRB proteins changes them to transcriptional repressor state. Phosphorylation and dephosphorylation of Rb control the binding of Rb to E2F during the cell cycle. Upon phosphorylation of pocket proteins by the cyclin-dependent kinases (CDK), heterodimer E2F-pocket protein complex is disrupted, and free E2F is released. E2F then translocates to the nucleus and activates the transcription of target genes (S-phase-promoting genes) such as cyclins, CDKs, checkpoint regulators, DNA repair, and replication proteins. On the other hand, in repressors (E2F4 and E2F5), p107 and p130 picking binding proteins mediate the recruitment of repression complexes to induce apoptosis via the p53 stabilization as well as through the p732 activation [28, 32, 124]. Transcriptional genes regulated during apoptosis are TP73, APAF1, CASP3, CASP7, CASP8, and MAP3K5. E2F6–8 factors act as repressors of E2F target genes through their interaction with mammalian polycomb complex (PcG) family proteins but not through pocket proteins [27].

Mammalian E2F family of TFs can function as tumor suppressors or activators, and the dual function depends on the tissue of expression. E2F1 acts as tumor suppressor in CRC and esophageal adenocarcinoma, whereas it has tumor-promoting role in pancreatic ductal adenocarcinoma and esophageal squamous carcinoma. E2F4 has tumor-promoting role in gastric, CRC, and liver carcinogenesis [40]. The function of E2F TFs especially E2F1 in CRC has been shown by a number of studies. Increased expression of E2F1 was found in CRC [116, 140], and the increased expression of E2F1 decreases the cell proliferation and induces apoptosis of CRC adenocarcinoma [14, 36, 136]. E2F1 expression levels are high in lung metastasis of colon adenocarcinoma and correlate with the thymidylate synthase expression levels coupled with poor response to 5-fluorouracil treatment [9, 66]. E2F1 promotes the aggressiveness of human CRC by activating the ribonucleotide reductase small subunit M2 (RRM2) [36]. Increased expression of E2F-1 sensitizes CRC cells to camptothecin, indicating the role of E2F1 in mediating the cytotoxicity of cells to DNA-damaging agents such as topoisomerase I and topoisomerase II inhibitors [31]. Recently, it has been shown that E2F2 plays a tumor suppressor role in colon cancer through inhibition of survivin and modulating the expression of CDK2, CCNA2, MCM4, and C-MYC. At tissue level, E2F2 expression is very low; however, E2F2 acts as tumor suppressor in colon cancer and plays an important role in microRNA-31 (miR-31) mediated proliferation of colon cancer [77, 79].

E2F4 TF has a tumor-promoting activity, and it is directly connected with cell proliferation of CRC cells. In agreement of this, in vitro and in vivo studies of CRC demonstrated higher nuclear expression levels of E2F4 in the replicating colon epithelium [85, 136]. Intriguingly, E2F4 contains a longer spacer segment of 13 consecutive serine amino acid residues in the transactivation domain region encoded by AGC trinucleotide repeat. This region of E2F4 is highly vulnerable to frameshift mutations in situations of genetic instability leading/contributing to the human CRC. Further, E2F4 mutations enhance the capacity of CRC cells to grow without anchorage, thereby contributing to tumor progression [94, 113, 142]. Microarray analysis of sporadic colorectal carcinoma tissues showed upregulation of E2F5 TF [74].

15.4.9 C-MYC

The proto-oncogene MYC family comprises of N-MYC, L-MYC, and C-MYC (MYC). MYC is a multifunctional, nuclear phosphoprotein that plays pivotal role in diverse cellular process such as cell cycle, survival, differentiation, apoptosis, energy metabolism, and cellular transformation [52]. MYC gene encodes a basic helix-loop-helix zipper (bHLHZ) protein that dimerizes with Max (small bHLHZ transcription factor) partner protein and binds the enhancer box (E-box) sequence CACGTG to transactivate target genes through recruiting histone acetyltransferases (HATs). MYC can also repress the gene expression via binding with another partner, Miz1. Thus, MYC protein is associated with both transcriptional activation as well as transcriptional repression. MYC protein through its leucine zipper TF-binding motif interacts with its partner proteins (Max or Miz1) to form heterodimers. L-MYC and N-MYC also encode transcription regulators whose expression is altered in a large variety of tumors [33, 51].

MYC is the most commonly mutated oncogene in human cancers. MYC expression is tightly controlled involving several transcriptional regulators, and its expression increases in cells requiring high MYC protein levels in response to mitogens. However, expression of MYC oncoprotein and/or its activity is often deregulated in different types of cancers by various mechanisms including mutations, gene amplifications, increased protein stability, activation of upstream mitogen signaling pathway and chromosomal translocations. In the context of CRC, deregulation of the RTK/RAS/MEK/ERK and WNT/APC/β-catenin pathways enhances MYC expression and increases protein stability [13, 23]. MYC amplification is observed in nearly 6% of the consensus molecular subtypes of CRC [65]. Amplification and increased expression of the MYC gene were reported in metastatic CRC [101]. Furthermore, frequent inactivating mutations in the SMAD, ARID1A (transcriptional regulators), and FBXW7 (E3 ubiquitin ligase) may result in increased mRNA transcription and protein stability, respectively. Deregulation of MYC often correlates with disease aggressiveness and poor patient prognosis and confers resistance to chemotherapy [15, 49, 138].

15.4.10 SOX Family of Transcription Factors

SOX proteins are characterized by the evolutionarily conserved approximately 80-amino-acid-residue-long DNA-binding motif called HMG (high-mobility group) box. SOX TF family consists of more than 20 members. SOX proteins bind to minor groove of DNA on a common 7-nucleotide-long (A/T)(A/T)CAA(A/T)G consensus sequence through the HMG box. Apart from regulating DNA binding of SOX proteins, HMG domain mediates nucleocytoplasmic shuttling and physical interaction of SOX proteins with other interacting proteins through nuclear localization signals (NLSs) and leucine-rich nuclear export signal (NES). SOX stands for SRY-related HMG box [108]. Based on the phylogenetic analysis of HMG domain sequence, structure, and functions of SOX proteins, SOX family is subdivided into A to J

subgroups. Members of the same subgroup possess similar biochemical properties and thus show functional redundancy. SOX TFs regulate diverse developmental events including cell type specification and cellular differentiation, and they work in concert with other TFs to regulate expression of their target genes [104, 130].

Gene amplification and/or overexpression of SOX genes (SOX4 and SOX11) are linked to a variety of malignant tumors including CRC. Immunohistochemical staining and RT-PCR studies have shown increased expression of SOX2, SOX4, and SOX9 in CRC tissues compared with adjacent normal tissues [35, 83, 126]. The increased SOX2 expression is correlated with mutated BRAF (V600E mutation) indicating that its expression is regulated by BRAF signaling pathway [84]. Wang et al. [126] have shown that increased expression of SOX4 plays a crucial role in the development and progression of CRC [126]. Increased expression of SOX9 correlates with tumor progression and connected with lower overall survival [17, 83, 110]). On the contrary, SOX7 has tumor suppressor activity in CRC. SOX7 expression is downregulated in primary tissues as well as CRC cells, inhibits proliferation, and induces apoptosis of CRC cells [145]. SOX proteins modulate tumorigenicity by modulating the expression of Wnt signaling pathway that targets genes either by enhancing (SOX2, SOX4, and SOX9) or by suppressing (SOX7) the β-catenin/TCF activity [71].

15.4.11 Activating Transcription Factor 3 (ATF3)

ATF3 (activating transcription factor 3) is a member of the ATF/CREB (mammalian activation transcription factor/cAMP responsive element binding) TF family which are implicated in the regulation of cellular stress response. ATF/CREB family members include ATF1-7, B-ATF, CREB, and CREM, which commonly contain basic region leucine zipper (bZIP) type of DNA-binding domain. The basic region part mediates sequence-specific DNA binding, whereas the leucine zipper region is required for forming homodimers or heterodimers with other bZIP domain containing proteins such as C/EBP, AP-1, or Maf families. ATF/CREB TFs bind to the TGACGTCA consensus sequence present in the cyclic AMP response element (CRE) of various promoters [118]. In vitro and in vivo studies have shown that ATF3 promotes growth and metastasis of colon cancer tumors [46, 134]. Further, it was shown that ATF3 downregulates the expression of retinoblastoma (Rb), Bcl-2, β-catenin, and EMT-inducing transcription factors, while ATF3 increases collective cell migration and expression of CD44 (cluster of differentiation 44). Therefore, ATF3 may play a complex dichotomous role in metastasis and apoptosis in human CRC cells [61].

15.4.12 Activator Protein 1 (AP-1)

The activator protein 1 (AP-1) TF is a dimeric complex consisting of basic region leucine zipper (bZIP)-containing proteins including members of the FOS (c-Fos,

Fra-1, Fra-2, and Fos-B) and JUN (c-Jun, Jun-B, Jun-D), Maf (Nrl, c-Maf, MafB, MafA and MafG/F/K), and ATF (B-ATF, JDP1, JDP2, ATF2 and LRF1/ATF3) protein. JUN family members can form homodimers, whereas the FOS family members require JUN family to form active transcriptional complex. Dimeric AP-1 complexes are bind to TPA response elements (TREs) and cAMP response elements (CREs) in the promoter and enhancer regions of target genes. AP-1 is a TF that regulates expression of target genes in response to bacterial and viral infections, growth factors, cytokines, and stress and controls cellular processes such as cellular proliferation, apoptosis, and differentiation [107]. AP-1 TF family members are differentially expressed in neoplastic and nonneoplastic colorectal tissues. Upregulation of c-Jun and Fra-1 is an early event in human CRC tumorigenesis [144]. Hypoxia, mutations in β-catenin and K-ras genes and LOH (loss of heterozygosity), or allelic imbalance of the adenomatous polyposis coli (APC) gene induce AP-1 in colon cells [5].

15.5 Conclusion

Colorectal cancer (CRC) is one of the most important causes of death worldwide. Despite the improvement in our understanding about CRC in recent years, current treatments are not effective in controlling the metastatic forms of CRC. CRC is a multistep process involving genetic alterations in both oncogenes and tumor suppressor genes. A very large number of tumor suppressor genes and oncogenes encode TFs. Under normal conditions, TF expression levels or activity is under tight control to maintain tissue homoeostasis. However, when the TF expression is altered or functionality is deregulated, TFs become oncogenic which in turn leads to dysregulation of genes implicated in tumorigenesis-related cellular process including proliferation, apoptosis, angiogenesis, and metastasis. A number of oncogenic TFs such as NFκB, Nrf2, c-MYC, and STAT-3 are over-activated in CRC, and on the other side, p^{53}, a tumor suppressor TF, is under-activated.

Many cancer malignancies require a subset of oncogenic and/or tumor suppressor TFs for their survival, proliferation, and disease progression. In contrary, these TFs appear to be dispensable for normal cells. Hence, targeting such TFs, either inhibition of oncogenic TFs or reactivation of tumor suppressor genes, may have highly synergistic anticancer activity against the tumor cells with little or minimal toxicity against the normal cells. Additionally, targeting TFs in tumor-associated immune cells may have the possibility to surmount the tumor-associated immunoresistance. Various novel strategies for targeting dysregulated TFs include blocking DNA-binding inhibition of protein-protein interactions (interaction with coactivators, corepressors, and other interacting proteins) and epigenetics. Direct inhibition of TF expression via RNA interference or antisense oligonucleotides or DNA decoys and blocking DNA binding via oligodeoxynucleotide decoys or pyrrole-imidazole polyamide have shown antitumor effect with minimal side effects and are succeeding into clinical trials.

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Activator Protein-1 Transcription Factors in Pathological Cancers

16

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Abstract

Activator protein-1 transcription factors play a critical role in the development of many cancers. Although many studies indicated the role of transcription factors driving the progression of cancer, there is still a knowledge gap in the process of better understanding the molecular mechanisms controlled by AP-1 transcription factors during cancer advancement. *In conclusion*, we have evaluated the role of transcription factors which provides new insights into mechanisms of regulation of colorectal cancer by AP-1 family transcription factors.

Keywords

AP-1 transcription factors · Colorectal cancer · Metastasis · Oncogenes

Abbreviations

%	Percent
AP-1	Activator protein-1
ATF	Activating transcription factor

The original version of this chapter was revised. The book was inadvertently published without Abstracts and Keywords, which are now included in all the chapters. An erratum to this chapter can be found at https://doi.org/10.1007/978-981-10-6728-0_39

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CRC Colorectal cancer
MAF Musculoaponeurotic liposarcoma
TPA 12-O-tetradecanoylphorbol-13-acetate
TRE TPA-response element

16.1 Introduction

16.1.1 Cancer

The oncogenic transcription factors drive vast majority of fatal courses of cancer. Despite therapeutic advances, the high mortality of patients with cancer has not been substantially reduced over the past years, largely because of the lack of understanding of the complex molecular network which enables tumour cells to invade tissues and metastasize. Therefore, an improved understanding of transcription factors and their molecular mechanisms which regulate metastatic transformation and progression of the cancer cells has to be better understood. Tumour metastasis involves a series of events which promote and regulate the migration of cancer cells to generate metastases at distant sites. The process is initiated in the primary tumour, where cancer cells dysregulate important cellular components like oncogenes and tumour-suppressor genes leading to alterations in the expression of other genes or errant activation of important signal transduction pathways; transcription factors regulating extracellular matrix degradation, angiogenesis and other processes are important for metastasis.

For this reason, identification of cancer-associated biomarkers and essential molecular interactions connected to this interplay between transcription factors and further downstream components will help to develop strategies to prohibit the metastatic spread of cancer cells.

16.2 Cancer Types

A large body of existing literature shows that cancers are of different kinds. Common types include tumours of the colon, lung, breast and prostate. Malignancies of the plasma and the lymphatic system include leukaemias, Waldenström's disease, Hodgkin's disease, lymphomas and multiple myeloma. Skin diseases include malignant melanoma. Tumours of the digestive tract include the pancreas, head and neck, stomach, oesophagus, liver, anal, colon and rectum. Malignancies of the urinary system include the bladder, prostrate, kidney and testis. Malignancies targeting women are breast cancer, ovarian cancer, gynaecological cancer and choriocarcinoma. Other miscellaneous malignancies include the retroperitoneal, brain, soft tissue, bone, thyroid, carcinoid tumour, nasopharyngeal and tumours of the unknown primary site. The two important hallmarks of cancer growth and progression are invasion and metastasis [40]. Cancer is defined to denote the kind of malignant growth or tumour. It is not limited to humans but occurs widely in animals and vegetable kingdom. Cancer is neither contagious nor infectious. The growth begins locally, spreads to different organs

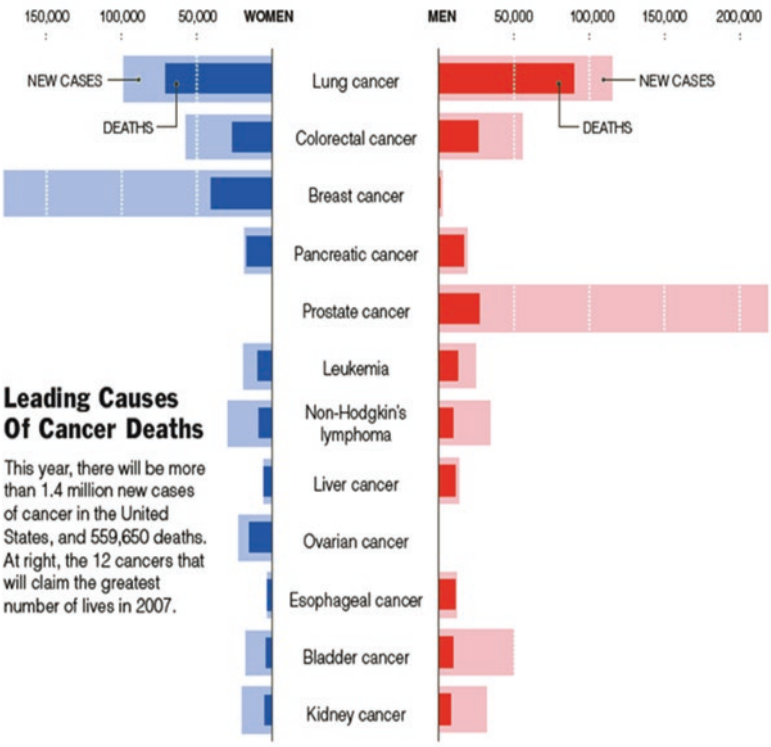

Fig. 16.1 Cancer incidence (Source: American Cancer Society)

and finally leads to death which is a multistep process. The cause of the cancer remains unknown. The probable estimation of stomach cancers is 30% in men and 22% in women [11]. Constitutive activation of transcription and growth factors also leads to malignant transformation [19]. The majority of the malignancies are triggered upon lost functionality of tumour-suppressor genes leading to the alterations in oncogenes [22, 36] (Fig. 16.1).

16.3 Colorectal Cancer

Colorectal cancer is the third leading cause of cancer death in each sex and second overall in men and women combined. Approximately 5–6% of individuals will develop a cancer of the colon or rectum within their lifetime (American Cancer Society. Cancer facts and figures 2009, Atlanta). Colorectal cancer is a major cause of mortality in the western population and accounts for 10% of all cancer deaths in the UK [43, 53]. The higher rates of risk factors for CRC include overconsumption of red and processed meat [16, 35], excess alcohol intake [15], excess body weight [5], physical inactivity [8, 9] and diabetes mellitus [20, 27–30]. Death due to cancer occurs in 1 of every 13 men and 1 of every 11 women. The estimation of cancer

incidence in the stomach is 30% among men and 22% in women [11]. Among different types of cancers, prostrate, bronchi, colon and rectum in men and women. Among women breast cancer accounts for half of total cancer deaths [23]. Usual consumption of fruits and vegetables combined is weakly inversely associated with risk of colorectal cancer, particularly colon cancer [48].

16.4 Transcription Factors

Transcription factors serve as an important constituent in regulating protein expression [50]. They either bind to the enhancer or the promoter region of the DNA, thereby regulating the proteins. These factors are vital and important for many cellular processes [14]. The transcription factors which generally bind to the DNA sequence is known as the response element or as the transcription factor binding site. The TPA-response element is called as TRE as it is strongly induced by the tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). Additionally the DNA binding of the AP-1 complex to the TRE sequence is induced by the cytokines, oncoproteins and growth factors which show an effect on proliferation, survival, differentiation and transformation of cells [13]. Various transcription factors are functional upon communication with other genes, for instance, the Fos transcription gene dimerizes with the Jun transcription factor, thereby increasing the expression levels of numerous genes that modulate cell division [10]. Increased expression of AP-1 family FOS proteins is associated with the acquisition of metastatic behaviour in many kinds of tumours [7, 21, 54]. Forced expression of Fra-I induces cell migration [2, 3, 26, 46] (Fig. 16.2).

16.5 AP-1 Family Transcription Factors

The first identified mammalian transcription family is AP-1 (activator protein) [4]. AP-1 is a dimeric complex consisting of members of JUN, FOS, ATF (activating transcription factor) and MAF (musculoaponeurotic liposarcoma) protein families. These complexes can form heterodimers and homodimers. These genes are well-known as the basic leucine-zipper genes since they interact with the DNA backbone with their basic domain and dimerize through a leucine-zipper motif. FOS and JUN are the main AP-1 proteins in mammalian cells. These two genes were initially recognized as viral oncogenes, v-Fos in the Finkel-Biskis-Jinkins osteosarcoma virus and v-Jun in avian sarcoma virus, respectively. JUNB and JUND are Jun family members, and FOSB, FRA1 and FRA2 are FOS proteins which have potent transactivation domains which induce target-gene transcription. The major role of AP-1 in transformation results in the regulation of cell morphology than cell proliferation [13]. The extracellular signals from growth factor receptors through mitogen-activated protein kinase convert into changes in the gene expression by AP-1 by targeting the AP1-responsive target genes [52]. AP1 activity has been implicated in a number of biological processes which include cell proliferation, differentiation, apoptosis and oncogenesis [24, 41] (Fig. 16.3).

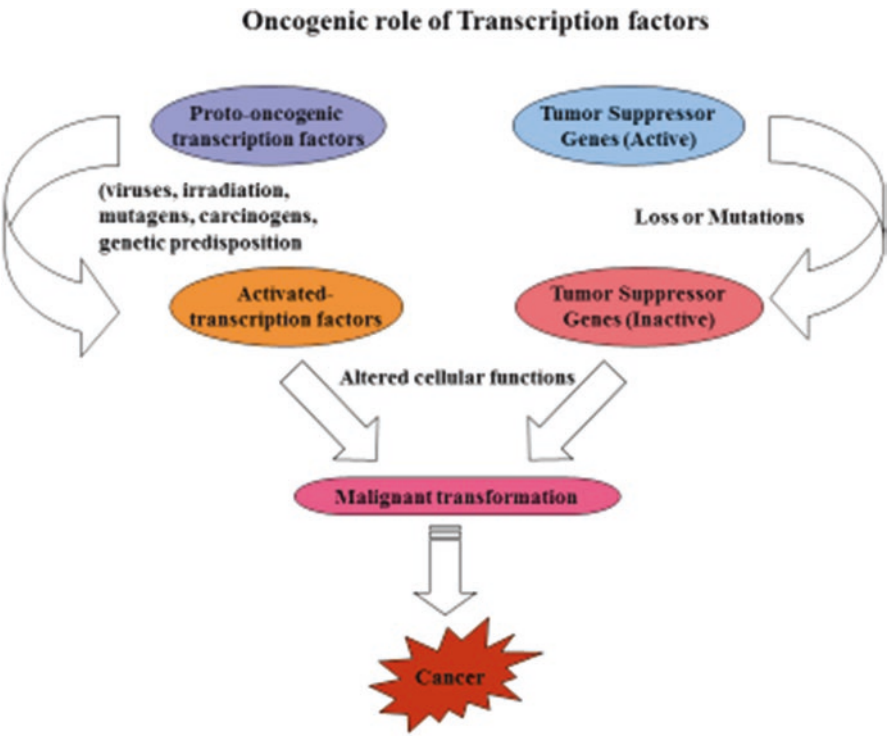

Fig. 16.2 Oncogenic transcription factors

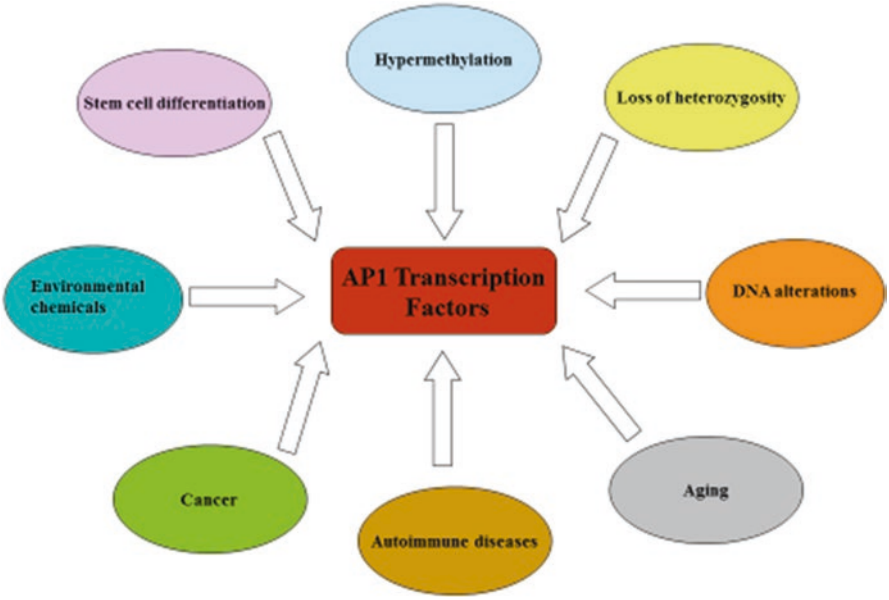

Fig. 16.3 Potential role of AP1 transcription factors

16.6 Role of Transcription Factors in Cancer

Cancer is commonly caused by the alterations in oncogenic transcription factors, which are typically somatic events, even though germ line alterations could predispose an individual towards inherited or familial cancer. A distinct genetic variation is not enough for developing a cancerous tumour. Many evidences points towards the involvement of a multistep process of consecutive alterations in many oncoproteins, tumour-suppressor genes or transcription factors in tumour microenvironment [17, 42]. Molecular pathways of invasion-related cellular activation include cell to cell adhesion, cell and matrix adhesion as well as proteolysis, ectopic survival and cell migration. The extracellular signal towards the receptor leading to the signal transduction and eventually to cellular response is vital for the modification of the invasive phenotype. Metastasis is a multistep process of invasion [32]. Proteolytic degradation of extracellular matrix is an important event for the activity of the invasive cells [51]. The activities of the normal cell behaviour and tissue maintenance mainly depend on ECM [31]. ECM along with its biological integrin and non-integrin receptors are known as a barrier, a substrate for invasion, a signal, a source of advancement and motility and also a modulator of survival in case of invasion [49]. The loss of E-cadherin is correlated with N-cadherin upregulation, eventually leading to migration and invasion [6, 18]. Cellular and biological activities are positively or negatively related to the invasive phenotypes comprising of cell-to-cell adhesion, cell and matrix adhesion, proteolysis, ectopic survival as well as migration [32]. The multi-phased invasion process of metastasis reveals that metastasis is an essential requirement for invasion [1]. Mainly the diagnosis of cancer includes grade of differentiation in terms of growth and invasion based on the staging of tumours. The TNM system, which is propagated by the International Union to fight against cancer, is used to evaluate and stage tumours for therapeutic resolutions. The root of clinical manifestations in the majority of invasive malignancies is the factors such as progression, invasion, survival and loss of differentiation [34]. Cell growth disturbances are implicated by oncogenes and tumour-suppressor genes which may differ in promoting or suppressing invasion, differentiation and survival [33].

The oncogenes were discovered in 1910 by Peyton Rous, who demonstrated the infectious filterable virus causing fibrosarcomas in chickens ([38, 39]. This virus was eventually known as Rous sarcoma virus [45], later shortened to v-Src [12]. This led to the final discovery of the first cellular proto-oncogene called as c-src in 1976 by Michel Bishop, Harold Varmus and Dominique Stehelin [44]. These are structurally and functionally heterogeneous groups of genes, whose protein products affect multiple regulatory cascades within the cell which finally decide cell fate [37]. Products of oncogenes are classified into six groups: transcription factors, chromatin remodellers, growth factors, growth factor receptors, signal transducers and apoptosis regulators [10]. Oncogenic transcription factors can control cell proliferation, apoptosis or both [25]. Proto-oncogenic transcription factors transform into activated oncogenic transcription factors by structural alterations resulting from gene translocations or mutations [25, 47] or by amplification. Oncogenes

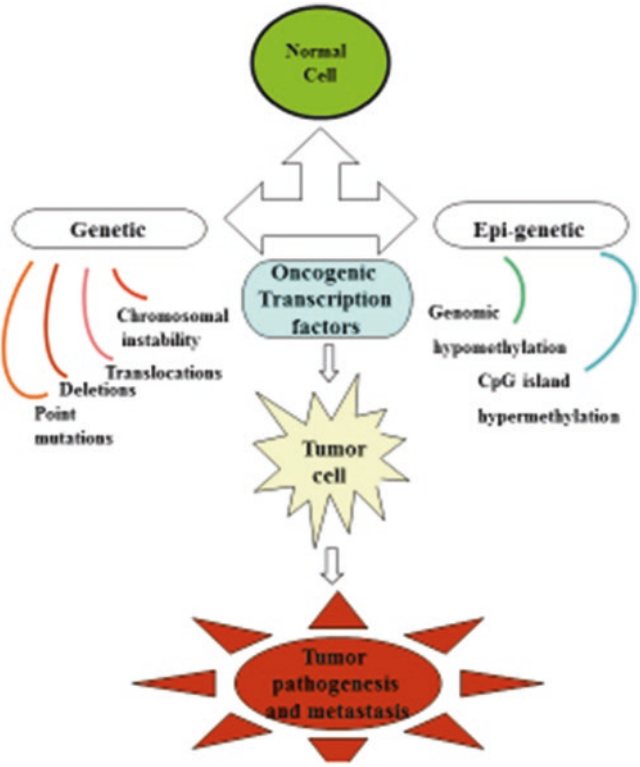

Fig. 16.4 Transcription factors in tumour pathogenesis

represent a class of genes which generally carry out critical functions to cellular signalling, growth and differentiation under normal conditions, but upon abnormal expression, these genes are able to induce tumorigenesis, ultimately leading to cancer [37] (Fig. 16.4).

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NF-κB: Its Role in Colorectal Cancer

17

A. Hartley, H. Wei, L. Prabhu, M. Martin, and T. Lu

Abstract

Colorectal cancer (CRC) is a major worldwide health problem and is the second leading cause of cancer-related deaths in the United States. Despite considerable progress in diagnosis and treatment, a high mortality rate persists, largely due to the complications associated with metastatic incidences. The pro-inflammatory transcription factor nuclear factor κB (NF-κB) is a central player in inflammatory responses and tumor progression. In CRC, constitutively activated NF-κB has been observed in the majority of patients. NF-κB significantly affects the process of tumorigenesis by promoting many aspects including tumor growth, proliferation, invasiveness, and angiogenesis. Importantly, the critical contribution of NF-κB to inflammation and tumorigenesis is due to its control of the expression of a large variety of target genes, many of which, when aberrantly expressed, help to orchestrate and promote CRC malignant potential. These NF-κB target genes include those vital to cell cycle regulation, cell proliferation, metastasis, and cell survival. Additionally, activation of NF-κB in both cancerous cells and inflammatory cells and subsequent induction of cytokines/chemokines within the tumor microenvironment also contribute to CRC cell malignancy in both

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autocrine and paracrine manners. These evidences implicate inhibition of NF-κB as an important approach for CRC therapy. Several recent combinatorial approaches using classical chemotherapeutics with NF-κB inhibitors seem to have resulted in very promising outcomes.

Keywords
Colorectal cancer · NF-κB · Transcription factor

17.1 Introduction

Colorectal cancer (CRC) is a serious worldwide health problem and remains the second leading cause of deaths from cancer in both men and women combined. Despite considerable advances in diagnosis and treatment, a high mortality rate persists, largely due to the complications associated with metastatic disease. CRC patients often present with a broad spectrum of neoplasms, ranging from benign adenomas to malignant carcinomas. While genetic factors such as mutations in DNA mismatch repair genes constitute a strong risk factor for developing CRC, they only account for 20–30% of all CRC cases. Notably, the majority of CRC cases (70–85%) are sporadic in nature and arise from the aggregate effects of multiple somatic mutations and epigenetic aberrations leading to the transformation of colon epithelial cells into adenocarcinomas [1].

Over the past decade, the pro-inflammatory transcription factor nuclear factor κB (NF-κB) has emerged as a central player in inflammatory responses and tumor development. In general, aberrant NF-κB activity seems to have a critical role in tumorigenesis and acquired resistance to chemotherapy. In CRC especially, NF-κB has been shown to be constitutively activated in nearly 60–80% of patients [2]. This elevated constitutive NF-κB activity is usually achieved by the induction of a local network of cytokines/chemokines and cell infiltration into affected sites. The continuous release of cytokines and growth factors by tumor cells and cells of the tumor microenvironment results in a tumor-promoting, feed-forward, and prolonged retention of nuclear NF-κB in malignant and tumor-associated immune/inflammatory cells. As a result, this sustained NF-κB-mediated inflammation significantly affects the process of tumorigenesis by modulating tumor growth and invasiveness, tumor-mediated angiogenesis, and the patterns of tumor-host interactions in the reactive tumor microenvironment [3].

The interconnection between inflammation and cancer was initially proposed by Virchow in the mid-nineteenth century when he hypothesized that cancer arose at regions of chronic inflammation brought about by irritants and tissue injury [4, 5]. There is now increasingly growing evidence to support the role of NF-κB as a critical mediator in the development of these inflammation-driven sporadic tumors. Perhaps the best-studied example is constitutive NF-κB activation in inflammatory bowel diseases (IBDs), which has been shown to significantly increase the risk of CRC development in patients with a number of years of active disease [6, 7]. IBD is associated with persistent NF-κB activation in cells such as the myeloid and epithelial cells located within the colonic mucosa. Such aberrant NF-κB activation was

shown to bring about the transformation of colon epithelial cells by upregulating the expression of proteins that mediate cellular proliferation (e.g., cyclin D1), anti-apoptosis (e.g., survivin, B-cell lymphoma 2 (Bcl-2), X-linked inhibitor of apoptosis protein (XIAP), inhibitor of apoptosis protein 1 (IAP1)), angiogenesis (e.g., vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), cyclooxygenase 2 (COX2)), and metastasis (e.g., matrix metallopeptidase 9 (MMP9), intercellular adhesion molecule 1 (ICAM-1)) [8, 9]. This chapter discusses recent evidence supporting the progression of CRC and briefly explores the implications of NF-κB as an important therapeutic target in this disease.

17.2 NF-κB Proteins and Signaling Pathways

As shown in Fig. 17.1, the family of NF-κB transcription factors consists of five proteins, namely, p65 (RelA), RelB, c-Rel, p105/p50 (NF-κB1), and p100/52 (NF-κB2). These proteins form distinct homo- or heterodimeric complexes, with the p65/p50 heterodimer being the most abundant. Both p50 and p52 are produced by proteasomal processing of their precursors p105 and p100, respectively. Although diverse, all NF-κB family members share a highly conserved domain – the N-terminal Rel homology domain (RHD), which is required for dimerization, DNA binding, interaction with the inhibitors of NF-κB (IκBs), and nuclear translocation. Unlike the RHD, the C-terminal transactivation domain (TAD) is conserved only among the Rel proteins, including p65 (RelA), RelB, and c-Rel. It confers positive regulation of gene transcription. In normal cells, NF-κB dimers are latent and are

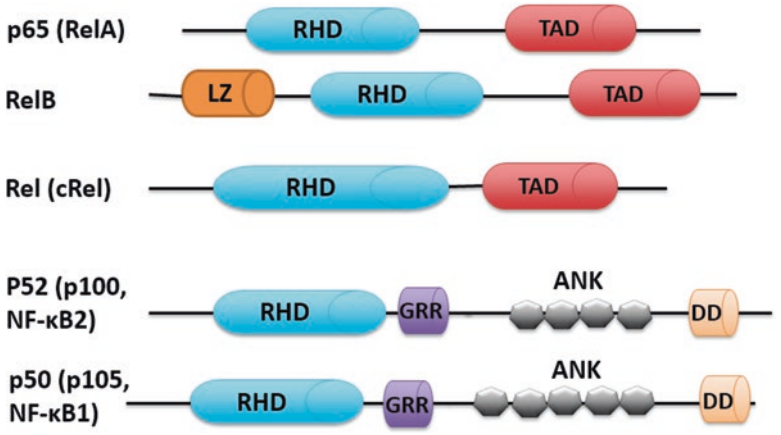

Fig. 17.1 Schematic of the NF-κB family members (Adapted from [10]). The NF-κB family members are defined by the N-terminal Rel homology domain (RHD), which is responsible for DNA binding and dimerization. All except p52 and p50 contain a transactivation domain (TAD), which confers positive regulation of gene expression. p52 and p50 also contain glycine-rich regions (GRR), which are necessary for their proteolytic cleavage and ankyrin repeats (ANK) similar to those found in IκB family of inhibitor proteins. Additionally, RelB contains a leucine zipper motif (LZ). Other abbreviation: *DD* dimerization domain

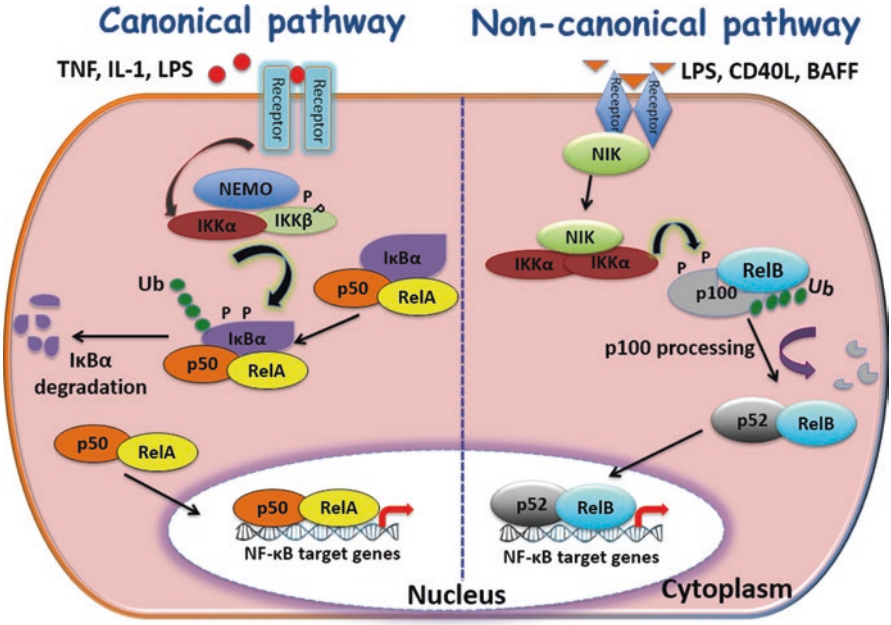

Fig. 17.2 Schematic of the canonical and non-canonical NF-κB pathways (Adapted from [11]). The canonical pathway (*left*) is induced by most physiological NF-κB stimuli and is represented here by TNF, IL-1, and LPS signaling. Stimulation of the corresponding receptor leads to the IKK complex activation comprised of two catalytic subunits, IKKα and IKKβ, as well as the regulatory IKKγ or NEMO subunit. IκBα is then phosphorylated in an IKKβ- and NEMO-dependent manner, which results in its polyubiquitination and subsequent degradation. The liberated p65/p50 heterodimer undergoes nuclear translocation where it engages in target gene transcriptional activation. The non-canonical pathway (*right*) is induced by a more selective family of cytokines, such as LPS, CD40L, BAFF, and lymphotoxin-β (LT-β). Upon activation, p100 processing depends on NIK, which triggers IKKα-mediated phosphorylation of p100, leading to partial processing of p100 and the generation of transcriptionally active p52/RelB complexes

mainly retained in the cytoplasm via association with the inhibitory IκB family of proteins. The IκB family also consists of several members (IκBα, IκBβ, IκBγ, IκBε, Bcl-3, p100, and p105) with IκBα and IκBβ being known for their prominent roles in binding to NF-κB heterodimers, effectively blocking their nuclear localization. All members of the inhibitory IκB complexes including p100 and p105 are characterized with the presence of ankyrin repeat domains (ANK) in their structure, which act to effectively mask the nuclear localization signals (NLS) of NF-κB heterodimers, keeping them sequestered in the cytoplasm [10].

Activation of NF-κB can be classified into two distinct pathways, commonly referred to as the canonical and non-canonical pathways (Fig. 17.2). In general, NF-κB can be activated by a divergent array of stimuli that lead to IκB kinase (IKK)-dependent phosphorylation, polyubiquitination, and subsequent proteasome-mediated degradation of IκB proteins. The liberation of NF-κB subunits then allows them to translocate to the nucleus, where they can bind to cognate κB sites in specific promoter regions and regulate target gene expression [10]. The canonical

pathway is typically simulated by factors such as tumor necrosis factor α (TNFα), interleukin 1 (IL-1), or lipopolysaccharide (LPS), leading to IKK activation (Fig. 17.2, *left panel*). The IKKβ subunit of the IKK complex phosphorylates serine residues in the signal responsive region (SRR) of IκBα, leading to its ubiquitination and subsequent proteasomal degradation. This results in the release of the p65/p50 heterodimer, which then translocates to the nucleus to induce transcription of target genes [11]. In contrast, the non-canonical pathway depends on NF-κB-inducing kinase (NIK)-induced activation of IKKα and is typically simulated by ligands such as cluster of differentiation 40 ligand (CD40L), LPS, and B-cell-activating factor (BAFF) (Fig. 17.2, *right panel*). In this pathway, p100/RelB complexes are retained in an inactive state in the cytoplasm. Signaling through a subset of receptors, including lymphotoxin-β receptor (LTβR), CD40, and BAFF receptor 3 (BR3), activates NIK, which in turn activates IKKα, leading to the phosphorylation and ubiquitination of p100 and its subsequent proteasomal processing to p52. This series of events creates a transcriptionally competent RelB/p52 complex that can translocate to the nucleus and induce target gene expression [12].

In addition to the association with their inhibitory IκB proteins, posttranslational modifications (PTMs) of NF-κB constitute another critical aspect of the extremely dynamic regulation of NF-κB activity. Besides the well-known PTMs, such as phosphorylation, acetylation, etc., our lab has recently demonstrated that protein arginine methyltransferase 5 (PRMT5), an epigenetic enzyme, positively regulates NF-κB activity through IL-1β-stimulated dimethylation of p65. This served as the first piece of evidence that NF-κB can be methylated on an arginine residue [13]. Previously, we also demonstrated that NF-κB can be methylated on lysine residues by the nuclear receptor-binding SET domain-containing protein 1 (NSD1) and demethylated by the F-box and leucine-rich repeat protein 11 (FBXL11). Overexpression of FBXL11 was shown to inhibit NF-κB activity, whereas high levels of NSD1 served to enhance NF-κB activity and rescued the inhibitory effect of FBXL11 [14]. These findings point to the highly sophisticated and fine-tuned regulation of the NF-κB pathway.

17.3 NF-κB Regulates Cell Cycle Progression and Promotes Cell Proliferation

The cell cycle, also known as the "cell-division cycle," is characterized by a series of coordinated events or phases that control the process of DNA replication and cell division. These phases include G0, during which the cell is quiescent; G1 and G2, in which the cell increases in size and prepares for DNA synthesis and mitosis [15]; the S phase, which involves duplication of a cell's DNA (DNA replication); and finally the M phase, during which the cell undergoes mitosis and subsequently divides to produce two daughter cells. In normal cells, the cell cycle is regulated by multiplex signaling. Phase transitions are tightly regulated by key regulatory proteins known as cyclin-dependent kinases (CDKs), a family of serine/threonine protein kinases activated during precise points of the cell cycle. Importantly, these regulatory proteins also govern a set of checkpoints responsible for monitoring completion of critical transitional events such that progression to the next phase is

delayed if necessary conditions are not achieved. CDK activation generally requires association with a set of secondary subunits known as cyclins, the levels of which fluctuate at the appropriate period of the cell cycle to create an active, CDK-cyclin complex that has unique substrate specificity [16].

NF-κB plays a critical role in the cell cycle via transcriptional control of cyclins. The best-studied example is cyclin D1, which is expressed early in the cell cycle. Cyclin D1 serves as a key regulator of G1 checkpoint control [15, 17–19]. Guttridge's group showed that inhibition of NF-κB activity decreased cyclin D1 expression causing cells to enter G1 cell cycle arrest [19]. In contrast, in transformed cells, dysregulated cyclin activity has been shown to accelerate cell cycle progression. For instance, cyclin D1 is overexpressed in a variety of human cancers, including CRC [20–22]. High expression of cyclin D1 plays a vital role in promoting tumor cell proliferation [16]. This aberrant expression of cyclin D1 in cancers may be attributed in part to the constitutively active NF-κB frequently observed in cancers. Several research groups have reported that NF-κB may directly induce cyclin D1 gene expression through binding to multiple consensus sites in the cyclin D1 promoter [19, 23]. However, enhanced cyclin D1 expression can also occur indirectly via the transcriptional activity of other oncogenes, such as the signal transducer and activator of transcription (STAT) 3/5, which are also activated by NF-κB-mediated production of cytokines, such as IL-6 [24, 25]. Overall, NF-κB-mediated dysregulation of cyclin D1 in tumor cells represents a crucial mechanism by which uncontrolled cell growth is achieved in cancer cells, among which are CRC cells, due to its promotion of the G1-to-S-phase transition [23].

In vivo, the direct relationship between constitutive NF-κB activation and CRC development was initially demonstrated in a colitis-associated colorectal cancer mouse model [11], in which various "irritants" of the gut biome promoted tissue injury and inflammation and increased intestinal epithelial cells (IECs) proliferation. As a consequence, malignant neoplasms were formed in the colon regions with NF-κB-mediated chronic inflammation [4, 5, 9]. Furthermore, conditional disruption of IKKβ-driven NF-κB activation within IECs attenuated the development of colonic adenomas [9]. NF-κB can accelerate the proliferative capabilities of cancer cells by dysregulating the expression of numerous target genes that are involved in cell growth and division. Similar to in vitro data, cyclin D1, once again, is an important gene among this list. Taken together, NF-κB activity has a critical role in regulating a variety of target genes that are required for cell cycle progression and cell proliferation in CRC.

17.4 Role of NF-κB in Metastasis

Metastatic CRCs are defined by their spread to distant organs and tissues, most often the liver, lungs, and abdominal cavity. Treating metastatic CRC is often more difficult and these patients tend to have a worse prognosis with an overall 5-year survival rate of ~11%. Constitutive activation of NF-κB has been increasingly recognized as a critical player in promoting initiation, progression, and metastasis of CRC. In fact, many of the distinct features of metastasis, such as cell migration, invasion, adhesion, and angiogenesis, can be attributed to the aberrant upregulation of several

NF-κB target genes that facilitate these processes [26]. Below, we will discuss some of the best-defined and prominent NF-κB target genes, whose upregulation has been shown to confer metastatic cells with their unique survival advantage.

17.4.1 Migration

Activation of NF-κB has been linked to tumor cell migration through NF-κB-mediated enhanced matrix metalloproteinase (MMP) production in tumor cells [27]. MMPs are essential to cell migration by facilitating rearrangement of the extracellular matrix (ECM). Perhaps the overall role of aberrant NF-κB activity in promoting tumor migration may be appreciated by the consequences of suppressing NF-κB activity. For instance, curcumin inhibited cell migration through its inhibition of NF-κB activity and subsequent downregulation of the expression of Cox-2 and MMP-2 in COLO 205 CRC cells [28, 29]. Another study also demonstrated the inhibitory effect of ginsenoside Rg3 on SW480 colon cancer cell migration via inhibition of MMP-9 and Cox-2 [30]. Furthermore, tumor migration and metastasis also correlated with enhanced NF-κB-dependent induction and secretion of a range of chemotactic factors that induce cell migration. These factors include chemokines and their receptors such as chemokine receptor CXCR4, monocyte chemoattractant protein-1 (MCP-1), ICAM-1, and migration inhibitory factor (MIF) [31]. Taken together, NF-κB has been shown to play a pivotal role in CRC cell migration.

17.4.2 Invasion

Invasion of cancer cells into surrounding tissue and the vasculature constitutes another early step in tumor metastasis. This is a multi-step process that requires chemotactic migration of cancer cells from their primary foci and their subsequent protrusive activity into the lymphatic or vascular circulation. One of the hallmarks of cancer cell penetrance into surrounding stroma involves degradation of and attachment to the ECM. It is well known that the major NF-κB target genes upregulated during these processes include MMP-2 and MMP-9. Both participate in the degradation of the ECM [30, 32]. There is also strong evidence supporting the idea that the mechanisms underlying tumor invasion tend to mimic the developmental process known as epithelial-to-mesenchymal transition (EMT). EMT is a highly dynamic process that allows epithelial-like tumor cells to assume a mesenchymal phenotype and is critical to the acquisition of invasive capability of CRC cells [33]. Constitutive NF-κB activity has also been implicated as an important factor in EMT of CRC cells. For instance, it has been reported that enhanced activation of NF-κB activity in tumor-associated macrophages (TAM) could lead to the increased expression of NF-κB target gene TNFα. When secreted, TNF-α activates the signal transduction of Wnt/β-catenin through inhibiting glycogen synthase kinase (GSK)-3β, promoting the EMT, which is deemed necessary for the invasion of CRC [34, 35]. Furthermore, it has been shown that NF-κB induces the expression of the mesenchymal-specific gene vimentin [35]. Therefore, inhibition of NF-κB is an

attractive therapeutic approach for counteracting the invasion and metastasis of CRC cells in part by limiting deregulated EMT.

17.4.3 Adhesion

Following protrusion, adhesion of escaped tumor cells to the substratum is largely accomplished by integrin and non-integrin molecules that facilitate binding of the leading edge of the cell to specific extracellular matrix protein domains. Additionally, selectins, integrins, and members of the immunoglobulin superfamily can also support CRC progression by promoting malignant cell attachment to secondary tissue sites. Cell adhesion molecules (CAMs) play a major role in the CRC metastatic potential and, therefore, significantly influence patient prognosis. Multiple reports have indicated that NF-κB activation increases expression of several adhesion molecules such as E-selectin, VCAM-1, and ICAM-1. Moreover, inhibition of NF-κB has been shown to reduce leukocyte adhesion and extravasation to the site of inflammation [36]. In contrast, NF-κB-mediated downregulation of cadherins (mainly E-cadherin) can facilitate tumor cell detachment from the primary site [37]. Ultimately, hyperactive NF-κB could facilitate CRC cells' detachment from the primary site, proliferation, and attachment at a new location to produce the secondary tumor.

17.4.4 Angiogenesis

Angiogenesis, which involves formation of new blood vessels from the preexisting vasculature, constitutes another important component of tumor growth and is considered a primary target in the treatment of metastatic CRC. VEGF and IL-8/CXCL8 are prominent pro-angiogenic and pro-metastatic proteins that serve as negative prognostic factors in CRC [38, 39]. Particularly, the VEGF family and their receptors represent the most important and widely studied pro-angiogenic factors. Among them, VEGF-A binding to VEGF receptor 2 (VEGFR-2) is believed to be the key mitogenic signaling pathway mediating angiogenesis [40]. Both VEGF and CXCL8 expression levels are upregulated in CRC via activation of the NF-κB pathway [38, 41, 42]. Of interest, a previous study also reported that endothelial cells not only respond to but also store and secrete VEGF, resulting in the autocrine activation of the VEGFR2, further enhancing tumor cells' migratory and invasive capabilities [42]. Another critical growth factor in CRC malignancy is epidermal growth factor receptor (EGFR), found on the surface of tumor cells. EGFR contributes to a number of processes involved in CRC development and progression and particularly angiogenesis. Approximately 80% of malignant tumors of the CRC are EGFR-positive [43, 44]. Furthermore, constitutive EGFR and NF-κB activities may combine to enhance the angiogenic potential of CRC cells, whereby EGFR- and NF-κB-dependent pathways establish positive loops to increase oncogenic potential, rendering CRC cells resistant to EGFR inhibitors [45].

Collectively, the above evidence affirms the critical involvement of NF-κB in four aspects of tumorigenesis, including migration, invasion, adhesion, and angiogenesis processes, which ultimately lead to the aggressive metastatic capabilities of CRC.

17.5 Role of NF-κB in Apoptosis

Apart from the various roles described above, NF-κB is also a salient player in apoptosis, a process by which cells undergo programmed cell death and is an essential part of normal physiology. Apoptosis is known to be critical for normal cell turnover, atrophy, immune defense, and embryonic development. Unsurprisingly, inappropriate apoptosis (either too little or too much) has been linked to many disorders including CRC. Failure of tumor cells to undergo apoptosis results in their evasion of death, further promoting their malignant potential and resistance to chemotherapeutic treatments. Here, we will briefly explore the role of constitutively active NF-κB in mediating evasion of apoptotic pathways in CRC cells as a major survival factor. Additionally, evidence for certain pro-apoptotic functions of NF-κB will also be mentioned.

17.5.1 The Predominant Anti-apoptotic Activity of NF-κB

Aberrant activation of NF-κB pathway is one of the key survival mechanisms for CRC cells because it results in the enhanced transcription of several NF-κB target genes that are known to impede the induction of apoptosis. These genes include IAPs, cellular FADD-like IL-1β-converting enzyme inhibitory protein (c-FLIP), and anti-apoptotic members of the Bcl-2 family (e.g., A1/BFL1 and B-cell lymphoma-extra-large (Bcl-xl)) [26]. In a significant portion of CRCs, one important means by which the NF-κB pathway becomes increasingly active is via the loss of p53 function. Normally, tumor suppressor genes like p53 play an important role to induce apoptosis of cancerous cells that would otherwise accumulate mutations and contribute to a transformed phenotype. However, in CRC cells, p53 is mutated and subsequently loses its pro-apoptotic function. This facilitates the uncontrolled growth of colon cells, thus leading to an aggressive CRC phenotype. The link between NF-κB and p53 has been well documented. Shao et al. demonstrated that overexpressed wild-type p53 markedly increased cytoplasmic expression of IκBα, resulting in a significant decrease in NF-κB nuclear localization and suppression of NF-κB-induced anti-apoptotic gene expression. This led to a rapid induction of apoptosis in CRC cells, therefore, leading to inhibition of CRC progression [46].

Moreover, Karin's group confirmed that the oncogenic role of NF-κB in colon epithelial cells was mediated through its anti-apoptotic function [47], which further prevented the apoptotic elimination of premalignant cells [9]. Furthermore, other researchers also reported that suppression of NF-κB activity could attenuate apoptosis in a number of CRC cell lines [48]. For instance, certain factors in our diet, such as genistein, a biologically active flavonoid found in high amounts in soy, were shown to inhibit NF-κB activity, leading to the downregulation of anti-apoptotic Bcl-2 as well as upregulation of pro-apoptotic Bcl-2-associated X protein (BAX) in CRC cells LoVo and HT-29 [49], resulting in increased apoptosis. Similarly, inhibition of NF-κB activity by a pharmacological agent, quercetin, also correlated with increased apoptosis in human CRC cells CACO-2 and SW-620, through downregulation of Bcl-2 and upregulation of BAX [50].

Taken together, these evidences support the notion that hyperactive NF-κB could lead to the induction of anti-apoptotic genes, while inhibition of NF-κB activity could abolish their expression, therefore impeding CRC progression.

17.5.2 The Potential Pro-apoptotic Activity of NF-κB

It is worth noting that the role of NF-κB in apoptosis is complicated. Although NF-κB activation in tumor cells is predominantly oncogenic, nevertheless, a few sporadic studies have shown that NF-κB may also function as a tumor suppressor by inducing apoptosis under certain specific conditions [51–53]. This is highly correlated to the induction of Fas in cancer cells. A pro-apoptotic role for NF-κB in CRC was reported by Kimura et al., in which cytokine-induced activation of NF-κB functioned as a potential pro-apoptotic factor through enhancement of Fas expression in a human CRC cell line, RPMI4788 [52]. The authors found that Fas expression was strongly potentiated by TNF/IFN-α-mediated activation of NF-κB and postulated that the pro-apoptotic tendency of NF-κB might be due to differences in downstream signal transduction induced by TNF/IFN-α or TNF-α alone. Similarly, another study showed that NF-κB directly regulated Fas transcription to modulate Fas-mediated apoptosis and tumor suppression, suggesting that canonical NF-κB signaling could serve as a transcriptional activator of Fas [53].

Based on the current knowledge, it is difficult to reconcile the potential dual roles of NF-κB in apoptosis in CRC, yet it is important to call attention to the above scientific findings. It may take researchers a long time to exactly figure out how such kind of dual functions of NF-κB in apoptosis may occur. Nonetheless, realizing the complexity of the function of NF-κB in CRC is crucial to studies that target inappropriate NF-κB activation. Collectively, we have presented strong evidence that NF-κB primarily exerts its oncogenic function via the upregulation of various anti-apoptotic factors, providing a rationale for developing anticancer strategies to inhibit NF-κB. Nonetheless, the complex nature of NF-κB-mediated apoptosis may offer clinicians better insight for developing more effective chemotherapeutic agents for CRC.

17.6 Conclusions and Perspectives

As shown in Fig. 17.3, the NF-κB family controls the expression of a large variety of target genes, many of which, when aberrantly expressed, help to orchestrate and promote CRC tumorigenic potential by affecting cell cycle regulation, cell proliferation, metastasis, and anti-apoptotic activity. Additionally, activation of NF-κB in inflammatory cells from the tumor microenvironment also contributes to CRC development by inducing expression of cytokines, chemokines, and growth factors. This leads to constitutive activation of NF-κB in tumor cells, which in turn release numerous factors that sustain the ongoing paracrine inflammatory process between the tumor microenvironment and tumor cells [3]. These evidences confirm that inhibition of NF-κB may be an important approach for CRC therapy. In fact, many efforts are already under way to develop NF-κB inhibitors. However, there remains some

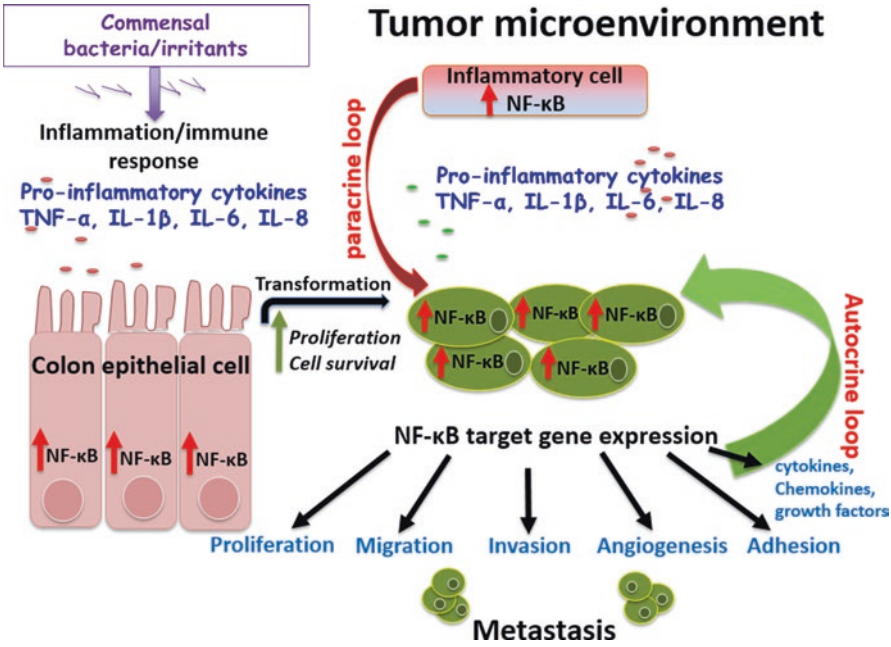

Fig. 17.3 Constitutive activation of NF-κB promotes inflammation and progression of CRC. Bacteria and various "irritants" of the gut cause tissue injury and inflammation, leading to excessive production of pro-inflammatory cytokines. These cytokines bind to cell surface receptors of colon epithelial cells, resulting in NF-κB activation in these cells. NF-κB activation in colon epithelial cells increases cell proliferation and survival via upregulation of proliferative and pro-survival NF-κB target genes, thus contributing to the malignant transformation of these cells. Activation of NF-κB in inflammatory cells from the tumor microenvironment also contributes to CRC development by inducing expression of cytokines, chemokines, and growth factors. This leads to constitutive activation of NF-κB in tumor cells, which in turn releases numerous factors that sustain the ongoing paracrine inflammatory process between the tumor microenvironment and tumor cells and the autocrine loop between tumor cells. Hyperactive NF-κB in tumor cells promotes expression of diverse NF-κB target genes, including cytokines, chemokines, and growth factors, as well as genes that are associated with metastasis, such as those promoting proliferation, migration, angiogenesis, adhesion, and invasion of tumor cells [3, 60]

reluctance to their use since NF-κB is also an essential player in the immune response against cancer. Prolonged immunosuppression via NF-κB inhibition is likely to have deleterious effects on patients. However, this can be largely overcome by the way that the drugs are administered. For instance, minimizing the dosage, with frequent but short-term administration, may help to alleviate the side effects. Furthermore, combinatorial approaches using classical chemotherapeutics with NF-κB inhibitors seem to have resulted in very promising synergies in recent studies [54].

Certain phytochemicals have also demonstrated beneficial effects in CRC patients. For instance, curcumin, ginseng extract, resveratrol, and green tea extract have been shown to inhibit IKK/NF-κB activity [11, 28]. C086 is another compound derived from curcumin and has recently been shown to be more potent than curcumin by downregulating NF-κB activity in both CRC cells and xenograft tumors

[11]. Proteasome inhibitors have also yielded some success as they act by blocking the degradation of IκB to enhance cytoplasmic retention of NF-κB heterodimers. However, very few documented reports exist to support their efficacy in triggering apoptosis when used as a monotherapy and may be more useful when employed in combination with either chemotherapeutic drugs or other death-inducing cytokines [55]. Other broad-acting agents such as sulfasalazine, methotrexate, and nonsteroidal anti-inflammatory drugs (NSAIDs) are also widely used to treat acute states of IBD and may lower the overall risk for colitis-associated CRC development [56, 57]. Finally, dexamethasone, a glucocorticoid, has been proven to be a potent NF-κB inhibitor [58]. Clinical trials have already been conducted to investigate the efficacy of this agent when used in combination with other therapeutics.

Taken together, these data implicate inhibition of the NF-κB pathway as a potent therapeutic approach for CRC [59]. Because NF-κB is involved in many physiological processes, not surprisingly, some adverse side effects have been noted upon systemic NF-κB inhibition. With the aid of nanoparticles however, the utility of NF-κB inhibitors may be significantly improved. This technology facilitates the use of drugs at minimal doses that can be delivered to restricted areas of the body [54]. While these efforts are promising, a concerted approach is required to develop the ideal NF-κB inhibitors, such that they target specific regions of the colon as well as the surrounding tumor microenvironment, with maximum efficacy and minimal systemic adverse effects for CRC patients.

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Curcumin Suppresses Colorectal Cancer Growth and Metastasis by Inhibiting NF-κB Activity

18

Ganji Purnachandra Nagaraju and Subasini Pattnaik

Abstract

The third most malignant disease diagnosed worldwide is colorectal cancer (CRC). CRC treatment by chemo- and radiotherapy is challenging due to the cancer's ability to activate major transcription factors such as NF-κB. Curcumin, a phytochemical, is known to downregulate NF-κB signaling in CRC cell lines and to exhibit anticarcinogenic properties. Therefore, curcumin and its analogues are novel therapeutic agents that could be used in the treatment of CRC growth and metastasis.

Keywords

Colorectal cancer · Curcumin · NF-κB · Growth

18.1 Introduction

Colorectal cancer (CRC) is a prominent cause of cancer-associated deaths. Approximately 40,000 CRC patients are diagnosed per year in the USA with a 40–50% death rate [1]. The standard of care for patients with locally progressive diseases (T≥T3 or N≥N1) is concurrent fluoropyrimidine and external beam

The original version of this chapter was revised. The book was inadvertently published without Abstracts and Keywords, which are now included in all the chapters. An erratum to this chapter can be found at https://doi.org/10.1007/978-981-10-6728-0_39

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radiation followed by surgical resection. The level of complete pathologic response with this treatment is 8–16% [2]. Attempts at improving the outcome of CRC by incorporating cytotoxic agents such as oxaliplatin or irinotecan have been disappointing [3]. These results indicate that in the majority of patients, the main challenge remains the primary resistance of CRC cells to cytotoxic chemotherapy. Due to the limited effectiveness of chemotherapy and radiation, other approaches have been sought to treat CRCs. Improvement in the outcomes of CRC is dependent on the introduction of agents that can modulate the intrinsic mechanisms of resistance.

18.2 Mechanism

The mechanisms of resistance to chemotherapy in CRC include the activation of nuclear factor kappa B (NF-κB) and hypoxia inducible factor (HIF-1α). NF-κB is a transcriptional factor that controls the expression of several oncogenes involved in tumor growth, angiogenesis, and inflammation [4–7]. NF-κB is normally activated in CRC [6]. Therefore, NF-κB is an important target for CRC therapy. In addition, epigenetic silencing of gene expression is another commonly known pathway that contributes to carcinogenesis and resistance in CRC [8].

18.3 Curcumin

Phytochemicals are compounds that come from natural resources. Many of these chemicals have anticancer properties, with curcumin being one of the more promising molecules. Curcumin is a phyto-phenolic molecule found in turmeric root [9]. These plants are native to certain parts of Asia and have been used for centuries for treating various diseases as learned from Indian Ayurvedic medicine [10]. Curcumin has been explored as an anticancer agent due to lower cancer rates found in countries where turmeric is a common ingredient in food. Curcumin displays extensive biological actions against metabolic diseases and cancer [11]. Extensive study over the last five decades has shown that curcumin can suppress various cancer cell activities [12]. The tumor growth process consists of multiple stages encompassing initiation, advancement, and development [13]. Excitingly, curcumin successfully acts at all three phases by targeting critical processes involved in cancer development and progression [14]. Curcumin stimulates chemopreventive and chemotherapeutic effects in different types of cancer cell lines [15]. The anticarcinogenic nature of curcumin has been reported in preclinical models of lymphomas, multiple myeloma, leukemia, and brain, pancreatic, gastric, and colorectal cancers [16].

Despite its anticancer properties, curcumin has low solubility in water and degrades easily, affording it low bioavailability in the body, where about 75% of the natural compound is excreted through the feces [17]. Due to these drawbacks, curcumin has limited efficacy as a cancer therapeutic. In preclinical studies, curcumin suppresses the development and progression of CRC cell lines (Fig. 18.1).

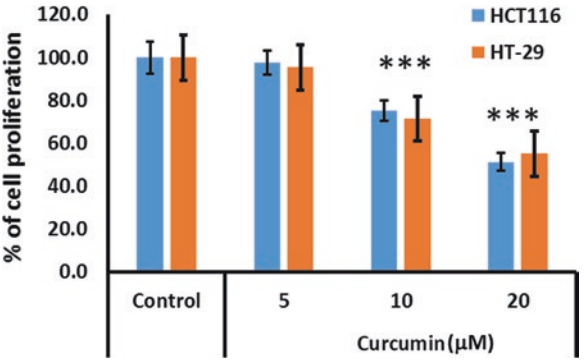

Fig. 18.1 Antiproliferative properties of curcumin in CRC cell lines (HCT116 and HT-29). Proliferation was evaluated by MTT viability assay. Cell lines were exposed to different doses of curcumin (5–20 μM) for 48 h ***$P < 0.001$

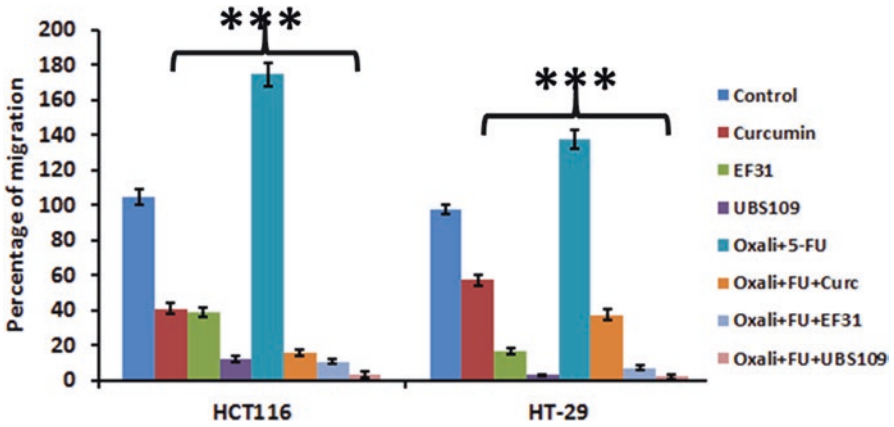

Fig. 18.2 Curcumin and its analogues EF31 and UBS109 increased the efficacy of oxaliplatin and 5-FU in both HCT116 and HT-29 cell line spheroid migration. For making spheroids, CRC cell lines were plated on agar-coated 96-well plate. After 48 h spheroids moved to 12-well plates and grown for 24 h with or without treatments. The outward distance of migration was shown in percentage as compared to controls. Combination of oxaliplatin+5-FU+curcumin or analogues significantly (*** $P < 0.001$) decreased the migration compared to the untreated or individual treatments. Each value denotes the mean ± standard deviation ($N = 5$)

Combination of curcumin and its analogues EF31 or UBS109 with 5-FU and oxaliplatin potentiates the decrease of spheroid migration and invasion (Figs. 18.2 and 18.3). The mode of action for curcumin or its analogues involves the downregulation of inflammatory signaling pathways, for example, NF-κB/cycloxygenase-2 (COX-2), inhibition of heat shock protein 90 (HSP90), and epigenetic regulation. The assessment of curcumin in clinical trials for cancer prognosis and prevention has yielded disappointing outcomes. Potential explanations for the lack of clinical activity of curcumin include its low potency and poor solubility [18].

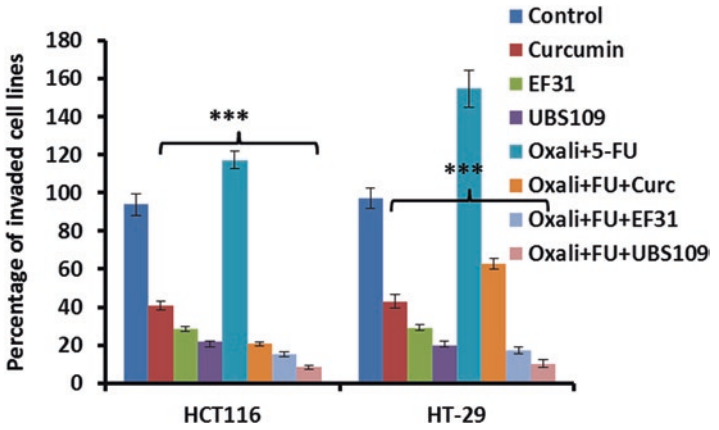

Fig. 18.3 Curcumin and its analogues EF31 and UBS109 increased the efficacy of oxaliplatin and 5-FU in HCT116 and HT-29 cell line invasion. HCT116 or HT-29 cell lines were plated on upper chamber of matrigel. Treatments were used in complete media contained in the lower chamber. Combination of oxaliplatin+5-FU+curcumin or analogues significantly (*** $P < 0.001$) decreased the invasion compared to the untreated or individual treatments. The results are expressed as the percentage of invaded cells compared to the control. Each value denotes the mean ± standard deviation ($N = 5$; ***$P < 0.001$)

18.4 Conclusion

CRC is one of the deadliest forms of cancer due to the often late onset of symptoms and the propensity of CRC cells to metastasize. In addition to the substantial ongoing efforts to improve early detection for this cancer type, death rates may be reduced by the development of treatment approaches that destroy cancer cells but do not affect healthy cells. Current treatments in CRC have not been effective at killing cancer cells; unfortunately, healthy cells have been harmed due to the low bioavailability and low water solubility of curcumin.

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Role of STAT3 in Colorectal Cancer Development

19

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Abstract

Colorectal cancer is the cancer of the colon, located at the lower part of the digestive system. Although the role of STAT3 in cancer is known, its role particularly in colon cancer is largely unknown. STAT3 is a cytoplasmic transcription factor that involves extracellular signaling to the nucleus regulating fundamental functions, like cell proliferation, apoptosis, differentiation, and angiogenesis. STAT3 is a key regulator; abrogated activation leads to several diseases, including cancer. Aberrant interleukin (IL)-6-mediated JAK/STAT3 signaling pathway is closely related to the advancement of several human solid tumors including colorectal cancer. With other upstream regulators, IL-6/JAK signaling can activate STAT3, and its role appears to be critical in various types of cancer. STAT3 has been traditionally recognized as an oncogene; more recently the dual role of STAT3 in cancer, either tumor inductive or suppressive, has been appreciated. This chapter describes the potential role of STAT3 in colon cancer based on in vitro, in vivo, and patient studies. Furthermore, we will discuss the mechanism of action and roles of the IL-6/JAK/STAT3 pathway in colorectal cancer and exploit current therapeutic strategies, to treat colorectal cancer. Understanding the complexity of STAT3 function in colorectal cancer has the potential to elucidate important molecular aspects of colorectal cancer with significant therapeutic implications.

The original version of this chapter was revised. The book was inadvertently published without Abstracts and Keywords, which are now included in all the chapters. An erratum to this chapter can be found at https://doi.org/10.1007/978-981-10-6728-0_39

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Colorectal cancer · STAT3 · IL-6 · JAKs · Proliferation · Migration · Angiogenesis · Therapeutics

19.1 Introduction

Colorectal cancer is the cancer of the large intestine (colon), positioned at the lower part of the digestive system. Colorectal cancer is the third most common cancer in the world, with nearly 95,270 new cases diagnosed and 49,190 deaths reported in 2017 ([113], Cancer statistics). Some of the known symptoms of colorectal cancer are change in bowel movements like diarrhea, constipation, change in the consistency of stool, rectal bleeding, and blood in stool; persistent abdominal discomfort, such as cramps, gas, or pain; weakness; fatigue; and unexplained weight loss. Many people with colorectal cancer experience no symptoms in the early stages of the disease, and gradually symptoms appear depending on size of the tumor and location in the large intestine. Colorectal cancer occurs when healthy cells in the colon undergo errors in their DNA mismatch repair mechanism. This leads to continuous division and accumulation of cells to form a tumor, which then migrates and destroys normal tissue of other parts of the body. Colorectal cancers occur due to gene mutations linked to increase the risk of cancer passed through families in small percentage. The genes responsible for chromosomal instability at the DNA level have been identified in colorectal cancer. Tumor cells typically have bizarrely abnormal karyotypes, with many losses, gains, and rearrangements of chromosomes which seem to be causally connected with the cancer. Inherited gene mutations do not make cancer inevitable, but they can increase an individual's risk of cancer significantly.

19.1.1 Types of Colorectal Cancer

Most common forms of inherited colorectal cancer are:

- Familial adenomatous polyposis (FAP or APC) is an autosomal dominant condition with hundreds or thousands of polyps that cause to develop cancer in the lining of the colon and rectum. The condition has been mapped to 5q21, and the gene responsible for APC was identified. Untreated FAP with patient's age around 40 years has enhanced risk of developing colorectal cancer.
- Hereditary nonpolyposis (HNPCC), also called Lynch syndrome, increases the risk of colorectal cancer and other cancers. It is autosomal dominant and highly penetrates, but, unlike FAP, there is no preceding phase of polyposis. HNPCC genes were mapped to two locations, 2p15-p22 and 3p21.3. People aged around 50 with HNPCC develop colorectal cancer. Patients with HNPCC are constitutionally heterozygous for a loss-of-function mutation. Loss of heterozygosity (LOH) studies on HNPCC using microsatellite markers show lacking alleles present in the constitutional DNA. Some tumor specimens appeared to contain

extra, novel alleles. Microsatellite instability (MIN) generally in tumors could be classified into MIN^+ and MIN^- in colorectal FAP and HNPCC, and other rare inherited colorectal cancer syndromes can be detected through genetic testing. Increased risk of colorectal cancer is associated with a typical western diet containing high fat and low fiber. In colorectal cancer malignant carcinomas develop from benign epithelial growths called adenomas which are classified into early (>1 cm in size), intermediate (<1 cm but without foci of carcinoma), or late (<1 cm with foci of carcinoma). The most common sequence of/in the development of colorectal carcinoma is as follows [42].

- Constitutional loss of one copy of the APC gene on 5q21 is sufficient to carpet the colon with adenomatous polyps, which suggest loss or mutation of APC, probably an early event in the development of sporadic cancers.
- In about 50% of intermediate and late adenomas, only 10% of early adenomas have mutations in KRAS oncogenes. KRAS mutations mediate in the progression from early to intermediate adenomas.
- About 50% of late adenomas and carcinomas have shown to exhibit loss of heterozygosity on 18q. This is relatively uncommon in early and intermediate adenomas. Characterized putative TS gene DCC (deleted in colorectal cancer) encodes a protein with homologies to cell surface glycoproteins involved in cell adhesion.

19.1.2 Risk Factors

Older age: Majority of people with colorectal cancer is older than 50, and colorectal cancers in younger people are generally less frequent.

African-American race: African-Americans have a greater risk of colorectal cancer compared to other races.

Inflammatory intestinal conditions: Chronic inflammatory diseases of the colon, i.e., ulcerative colitis and Crohn's disease increase the risk of colorectal cancer.

Inherited syndromes: Genetic syndromes such as familial adenomatous polyposis and hereditary nonpolyposis (Lynch syndrome) passed through generations of family increase the risk of colorectal cancer.

Family history: If the parent, a sibling, or child in the family has colorectal cancer or rectal cancer, the risk to develop colorectal cancer is greater.

Low-fiber, high-fat diet: Colorectal cancer and rectal cancer may be related to diet; low in fiber and high in fat and high in red meat and processed meat cause more risk of colorectal cancer.

Diabetes: People with diabetes and insulin resistance may have an enhanced risk of colorectal cancer.

Obesity: People with obesity have increased risk of colorectal cancer when compared to people with normal weight.

Smoking: People who smoke have an increased risk of colorectal cancer.

Alcohol: Heavy use of alcohol increases risk of colorectal cancer.

Radiation therapy: Radiation therapy directed at the abdomen to treat previous cancers can increase the risk of colorectal cancer.

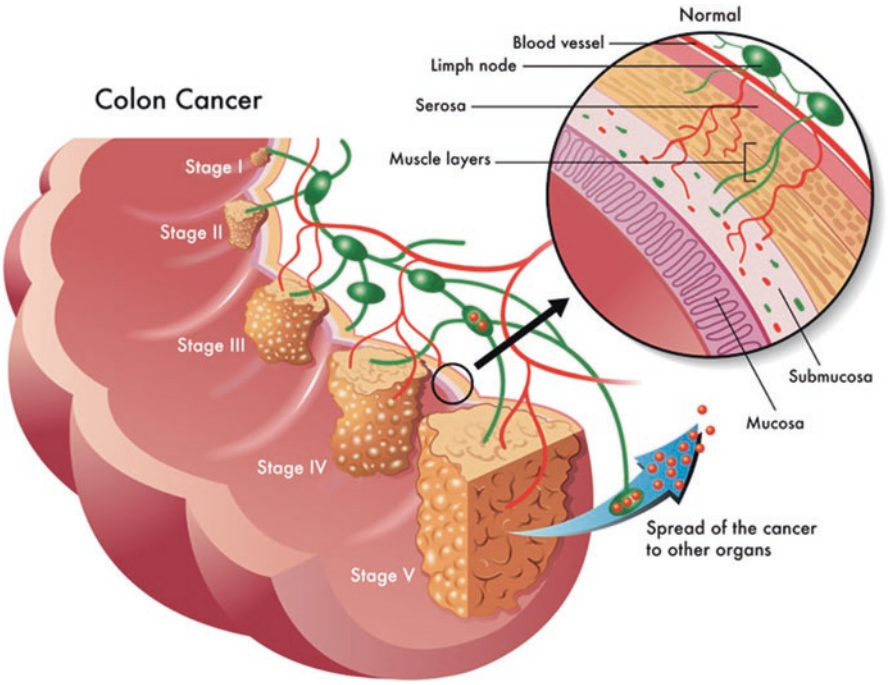

Fig. 19.1 Colon epithelium showing different progressive stages in colorectal cancer (Adopted from Colorectal cancer and Mistletoe-A review by Dr. Becky Lee ND Naturopathic Doctor)

19.1.3 Stages of Colorectal Cancer

Four stages of colorectal cancer are described as shown in Fig. 19.1.

Stage I: Cancer has grown through the superficial lining (mucosa) of the colon or rectum and has not spread beyond the colon wall or rectum.
Stage II: Cancer has grown into or through the wall of the colon or rectum and has not spread to nearby lymph nodes.
Stage III: Cancer has invaded nearby lymph nodes and has not affected other parts of the body.
Stage IV: Cancer has spread to distant sites and organs such as the liver or lung.

19.2 Signal Transducer and Activator of Transcription 3 (STAT3)

It is a transcription factor with oncogenic potential, initially found in interferon (IFN)-regulated gene transcription as a transducer of signal from cell surface to nucleus [2]. STAT3 belongs to a highly conserved family of protein, composed of seven members

and activated by several cytokines, growth factors, and oncogenic proteins. STAT3 constitutively phosphorylates in several cancer cell lines and directs cell transformation and tumorigenesis by transducing signals from extracellular stimuli to different interferon's (IFN-α, IFN-β, and IFN-ɣ) cytokine families, such as gp130 cytokines like IL-6, IL-12, and IL-23 and also ɣC cytokines such as IL-2, IL-15, and IL-21.

STAT3, located on chromosome 12, ranges between 750 and 850 amino acids. STAT3 phosphorylation allows nuclear translocation, and STAT3 dimer binds to DNA and directs upregulation of many genes involved in the cell cycle and survival such as CCND1, MYC, or BCL2L1. STATs have structurally and functionally conserved domains like amino-terminal domain (NH2), coiled-coil domain, DNA-binding domain (DBD), linker domain (Lk), SH2 domain, tyrosine activation domain (Y), and transactivation domain (TAD). Constitutive activation of STAT3 along with STAT5 have been observed in majority of human cancer cell lines and tumor tissues [17]. STAT3 and NF-κB in the colon are the main factors controlling the ability of preneoplastic and malignant cells to antagonize apoptosis-based tumor surveillance, which allocate tumor angiogenesis and invasiveness. The communication between cancer cells and inflammatory cells is due to the significant role in regulation of the interaction between STAT3 and NF-κB.

19.2.1 Mechanism of STAT3 Activation

Phosphorylation of tyrosine residue is crucial for STAT3 activation which later dimerizes to other STATs by reciprocal SH2 phosphotyrosine interaction important to its translocation into the nucleus, resulting in binding specific enhancer elements for initiation of transcription [31] as described in Fig. 19.2. Activation of STAT3 is highly regulated and important for normal biological functions like embryonic development, organogenesis, cell differentiation, and immune response [77]. STAT3 is persistently activated in many cancers and plays a central role in tumor growth and metastasis. STAT3 controls cellular proliferation, invasion, migration, and angiogenesis, suggesting STAT3 integrally participates in tumor initiation, progression, and maintenance.

Cell survival and cell cycle progression direct by STAT3-mediated transcription in cells. Three major mechanisms explain the STAT3 activation:

- Receptor associated tyrosine kinases (JAKs)
- Cytokine stimulation of membrane receptors with innate kinase activity (EGFR and PDGFR)
- Cytoplasmic kinases (Src and Abl) family [14]

STAT3 is activated by phosphorylation of tyrosine residue at position 705, catalyzed by JAK family tyrosine kinases, like epidermal growth factor (EGFR), vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), and colony-stimulating factor-1 (CSF-1). Conformational changes occurred due to receptor and ligand binding which bring JAKs into

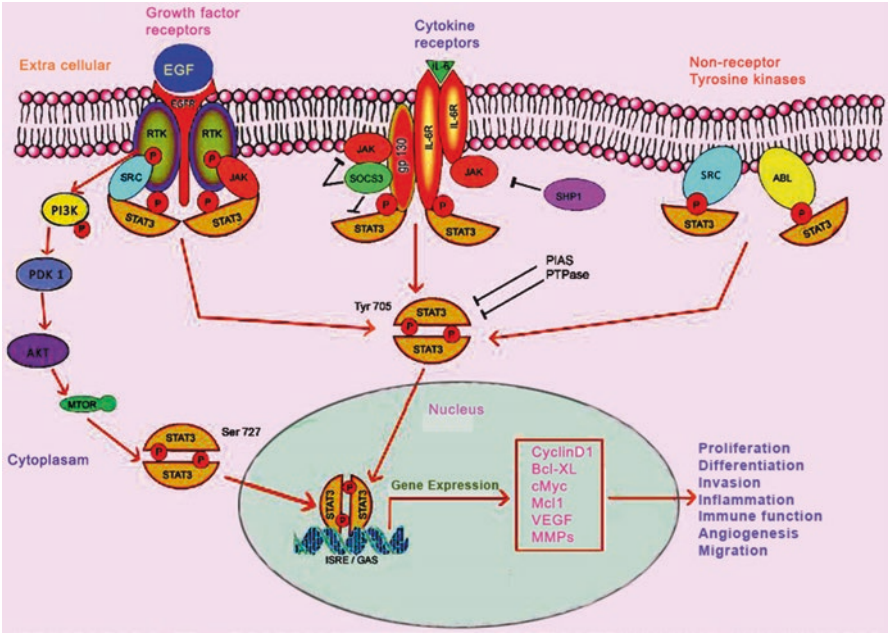

Fig. 19.2 Mechanism of IL-6/ JAK/STAT3 signaling pathway. Different ligands, usually cytokines, like interleukins (IL-6) bind to the IL-6R on target cells in association with gp130 complex, and growth factors bind to cell surface receptors and activate associated JAKs, enhancing kinase activity. Activated JAKs phosphorylate tyrosine residues on the receptors to form STAT3 homodimers and translocate into the nucleus and bind to members of ISRE/GAS family of enhancers to induce transcription of target genes (like Cyclin D1,Bcl-xl,c-myc Mcl1, and VEGF) by combining with consensus DNA elements. STAT3 tyrosine phosphorylation by membrane receptors with innate kinases like EGFR and by nonreceptor (cytoplasmic) tyrosine kinases like c-Src, ABL, and cytokine receptors (IL-6R). Serine kinases like mTOR phosphorylate STAT3 at serine position727 to increase kinase activity. STAT3 mediates in cancer cell proliferation, differentiation, invasion, inflammation and immune function, and angiogenesis. The protein inhibitors of activated STATs (PIAS) family of proteins are negative regulators of STAT3-involved gene transcription. In addition, the suppressors of cytokine signaling (SOCS) protein family affect the JAKs, and inhibit the phosphorylation of gp130, STATs, and the JAKs themselves. (Adobe photoshopCS version 8.0)

proximity with each other, enabling activation by transphosphorylation [104]. Phosphorylated JAKs provide as docking sites for STAT molecules. Phosphorylation results STAT dimerization followed by binding on the SH2 domain of one molecule containing phosphotyrosine of another STAT molecule and represents dimers stabilized by bivalent bonds. Activated STATs detach from the receptor, dimerize and translocate into the nucleus, and bind to members of the GAS (gamma-activated site) family of enhancers. The non-receptor tyrosine kinases like Src and Abl and cytokine receptors (IL6R) in association with JAKs catalyze tyrosine phosphorylation [148]. In canonical activation of IL-6-mediated STAT3, activation directs tyrosine phosphorylation of STAT3 and basically activates many genes. Maximal transcription activity of STAT3 is mediated by serine phosphorylation at position

727 by serine kinases such as MAPK (p38MAPK, JNK, ERK), PKC$^{\delta}$, mTOR, and NLK [72]. Acetylation of STAT3 regulates transcriptional activity and homodimer ability [151].

Other factors including UV radiation, sunlight, carcinogens, stress, smoke, and infection play a significant role in STAT3 activation. Blocking JAK/STAT3 signaling with inhibitors causes low viability of colorectal cancer cells due to apoptosis and cell cycle inhibition via downregulation of Bcl-2, Bcl-xL, Mcl-1, and cyclin D2 and upregulation of p21$^{waf1/cip1}$ and p27^{kip1}. Histological analysis of colorectal cancer tissues revealed several STAT3- and JAK3-activated forms in oncogenesis of colorectal cancer malignant neoplasms. In tumor microenvironment, STAT3 negatively controls the activity of helper T cells and dendritic cells by partial differentiation and absence of MHC class II molecules mediated in antitumor activity [132]. STAT3 activation via acetylation directs protein–protein interactions and transcriptional activity in noncanonical signaling pathway. The CD44 in association with acetylated STAT3 translocate into the nucleus and controls cyclin D transcription which leads to increased cell proliferation [146]. It has been demonstrated that STAT3 and JAK3 are continuously activated in two human colon carcinoma cell lines SW480 and HT29.

19.2.2 STAT3 in Colon Cancer

In normal cells STAT3 phosphorylation is temporary and transient which is essential for maintenance of gastrointestinal (GI) homeostasis. However, in different primary cancers and tumor cell lines, continuous activation of STAT3 has been observed [78]. STAT3 signaling acts as a molecular link between chronic intestinal inflammatory diseases and CRCs [85]. Activated STAT3 by phosphorylation translocate into the nucleus and bind to DNA, to upregulate several genes involved in the cell cycle and survival like CCND1, MYC, and BCL2L1 and control innate immunity activation in colorectal cancer oncogenesis due to inhibition of ligands [9, 12]. Cytoplasmic transcription factor STAT3 translocate into the nucleus after growth factor stimulation which intricate both in metastatic CRC oncogenesis and EGFR signaling. Different mechanisms driven by the transcriptional activity of STAT3 might involve cancer progression.

Wnt, Notch, TGF-b, and Hedgehog signaling pathways are aided with STAT3 and crucial for self-renewal of the intestine stem cell maintenance. Toll-like receptor (TLR) signaling directs tolerance across the intestinal microbiota and growth factor signaling pathways such as MAPK or Akt/PKB which contribute mitogenic and survival signals. STAT3 overexpression is correlated with higher CRC-specific mortality with a direct role in metastatic CRC prognosis. The functional role of STAT3 in mCRC might be the activation of molecular networks governing chemotherapy resistance and Eme1 endonuclease to diminish DNA damage [52, 133]. EGFR and different other signaling pathways like interleukin-6/Src kinases stimulate STAT3 activation in cancer cells and control the ability of anti-EGFR to hinder STAT3-related oncogenesis [52]. Presence of KRAS mutations is a biomarker for

lack of anti-EGFR monoclonal antibody efficacy. Activated STAT3 (pSTAT3) is combined with KRAS mutations correlated to high nuclear expression of pSTAT3 in metastatic CRC. The negative regulation of pSTAT3 has a central role in STAT3 involved in angiogenesis [89].

Cyclin D1 and VEGF (vascular endothelial growth factor) transcription in tumor cells is stimulated by STAT3 directly, resulting in proliferation and angiogenesis in mCRC [3, 110]. BCL2L1and MCL1expression elevates STAT3 transcriptional activity and advances to drug resistance by preventing the cell death pathways aided with genotoxicity treatment.

19.2.3 STAT3 Negative Regulation Mechanism

STAT3 is known to be constitutively activated in many cancers including colon cancer. Hence, inhibiting its activity would be a therapeutic approach for treating cancer. Some of the negative regulators for STAT3 activity are given below.

19.2.3.1 Tyrosine Phosphatases

Tyrosine phosphatase enzymes play an important role in STAT3 deactivation. These enzymes are divided into classical protein tyrosine phosphatases (PTPs), dual-specificity phosphatases, and low molecular weight phosphatases [21]. PTPs display homology with signature motif VHCSXGXGR [T/S] G and also with tertiary structure [5, 86]. These PTP enzymes are divided into two groups: the transmembrane tyrosine phosphatases CD45 and non-transmembrane PTPs, such as SH2 domain comprising SHP1 and SHP2, phosphotyrosine phosphatases 1B (PTP1B), and T-cell-protein tyrosine phosphatases (TC-PTP). It has been shown that enhanced JAK2 and STAT3 phosphorylation was observed in the absence of CD45 gene [66]. Dual-specificity PTPs dephosphorylate both phosphotyrosine and phosphoserine/phosphothreonine. PP2B, a dual-specificity phosphatase, dephosphorylates STAT3 tyrosine phosphorylation, and protein phosphatase 2A (PP2A) dephosphorylates STAT3 serine phosphorylation [21].

19.2.3.2 Protein Inhibitors of Activated STATs (PIAS)

The PIAS family is composed of five members of proteins, PIAS1, PIAS3, PIASy, PIASxa, and PIASxb. These PIAS regulates the activity of STATs and also other transcription factors [6, 112]. PIAS proteins have conserved LXXLL signature motif represented by amino-terminal region [111]. PIAS proteins have zinc-binding domain, acidic domain, and serine/threonine-rich regions. PIAS involved in gene regulation directly blocking the DNA-binding activity of transcription factors result in anchoring of transcriptional corepressors or co-activators and promote protein sumoylation [112]. PIAS3 interact with STAT3 to suppress transcriptional activity of STAT3 [23].

19.2.3.3 Suppressors of Cytokine Signaling (SOCS) Proteins

SOCS protein family is composed of eight protein members; CIS along with SOCS1 to SOCS7 are inducible and interact with SH2 domain containing JAK2 kinase inhibitors of cytokine signaling [37]. SOCS proteins hinder signaling in three ways:

either by binding their SH2 domain to JAKs (SOCS1), by binding to receptor cytoplasmic domain (SOCS3), or by competing with STAT–SH2 domains for the recruitment to the receptor complex (CIS, SOCS2) [27]. Activation of SOCS box by SOCS proteins is involved in proteasomal degradation pathway [88].

19.3 STAT3 Crosstalk with Other Pathways in Cancer

19.3.1 IL-6 Family Cytokines

Tumor progression in solid tumors significantly correlated with stimulation of IL-6 signaling family members. Continuous JAK–STAT3 stimulation by IL-6 and IL-11 results in a chronic inflammatory state in the intestine and influences intestinal epithelial cell turnover, contributing to increased incidence of gastric tumorigenesis [38, 68]. Abrogated LIF corresponds to the activation of the JAK–STAT3 pathway throughout stem cell-induced tumor progression. LIF expression levels are controlled highly by transforming growth factor-β (TGF-β) defends crosstalk between TGF and LIF–JAK–STAT3 is crucial for tumor development [97] and encompassing contribution of IL-11 and LIF to tumor development. IL-6 is a significant driver of JAK–STAT3 activation in different tumors, and its expression can be stimulated by oncogenes. Upregulation of IL-6 expression stimulates JAK–STAT3, suggesting that STAT3 can contribute as an important target to prevent the cancer-promoting effect of oncogenes. JAK–STAT3 signaling in epithelial cells and immune cells is directed by IL-6 inflammation and contributes to oncogenesis [53]. IL-6 acts as an activator of STAT3, binds to IL-6 receptor (IL-6Rα) on the cell surface, and is involved in conformational changes; results in the formation of a hexameric signaling complex consist a gp130 homodimer and two IL-6 (IL-6Rα) heterodimers. Continuous activation of JAKs along with a proline-rich, membrane-proximal cytoplasmic domain of gp130 and activated JAKs controls phosphorylation of gp130, interferes in docking and stimulation of cytosolic STAT3, and leads translocation into the nucleus. IL-6 family members, like IL-11, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin M (OSM), and IL-31, activate receptors involved in signal transduction via JAK–STAT3. Myeloid cells produce IL-6 family cytokines like IL-6 and IL-11 [54, 99], and these cytokines control different inflammatory processes, which play a pivotal role in the homeostasis of pro-inflammatory and anti-inflammatory responses [115].

19.3.2 GPCRs

GPCRs upregulate the inflammatory mediator release and directs the stimulation of STAT3 leading to cancer progression, but GPCR-mediated STAT3 activation is essential for JAKs. Stimulation of STAT3 by adrenoreceptors results in the activation of IL-6 production, and receptor activation in signaling cascade suggest that GPCR signaling can crosstalk with IL-6 signaling [122]. Sphingosine-1-phosphate

receptor 1 (S1PR1) is a GPCR responsible for the signaling of lipid metabolite S1P [105]. Nuclear factor-κB (NF-κB) stimulation by S1P is involved in an increased production of IL-6, STAT3 activation, and CAC tendency in vivo. S1P–S1PR1 extends STAT3 activation on an inflammation-associated tumor development, and S1P–S1PR1 signaling plays a significant role in T-cell and B-cell development [24]. S1PR1–STAT3 signaling mediates in the formation of a pre-metastatic niche in myeloid cells and causes increased metastasis [33]. S1PR1–STAT3 intrinsic pathway modulates regulatory T cells and tumor regulatory T-cell migration and accumulation in tumors, resulting in inhibition of antitumor CD8+ effector cells [60]. S1PR1 inhibits migration of regulatory T-cells in the mouse and thus facilitates with S1PR1 regulatory T-cell accumulation in tumors [60]. S1P–S1PR1 pathway plays a significant role in the physiology of cancer cells and link between JAK–STAT3 and S1P–S1PR1, leading cancer cells through disrupted lipid signaling pathways. GPCRs, like S1PR1, activate JAK–STAT3, SRC–STAT3, and NF-κB and interpret to block the effects of the drugs against the GPCRs.

19.4 STAT3 Role in Tumorigenesis of Colorectal Cancer

19.4.1 Cellular Proliferation

STAT3 plays an important role in cellular proliferation and cell survival by enhancing the expression of growth-promoting genes such as c-Myc, Pim-1, and cyclin D1 [30]. Additionally, constitutive activation of STAT3 promotes elevated cell cycle progression by upregulating cyclin D1 and c-Myc expression. Shirogane et al. demonstrated that STAT3 upregulated the expression of growth-promoting gene Pim-1 in tumor cells [110]. In cancerous cells, STAT3 promoted survival signals and suppressed the apoptosis by inhibiting the expression of Bcl2, Bcl-xL, Mcl1, survivin, and cIAP2 [102]. Further, it was shown that the expression of p53 is negatively regulated by STAT3, a well-known inhibitor of cellular proliferation and inducer of apoptosis [90]. C-Myc protein is an important regulator of the transition from G1 to S and behaves as stimulator of cdc25A gene, which controls the activity of cyclin dependent kinases [47]. Docking of STAT3 to the c-Myc promoter is crucial for stimulation of c-Myc transcription upon IL-6 and Src activation. STAT3-dependent cell cycle progression controlled by Pim-1 cooperates with c-Myc [110]. Upregulation of cyclin D1 and c-Myc expression at transcriptional level by persistently activated form of STAT3 contributes to increased cell cycle progression.

19.4.2 Cellular Invasion

Rate of cellular invasion directed by STAT3 actively in extracellular matrix is an important process in tumor progression and formation of metastasis. STAT3 plays a significant role in complex metastasis multistep process by controlling the

expression of matrix metalloproteinases (MMPs). Elevated invasion and metastasis processes are associated with overactivation of STAT3 by phosphorylation [120]. Further silencing of STAT3 by shRNA decreases cancer cell invasiveness and MMP-7 expression in pancreatic cancer cells. These data showed that STAT3 plays an important role in regulation of tumor growth, invasion, and angiogenesis, which was due to reduced expression of MMP-7 in pancreatic cancer cells. Constitutively activated STAT3 regulates MMP-2 gene expression directly by binding to the promoter region of MMP-2 gene and the expression of matrix metalloproteinase's MMP-9 and MMP-1 controlled by STAT3 activation [141].

19.4.3 Cellular Migration

STAT3 plays a significant role in cellular migration and cell motility in vitro during the progression of cancer. The evidence was obtained with the depletion of STAT3 using siRNA resulting in decreased rate of cellular migration [114]. Stathmin is shown to be an oncoprotein 18, anchors α/β-tubulin heterodimers, and is involved in microtubule depolymerization. STAT3 specifically interacts with stathmin for controlling cell migration and microtubule dynamics. STAT3 inactivation leads to a random mode of migration and thus altering Rac1 activity to balance directional persistence during migration [124]. Studies from Debidda et al. demonstrated that STAT3 activation increases Rho GTPase-regulated cell migration and proliferation [32]. Constitutive activation of STAT3 enhanced migratory potential of epithelial cells via integrin β6 [8].

19.4.4 Tumor Cell Adhesion

Tumor cells damage the basement membrane by decreasing cell–cell and cell–matrix adhesion. These tumor cells travel in the circulatory or lymphatic system (intravasation), extravagate into the other vital organs, and adhere to form metastasis. Metastatic tumor cells' circulation in the blood vessels is controlled by different nonspecific forces including mechanical stress, hemodynamic turbulence, loss of adhesion-induced cell death, and cell-mediated cytotoxicity [50]. Hence, very low percentage of tumor cells survive during circulation due to mechanical stress and shear flow in the blood vessels to form metastasis in distal organs. In protecting the tumor cells from the body's immune surveillance, STAT3 plays a crucial role during their transportation through circulation. STAT3 activation in tumor cells causes increase in the probability of survival of tumor cells by secretion of different inflammatory factors that act as immune suppressors such as IL-6 and TNF alpha [87]. Wang et al. showed that constitutive activation of STAT3 in tumor cells suppresses the tumor expression of pro-inflammatory mediators [135]. The results obtained by McCarty et al. suggest that in association with platelets, tumor cells shielded from the stresses of shear flow [82].

19.4.5 Angiogenesis

Angiogenesis is an important process in tumor growth and metastasis. Vascular endothelial growth factor (VEGF) is one of the downstream target genes of STAT3. VEGF is a significant angiogenic molecule which mediates neovascularization during tumor growth [58]. As mentioned above, STAT3 gene directs transcriptional activation of VEGF [22]. VEGF expression in melanoma cells of tumor angiogenesis is controlled by continuous stimulation of STAT3 [89]. VEGF expression is regulated by STAT3 activation in human cancer cells and inhibition of both HIF-1 and VEGF expression by targeting to STAT3 activation [143]. VEGF receptor signaling is controlled by STAT3 activation in endothelial cells [10]. Hindrance of endothelial cell migration and blood vessel formation is achieved by blocking STAT3 signaling [144]. Hypoxia-inducible factor-1α (HIF1α) is a significant mediator of angiogenesis controlled by STAT3 expression. Under hypoxic conditions, STAT3 and HIF1α bind simultaneously to the VEGF promoter resulting in maximum transcriptional activation which leads to angiogenesis [107].

19.4.6 Metastasis

Metastasis is a complex and multistep process of cancer, initially advances surrounding tissue and gradually basement membrane of intestine and then enters into the blood circulation. STAT3 targets the genes associated with invasion, cell survival, self-renewal, angiogenesis, and tumor cell immune evasion leading to the promotion of metastasis. Hence inhibiting the STAT3 activation would be an excellent therapeutic approach for controlling tumor metastases.

19.4.7 Cell Transformation

Bromberg et al. elegantly demonstrated that STAT3 activation controls cellular transformation [15]. Activated STAT3 controls interleukin-6-directed transformation in mouse skin epithelial cells [149]. Furthermore, TRK oncogenic transformation in vitro is induced by STAT3 [83]. Hwang et al. showed that STAT3 activation regulates transformation of the NIH3T3 fibroblasts via RET/PTC tyrosine kinases [64]. Malignant transformation susceptibility was sustained by targeting STAT3 in different cell types [44] and enhances the significance of STAT3 in malignant transformation.

19.5 Role of STAT3 in Intestinal Epithelial Cells and Immune Cells

Induced IL-6 signaling controls the translocation of STAT3 into the nucleus of intraepithelial lymphocytes (IELs) and subsequently activates pro-survival genes in IELs which increases inflammation [7]. Immune-regulatory signals in intestinal

epithelial cells (IECs) controlled by IEL–STAT3 activation in myeloid progenitor cells contribute to decreased inflammation [63]. IEC–STAT3 signaling is crucial for the prevention of intestinal epithelium from inflammation-induced damage and serves IEC-mediated barrier function. Continuous activation of STAT3 in intestinal environment increases the generation of cytokines simultaneously in chronic inflammation. Cell-autonomous stimulation of oncogenic gene expression causes increased cell proliferation due to deregulated STAT3 activity in IECs [12]. Malignant transformation of IECs was controlled by STAT3 by decreasing the development of antitumorigenic gut inflammation [98]. A significant biomarker for determining therapeutic response in human cancer cells is Let-7–IL-6–STAT3 signaling. miRNAs derived from tumor cells are wrapped into microvesicles and delivered to the adjoining endothelial cells. miR-9 produced by tumor cells potentially stimulates endothelial cell migration and tumor angiogenesis by blocking suppressor of cytokine signaling 5 (SOCS5) via JAK–STAT3 activation [153].

In colon epithelial cells, IL-6–STAT3 signaling is important for the development of obesity-associated colorectal cancer [43]. Increased tumor cell proliferation, survival, invasion, and stimulation of antitumor immune suppression in cancer are observed due to STAT3 signaling. In the tumor microenvironment, continuously activated JAK–STAT3 facilitates a proliferative chance to immune cells and directs pro-oncogenic inflammation. Formation of pre-metastatic niche at distal metastatic sites causes the increased myeloid cell survival. In human cancers induced JAK–STAT3 signaling directs cancer stem cells self-renewal.

19.6 Role of STAT3 in Development of CRC: Evidence from In Vivo Studies Using Mouse Models

STAT3 induced epithelial expression of inflammatory mediator, Toll-like receptor (TLR) 2 in gastric tumors. Inhibition of TLR2 regulated gastric tumorigenesis but not inflammation illustrating the significance of STAT3 in oncogenesis of gastric cancer [131]. STAT3 and NF-κB controlled each other in paracrine cell-autonomous mechanisms. Human intestinal mouse model $gp^{130757F/F}$ exhibits the features of tumor development stimulated by STAT3 transcription factor overexpression. Diminished STAT3 activity is observed in $gp^{130Y757F/Y757F}STAT3^{+/-}$ mice. Loss of one STAT3 allele in $gp^{130757F/F}$ mice affects the reduced frequency and rate of tumor development due to hindrance of proliferation-induced glandular hyperplasia. $gp^{130757F/F}$ mice treated with antimicrobials show reduced tumor growth and macrophage production with neutrophils penetration. gp130 chain (the signal-transducing subunit of the IL-6 type cytokine receptor) mutant form is expressed in mice, enhances IL-11–induced stimulation of STAT3, and leads to the formation of gastric polyps [69]. CAC-associated mutations in p130 direct STAT3 activation by IL-6 and IL-11, prevent epithelial damage and inflammation from dextran sodium sulfate, but increases tumorigenesis in mice [12]. Induced STAT3 in progenitor cells interferes in proliferation and survival of epithelial cells and regulates the expression of inflammatory cytokines and elevated secretion of immunosuppressive factors preventing dendritic cell (DC) maturation [135]. In immune cells STAT3 activation

infiltrates tumor cells and leads to the formation of the tumors. STAT3 activates transcription of IL-23 in TAMs and dendritic cells (DCs) and also blocks NF-κB-dependent expression of the antitumor cytokine IL-12 [74]. Macrophages produce IL-23, activate STAT3 in IL-23R expressing Treg cells, direct upregulation of the immunosuppressive cytokine IL-10, and involve immune evasion. Generation and expansion of the tumorigenic Th17 cells and secretion of IL-17 are controlled by IL-23 and STAT3 [56]. Non-phosphorylated STAT3 with NF-κB enhances transcription of genes by involving acetylation of RelA and possesses NF-κB in the nucleus [145]. Activated STAT3 involves transcription of the TGF-b signaling antagonist Smad7 [69].

In the intestinal STAT3 signaling, TLRs are expressed through MAPK and NF-κB with several adaptor proteins like MyD88. The production of chemokines, inflammatory cytokines, and antimicrobial peptides is controlled by the activation of MAPK and NF-κB pathways [71]. Decreased tumor growth and tumor number in ApcMinþ/‑ mice due to removal of the adaptor protein MyD88 alters TLR signaling [100]. Mice develop increased inflammation and colitis-induced tumor growth due to MyD88 deficiency [106]. In inflammation-induced intestinal tumorigenesis and sporadic or familial cancers, MyD88 signaling plays a significant role. In inflammation, for efficient tissue repair, MyD88 signaling is necessary, and the absence of Myd88 signaling contributes to increased inflammation and tumor development. MyD88 mediates tumor growth of sporadic or familial CRC, by stimulating an unsuitable tissue repair response, directing tumor cell proliferation, and expanding tumor growth. Mice lacking IL-18 signaling requires inflammasome alike Myd88-deficient mice adaptable to CAC [106]. Reducing inflammasome components like Nlrp3, Nlrp6, caspase-1, and ASC decreases levels of IL-18 and inflammation-associated tumorigenesis and produces observed phenotype in IL-18-deficient mice [152]. IL22BP (IL-22 binding protein) is a soluble, high-affinity receptor for IL-22 and requires IL-18 for down regulation, ultimately decreasing the availability of IL-22 under steady-state conditions.

In gastric tumor initiation and development of microbial environment play significant role due to STAT3 activation in CRC of $gp^{130757F/F}$ mouse. STAT3 involves in signaling via gp130 receptor subunit for the interleukin (IL)-6 family of cytokines and results both in embryologic and adult tissue regulatory processes [17]. STAT3 aberrantly overexpressed in many human cancers acts as an oncogene. T cells show impaired IL-6-induced proliferation and anticipate apoptosis and proliferation due to characteristic disruption of STAT3.

Intrinsic TK receptor activity like EGF and PDGF directly phosphorylates STAT proteins through cytokine receptor signaling which activates growth factor receptors. Cytoplasmic kinases like Src and Abl activate growth factor receptors, JAKs and TKs, and phosphorylate STATs directly. C-Src along with IL-3 receptor is involved in STAT3 activation through EGF and PDGF receptors [94]. TKs participate in initiation of STAT signaling through cytokine and growth factor receptors called “TK–STAT” pathways.

In oncogenesis, cell cycle progression, apoptosis prevention, tumor angiogenesis, and immune escape responses, STAT3 plays a significant role to enhance the expression of a sequence of cancer-associated genes like cyclin D1, c-Myc, VEGF,

and survivin [76]. In CRCs and noncancerous lesions, nuclear translocation of STAT3 was detected. CRC formation and expression of the genes are closely correlated with Wnt and NF-κB signaling mechanisms. In CRC formation, Wnta/NF-κBa, VEGF, c-Myc, survivin, MMP-7 and cyclin D1 expression detected by STAT3 activation. Wnt signaling is uncommonly stimulated in noncancerous lesions and exhibits decreased effect in tissues at gene transcription level and in chronic gastric anomalies; Stat3 and NF-κB mechanisms are functional.

19.6.1 GPCRs/Rho GTPase Family/Cadherin Engagement

Angiotensin II and S1PR1/2 GPCRs manipulate STAT3 activation by JAKs. In STAT3 tyrosine phosphorylation Rho GTPase family member, Rac1 plays a crucial role by binding at the DNA-binding region of STAT3. STAT3 phosphorylation is triggered by interleukin-6 regulated by the male germ cell RacGAP–STAT3 associated with the interleukin-6R/gp130 complex [81, 130]. The Rac1/male germ cell RacGAP complex activates STAT3 by tyr705 phosphorylation and translocates into the nucleus. Cadherin augmentation directs to activate STAT3 phosphorylation, resulting in the rapid enhancement of cell density in tumor microenvironment and also normal cells. Rac1-Cdc42 (Rho GTPase family) is stimulated by cadherin addition and activates NF-κB to promote the increased levels of IL-6, which corresponds to STAT3 activation [101].

19.6.2 Toll-Like Receptors

In tumor tissue, Toll-like receptors like TLR-2 elevated expression levels correlated with the STAT3 activation [131]. Classical stimulation of STAT3 by TLR4 ligation associated with Notch, NF-κB, and MAP kinase mechanisms [92]. STAT3 directly activates TLR function by IgG generating human B cells and TLR-mediated antibody and IL-10 production [80]. Lipopolysaccharide (LPS) increases the level of phosphorylated STAT3 and exploits the activation of TLR4 signaling [147].

TLR3 activated by STAT3 during injury hinders photoreceptor survival and visual function throughout oxidative stress. Activation of STAT3 is within minutes through TLR9 directly by cytokines, growth factors, and CpG. STAT3 prevents immunosuppression by acting as an inhibitor of antitumor immunity. JAK2 is placed by Frizzled 4 (FZD4) and activated through TLR9 engagement with CpG oligodeoxynucleotides (ODNs) and finally connects CpG–TLR9–FZD4 signaling cascade for subsequent STAT3 tyrosine phosphorylation [150].

19.6.3 MicroRNAs

MicroRNAs act as potential biomarkers for colorectal cancer diagnosis, prognosis, and drug-response prediction. There is a controversy in the literature for using

microRNA as biomarkers in colorectal cancer. This controversy could partially be due to heterogeneity between the different series associated with tumor stage, tumor location, genetic background of the tumors, and technical issues. More progress is needed before microRNAs can be used in clinical practice. Accumulation of further data will allow to determine the most relevant microRNAs as biomarkers and also to better understand their role in colorectal carcinogenesis [26]. In STAT3 signaling, miRNAs act as critical regulators during the progression of cancer. In the case of breast cancer, MiR-519d acts as a tumor suppressor by inhibiting STAT3 expression [34]. miR-20a is considered as an important therapeutic target, biomarker, and negative regulator of STAT3 expression, and its activation at low expression levels increases proliferation in the survival of hepatocellular cancer patients [39].

Let-7 is a tumor suppressor miRNA family member, promotes the cytoplasmic expression of the suppressor of cytokine signaling 3 (SOCS3), and blocks STAT3 activation by JAK2 downstream signaling events, leading to the decrease in growth and migration of poorly differentiated PDAC cell lines [95]. Activated Src stimulates NF-κB-mediated inflammatory response and initiates LIN28 transcription resulting in let-7 inhibition and causes an elevated level of IL-6 associated with STAT3 activation. The results from this study showed that let-7, IL-6, and STAT3 create a negative feedback loop for the transformation of cells by epigenetic control during the progression of inflammation and cancer [65].

19.6.4 Tyrosine Phosphatases Combine with Negative Regulatory Mechanism

STAT3is activated by tyrosine phosphorylation through protein tyrosine kinases in colorectal cancer [62]. STAT3 activation is controlled by dephosphorylation by protein tyrosine phosphatases [79]. In skin and brain cancer cells, STAT3-negative control mechanism by SHP2 and prevention of STAT3 tyrosine 705 phosphorylation due to activation of protein tyrosine phosphatase SHP1 by morin in tumor cells are observed [59]. In esophageal cancer cells, leptin-induced JAK2 activation controlled by adiponectin and PTP1B protein increases transcriptional activity of STAT3 [11].

19.6.5 PIAS Protein Family Combines with Negative Regulatory Mechanism

PIAS are protein inhibitor family in association with activated STAT proteins ranging from 507 (PIASy) and 650 (PIAS1) amino acid residues having four genes, PIAS1, PIASx (PIAS2), PIAS3, and PIASy (PIAS4). These proteins control STAT family members with PIAS1 expression in colon cancer [25]. PIAS proteins, through different mechanisms, control transcription by inhibiting the DNA-binding activity of transcription factors and anchor transcriptional corepressors, which lead to enhanced protein sumoylation. STAT3 activity is degraded by PIAS3 proteins [29].

19.6.6 SOCS Protein Family Combines with Negative Regulatory Mechanism

Suppressor of cytokine signaling in short SOCS comprises eight members: CIS and from SOC1 to SOC7 [138]. JAK–STAT3 signaling mechanism is negatively prevented by these SOCS proteins by three different mechanisms:

- Hindering JAK activity or degrading JAKs with proteasome
- Concealing the binding sites of STAT3 on cytokine receptor
- Proteasome degradation of targeting proteins through ubiquitination

Enhanced myelogenic differentiation was observed through restriction of JAK1 and gp130 by SOCS1 and SOCS3 [36]. IL-17/STAT3 mechanism is controlled by Platelet factor 4 (PF4) by changing the expression of SOCS3 [41].

19.7 Different Control Mechanisms of STAT3

In skin and prostate cancers, STAT3 is crucial for the constitutive NF-κB activity and exploits a STAT3 → NF-κB → IL-6 signaling loop links inflammation to carcinogenesis [40]. STAT3 phosphorylation at Tyr705, initiated through Ser727 phosphorylation by several serine kinases such as MAPK, PKC, mTOR, NLK, etc., to phosphorylate STAT3 at serine 727, is needed for enhanced STAT3 transcriptional activity [118]. Induced STAT3 activation is directed by NF-κB by increasing IL-6 secretion [67].

19.7.1 Epigenetic Modifications

In various cancers, the constitutive activation of STAT3 leads to increased proliferation of cells, prevention of programmed cell death, tumor angiogenesis, invasion, and migration. In cancer cells, epigenetic effect of gene promoter region interferes through the CpG methylation by DNA methyltransferase 1 (DNMT1) enzyme and regulates tumor-suppressor gene expression. STAT3 acetylation serves to control DNMT1 anchoring on different tumor-suppressor gene promoters which leads to enhanced methylation of relevant genes and progression of cancer. In response to cytokine treatment like IL-6 and LIF, OSM–STAT3 acetylates on a single lysine residue, Lys685, and its coactivator p300/CREB-binding protein (CBP) [93, 134, 151]. In cancer cell lines, other tumor-suppressor genes, like CDKN2 cell A, DLEC1, STAT1, and PTPN6, enhance promoter methylation by STAT3 acetylation [75]. The mechanism of unphosphorylated STAT3 target DNA binding is by regulating chromatin organization and binding to AT-rich DNA sequences, which plays a significant role in control of gene expression and/or chromatin organization, because of their special structure with a narrow minor groove that can be detected by proteins [127]. Unphosphorylated STAT92E proteins can manage heterochromatin through the regulation of histone H3 Lys-9trimethylation (H3K9me3) in Drosophila [108]. Olga A. Timofeeva et al. demonstrate epigenetic modification in

cancer cells by unphosphorylated STAT3 function detected in DU145, and MCF-7 cells are more accessible chromatin conformation than the non-transformed MCF-10A cells, which facilitate for unphosphorylated STAT3 binding, suppression of CHOP expression, and successive prevention of the apoptosis of cancer cells with the involvement of the N-terminal domain of STAT3 [128].

19.8 Colon Tumor Microenvironment

Tumor microenvironment is a complex network of cells consisting of tumor-associated macrophages (TAMs), neutrophils, granulocytes, myeloid-derived suppressor cells (MDSCs), immature myeloid cells (iMCs), mast cells (MCs), and dendritic cells (DCs) in the T and B cells [55]. Galon et al. demonstrated that the density, location, and type of tumor-infiltrating immune cells within colon tumors would provide a valuable prognostic tool in the treatment of colorectal cancer [48]. Tumor biology is influenced by a complex network of immune cells in the tumor stroma. Increase in innate immune cells, like TAMs, MCs, neutrophils, and MDSCs, T-helper (Th) 17 and Th2 subsets of adaptive immune cells controls enhanced tumor development direct tumorigenesis of colorectal cancer [96].

Formulation of humoral factors: The IL-6 activates the STAT3, both in autocrine and paracrine origin of IL-6 from cancer-related fibroblasts, adipocytes, or myeloid cells on the edge of the tumors. In the generation and progression of colorectal cancer, pSTAT3 plays a crucial role and successively enhances the expression of IL-6, leads the formation of amplification loops, and activates the elevated expression of autocrine and paracrine cytokines and growth factors, like IL-8, CCL5, CCL2, CCL3, IL1-β, GM–CSF, VEGF, and MCP-1.

- Role of fibroblasts, adipocytes, and macrophages in tumor microenvironment: Cancer-associated fibroblasts (CAFs) increase cancer progression via remodeling the ECM and mediate initiation of angiogenesis and anchoring of inflammatory cells and directly activate cancer cell proliferation through production of growth factors. IL-6–STAT3–Twist signaling pathway controls mesenchymal–epithelial cell interactions and the expression of CXCL1260, associated with the regulation of the CAF phenotype. Coculturing cancer-related adipocytes with cell lines can inverse an immature and proliferative phenotype which leads to elevated cancer cell migration via increased expression of IL-6 and MCP1 [45]. Triterpenoid compounds prevent STAT3 activation and depletion of macrophage polarization to M2 phenotype, which mediates in tumor development and lacks clinical diagnosis [46]. Cancer cells suppress immunological environment, docking immune cells, invert functions advantageously, and anticipate them from mounting direct immune response instead of alternating cancer progression. Molecular hub of immune suppression is STAT3 and controls VEGF and IL-10-mediated crosstalk between cancer cells and immune cells [143]. In cancer, STAT3 promotes myeloid-derived suppressor cell (MDSC) expansion and immune suppression [139]. Exosomal Hsp70 in association with pSTAT3 mediates immune suppression activity of MDSCs [35]. Sun et al. showed that inhibit-

ing STAT3 activity to extricate the hepatocellular carcinoma-activated immune suppression causes elevated levels of the NK cell functions [121].

- Linking inflammation to cancer: In cancer progression and inflammation, STAT3 and NF-κB are crucial active transcription regulators that interfere communication between cancer cells and inflammatory cells. In tumor progression and inflammation, STAT3 and NF-κB uniformly bind to gene promoters and stimulate pro-inflammatory cytokine secretion which leads to enhancement in target gene expression and function by positive feedback mechanism [125].

Tumor angiogenesis: Activated STAT3 plays a significant role in angiogenesis regulation involved in VEGF and bFGF endothelial cell proliferation, extracellular matrix degradation, and endothelial cell migration, and alteration of junction adhesion molecules directs mediators of angiogenesis by stimulated STAT3 and increases the formation of new blood vessels and progression of cancer [16]. Targeting STAT3 is a suitable method to weaken the promoting function of tumor microenvironment and evolves the therapeutic consequence for cancer.

19.9 Biomarkers of Colorectal Cancer

- Elevated levels of circulating cell-free DNA (cfDNA) in cancer patients are a potential biomarker of the colorectal cancer. Quantitative real-time PCR detects ALU repeats in the plasma or serum of operated and nonoperated CRC patients and compares the levels of detected circulating cfDNA with normal healthy controls [28]. Initial wild-type KRAS status with epidermal growth factor receptor (EGFR) inhibitors to develop KRAS mutations detectable in cfDNA in the serum of CRC patients [84].
- Intestinal-specific transcription factors CK20 and CDX2 are generally expressed frequently in cells and negative for CK7. The mismatch repair proteins are candidate markers like, MLH1 and MSH2 with many other molecules mediating various cellular processes, such as matrix degradation, oxidative metabolism, or protein folding [91].
- Circulating tumor cells (CTCs) are malignant cells derived from a primary tumor or its metastases and enters into the bloodstream. For targeted therapy in molecular genetics, characterization of CTCs has potential implications. Identification of CTCs in metastatic CRC patient's peripheral blood is correlated with prognosis [57].
- MicroRNAs (miRNAs) are small noncoding RNA strands that can posttranscriptionally control the expression of multiple target genes, implicated in several steps in carcinogenesis. miRNA levels in serum of CRC patients have shown early disease detection and monitoring of cancer recurrence after surgery [4]. miRNA-29 is to suppress tumorigenesis by inhibiting cell proliferation and migration. The oncogenic miRNA and miRNA-21 negatively control tumor-suppressor genes depending on manageable diagnostic and prognostic marker in CRC. Elevated levels of miRNA are significantly found in CRC patients with colonic adenomas compared with controls. Increased levels of miRNA detected

in both tumor tissue and patients serum are correlated with tumor size, presence of metastasis, and reduced survival [129].

- Analysis of serum samples using Multiplex protein array from adenoma-bearing patients and healthy control, showed that combinations of CEA and IL-8/CEA and C-reactive protein are best screening for early CRC/adenoma detection [18]. Combinations of serum CEA, cytokines, and CA19.9 are used to differentiate adenoma from healthy controls. Combinations of tumor markers like CEA and CA19.9 are indicative of the disease processes such as inflammation, immune response, angiogenesis, and metastasis [61, 109].

19.10 Therapeutics

Given the pivotal role of constitutive STAT3 activation in colon cancer, targeting to inhibit its activity would be a therapeutic approach for treating cancer**.** For blocking aberrant STAT3 activation and for drug development, growth factor receptor Tyr kinases and non-receptor Tyr kinases (NRTKs) are targeted. Different types of Tyr kinase inhibitors are developed against aberrant STAT3 activity and tumor growth in animal models. Downregulating the Tyr phosphorylation is an alternative approach which targets directly the STAT3 protein, mimicking the physiological negative modulators.

19.10.1 STAT3 Inhibitors

In cancer therapy, STAT3 is a good molecular target due to its crucial role in tumorigenesis and cancer cell biology. Two types of STAT3 inhibitors are classified based on the activity of STAT3 inhibition.

19.10.1.1 Indirect Inhibitors

In STAT3 activation, indirect inhibitors mediate in blocking of upstream effectors, like cytokine and kinases. JAK2 inhibitor, WP1066, conceals cancer growth, migration, and invasion which leads to enhanced chemosensitivity of ovarian cancer cells and reduce the rate of STAT3 phosphorylation [123].

19.10.1.2 Direct Inhibitors

Direct inhibitors block the domains of SH2, DNA-binding, and N-terminal of STAT3 by interrupting protein dimerization, DNA binding, and to prevent nuclear translocation, respectively. Direct inhibitors target the SH2 domains treated as the most commonly studied sites because of its significant participation in STAT3 activation.

Inhibitors are classified into three groups of compounds on the basis of structure:

One of these classes comprises peptides and peptidomimetics. Both Peptides and peptidomimetics can directly afflict the dimerization of STAT3 inhibits effectively transcriptional activity associated to low cell permeability and stability.

Another class of compounds, novel, synthetic STAT3 small-molecule inhibitors, are used in several approaches, such as medicinal chemistry and structural

applications based on high-throughput virtual screening in preclinical models. Site-directed computational fragment-based STAT3 small-molecule inhibitors alter problems correlated with cell permeability and feasibility to suppress the STAT3 activity. A novel non-peptide, cell-permeable small-molecule inhibitor LY5 has great binding affinity with the SH2 domain of STAT3 and disrupts dimerization leading to abrogated STAT3 activation with low IC50 values (0.5–1.4 M). Selective inhibition of persistent STAT3 activation by LY5 stimulates the apoptosis, and it is a promising therapeutic drug candidate approach for human medulloblastoma [140]. In clinical trials, OPB-31121 inhibitor actively interacts and shows a high affinity to the SH2 domain of STAT3 [51] and evolves a significant antitumor effect on gastric cancer. A natural polyphenolic flavonoid compound silibinin is isolated from the seeds of milk thistle (*Silybum marianum*), and it shows moderate inhibition on pSTAT3 in preclinical studies and clinical trials of gastric [136] and prostate cancers [1]. In cancer patients therapeutic effects remain unsatisfactory, due to low bioavailability of its flavonolignan structure [13]. Homoharringtonine (HHT) is a natural compound isolated from *Cephalotaxus harringtonia*, which effectively inhibits the STAT3 activity by interrupting the IL-6/JAK1/STAT3 signaling pathway and stimulates the apoptosis of gefitinib-resistant lung cancer cells. HHT remarkably suppresses the tumor growth in vivo, but gefitinib does not show a similar effect in nude mice injected with H1975 cells and in NSCLC in an EGFR-independent manner [19].

19.10.2 Oligonucleotides

Oligonucleotide inhibitors selectively target STAT3 and control viable STAT3 activity. Decoy oligonucleotide (ODN) is a double-stranded 10–20 base pair DNA which comprises a TF's consensus and particularly inhibits STAT3 activity by binding competitively to the DNA-binding domain of STAT3 which leads to competent attenuation of specific gene expression. G-quartet oligonucleotides are G-rich oligodeoxynucleotides having four-stranded potassium-dependent intramolecular G-quartet structures and engage sites within the SH2 domains of STAT3. These oligonucleotides strongly block STAT3 activation and tumor growth in NSCLC [137] and prostate cancer [103], and their large size and potassium dependence limit their cellular delivery and possibility to be determined in clinical trials and to turn off unwanted genes.

Small interfering RNA (siRNA) is a natural posttranscriptional gene-silencing mechanism targeting STAT3 which is considered as a useful method for the treatment of breast cancer [73] and lung adenocarcinoma [119].

19.10.3 Receptor Antagonists

In many human tumors, aberrant activation of Tyr kinases induces continuous activation of STAT3 and other signaling molecules mediated in tumorigenesis. Attractive therapeutic targets for controlling the malignant phenotype are cell surface receptors. Growth factor receptors, such as EGFR family, and receptors for

cytokines, such as gp130/IL-6 receptor family, are targets for improving effective therapeutic models to control cancer. Receptors either have intrinsic Tyr kinase activity, with growth factor receptors, or associated cytoplasmic Tyr kinases. In experimental models, to modulate cell surface receptors, antibodies or other molecular entities could be designed that specifically compete against the physiological ligands for binding to the receptor [70, 142]. EGFR family of receptors are overexpressed in breast, pancreatic, ovarian, colon, and lung cancers, as well as HNSCC and other tumors [142]. Monoclonal antibodies are more extensively used to inhibit the EGFR family, and the first human anti-mouse EGFR antibody had low efficacy and directs the improvement of chimeric human mouse MAb 225 (monoclonal antibody, IM-C225, Cetuximab) and proved to be an effective therapy, used in combination with chemotherapy or radiation therapy in the treatment of HNSCC, colorectal cancer, and NSCLC [70]. Preventing the binding of IL-6 ligands to its receptor results in the decrease in STAT3 activation and alters the malignant phenotype. In U266 MM cell line, blocking of the IL-6 receptor by the IL-6 "super antagonist" Sant7 suppressed aberrant STAT3 activation and the viability of cells [20].

19.10.4 Tyrosine or Serine Kinase Inhibitors

STAT3 is a downstream receptor and non-receptor for Tyr kinases (RTKs and NRTKs). The abundant aberrant TK activity was observed in human tumors, such as breast, lung, prostate, colon, pancreatic cancer, glioblastoma, and other cancers [126]. The aberrant Tyr kinase of RTK or NRTK activity was inhibited by persistent activation of STAT3. Decreased levels of persistently activated STAT3 lead to the inhibition of the intracellular Tyr kinase activities of RTKs and exploits stimulation of apoptosis and tumor growth modifications [116]. The NRTKs, Src, and JAKs are involved in STAT3 activation [116], for developing small-molecule inhibitors as novel therapeutic agents, PD166285, SU6656, and PD180970, and regulate cell cycle arrest and apoptosis of tumor cells by the prevention of aberrant STAT3 and downregulation of STAT3 target genes, such as for Bcl-xL and Mcl-1 anti-apoptotic proteins [49]. AG490 blocked STAT3 activation [117] that inhibits Bcl-xL expression and induced apoptosis of malignant cells [20].

19.11 Summary

STAT proteins transmit signals from the cell surface to the nucleus and directly participate in the regulation of genes. STAT proteins are activated by many cytokines, such as IL-6 family cytokines and numerous growth factors, including EGF and PDGF in mammalian cells. STAT3 activation by tyrosine/serine residue phosphorylation in the transactivation domain results in maximal transcriptional activity.

Cytokine receptors such as interferons or IL-6 receptors lack intrinsic TK activity and recruit members of the Janus kinase (JAK) family of cytoplasmic TKs to act as intermediaries for activation of STAT. Cytokine receptor signaling activates growth factor receptors with intrinsic TK activity such as EGF receptor and PDGF receptor directly phosphorylates STAT proteins. Growth factor receptors, JAKs and

TKs directly phosphorylate STATs are cytoplasmic kinases including Src and Abl. C-Src is involved in STAT activation by the IL-3 receptor, EGF, and PDGF receptors. TKs are involved in activation of STAT signaling by both cytokine and growth factor receptors called TK–STAT pathways. STAT activation controls normal biological processes which results in expression of genes that control critical cellular functions such as cell proliferation, survival, differentiation, and development.

In the case of cancer, STAT activation is rapid yet transient, and constitutive signaling of STATs is increasingly associated with malignant progression. STAT protein activation requires tyrosine phosphorylation and serine phosphorylation of the kinases that are responsible for catalyzing molecular mechanisms involved in the activation of human tumors. STAT3 plays an active role in oncogenesis, cell cycle progression, apoptosis prevention, tumor angiogenesis, and immune escape by promoting the expression of a panel of cancer-associated genes such as cyclin D1, c-Myc, VEGF, and survivin. Nuclear translocation of STAT3 was observed in CRCs and noncancerous lesions. In addition to STAT3 signaling, Wnt and NF-κB signaling pathways are also closely associated with CRCs formation and expression of the genes. STAT3 and Wnta/NF-κBa activation results in gene expression of VEGF, c-Myc, survivin, MMP-7, and cyclin D1. Wnt signaling was rarely activated in noncancerous lesions and shows less effect on the gene transcription in tissues. STAT3 and NF-κB pathways are active in chronic gastric abnormalities.

STAT3 controls the expression of genes resulting in tumor progression, cell proliferation, and survival by epigenetic mechanisms, like DNA methylation and chromatin modulation. Many biological functions of activators of STAT3 play a crucial role in cancer by inhibiting mitochondrion functions, leading to tumor cell proliferation, survival, tumor invasion, angiogenesis, and immune suppression. Targeting STAT3 for cancer therapy has a pivotal role in stromal cells and immune cells, which are recruited to the tumor microenvironment to promote tumor progression. STAT3 activation is a potent immune checkpoint for multiple antitumor immune responses. JAK–STAT3 signaling has a pivotal role in inflammation-mediated cancer, obesity, metabolism, cancer stem cells (CSCs), and pre-metastatic niche formation. In JAK–STAT pathway, interferon-α (IFN-α), IFN-γ, and intereukin-6 (IL-6)-mediated downstream signaling are crucial for transducing signals from various receptor and non-receptor tyrosine kinases activated in cancer cells. Toll-like receptors (TLRs), such as TLR9 and TLR4, are activators of the JAK–STAT3 pathway. STAT3 upregulates the expression of TLRs in malignant cells and promote tumor progression. MicroRNAs (miRNAs) play a major role in cancer and regulate the JAK–STAT3 pathway. G-protein-coupled receptors (GPCRs) control STAT3 through JAK and Src family kinases. Upstream activators of JAK–STAT3 pathway and the biological functions in cancer are crucial for designing novel and more effective cancer therapeutics.

19.12 Conclusions and Future Directions

STAT3 is a significant transcription factor in the pathogenesis of several human cancers, constitutively active in the large majority of human colorectal cancer tissues. Cell cycle promoting the effect of STAT3 may contribute to oncogenesis in the colon epithelium. In colon carcinoma cell lines, cell proliferation is triggered by

STAT3 heterologous overexpression or cytokine-mediated activation endogenously. In colon carcinoma cells, STAT3 function may contribute to an improper anti-apoptotic signaling. In cancer, overexpression of growth factor receptors and inhibition of negative regulators occur as a result of STAT3 activation. In colon carcinoma tumor cell lines, due to the absence of cytokine stimulation for negative regulation of STAT3 mechanism, this leads formation of the tumors. One of the future directions is to recognize the factors corresponding to activation of STAT3 in CRC and illustrate their possible suitability as diagnostic markers or therapeutic targets.

Several significant STAT3 gene pathways are variously upregulated in CRC and contribute to the formation of a pro-inflammatory feedback loop that is significant for malignant transformation. Downstream cytokines and chemokines are mediated at every step of tumorigenesis, such as initiation, transformation, proliferation, cancer cell survival, invasion angiogenesis, and metastasis. Pharmacological compounds targetting the inflammatory signaling pathways are currently being tested in the laboratory and clinical setting. There is a demand for the development of prominent immunocompetent CRC mouse models for more accurate in vivo testing and thus for development of drugs to treat CRC. Proteomic analysis of CRC offers the opportunity to conduct a quantitative and functional evaluation of protein activity in different signaling networks involved and allows assessment of posttranslational modifications, complementing gene expression studies in CRC. New approaches like high-throughput screening may help to recognize novel agents that block key signaling pathways. Further research might shed more light on the clinical role of targeting inflammation during the progression of CRC.

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E2F1: Transcriptional Machinery in Colon Cancer

20

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Abstract

E2F family of transcription factor acts as central modulator for important cellular events including cell cycle progression, apoptosis, and DNA damage response. E2F1 regulates G_1/S-phase transition of cell cycle transactivating a variety of genes involved in chromosomal DNA replication, including its own promoter. E2F1 is regulated in a cell cycle-dependent manner, principally through its temporal association with pocket protein family member, retinoblastoma. Pocket proteins, in turn, regulated through phosphorylation by cyclin-dependent kinase (CDK). Different E2F family exhibits distinct cell cycle and apoptotic activities. This review focuses on E2F1 function and its putative role in colon cancer.

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20.1 Introduction

Colon cancer is the second leading cause of cancer mortality in the United States, and worldwide estimates are at least half a million. Genetic mutations are reported as one of the causes of inherited cancer risks in colon cancer. Nevertheless these mutations are expected to account for only 5–6% of CRC cases overall [1]. The expediency of premalignant stages, hereditary disorder recognition, and high occurrence of cancer disease allowed comprehensive genetic studies that have established colorectal cancer as an exemplar for understanding the tumorigenesis. Some of the recent studies shed a new light and emphasized on transcription factor E2F1 as a prospective driving force in metastasis. The E2F transcription factors are downstream targets of retinoblastoma (RB), which mainly regulates G1/S cell cycle and influences the cell proliferation in several mechanisms. However previously it was believed that E2F regulates the genes which were also involved in DNA synthesis. Interestingly, the diverse role of E2F in the cell cycle was established by modern techniques, viz., DNA microarray, CHIP (chromatin immunoprecipitation), as well as computational tools [7].

According to Dimova and Dyson the E2F families of transcription factors are of two groups: traditional or well-studied group (E2F1 to E2F5) and the novel discovered group (E2F6, E2F7, and E2F3b). The traditional group was divided based on their transcription properties, cell cycle properties, interaction with pRB, and related pocket proteins (p107, p130). E2F1, E2F2, and E2F3a are expressed during the cell cycle act as transcription activators through interaction with pRB. E2F4 and E2F5 are poor transcription activators which seem to act as poor repressors by recruiting pocket proteins. E2F3b function as a transcription repressor. However the novel discovered groups E2F6 and E2F7 lack transactivation, and pocket-binding domain thereby represses the transcription in pocket protein-independent manner. E2F1-6 forms heterodimers with DP proteins to attain high-affinity DNA binding. Notably, E2F7-8 has no cofactors to bind E2F target genes. The E2F proteins are downstream targets of growth factor signaling cascade and eventually regulate genes involved in cell cycle progression.E2F1 function as both transcriptional repressors and activators depending on their association with pocket proteins. Thus, E2F family proteins are also critical in regulating oncogenic transformation [4]. Six E2F family members are divided into three groups based on their oncogenic ability in fibroblasts: strong oncogenes (E2F2 and E2F3a), weak oncogenes (E2F1 and E2F6), and antioncogenes (E2F4 and E2F5).

20.2 Role of E2F1 in Cancer

E2F1 is an important downstream target of tumor suppressor pRB. The retinoblastoma (RB) pathway is inactivated in human tumors, which affect E2F activity. This leads to increased proliferation and inhibition of apoptosis resulting in

tumorigenesis. E2F-induced apoptosis occurs via both p53-dependent and p53-independent pathways. Several E2F-regulated genes, viz., CDKN2A, p73, Apaf-1, and caspases, contribute to E2F-induced apoptosis [12]. Hyperphosphorylated pRB interacts with E2F1 at specific site in the C-terminal domain of pRB. At S-phase checkpoint, pRB gets dephosphorylated and frees the bound E2Fs to initiate the expression of genes necessary for S-phase progression. Notably, a single substitution (valine to proline) in RB-binding factor alters E2F1 ability to interact with hyperphosphorylated pRB. Additionally interaction between E2F1 and pRB1 regulates the transcription activation of E2F1 target genes [3].

20.3 Role of E2F1 in Cell Cycle

E2F1 and the tumor suppressor RB genes are the central modules of cell proliferation. Specifically, the G_1 cyclins with their associated kinases, CDK4 and CDK6, phosphorylate RB, p130, and p107. This inactivates their capacity to interact with the E2F transcription factors. Phosphorylation accumulates E2F1, E2F2, and E2F3a that activates the transcription of genes essential for DNA replication and cell cycle progression. Moreover the phosphorylation of RB and p130 disrupts complexes with E2F3b, E2F4, and E2F5 found in quiescent cells that function as transcriptional repressors of S-phase genes (Figs. 20.1, 20.2, and 20.3) [19].

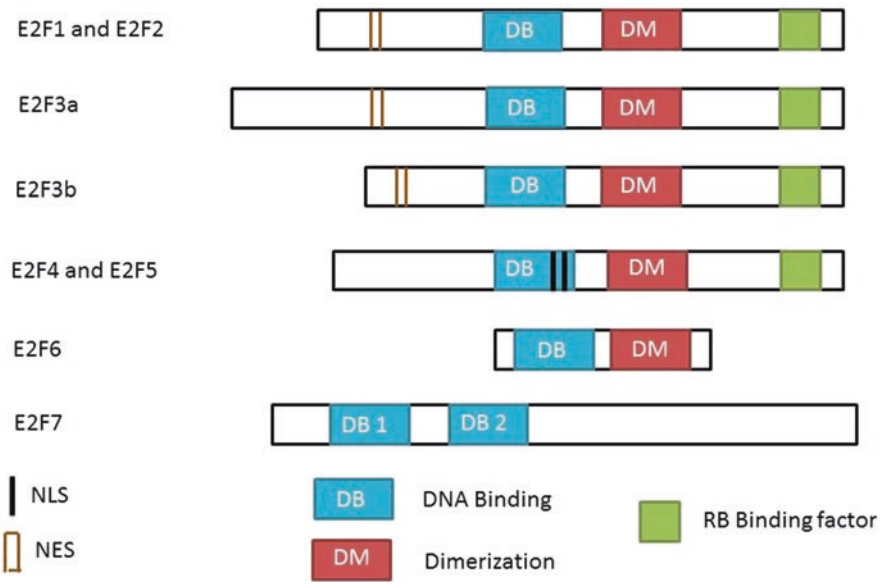

Fig. 20.1 Domain structure of E2F transcription factor family. E2F1-6 has one DNA-binding domain and one dimerization domain (DIM). E2F1-5 has pocket protein-binding sequences and transactivation domains. Nuclear localization signal is present in E2F1-3 genes and the nuclear export signal (NES) in E2F4-E2F5. E2F7 gene has two DNA-binding domains and lacks dimerization domain [7]

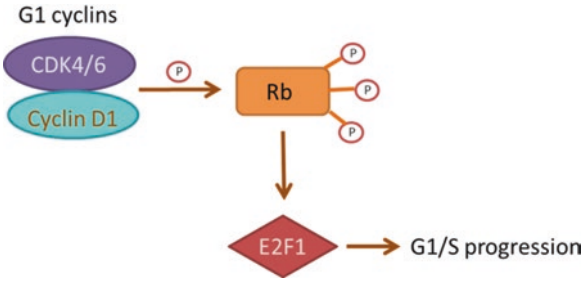

Fig. 20.2 Rb pathway. G_1 cyclins hyperphosphorylate retinoblastoma (RB), which accumulates E2F1 and promotes the phase transition of cell from G_1 to S. In G_1 arrest of cell cycle, Rb is the downstream target for growth inhibitory signals. Interestingly the pRB is expressed throughout the cell cycle, but its antiproliferative activity is counteracted by phosphorylation during the G_1/S transition

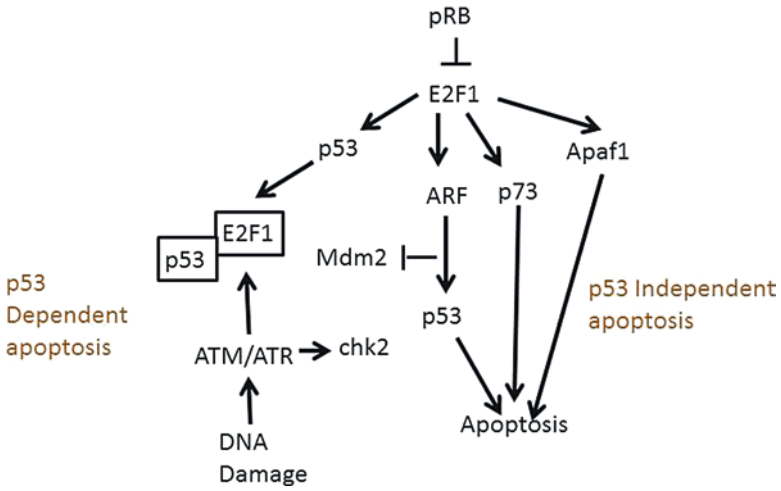

Fig. 20.3 Role of E2F1 in apoptosis via p53 dependent and independent**.** E2F1 promotes apoptosis via tumor suppressor p53 and independent of p53. E2F1 activates ARF, which stabilizes p53 by alleviating its proteosomal degradation through MDM2. In response to DNA damage, ATM/ATR and CHK2 kinase phosphorylates E2F1 and increases its stability. E2F1 also interacts directly with p53 via the cyclin A binding domain, thereby inducing p53-mediated apoptosis. E2F1 acts via direct upregulation of genes including p73 and APAF-1 Srt [13]

In addition to the role of E2F1 in cell cycle progression, overexpression has been reported in various types of tumors, including GC (gastric cancer). Plethora of studies reported that the knockdown of LINC00668 could perhaps abrogate E2F1-induced cell cycle acceleration and proliferation. LINC00668 may be key downstream effectors of E2F1 in gastric cancer [20]. Interestingly the knockdown of UHRF1 inhibits cellular proliferation in colon cancer cell lines. Knockdown of E2F1 was also reported to decrease UHRF1 expression. Notably UHRF1 may be used as a potential therapeutic target for colorectal cancer patients with high UHRF1 expression [16].

20.4 Role of E2F1 in Metastasis

E2F1 promote CRC migration, invasion, and metastasis by controlling RRM2 transactivation. Fang et al. [10] validated that both E2F1 and RRM2 were elevated in most CRC tissues compared to normal tissues. Protein expression levels of E2F1 and RRM2 were comparable and positively correlated with tumor stage and lymph node metastasis (LNM). Constantly, patients with low E2F1 and RRM2 levels were demonstrated with a better prognosis as compared to high levels [10]. Several reports in colorectal carcinogenesis depicted that overexpression of E2F1 is associated with the progression of adenomas to adenocarcinomas and metastasis. Banerjee et al. [2] demonstrated that high levels of thymidylate synthase (TS) and E2F1 in colorectal cancers lead to lung metastasis and even higher levels to pulmonary metastasis. Iwamoto et al. [14] in their study stated that the high expression of E2F1 is due to amplification of E2F1 gene. However increased levels of E2F1 enriched with an advantage of proliferation in tumor cells could lead to tumor progression. Despite that it was previously demonstrated that E2F1 expression levels may be more informative than TS in order to determine prognosis of colorectal cancers and also lead to selection of personalized therapy.

20.4.1 Adhesion

Chen et al. [5] reported that E2F1 downregulates expression of cell adhesion molecules, viz., VCAM-1, ICAM-1, and E-selectin in TNF-alpha pretreated (sublethal concentrations) human aortic endothelial cells (HAECs). This inhibition correlates with suppressed NF-κB activity and hypophosphorylation of IκB-alpha. The expression levels of TNF-alpha and IκB-alpha are stabilized in E2F1overexpressing HAECs. Remarkably, experimental evidence on endothelial cells depicted that diminished adhesion molecule expression in the E2F1-transduced endothelial cells markedly reduces adhesion of a monocytic cell line (U937) to transduced endothelial cells. Accordingly, E2F1 plays an unexpected role in regulating NF-κB-dependent cell adhesion in cytokine-stimulated cells.

20.4.2 Invasion and Migration

Ribonucleotide reductase small subunit M2 (RRM2) contributes to tumor growth and invasion in several cancers including colorectal cancer (CRC). Silencing RRM2 a downstream target of E2F1 abrogated migration and invasion in CRC cell lines [10]. Increased expression or amplification of E2F1 along with RB1 loss is associated with metastasis and resistance to chemotherapy. Thus, high levels E2F1 in several tumors are marker for unfavorable for patient survival and prognosis [18].

20.4.3 Angiogenesis

RTKs (receptor tyrosine kinases) like VEGFR3 are overexpressed in cancer cells and play an important role in tumor progression, interacting with corresponding ligands. Upregulation of receptor and ligand concentration depend on E2F1 interaction with their promoters. Parallel regulation of VEGF-C and VEGFR-3 by E2F1 stimulates cultured cells to new blood vessels in vivo [9]. RB-E2F1 pathway promotes expression of VEGF receptors facilitating angiogenesis, tumor growth, and progression in response to aberrant signaling events. Cooperation between mutant p53 and E2F1 leads to ID4 transcriptional activation, which in turn promotes angiogenesis through pro-angiogenic soluble mediator induction [17]. New transcriptional axis E2F1/p53/ID4 revealed the existence of undefined mechanism underlying tumor neo-angiogenesis [11].

20.5 Role of E2F1 in Apoptosis

Apoptosis is a vital process in various pathways, viz., normal cell turnover, proper development, immune system functioning, hormone-dependent atrophy, chemical-induced cell death, and embryonic development. Deregulated apoptotic signaling is the significant cause for many diseases including neurodegenerative, autoimmune disorders and cancers. Deregulated retinoblastoma protein pathway is one of the cancers hallmarks. However, tumors harboring genetic alterations in the pRB pathway also affect apoptotic pathway [15]. The hypophosphorylated RB binds to E2F and negatively regulates E2Fs activation, leads to increased expression of E2F-regulated genes. Strikingly, overexpression of E2F results in apoptosis in vivo and in vitro [6]. E2F1 overexpression causes G_2/M arrest followed by increased protein levels of c-Myc and p14ARF. It also initiates apoptosis and suppresses the growth of colon adenocarcinoma cells. Overexpression of E2F1 is associated with downregulation of Mcl1 and upregulation of c-Myc and p14ARF proteins. Therefore, E2F1 is a potential target for gene therapy in treatment of colon cancer [8].

20.5.1 p53 and Apoptosis

In p53-dependent pathway, E2F1 activates ARF, which stabilizes p53 by alleviating its proteosomal degradation through MDM2. ARF negatively regulates E2F1 in feedback mechanism. In response to DNA damage, ATM/ATR and CHK2 kinase phosphorylates E2F1 and increases its stability (CHK2 also stabilizes p53 by phosphorylation). Subsequently, E2F1 directly interacts with p53 via the cyclin A binding domain, thereby inducing p53-mediated apoptosis. Interestingly p53-independent apoptosis by E2F1 occurs via direct upregulation of p73 and Apaf-1 genes. The assembly of cytochrome C and Apaf1 released from the mitochondria leads to formation of apoptosome that catalyzes caspase-9 and initiates the proapoptotic effector caspases including caspase-30 [18].

20.6 Conclusion and Future Perspectives

E2F1 functions in tissue-specific manner as an oncogene and tumor suppressor. E2F1 targets genes involved in angiogenesis, invasion, and metastasis. This supports a vision that E2F1 play an ambiguous role in many aspects of cancer development. Present review provides a new insight on E2F1 role in tumorigenesis and for development of novel therapeutics. E2F transcriptional activity is regulated at different levels, through association with RB proteins. Beyond their role in cell cycle, E2F1 is implicated in regulation of apoptosis, development, and differentiation. Despite the fact that the role of RB and E2Fs in cell proliferation is a well-proven hypothesis, however their role in regulation of apoptosis, invasion, and angiogenesis that contribute to tumor growth is not well characterized. The role of E2F family members in colorectal malignancies is yet unclear. Hence there is need to investigate the underlying mechanisms of E2F1 in diagnosis, prognosis, and cancer therapy.

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Role of Notch Signaling in Colorectal Cancer

21

Divya Thirumalaipillai Rajendran, Boopathi Subramaniyan, and Mathan Ganeshan

Abstract

Notch signaling is a simple pathway in its mechanism and complex because it activates many genes at transcriptional level. Notch plays an imperative role in embryogenesis and progenitor/stem cell maintenance and critically preserves the balance between a cell proliferation, differentiation, and apoptosis. Mutation and deregulation of Notch will be a reason for many diseases including cancer. Aberrantly expressed Notch involves in the carcinogenesis of many cancers such as colorectal cancer (CRC), breast cancer, pancreatic cancer, prostate cancer, liver cancer, cervical cancer, Ewing sarcoma, Kaposi's sarcoma, lung cancer, ovarian cancer, and lymphoma. Among these, Notch plays a major role in CRC from development to metastasis. CRC occurs when the lining of colon epithelial cells becomes neoplasm, and this happens when the poise is missing between the normal and cancerous condition due to the overexpression of Notch. In CRC Notch promotes the stemness and epithelial to mesenchymal transition (EMT) which are requiring in aberrant crypt formation, invasion, and metastasis, respectively. Moreover, overexpression of Notch in CRC is connected with poor prognosis and chemoresistance. Notch counteracts with Hippo/YAP, WNT, NFkB, PI3K/Akt, and EGFR pathways for tumor initiation and development. Tumors are dependent on angiogenic switch for development and invasion which is activated by Notch ligand Dll-4 by overexpressing vascular endothelial growth factor (VEGF). This review will be focusing on the uniqueness of Notch in CRC.

Keywords

Notch · CRC · Chemoresistance · Transcription factor and EMT pathway

The original version of this chapter was revised. The book was inadvertently published without Abstracts and Keywords, which are now included in all the chapters. An erratum to this chapter can be found at https://doi.org/10.1007/978-981-10-6728-0_39

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21.1 Introduction

Colorectal cancer (CRC) is the third most common cancer and reason for 610,000 deaths per year worldwide. The death in CRC is mostly due to liver metastasis, tumor recurrence, chemoresistance, and cancer stem cells (CSCs). Besides, the deregulated signaling pathways include WNT, Notch, EGFR, and TGF-β acting a key role in CRC development [26]. Among these, Notch pathways poise a pivotal role in CRC tumor initiation, tumor recurrence, metastasis, poor prognosis, chemoresistance, and maintains of CSCs [8]. Notch is a single transmembrane receptors composed of four receptors Notch 1–4, along with five canonical ligands, Jagged (Jag)1, Jag2, Delta like (DII) 1, Dll3, and Dll4. Notch plays an imperative role in embryogenesis and progenitor/stem cell maintenance and critically preserves the balance between a cell proliferation, differentiation, and apoptosis [8]. Mutation or deregulation of Notch signaling pathway leads to many diseases including cancer. Aberrantly expressed Notch involves in the carcinogenesis of many cancers such as CRC, breast cancer, pancreatic cancer, prostate cancer, liver cancer, cervical cancer, Ewing sarcoma, Kaposi's sarcoma, lung cancer, ovarian cancer, and lymphoma [23]. Among these, Notch role in CRC is crucial from its initiation to metastasis. So understanding the role of Notch in CRC will pave a way to find new therapeutic targets for the treatment of CRC.

21.2 Notch Structure

Notch is a single transmembrane juxtacrine-mediated signaling pathway. Notch family consists of four type-1 transmembrane receptors, Notch 1–4. These are synthesized in precursor form and undergo subsequent cleavages to form mature active Notch receptor. The first cleavage is by furin-like convertase at trans-Golgi apparatus and forms extracellular domain, intracellular domain, and transmembrane domain. The extracellular domain is made up of 36 EGF-like repeats. The C-terminal of the EGF repeats consists of three cysteine-rich lineage defective-12 (LIN-12) and Notch repeats (LNR) which prevents receptor activation in the absence of ligand. Following this, it consists of heterodimerization domain and transactivation domain. Intracellular domain consists of RBPJ-associated molecule (RAM) domain, two nuclear localization signals, 6 ankyrin (ANK) repeats, and a PEST (proline-glutamine-serine-threonine) sequences which are collectively called as Notch intracellular domain (NICD) (Fig. 21.1) [3, 11, 15]. The sequence of proteolytic cleavage, cleavage site, and enzymes involved in cleavage are given in Table 21.1.

21.3 Notch Mechanism

Notch is a single transmembrane receptor with extracellular, transmembrane, and intracellular regions, and it is activated by sequence of proteolytic cleavages. The pathway activation is initiated by binding of one of the five (Jag1 and Jag2, DLL-1,

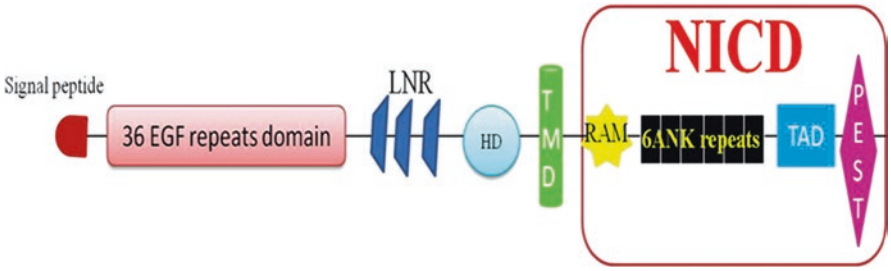

Fig. 21.1 Notch structure: EGF, epidermal growth factor: LNR, Lin12 Notch repeats; HD, heterodimerization domain: TMD, transmembrane domain:, ANK, ankyrin repeats: TAD, transactivation domain: and PEST, proline-glutamic acid-serine-threonine

Table 21.1 Notch cleavage site and enzymes

Cleavage	Cleavage site	Enzyme	References
S1 cleavage	1664/1665	Furin-like convertase	[3]
S2 cleavage	1710/ 1711	A disintegrin and metalloprotease (ADAM)	[3]
S3 cleavage	1743/1744	γ-secretase	[3]

DLL-3, and DLL-4) ligands which leads to the conformational changes in Notch receptors. Subsequently, S2 cleavage site is exposed for ADAM to remove the extracellular region. Following this, S3 cleavage occurs by γ-secretase (complex of nicastrin and presenilin-1) which removes the transmembrane region and releases Notch intracellular domain (NICD). The NICD is accumulated in cytoplasm and then translocated to the nucleus by concentration gradient where it binds with transcriptional regulator termed as C-protein binding factor 1 [(CBF1) also known as RBP-Jk or CSL for CBF1/Su(H)/Lag1)] leading to the displacement of corepressor and brings coactivators such as MAML-1 to activate the expression of hairy and enhance of split (Hes) and Hes-related repressor protein (Hey) families [1, 6, 7] (Fig. 21.2).

21.4 Notch and Transcription Factors

Once ligand binds to the Notch receptor, it can activate proteolytic cleavage mediated by gamma-secretase lead to release NICD. NICD is acting as a transcription coactivator, but it can't bind to DNA, because it doesn't have a zinc finger domain. Consequently, they can recruit RBP-J and MAML1–3 to activate transcription genes through binding with DNA. Once they are formed, it can recruit chromatin remodeling complex, which consists of histone deacetylase or histone acetylase proteins. In the absence of ligand or transcription coactivator of NICD, the RBP-J acts as a repressor. Notch activates many transcription factors directly and indirectly such as HES1, HEY1, TGFβ, NF-κB, snail, slug, PTEN, and cyclin D3 [14].

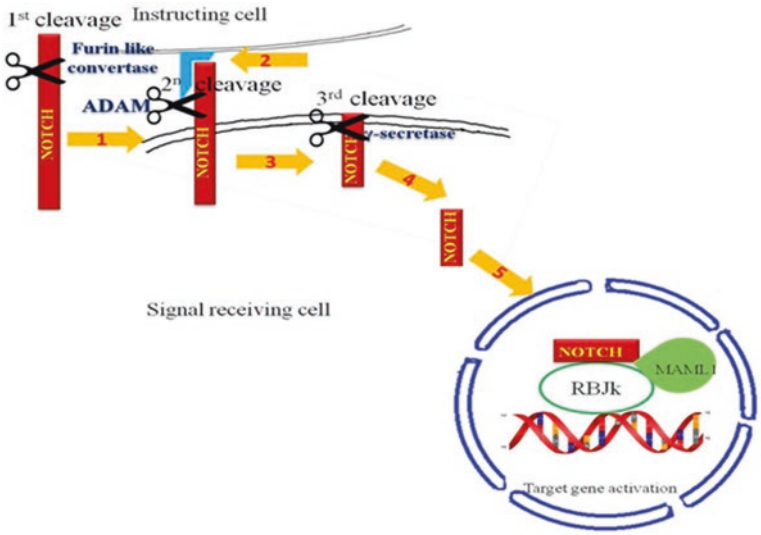

Fig. 21.2 Notch signaling pathway. *1*. Notch receptor is cleaved by furin-like convertase in trans-Golgi to produce mature Notch receptor. *2*. One of the five (Jag1, Jag2, Dll-1, Dll-3, and Dll-4) binds from instructing cell to activate signaling pathway. *3*. Exposure of S2 cleavage site and cleaved by ADAM enzyme to extracellular region. *4*. S3 cleavage carried out by γ-secretase to release Notch intracellular domain (NICD). *5*. NICD is then translocated to nucleus and activates target gene expression

21.5 Notch in Colorectal Cancer

Colon is the part of digestive system is lining with absorptive and secretory epithelial cells interspersed with deep crypts which consists of 2000 cells with 5–10 stem cells [19]. CRC is identified with aberrant crypt formation intervened by Notch pathway. Notch signaling regulates the cell proliferation and differentiation of epithelial cells to absorptive or secretory cells, maintaining apoptosis in the colon. The deregulated Notch will fail to spot the poise and leads to the formation of cancer [26]. Notch-mediated tumor initiation mechanism in CRC is not well understood, but the possible reason will be overexpression of mutated Notch receptors, ligands, and Notch signaling interplay between other signaling pathways. Notch is highly expressed in the primary stage of CRC relatively than the later stage. Notch1 and Hes1 are highly expressed in primary and metastatic CRC than colonic mucosa [19]. The other reasons could be (i) mutation of E3 ubiquitin ligase which is responsible for NICD degradation; (ii) mutation in APC leads to the overexpression of NICD [28]; (iii) the decreased expression of atonal homolog1 (ATOH1) which is essential for intestinal secretory cell commitment; and (iv) Hes1, the direct target of Notch, is repressor for KLF4 which inhibits CRC cell proliferation when overexpressed [2]. Other than this Notch interplays with many signaling pathways in CRC such as WNT, NF-κB, EGFR, and TGFβ. Notch signaling can be an oncogenic or tumor suppressor; it depends on the context of cells in where cancer occurs [24].

21.5.1 Notch Interplay with Signaling Pathways in CRC

Wnt/β-catenin signaling is the essential pathway which is highly articulated in 80% of sporadic CRC, and it is the main reason for inherited familial adenomatous polyposis (FAP)-related CRC [23]. Wnt/β-catenin signaling is the key pathway involved in the transformation of epithelial cells into self-renewing CSCs for CRC development. Wnt/β-catenin signaling activates its target Jag1 that trigger the Notch and increase its expression in CRC. Most of the CRC carries the mutation of APC which is the component of Wnt signaling pathway. In Apc-mutated condition, Notch signaling is requisite for consequent development of CRC [4, 26, 27]. PI3/AKT pathway is upregulated in 40% of human CRC, and it is important for cell survival and growth. Notch activates its target HES1 which inhibits PTEN leading to the activation and overexpression of PI3/AKT pathway in CRC. Notch involves in colonic carcinogenesis by stimulating EGFR pathway. Notch signaling arbitrated activation of transcription factor NF-κB in CRC results in chemoresistance of CRC cells [10]. Notch also inhibits the activity of transcription factor TGF-β pathway which is important for tumor suppressor and cell growth inhibition. In CRC, TGF-β-mediated induction of Jag1 results in the overexpression of Notch [30]. Angiogenesis is the formation of new blood vessels which is an important characteristic of growing tumor. Inhibiting or disrupting angiogenesis will be the great therapeutic way to inhibit or treat cancer [13]. Jag1 Notch ligand activates the Notch-dependent angiogenesis by activating VEGF in CRC [21]. Notch signaling upregulates the expression of anti-apoptotic genes including Bcl2 and BclXL. With these Notch also upregulates the expression of cyclin D1 and p21/WAF1 which are involved in cell cycle regulation and cell proliferation [23].

21.5.2 Notch Interaction with EMT Pathway

An epithelial mesenchymal transition (EMT) pathway plays an important role in key biological process of embryogenesis and metastatic cancer development [31]. The basement-degraded epithelial cells migrate to form new types of mesenchymal cells in order to increase the motility and invasion [16]. During this EMT process, the epithelial cell loses the cell junction followed by the reorganization of the actin cytoskeleton to initiate the transition by the relocation of epithelial markers such as E-cadherins and integrins from cell membrane to the nucleus. These could gain the expression mesenchymal markers such as N-cadherins, vimentin, and fibronectin [30]. Moreover, the growing report indicates that cancer stemness and chemoresistant mechanism are achieved by high rates of EMT signaling pathway [8], especially its interaction with oncogenic signaling pathways like MMP-3, BCL9-2, EGFR, met, goosecoid, kaiso, TGF-β, FOXC2, GSK-3β, Smad-3, Pez, Snail1, Snail2, ILK, and Notch [30]. Interestingly, the recent report revealed that the growing incidence of colon cancer is due to overexpression of Notch and its regulation of EMT pathway [9]. Notch signaling can regulate Snail1 expression through the induction of hypoxia inducible factor 1α (HIF-1α). Subsequently the HIF-1α binds the promoter region of LOX (lysyl oxidase) for stabilization of Snail1 and its

transcription. Likewise Notch interact Snail2 leads to repression of E-cadherin and β-catenin activation [25]. Moreover, targeting of Notch signal leads to downregulation of EMT and induction of p21 in human colon cancer cells [5].

21.5.3 Targeting Notch Signal for CRC Treatment

The gamma-secretase inhibitors (GSI) compounds treated CRC showed inhibition of the γ-secretase, which is a mandatory enzyme to activate Notch internal signaling, but it induces extracellular signal-regulated kinases (Erk) for continued cell proliferation. However, the GSI combined with cisplatin drug-treated cells exhibited the downregulation of Erk and activation of cell death [2]. The combined treatment of biphenolic compound and ionizing radiation (IR) treated in CRC results revealed the downregulation of the Notch, CRC stem cell protein marker DCLK1, and downstream target gene of Hes1. These downregulation genes can activate the apoptosis pathway. A similar result has observed in tumor xenograft animal model [22]. Interestingly the flavonoid compound epigallocatechin-3-gallate-treated cells has showed promising activity toward the inhibition of β-catenin, but quercetin showed weak inducer for downstream target genes reported in human colon carcinoma cell line HT29 [20]. The report of [12] indicates that the bioactive compound of Withaferin-A isolated from *Withania somnifera*-treated colon cancer cells exhibited downregulation of Notch signal by induction of c-Jun-NH2-kinase-dependent apoptosis. These mechanisms can reduce the expression of Akt/NF-κB/Bcl-2 and mammalian target of rapamycin signaling components (pS6K and p4E-BP1). Likewise, a Y-secretase inhibitor can induce the differentiation of goblet cell from the proliferative crypt in mice carrying mutated gene for an Apc tumor suppressor. Similar result has seen in blocking of Notch signal with Y-secretase inhibitor [29]. These reports further support the interaction of Wnt and Notch signaling pathways. Moreover, the Jagged1 was silenced by lentiviral Jagged1-shRNA resulted in decrease of cell viability. Besides, the knockdown studies showed the induction induced cell cycle arrest at G0/G1 phase with reduced expression of cyclin D1, cyclin E, and c-Myc in vitro colon cancer cell. However, the knockdown studies of xenograft mouse model have reflected the same results of in vitro [6]. The small molecular inhibitor FLLL32-treated colon cancer stem cell has seen the inhibition of STAT3 through the downregulation of survivin, Bcl-XL, and Notch signal. Moreover, the significant inhibition of tumorsphere formation and induction of cleaved caspase-3-mediated apoptosis observed in small molecule-treated cells are compared to curcumin-treated cells [17].

Curcumin analogue GO-Y030 treatment has shown the inhibition of activated STAT3 followed by reduction of cyclin D1, survivin, Bcl2, Notch, and p-RP in colon cancer cells. These reductions can facilitate the cleaved caspase-3-mediated apoptosis. Moreover, GO-Y030 treatment reduces the tumor growth in mouse xenografts with SW480 and HCT-116 colon cancer stem cells in dose-dependent studies [17]. However, Meng et al. [18] reported that the oxaliplatin, 5-fluorouracil (5-FU), and SN-38 compound-treated cell revealed the induction of the Notch-1

intracellular domain (NICD)-mediated chemoresistance toward cancer cell progression through the activation of Y-secretase. Subsequently, silencing of Y-secretase subunit nicastrin by small interfering RNA (siRNA) prevented activation of NICD after the treatment of oxaliplatin. Concurrently, the silencing or blocking of NICD by sulfonamide GSI (GSI34)-treated colon cancer cells sensitizes to chemotherapy compounds through downregulation of phosphoinositide kinase-3/Akt. The inhibition of Notch signaling leads to suppression of tumor invasion and intravasation in genetic depletion Aes in ApcD716 mice. These reports further confirm the inhibition of Notch potential target for control and treatment of metastasis colon cancer [28].

21.6 Conclusion

Notch plays a major role in normal development and cancer condition. Its role in CRC cancer is very important from cancer progression to metastasis. This review explained about Notch and its transcription factor role in CRC mainly in metastasis and targeting Notch pathway in CRC. Targeting Notch will be a great therapeutic source to treat the CRC.

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LEF/TCF: Its Role in Colon Cancer

22

Amanda Joy Bastien

Abstract

Many advances have been made in understanding the role of transcription factors in different types of cancer. With colorectal cancer being the third most commonly diagnosed cancer, studying critical transcription factors such as the lymphoid enhancer factor/T-cell factor (LEF/TCF) transcription factor family may assist in understanding its growth and progression but also serve as a therapeutic target in hopes for better patient outcomes. This chapter will discuss the pathway LEF/TCF transcription factors are known to be involved in, their regulation, and current studies that have investigated their role and function in colorectal cancer and normal cell maintenance.

Keywords

LEF/TCF · Colorectal cancer · Wnt signaling · Transcription factors

22.1 Introduction

The Wnt/wingless pathway is associated with several roles including but not limited to cell fate in embryonic development and adult organ maintenance. When there is improper cell maintenance, abnormal cell growth occurs and a tumor develops. Proteins of the canonical Wnt pathway are conserved among diverse organisms from slime mold and nematodes to mammals and are notably known for its central mediator β-catenin [1–4]. Mutations in the stabilization of β-catenin and the constitutive activation of Wnt signaling occur in more than 80% of sporadic colon tumors [5].

This constant activation of Wnt signaling causes an accumulation of β-catenin levels in the nucleus where it then interacts with the N-terminus of the family

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transcription factors lymphoid enhancer factor/T-cell factor (LEF/TCF) to promote cell growth and/or tumor development [6].

In knockout studies of LEF/TCF, the family of transcription factors has been implicated in regulatory roles in nearly all body tissues [7–11]. With colorectal cancer being the third most commonly diagnosed cancer and the third leading cause of cancer-related deaths in both men and women in the United States [12], inhibiting parts of the Wnt pathway such as the end point mediators LEF/TCF family transcription factors may be useful targets in treating many cancers including colon cancer. This chapter will discuss the LEF/TCF transcription factors, focusing on LEF-1 and TCF-4 and their role in colon cancer.

22.2 LEF/TCF Proteins: Structure and Signaling Pathway

Since the discovery of LEF/TCF family transcription factors, founding members being TCF-1 and LEF-1, the family of proteins has been extensively studied in the field of cancer biology [13–15]. Interestingly, the majority of invertebrates have a LEF/TCF ortholog, and it seems to have expanded to a family of four in most vertebrates [16]. The four proteins being TCF-1, LEF-1, TCF-3, and TCF-4, [also known as TCF7, LEF1, TCF7L1, and TCF7L2, respectively]. The class is characterized by a high-mobility group [HMG] domain. Its role is to bind DNA. Many studies have shown that the HMG domains among LEF/TCF are highly similar; for example, LEF-1 and TCF-1 HMG domains are almost identical, differing by only three amino acids [17, 18] and bind the same DNA sequence, [A/T][A/T] CAAAG [7, 19].

In the nucleus, any of the four mammalian LEF/TCF proteins can bind β-catenin through its N-terminal binding domain. This interaction was found using a yeast two-hybrid screen [20]. The interaction between β-catenin and its N-terminus binding region replaces transcriptional repressors and recruits other transcriptional coactivators [21]. When the LEF/TCF transcription factors are activated, downstream targets which include proto-oncogenes such as cyclin D1 [22, 23] and c-*myc* can be turned on [24]. Regardless, this pathway is highly studied, and the β-catenin-LEF/TCF complex regulates at least 50 genes [25–29].

The isoforms of LEF/TCF family transcription factors play important roles as well. In both in vivo and in vitro studies, TCF naturally occurring N-terminal deletion negatively regulates Wnt signaling [30]. This trend is not just prevalent for TCF N-terminus deletions but for LEF as well [31]. When overexpressed in HT29 cells, full-length LEF-1 increases cellular proliferation in vitro, but its truncated isoform, lacking the β-catenin interaction domain, inhibits cellular growth [30–33]. In fact, these dominant-negative isoforms have been found to occur naturally for TCF-1, LEF-1, and TCF-4 and are all believed to be involved in negative feedback of the Wnt pathway. Complicated, but critical, the LEF/TCF family and their isoforms play an important role in several processes such as the cell cycle, tumor formation, and apoptosis. For basic structure of LEF/TCF family proteins and their common isoforms, see Fig. 22.1.

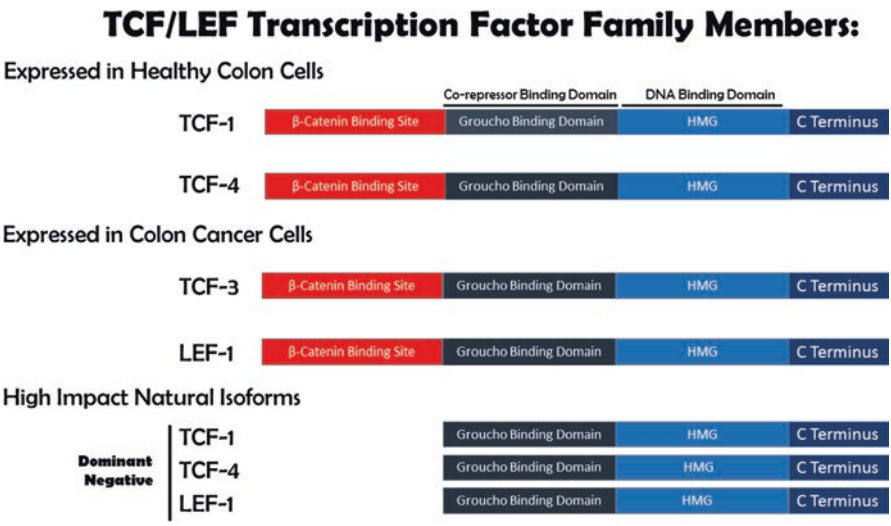

Fig. 22.1 Schematic of the LEF/TCF transcription family members. On the N-terminus, LEF/TCFs have a β-catenin-binding domain. The binding of β-catenin is required for LEF/TCF function. On the C-terminus is a high-mobility group [HMG] domain mediating sequence-specific DNA binding. Many common isoforms occur naturally due to activation at different promotors such as the dominant-negative isoforms above or due to alternative splicing at the C-terminus [30] (Figure adapted from [39])

Once LEF/TCF is bound to β-catenin, many post-translational modifications can take place. Phosphorylation, sumoylation, ubiquitination, and acetylation can effect whether LEF/TCF interact with coactivators, repressors, or DNA [34]. For example, when NLK/Nemo phosphorylates TCF/LEF, the modification seems to destroy the DNA-binding affinity of the β-catenin/TCF/LEF complex and as a result negatively regulates later Wnt target genes [35, 36].

In contrast, when there is an absence of Wnt, then TCF/LEF binds to Wnt response elements (WRE) and corepressors such as Groucho, CtBP, and HDACs. Many studies have shown that the absence of Wnt signaling in the nucleus, TCF acts as a repressor of Wnt target genes [37]. TCF negatively regulates gene expression by interacting with Groucho (human TLE1); this TCF/Groucho interaction increases histone deacetylation and chromatin compaction, leading to transcription deactivation. However, it has been shown that the presence of β-catenin in the nucleus displaces Groucho for gene activation [38]. See Fig. 22.2.

LEF/TCF full-length expression and their isoforms vary between normal cells and colon cancer cells. In the normal colon, TCF-1 and TCF-4 are expressed, and LEF-1 and TCF-3 are silenced [14, 32]. Moreover, they also have different biological roles. In general LEF-1 is viewed as an activator and TCF-3 as mostly a repressor but can be an activator [39, 40]. Both TCF-1 and TCF-4 act as both inhibitors and activators, but TCF-4 is the dominant interactor of β-catenin in the colon**.** TCF-4 mediates the transformative process of colon epithelial cells when tumor-suppressor protein adenomatous polyposis coli (APC) is loss. The family of proteins has also

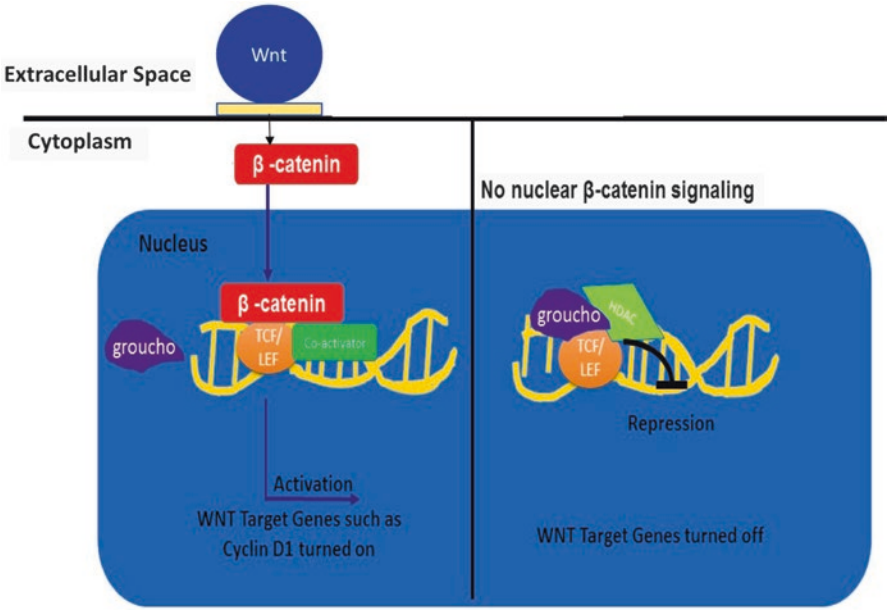

Fig. 22.2 Schematic of the LEF/TCF activation and repression. When there is an absence of Wnt signaling and thus no nuclear β-catenin, TCF/LEF binds to Wnt response elements (WRE) and corepressors such as Groucho, CtBP, and HDACs. When there is Wnt signaling and β-catenin is located into the nucleus, it binds to the TCF/LEF N-terminus binding region displacing transcriptional repressors and recruits other transcriptional coactivators [21]. When the LEF/TCF transcription factors are activated, downstream targets include proto-oncogenes such as cyclin D1 and other genes [22, 23] (Figure adapted from [38])

been found to regulate one another. For example, transcription of LEF-1 can be directly regulated by TCF4-β-catenin [31]. This shift may be a pivotal role during initial cancer formation as expression of LEF-1 is found only in colon cancer cells.

22.3 LEF/TCF: Role in Cell Cycle and Cellular Proliferation

The cell cycle is highly complex with many regulatory proteins involved in the timing and frequency of DNA replication and cell division. It is important for cells to have proper cell cycle checkpoints and regulation in order to remain healthy and for the prevention of cancer.

The cell progresses through the following stages: G1, S, G2, and then mitosis during the cell cycle. Levels of the β-catenin-TCF/LEF complex are thought to be at its peak during the S-G2 phase and during G2 binds to the promoter of its downstream targets [41], a notable target being cyclin D1.

The central role of cyclin D1 is the regulation of G1 to S phase transition during the cell cycle [42, 43]. It complexes with cdk4 and cdk6 and incorporates incoming signals and other mitogens into the cell cycle [44]. In colon cancer, 44% of tumors

overexpress cyclin D1 [45], and its overall high expression has been highly characterized in multiple studies [46–48]. Cyclin D1 expression in colon cancer is probably linked to its cell cycle-promoting activity since lessening the expression of cyclin d1 slows growth of human colon cancer cells [49]. It also has been shown that cyclin D1 is a downstream target of the β-catenin-TCF complex and therefore when continually activated allows cells to proceed through the cell cycle and continue tumor growth. In support of this, expression of the dominant-negative form of TCF, lacking the β-catenin-binding domain, in colon cancer cells strongly inhibits expression of cyclin D1 causing cells to arrest in the G1 phase of the cell cycle due to the lack cyclin D1 activation [23].

The β-catenin/LEF-1 pathway also shows cyclin D1 as a direct target; LEF-1 binds directly in the cyclin D1 promoter [22]. In addition, antisense cyclin D1 cDNA inhibits growth of SW480 colon cancer cells in nude mice; this suggests a critical role for cyclin D1 in tumorigenesis and that targeting the TCF/LEF family can downregulate its specific activity [49].

The end point of the cell cycle is mitosis; this is when one cell becomes two. Several studies have shown that TCF-4 maintains the crypt stem cells of the gut, and its continuous activation causes uncontrolled cell proliferation [50]. In support of this finding, another lab determined the disruption of β-catenin/TCF-4 complex resulted in decreasing the expression of c-MYC and downstream targets causing G1 arrest [9]. TCF-4 may be an important switch in cell cycle regulation and proliferation and when constitutively activated a monumental event in colon cancer tumorigenesis.

22.4 Role of TCF/LEF in Metastasis

22.4.1 Adhesion

The ability of cells to adhere to each other is essential for the makeup of multicellular organisms. When cell-cell disruption occurs, tumor cells can migrate and proliferate, leading to metastasis. Disruption is common when expression of cadherin/catenin family members becomes low or by improper assembly of adherens junctions [51].

Studies previously discussed have implicated both cyclin D1 and LEF/TCF in the cell cycle but also in cell-cell interaction maintenance. In a study, both cyclin D1 and β-catenin were silenced by siRNA transfections. Using SW80 cells, they looked at the formation of E-cadherin-mediated adherens junctions by staining with a Cy3-coupled anti-E-cadherin antibody and imaged using a fluorescence microscope. They concluded that there was an increase in the number of adherens junctions after the depletion of cyclin D1 and β-catenin [52].

Furthermore if there is constitutive activation of β-catenin and the β-catenin-LEF/TCF complex and its target cyclin D1, then there may be less cell-cell adhesion allowing colon tumor cells to detach and enter the circulatory system allowing cancer to spread throughout the body.

22.4.2 Invasion

Once cancer cells move into tissues surrounding the tumor, the first stage of metastasis has occurred: invasion. Often invasion is accompanied by epithelial-to-mesenchymal transition (EMT) where epithelial cells are transformed into migrant mesenchymal cells. Transcription factor, ZEB1, is the key inducer of EMT, and its levels are affected by endogenous β-catenin or TCF4 knocked down in SW480 cells [53]. Furthermore, β-catenin/TCF-4 complex actually binds directly to the ZEB1 promoter activating its transcription and thus the EMT process. Also shown in vitro, LEF-1 increases the invasiveness of cancer cells when upregulated [54, 55].

In general, many studies show upregulation of the LEF/TCF transcription family levels and its downstream targets increases the invasiveness of colon cancer.

22.4.3 Migration

Metastasis occurs when cells migrate from the initial site of disease into the circulatory system invading a new site [56]. Approximately 50% of patients with advanced local colon cancer will also have metastasis into the liver. When this occurs, life expectancy is predicted to be less than 1 year [57, 58]. Recently HEF1, a multi-domain scaffolding protein of the Cas family has been identified as a novel target of TCF/β-catenin signaling. In ten colorectal cancer cell lines, high expression of HEF1 was observed. Furthermore, when comparing colon primary tissues, 94% had high HEF1 levels, whereas the control tissue only had 8% [59]. Moreover, many targets of TCF/β-catenin complex have important roles in proliferation and migration. Preventing their interaction with one another may be useful in preventing or controlling the spread of colon cancer.

Furthering this reasoning, FOXN3 and other proteins upstream of the β-catenin/TCF interaction may be potential therapeutic targets. In several colon cancer tissues, it was found that FOXN3 (Forkhead box N3) was downregulated. This was further investigated with in vitro studies. When stably knocked down in colon cancer cell lines SW480 and SW620, FOXN3 increased the activity of β-catenin/TCF. When overexpressed, the opposite was found. Using a Boyden chamber assay, a tool to measure cellular migration, it was found that overexpression of FOXN3 also inhibited migration of colon cancer cells [60]. Taken together, targeting β-catenin/TCF complex and down regulating it, like FOXN3 has shown to do, may restore normal biological function in colon cancerous cells.

22.5 Angiogenesis

First approved in the United States in February 2004, Avastin became a widely used treatment for colon cancer in conjecture with a chemotherapy regimen; Avastin is often used in parallel with chemotherapeutic 5-fluorouracil [5-FU] [61]. Avastin (bevacizumab) and similar drugs have been extensively used as a treatment option for patients

with metastatic colorectal cancer. Avastin was the first product approved in the United States that prevents angiogenesis, formation of new blood vessels, in cancer treatment.

Avastin works by inhibiting the protein "vascular endothelial growth factor" (VEGF). VEGF is secreted by tumor cells and acts on endothelial cells of existing blood vessels to promote new blood vessel formation [62]. Many studies have shown that VEGF is an important angiogenic factor in both colon cancer and metastatic colon cancer [63]. In fact its overexpression correlates with the development of metastases in colon cancer [64]. However, when VEGF is targeted and bound to Avastin, it cannot stimulate the growth of blood vessels. When blocked, the tumor cannot receive blood, oxygen, and other needed nutrients. This limits the tumor's ability to replicate and grow. Overall VEGF inhibitors like Avastin have shown to be significant in cancer treatments in both in vivo and in vitro studies, but challenges still persist. It remains elusive why some cancers have little response to VEGF-specific inhibitors, and many patients experience relapse. In search of other therapeutics, inhibition of the LEF/TCF transcription family proteins remains another hopeful option.

Interestingly, TCF-4 binds directly with VEGF and also has a critical role in regulating downstream targets of the Wnt signaling pathway. Mutation of TCF sites and expression of the dominant-negative TCF-4 isoform abolished transforming growth factor-β-induced VEGF promoter activity [65]. Furthermore, depleting the TCF-4 and VEGF interaction may decrease levels of angiogenesis and metastasis since high levels of both are seen in many cancers, and the two proteins directly interact with one another.

22.6 Apoptosis

A cell's inability to undergo apoptosis is a hallmark of cancer. Apoptosis is the biological process where a cell commits programmed cell death; a process that is a double-edged sword: too much is harmful permitting growth and renewal and too little allowing unhealthy cells to propagate and spread. Initially described by Kerr et al. in 1972, apoptosis has continued to be a highly studied biological process into today's world [66].

A common laboratory test for live cell apoptosis is annexin V staining. In healthy cells phosphatidylserine (PS) is localized on cell membrane's cytoplasmic side. In apoptosis, PS is translocated to the outer cell membrane, thus allowing it to be stained for in annexin V. In a study using SW480 and HT29, colon cancer cell lines, the cells were made to stably express LEF full length or dominant-negative LEF-1 [lacking the β-catenin-binding terminal]. The level of cells undergoing apoptosis and therefore staining annexin V positive were approximately twice of the controls in both cell lines for the dominant-negative LEF-1 isoform, but not for full-length LEF-1.The authors concluded that the shorter isoform, lacking the β-catenin-binding domain, is likely to lower survivability of colon cancer cells, and the isoform variant is likely to be involved in apoptosis [67].

TCF-4 knockdown in SW80 and HCT116 cells inhibited proliferation and increased apoptosis even more so than β-catenin knockdown. Knockdown also increased cytotoxicity sensitivity to many chemotherapeutics such as 5-FU and oxaliplatin [68].

Furthermore, these studies support that isoforms of LEF may induce apoptosis taking part in a negative feedback loop in Wnt signaling preventing cell cycle progression and cellular proliferation, and in contrast its full length, expressed in colon cancers, having no significant impact on inducing apoptosis. Taken together with TCF-4 knockdown inducing apoptosis perhaps higher levels of isoform LEF-1 and downregulation of TCF-4 and full-length LEF-1 may help impede colon cancer cellular progression.

22.7 Conclusion and Future Perspectives

The upregulated transactivation of β-catenin/TCF target genes is a major event in the progression of colon cancer. Constitutive activation of the complex and its target genes promotes and enables colon tumorigenesis and causes defect in a cell's ability to regulate its cell cycle, proliferation, and the ability to commit to apoptosis.

Given the important roles of the TCF/LEF transcription protein family and its complexity with being an activator but also an inhibitor, this makes them a potential therapeutic target. Potential inhibitors used today in cancer treatment include antibodies, small molecules, antisense RNA, and dsRNA for use in RNAi. The use of these targeted therapies has significantly improved the treatment of cancer over the past 10 years. However, the use of monoclonal antibodies in targeting TCF/LEF would be deemed useless as the complex occurs in the cell nucleus, but small molecule inhibitors (molecular weight approximately 500 Da) can enter thereby blocking receptor signaling and interfering with downstream intracellular molecules.

Many efforts are already under way to target the LEF/TCF transcription factor family. Using the crystal structure of the β-catenin-TCF complex (PDB ID:1JPW), a derivative of coumarin called esculetin (6,7-dihydroxy-2-chromenone) has been identified as a potential inhibitor of the complex. Coumarin is found in several medicinal plants [69, 70], and its ability to inhibit cancer proliferation has been reported previously in the rat colon [71]. In efforts to target human colon cancer cells, Sung-Young Lee investigated the molecular mechanism by which esculetin works. His findings show that esculetin binds directly to β-catenin, leading to the disruption of the β-catenin-TCF complex and its downstream targets. Other potentially useful small molecule inhibitors include PKF118-310, CGP049090, PKF115-584, PKF222-815, and PKF118-744 [72].

Although promising, the majority of Wnt inhibitors are still in laboratory testing and have not reached the clinical testing stage. The β-catenin/TCF interaction plays such an important role in regulating homeostasis that its interference may have some serious adverse effects. Studies have shown when Wnt is inhibited, it negatively affects hematopoietic stem cell homeostasis in vitro and in vivo [68]. In addition, many small compounds identified as potential inhibitors of the N-terminus of the β-catenin-TCF binding region may be very similar to other important binding partners including E-cadherin and APC [73, 74]. Future studies need to determine if these small inhibitor molecules like esculetin are specific to the β-catenin complex or if it also disrupts other interactions such as E-cadherin. Effecting E-cadherin could cause harmful consequences such as affecting cell-cell adhesion and other biological

processes. Although further testing and investigation are required, targeting the TCF/LEF family still remains a hopeful target in colon cancer treatment.

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NF-κB: Its Role in Pancreatic Cancer

23

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Abstract

Nuclear factor κB (NF-κB) is one of the transcription factors involved in the progression of pancreatic cancer. Pancreatic cancer (PC) is a deadly cancer in today's world, and treatment approaches become critical due to poor prognosis and chemoresistance of the cancer. The NF-κB signaling pathway is known to induce cell proliferation and metastasis, including migration and angiogenesis. Furthermore, the NF-κB pathway is also involved in the prevention of apoptosis in PC. In this present chapter, we discuss the role of NF-κB in the progression of PC, resistance to chemo drugs such as gemcitabine, and the influence of downstream targets on the metastatic characteristics of cancer. Therefore, targeting the NF-κB signaling pathway stands as a rational approach for future treatment of pancreatic cancer.

Keywords

Pancreatic cancer · NF-κB · Cell growth · Metastasis · Epigenetics

23.1 Introduction

Nuclear factor κB (NF-κB) is an important transcription factor in the cell signaling pathway of an inflammatory response to any stimulus such as a pathogen, stress, or cytokine. NF-κB also plays a crucial role in cell proliferation and cancer. The transcription factor is activated through two pathways: the classical pathway and the

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nonclassical pathway. Its activation is regulated by a class of proteins known as inhibitors of κB (IκB). The degradation of these inhibitor proteins results in the movement of NF-κB into the nucleus for transcription of a target gene. Five proteins, sharing a common Rel homology domain, constitute the family of NF-κB [72]. The major component in the family of NF-κB is Rel 1A (p65) protein [60], and its expression levels are varied through different species [52]. Rel A plays a major part in the proliferation of different cancers. One study showed that p65 was involved in the proliferation of ovarian cancer cells [69]. Other proteins include Rel 1B, p52/p100, p50/p105, and c-Rel [16, 26, 54]. In the classical pathway, the stimuli that trigger a NF-κB response are extracellular signals such as cytokines. In this signaling cascade, the IκB kinase degrades the IκB protein that reduces the p65 in the cytoplasm. Rel 1A is translocated into the nucleus to start the transcription of the target DNA sequence [31]. The activation of the nonclassical or noncanoncial pathway occurs through receptors such as lymphotoxin β receptor (LTβR), which is known to activate the inflammation process in various cancers [20, 63, 87] and thereby stimulating the NF-κB-inducing kinase (NIK). NIK degrades the IκB proteins in the heterodimer complexes to allow translocation of the NF-κB transcription factors into the nucleus [54] (Fig. 23.1).

NF-κB transcription factors are constitutively activated in cancers, and they are invariably involved in inflammation-associated carcinomas [15, 21, 61]. NF-κB factors are essential contributors in the progression and invasion of any cancer [36].

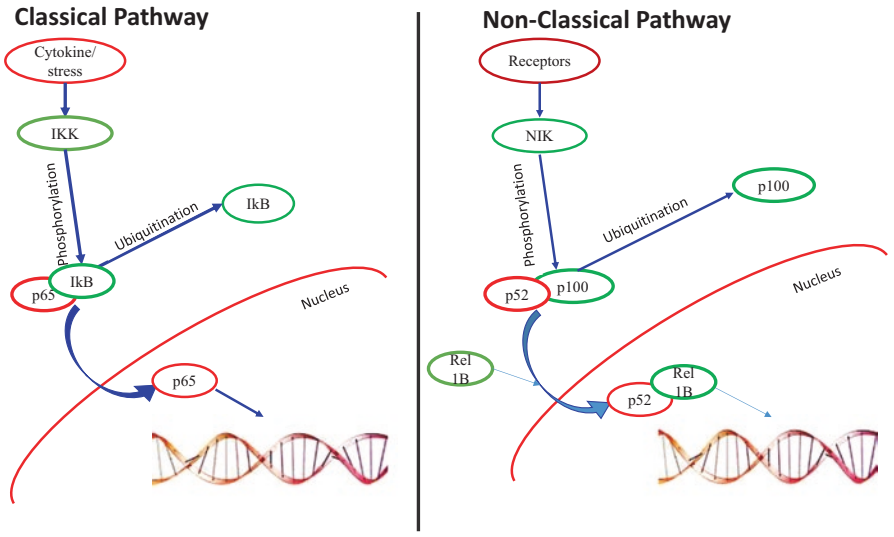

Fig. 23.1 Activation of NF-κB through different pathways. In the classical pathway, cytokines or stress induces the translocation of p65 from the cytoplasm into the nucleus, whereas the receptors in the nonclassical pathway induce the translocation of the p52 and Rel1B complex from the cytoplasm into the nucleus. Ubiquitination of certain proteins also occurs in both pathways: IkB in the classical pathway and p100 in the nonclassical pathway

The development of drug resistance has been a concern in prostate cancer, due to NF-κB [14]. Moreover, the induction of angiogenesis and the downregulation of tumor suppressor maspin [29] are evidence of a major role of NF-κB in cancer progression [49].

Less than 5-year survival rates, bad prognosis, and chemoresistance make pancreatic cancer one of the deadliest cancers worldwide [19, 68]. The cases of pancreatic cancer have been steadily increasing every year in Caucasian men as the Western lifestyle increases susceptibility to the cancer [45, 57]. The mortality of people depends on race as observed in the case of pancreatic cancer where the mortality in African-Americans is higher than the rate in Caucasians [45]. Having high mortality rates in its advanced stages, pancreatic cancer has emerged as a critical area of research for the identification of any biomarkers in blood or the recognition of any signaling pathways during the initial stages of the cancer.

In this review, we discuss the measures by which NF-κB could be used as an efficient biomarker in the progression of pancreatic cancer.

23.2 Role of NF-κB in Cell Proliferation

Cell proliferation is the signature characteristic of a cell, and many cytokines aid in this process. Unfortunately, cytokines cannot regulate the cell proliferation of a cancer cell as they recognize the cancer cell as a normal cell in our bodies. Identifying novel regulators in the proliferation of pancreatic cancer is important as silent symptoms continue until the latter stages of the cancer. One such protein is carboxypeptidase E (CPE) from the family of pro-protein convertases, which regulate cell proliferation when CPE is downregulated [43]. FAM21, a part of WASH complex (Wiskott–Aldrich syndrome protein and Scar homologue), is another protein that could act as a target as its reduction has made pancreatic cancer cells sensitive to gemcitabine [18]. The major role, however, is played by the master cytokine NF-κB in the regulation of the other cytokines and receptors in cell proliferation. NF-κB is mediated in all cancers by various proteins, such as p21-activated kinase (PAK4) [76], Th17-type cytokines [17], and MAP3K7 [88]. The downregulation of NF-κB with its inhibitors maintained the growth of pancreatic cancer, indicating a critical part played by NF-κB [38, 91].

In vitro studies have shown that NF-κB is inhibited by a nonsteroidal drug tolfenamic acid (TA) [59] through its translocation to the nucleus to promote transcription of cell proliferative protein BCL-2 [6, 86]. BCL-2 is also inhibited by the knockdown of a multifunctional protein, clusterin (CLU), through the NF-κB signaling pathway in order to sensitize the pancreatic cells to gemcitabine [86]. Expression of clusterin in pancreatic cancer was significantly higher than normal in patients who were on gemcitabine, indicating that clusterin induces resistance to the drug [11].

NF-κB is constitutively activated in pancreatic cancer by KRAS mutations. The KRAS oncogene is a protein from the RAS family and is well known to initiate pancreatic cancer [33, 54]. Regulation of the mutant KRAS gene has been a challenge; therefore, the focus has now been diverted to downstream targets of

KRAS. Membrane-bound mucin MUC4 is an early downstream target in pancreatic cancer. MUC4 transcription is mediated by NF-κB and AP-1 [78]; thus, regulation of NF-κB expression is involved with the expression of MUC4 and thereby reduces the KRAS mutations. MUC4 has two splice variants out of which MUC4/Y induces cell proliferation in pancreatic cancer cells: either through the JNK or the AKT signaling pathway [85]. TAK-1 (transforming growth factor beta-activated kinase 1) and GSK-3 (glycogen synthase kinase) are also downstream targets of KRAS that promote pancreatic cancer through the noncanonical pathway of NF-κB [5].

23.3 Role of NF-κB in Apoptosis

Programmed cell death, also known as apoptosis, is the natural way for a cell to cease existence. Its dysregulation, however, is one of the hallmarks of cancer [13]. NF-κB, a protein that helps in the survival of a cell, plays a huge role in the dysregulation of apoptosis during cancer [72]. Papademetrio et al. [53] showed that inhibition of the NF-κB pathway leads to apoptosis in pancreatic cancer. Tenascin-C, a glycol-protein located in extracellular matrix (ECM), is highly expressed in pancreatic cancer as opposed to its absence in noncancerous cases [64]. This glycol–protein induces resistance of pancreatic carcinogenesis and allows apoptosis through the NF-κB pathway by translocating the p65 to the nucleus through phosphorylation of ERK1/ERK2 [64]. Radiation therapy with nafamostat mesilate enhances cell apoptosis, and the combination with the inhibition of NF-κB increases the effect of the therapeutic approach [66]. In order to reduce chemoresistance due to the gemcitabine-induced nuclear factor κB (NF-κB) activation, another therapeutic approach includes the combination of nafamostat mesilate and gemcitabine, which was shown to reduce the progression of pancreatic cancer [77]. TRAIL-induced (tumor necrosis factor-related apoptosis-inducing ligand) apoptosis is an effective way to eliminate cancer cells as it selectively induces cell death in carcinogenic cells [25]. PDAC, however, has the ability to develop resistance to TRAIL, and TRAIL-resistant cells have a high amount of NF-κB activity in the cells [37, 75]. C-Rel is the main contributor to TRAIL resistance in PDAC [25]. Regulating c-Rel along with GSK-3α—glycogen synthase kinase-3—could control the TRAIL resistance to induce apoptosis in pancreatic carcinogenesis [92]. Targeting the NF-κB also reduced the TRAIL resistance and enhanced apoptosis [74].

23.4 Role of NF-κB in Cell Cycle

NF-κB mediates a lot of proteins and proteases from escaping the cell cycle arrest at various stages. P65, from the NF-κB family, promotes pancreatic cancer through activation of stathmin. Stathmin is a protein regulated by p53, and it dysregulates the microtubules in the malignancies and, therefore, leads to chronic hypoxia [44]. Inhibition of dysregulated stathmin arrested cells at the G2/M stages in the cell

cycle [44]. NF-κB also silences protease inhibitors such as the histone deacetylase inhibitor belinostat in order to enhance pancreatic cancer [12].

The effect of NF-κB could be reduced by inhibiting its translocation to the nucleus to transcript survival proteins. Drugs specific to the target translocation of NF-κB have become increasingly available nowadays. Chen et al. [9] showed that dihydroartemisinin (DHA), derived from an anti-malaria drug artemisinin, inhibited the NF-κB translocation and decreased the expression of cdk2 (cyclin-dependent kinase), cdk4, and cyclin E. Pristimerin, a triterpenoid [40], caused cell cycle arrest at the G1 stage, and a significant decrease in the expression of the aforementioned cell cycle proteins was observed [82]. This triterpenoid also cleaves caspase 3 and sensitizes the pancreatic cancer cells to gemcitabine by inhibiting the localization of NF-κB to the nucleus [82]. Similarly, gemcitabine-induced NF-κB could also be reduced by another medicinal drug, honokiol, thereby increasing the effects of gemcitabine and arresting the cell cycle at the G1 phase [2]. Benzyl isothiocyanate (BITC), another plant drug [34], arrested the cell cycle at the G(2)/M phases by targeting the nuclear localization of NF-κB transcription factor and lowering the expression of cyclin D1, cyclin B1, and cyclin-dependent kinase 1 [70].

β-adrenoceptors are cell membrane receptors whose inhibitors are epinephrine, and these receptors increase the uptake of glucose by the muscle in the pancreas. β-adrenoceptors are considered to be a part of cancer cell invasion, and therefore Zhang et al. [89] reviewed the inhibition of these receptors. It was observed that the β2-adrenoceptor antagonist, ICI118,551, significantly decreased the expression of cell cycle kinases such as cyclin D1, cyclin E, and Akt but increased pro-apoptotic proteins. ICI118,551 also significantly reduced the activation of NF-κB through inactivation of the cAMP/PKA pathway and the MAPK pathway [90], marking reduced cell invasion and cell proliferation in pancreatic cancer [75].

23.5 Role of NF-κB in Migration

Caused by TGF-β signaling, epithelial–mesenchymal transition (EMT) as a characteristic of metastasis TGF-β also activates NF-κB (p65) through TAK1, which is involved in cell proliferation, migration, and cell invasion [24, 27]. TGF-β, when overexpressed in malignancies, promotes the cell to migrate from one area to another. Maier et al. [46] also covers how defective TGF-β signaling could act as an inducer of EMT and a promoter of pancreatic cancer progression.

Targeting NF-κB for EMT reduction is a way of decreasing the migration of pancreatic cancer cells to other areas [57]. CARD-containing MAGUK protein 3 (CARMA3) affects the NF-κB pathway through Bcl10; overexpressed CARMA3 is silenced in pancreatic carcinoma, and the NF-κB signaling is also significantly decreased in the cancer along with migration, cell invasion, and cell proliferation [22]. Blocking the NF-κB through the aforementioned various drugs reduces the EMT of a pancreatic cancer cell. Resveratrol, a natural polyphenolic compound detected in grapes, inhibits the EMT of PC cells through suppression of the NF-κB

pathway [42]. Similarly, grape-seed proanthocyanidins inhibit the metastasis of pancreatic cancer through reversal of EMT, promotion of mesenchymal to epithelial transition, and targeting NF-κB [55]. Metastasis of pancreatic cancer is observed by significant changes in the expressions of E-cadherins, N-cadherin, and vimentin [55].

Superoxide dismutase (SOD) enhances the production of reactive oxygen species (ROS) that aids in the invasiveness of the carcinogenesis. SOD aids in this process by activating the ERK/NF-κB pathway and promotes expressions of EMT-related proteins such as E-cadherins, N-cadherin, and vimentin [41]. Further research is required on the role of NF-κB in the invasion and migration of pancreatic cancer.

23.6 Role of NF-κB in Angiogenesis

Overexpression of the vascular endothelial growth factors (VEGF), interleukin 8 (IL-8), matrix metallo-protein (MMP) 9, and cyclooxygenase (COX)-2, are observed during angiogenesis of any type of cancer. All these proteins are transcript products of NF-κB, so inhibition of NF-κB would reduce the formation of angiogenesis in malignancies [47]. There are various drugs identified to block NF-κB in angiogenesis. In 2011, dihydroartemisinin (DHA) was found to have an anti-angiogenic effect by targeting the NF-κB pathway and inhibiting the DNA-binding activity of NF-κB [80]. Initiation of transcription by NF-κB through DNA binding was also reduced by a proteasome inhibitor MG132 and thus decreasing the expression levels of VEGF and IL-8 [47]. A more recent study has shown that zerumbone, a component of subtropical ginger, inhibits the expression of pro-angiogenic proteins by blocking the activity of NF-κB [62]. Another vegetative compound, oxymatrine, from Chinese herbs has decreased the expression of VEGF in addition to the expression of NF-κB in an anti-angiogenic effect [10]. Blocking NF-κB to reduce angiogenesis may be a vital strategy because all pro-angiogenic proteins are gene products of NF-κB; therefore, the dysregulation of NF-κB has to be targeted in order to stop angiogenesis and cell invasion.

23.7 Epigenetics of NF-κB in Pancreatic Cancer

NF-κB is critical to the invasiveness of pancreatic cancer [71]. Two inhibitors of NF-κB, namely, JSH-23 and SM-7368 [39, 65], were identified to block the invasiveness of pancreatic cancer cells and the migration of cancer [71]. SOX9 is an essential transcription factor in the development of pancreatic cancer [67]. Epigenetic changes in this biomarker significantly inhibit the invasiveness of pancreatic cancer cell lines, PANC-1 and HPAC [71]. Expression of SOX9 is regulated by the p65 subunit of the NF-κB family [71]. Regulated by NF-κB, another biomarker is MMAC/PTEN, which is involved in the PI3K/Akt signaling pathway [3]. Decreased expression of MMAC/PTEN activates the PI3K/Akt pathway, which subsequently activates NF-κB in pancreatic cancer [3]. The introduction of NF-κB inhibitors has shown improved results in both cases. Benzyl isothiocyanate inhibits

histone deacetylases, resulting in a significantly reduced expression of NF-κB [7]. Therefore, identification of upstream biomarkers in the NF-κB signaling pathway carries significance in diagnosing pancreatic cancer in its early stages.

23.8 Molecular Mechanism of NF-κB in Pancreatic Cancer

Alteration in the NF-κB signaling pathway mediates carcinogenesis through VEGF and MAPK [30]. Dysregulation of the NF-κB pathway is accompanied by alterations in the expressions of proteins such as Bcl2, survivin, and XIAP [1]. A previous study showed that the activation of the NF-κB pathway may be prolonged by the use of eicosapentaenoic acid (EPA) in pancreatic cancer [58]. The usage of fatty acids in combination with gemcitabine resulted in the proliferation of pancreatic cancer through the NF-κB pathway [32]. This indicates the usage of fatty acids and other novel proteins to regulate the signaling pathway of NF-κB in pancreatic tumorigenesis [23].

The nuclear localization of NF-κB in pancreatic cancer in order to initiate the transcription of anti-apoptotic proteins is regulated by pro-inflammatory proteins such as angiotensin II [8]. Angiotensin activates the extracellular signal-regulated kinase 1/2 (ERK1/2), which induces monocyte chemoattractant protein 1 (MCP-1). This initiates the process of inflammation and thereby induces nuclear localization of NF-κB [8]. Wang et al. [84] showed that there is cross talk between Notch-1 signaling and NF-κB signaling in pancreatic cancer, which could be inhibited by an isoflavonoid known as genistein found in soy products [4]. NF-κB is also activated by the nuclear localization of glycogen synthase kinase 3 (GSK3β) in pancreatic cancer [51]. One of the downstream targets of NF-κB in pancreatic cancer is the urokinase-type plasminogen activator, and the gene transcript was shown to be inhibited by neutralization of interleukin-1 [50]. The urokinase-type plasminogen activator is expressed along with VEGF and IL-8 through IL-1 alpha-activated NF-κB transcription, which is observed in metastatic pancreatic cancer [48]. CCN1 or CYR61 (cysteine-rich angiogenic inducer 61) activates the NF-κB pathway in pancreatic cancer through the activation of the Ras-related c3 botulinum toxin substrate 1 (Rac3) and the V-akt murine thymoma viral oncogene homologue (Akt) signaling pathway [81]. All the identified proteins until now only represent a small number of activators in the activation mechanism of NF-κB. Further understanding of the complete mechanism by which the NF-κB and its target genes are activated in pancreatic cancer cells is essential for identifying a better biomarker in the early stages of cancer progression.

23.9 Conclusion

Pancreatic carcinoma (PC) is one of the deadliest cancers in the world with reduced time after diagnosis due to the silent progression of the disease. Thus, a therapeutic agent that can identify PC before migration to other parts of the body is essential. NF-κB is a target that participates in all the hallmarks of a cancer from cell

progression to angiogenesis [30, 47]. Hence, upstream or downstream targets in the NF-κB pathway may stand as suitable factors to diagnose pancreatic cancer. One of the major problems in PC is the intrinsic ability of the cells to develop resistance to gemcitabine, the most widely used drug to target PC [73]. Gemcitabine resistance is reduced by inhibiting the NF-κB pathway and other proteins, making the drug much more effective on the cancer cells [84]. Other drugs are used in combination with gemcitabine in order to enhance the effect of the drug. Dihydroartemisinin, a drug used to treat malaria, enhances the effect of gemcitabine by inactivating NF-κB and its downstream targets, c-myc, Bcl-2, etc. [79], similar to vitamin E δ-tocotrienol [35]. A compound found in a Chinese plant, escin, sensitizes pancreatic cancer cells to gemcitabine [56, 83]. A more effective way to target PC is to target a combination of proteins such as Stat3, NF-κB, Cox-2, and EP4 [28].

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Role of STAT3 in Pancreatic Cancer: A Target for Therapy

24

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Abstract

STAT3 is a member of the signal transducer and activator of transcription (STAT) family of proteins. Activation of the STAT3 signaling cascade results in the transcription of several pro-proliferative and anti-apoptotic proteins. STAT3 is also a potent modulator of the immune system, and its activation results in the suppression of the immune response. Dysregulation of STAT3 is implicated in the development and progression of a number of malignancies, including pancreatic ductal adenocarcinoma. Because of its involvement in tumorigenesis and the evasion of the immune response, STAT3 is emerging as a potential therapeutic target for pancreatic cancer, with several therapies currently under development.

Keywords

Pancreatic cancer · STAT3 · Immunotherapy

24.1 Introduction

Pancreatic cancer is one of the most fatal malignancies worldwide. It is the fourth leading cause of cancer-related deaths in the United States [1]. Pancreatic ductal adenocarcinoma (PDAC) is both the most common and lethal form of pancreatic cancer, making up 85% of all pancreatic cancer diagnoses. This malignancy has a

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close association with inflammation; it often arises from a chronic inflammatory state, such as chronic pancreatitis. Like most other forms of cancer, PDAC enables its growth by cloaking itself from the body's immune system [2]. Despite the many advances made in the field of oncology and the increase in life expectancy following the diagnosis of many forms of cancer, the prognosis of PDAC remains poor. This is partially attributable to both late-stage diagnosis and resistance to current treatment. Because of treatment resistance, researchers continue to search for new and more effective therapies. Recently, among other promising pursuits, the search has turned to transcription factors as potential therapeutic targets.

The signal transducer and activator of transcription (STAT) proteins are a family of seven transcription factors with well-established roles in a diverse array of vital cellular functions including the immune response, cell cycle, cell growth, and apoptosis. Because these proteins activate gene expression, dysregulation of any step along the STAT activation cascade can incite tumorigenesis. There are seven currently identified members of the STAT family of proteins: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 (Fig. 24.1) [3–5]. All STATs share a similar structural organization with six functionally conserved domains including the DNA-binding domain, the SH2 domain, and the transactivation domain (TAD) [6, 7]. STATs are cytoplasmic proteins that are activated following the binding of ligands, including cytokines and growth factors, to cell membrane-associated receptors. Receptor-associated tyrosine kinases such as Janus kinase (JAK) then phosphorylate a conserved tyrosine residue in the TAD (Fig. 24.1). Phosphorylation of this

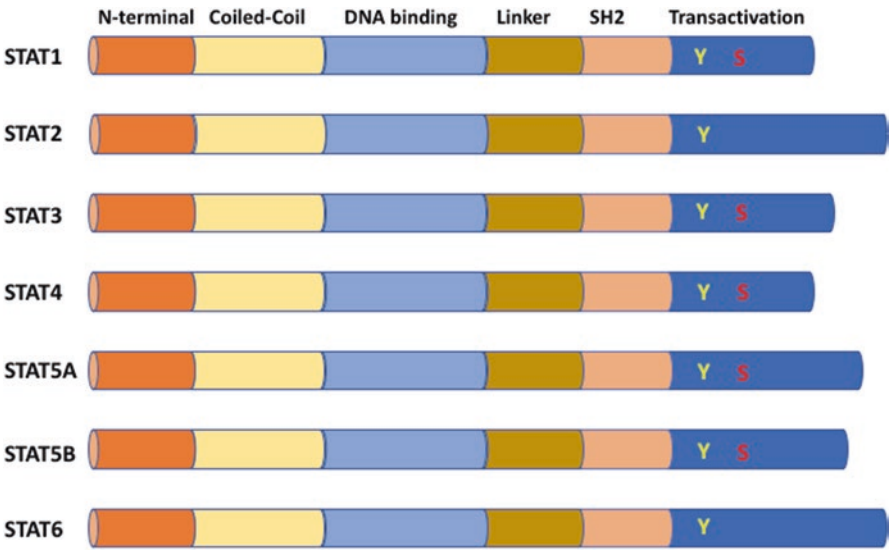

Fig. 24.1 A structural organization of STAT family proteins. The STATs have six common domains. While all domains are well conserved, their transactivation domain varies in size and existence of important residues. Tyrosine (Y), a phosphorable residue, exists in TAD domain of all STATs, while serine (S), another phosphorable residue, is not present in STAT2 and STAT6 TAD

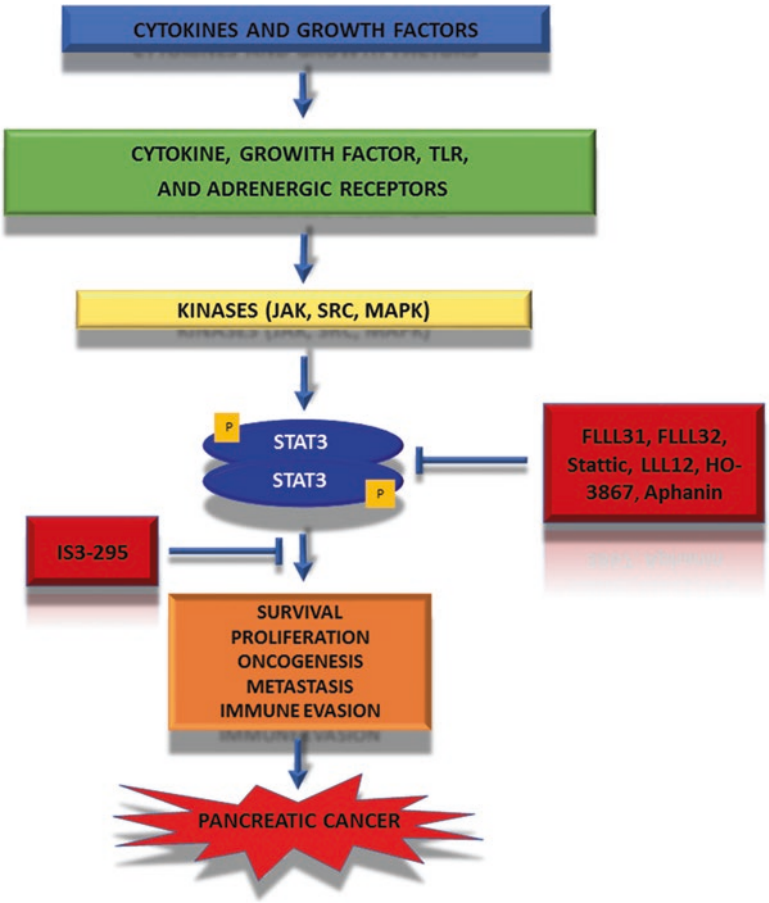

Fig. 24.2 Schematic description of STAT3 activation pathways and contribution to pancreatic cancer. Red boxes depict inhibitors currently under investigation for therapeutic use in pancreatic cancer and their sites of action

tyrosine residue results in dimerization through SH2 domain interactions, which activates the STAT protein (Fig. 24.2). Ultimately [8], the activated STAT protein will translocate to the nucleus. The DNA-binding domain is involved in the direct binding of STATs to their corresponding gene promoter sites within the cell's DNA. The TAD also regulates transcriptional activation of target genes through interaction with other transcriptional regulators. In addition to the tyrosine phosphorylation mentioned above, STATs can be phosphorylated at a serine located in the TAD. Serine phosphorylation at this site has been shown to enhance transcriptional activity and DNA binding [9].

Specifically, STAT3 has well-established links to numerous cancers, including PDAC. STAT3 has been shown to be constitutively activated in PDAC and is thought to contribute to tumorigenesis through its pro-proliferative and anti-apoptotic effects [10]. STAT3 also plays an important role in modulating the immune response to

malignancy. While an increase in STAT1 activity has been shown to cause a more robust antitumor immune response, an increase in STAT3 decreases the immune response to cancer [11, 12]. Both in vitro [13] and in vivo [14, 15] studies have demonstrated that blocking STAT3 inhibits cancer cell growth and induces cell death. Not surprisingly, overexpression of STAT3 has been shown to be a poor prognostic factor in pancreatic cancer [16]. This chapter will focus on STAT3 and its role in pancreatic tumorigenesis and progression and in immune response modulation. Furthermore, the chapter discusses STAT3 as a potential therapeutic target in the treatment of patients with pancreatic cancer.

24.2 The Role of STAT3 in Transcription

Cell-cell signaling pathways are critical processes that allow cells to communicate information to one another and thereby elicit a response. Such responses are commonly mediated through transcription factors, which are regulators of gene expression. Dysregulation of cell-cell signaling pathways and of transcription factors is known to contribute to many diseases, including cancer.

In the canonical JAK-STAT pathway, STAT3 activation is promoted by the binding of cytokines and growth factors to their receptors. Cytokines and growth factors that have known involvement in this pathway include IL-6, IL-11, LIF, IFNs, EGF, IL5, IL6, HGF, LIF, and BMP2. Their corresponding receptors include cytokine receptors such as the IL-6 receptor, G-protein-coupled receptors such as adrenoceptors and S1PR1, and Toll-like receptors such as TLR9 and TLR4 [17, 18]. The binding of these ligands activates the receptors which, in turn, activate receptor-associated tyrosine kinases. Among the most well studied of these receptor-associated kinases are the Janus kinase (JAK) proteins, which phosphorylate and activate STAT3 [19]. In addition to JAK, alternative kinases within the cell, including MAPK and Src, can phosphorylate STAT3. When the JAK-STAT signaling pathway is inactive, STAT proteins are normally located in the cytoplasm of the cell. Phosphorylation of STAT proteins at Tyr705 results in dimerization. Following dimerization, STAT translocates to the nucleus. Once in the nucleus, STAT proteins will bind directly to DNA at the promoter regions of their target genes, directly regulating the transcription of these target genes.

STAT3 is known to regulate the transcription of a broad range of target genes and, consequently, a number of cellular processes. Key target genes of STAT3 include genes involved in promoting hallmark properties of cancer, including proliferation (cyclin D1, HSP-70, HSP-90), apoptosis (BCL-2, BCL-XL, MCL-1, survivin), angiogenesis (VEGF-A, HGF), inflammation and immunosuppression (IL-10, IL-23, TGF-β, COX-2), and metastasis (MMP proteins, fascin, Vimentin, I-CAM-1). STAT3 also promotes the transcription of other transcription factors that are implicated in tumor promotion (c-Fos, HIF1-a, c-Myc, Sox2, p53, Twist, FOXO1). Thus, STAT3 promotes tumor formation and progression through a number of mechanisms [20].

STAT3 signaling can be switched off by regulatory events including inactivation of JAK-STAT signaling receptors, disruption of the binding interface between JAK proteins and STAT3, and through STAT3 dephosphorylation mediated by protein tyrosine phosphatases such as SHP-1, SHP-2, PTP1B, PTPεC, and TC45. In particular, TC45 is a phosphatase that localizes to the nucleus, where it dephosphorylates STAT3, thus inhibiting its role in transcription. Dephosphorylated STAT3 inhibits its dimer formation, which promotes nuclear export and rapid termination of STAT3 signaling [21].

24.3 The Role of STATs in Proliferation and Apoptosis

STAT3 has been implicated in cellular proliferation and resistance to apoptosis, both hallmarks of cancer. Overexpression of STAT3 in mouse models activates genes known to directly promote proliferation, which increases the risk of developing spontaneous cancers [22]. STAT3 is also known for its role in promoting the proliferation of immune cells. In murine models, T-cell-specific depletion of STAT3 impairs IL-6-mediated induction of cellular proliferation and prevents proapoptotic signaling independent of BCL-2 [23]. Similarly, STAT3 activity mediated through Gp130-like receptors is known to activate genes including those promoting proliferation in B lymphocytes [24, 25].

Disruption of JAK/STAT3 signaling promotes cell cycle arrest in G1 phase, revealing a role for STAT3 in the G1/S phase transition [26, 27]. In detail, STAT3 promotes cell proliferation through its transcriptional activity by upregulating expression of target genes such as cyclin D1, c-Myc, and pim-1, thus indirectly accelerating cell cycle progression [28, 29]. Interestingly, when constitutively expressed together, pim-1 and c-Myc can compensate for STAT3-mediated cell cycle arrest [28].

STAT3 also regulates the transcription of anti-apoptotic target genes including MCL-1, survivin, BCL-XL, and BCL-2 [20]. Additionally**,** STAT3 can promote cell survival by downregulation of p53, a gene which is well established for its role in slowing cell cycle progression and inducing apoptosis [29]. Downregulation of STAT3 has been shown to induce apoptosis, including in pancreatic cancer cell lines [30–32].

24.4 The Role of STAT3 in Pancreatic Cancer

It has recently been reported that STAT3 plays a critical role in tumorigenesis and metastasis in a variety of malignancies, including pancreatic cancer (Fig. 24.2). In KrasG12D-driven mouse models of pancreatic neoplasia, myeloid cells found in the tumor stroma showed enhanced secretion of IL-6, which activates the JAK-STAT pathway in pancreatic epithelial cells. This promotes the progression of pancreatic intraepithelial neoplasia (PanIN) and ultimately leads to the development of pancreatic ductal adenocarcinoma [33]. Additionally, there is often a complex cross talk between pancreatic tumors and their stroma that can lead to progression of the

malignancy. Pancreatic stellate cells (PSCs) are a type of pancreatic cancer-associated fibroblasts. PSCs promote the activity of myeloid-derived suppressor cells (MDSCs) via the IL-6/STAT3 signaling pathway. This results in an immunosuppressive and tumor-promoting environment [34].

In metastasis, STAT3 is implicated primarily through the functions of its target genes. STAT3 target genes have roles in many of the steps of the metastatic cascade such as cell survival, invasion, angiogenesis, and evasion of the immune system. Angiogenesis describes the formation of new blood vessels, which helps increase blood flow to a tumor. Recently, STAT3 has been implicated in angiogenesis by promoting the transcription of vascular endothelial growth factor (VEGF). VEGF is a key molecule that, when overexpressed, promotes angiogenesis. VEGF is frequently overexpressed in PDAC and other highly metastatic cancers. One group recently reported that overexpression of STAT3 correlates with overexpression of VEGF in pancreatic cancer. This corresponds with the evidence that VEGF is a direct transcriptional target of STAT3 [35].

STAT3 is also known to influence metastasis through the regulation of expression of matrix metalloproteinase (MMP) proteins. Several MMP proteins are predicted to have a STAT3-binding site in their promoter, and, in some cases, it was found that their transcription is positively regulated by STAT3 binding [6]. MMPs play a role in the metastasis of cancers, including pancreatic cancer, as MMP proteins are frequently overexpressed in PDAC.

In addition to its role in cancerous cells, STAT3 and its associated signaling pathway can play critical roles in tumor stromal cells, including cells of the innate and adaptive immune systems. The intricate cross talk between tumor cells and immune cells can greatly influence cancer progression. It has been reported that STAT3 is a negative regulator of the immune inflammatory response. Recent reports have shown that depletion of STAT3 in macrophages and neutrophils enhances the inflammatory response to toxins in mice [36]. The adaptive immune response is also affected by modulation of STAT3. Hyperactivation of STAT3 in APCs results in the mitigation of T-cell activation and leads to T-cell tolerance of antigens. Conversely, the inhibition of STAT3 in APCs results in increased activation of anergic T cells, both in vitro and in vivo [37]. T cells with constitutive expression of the protein demonstrate an inability to infiltrate tumor stroma [38]. STAT3 signaling inhibition in hematopoietic cells improves T-cell and natural killer cell antitumor responses [11].

In metastasis, before tumor cells colonize a secondary site, the site of colonization is first infiltrated by immune cells, including tumor-associated macrophages and other hematopoietic precursors. These cells provide a homing signal for metastatic tumor cells, which is known as the premetastatic niche [39]. The JAK-STAT pathway has been implicated in the formation of the premetastatic niche, where STAT3 promotes proliferation and survival of myeloid cells at these secondary sites. Furthermore, inhibition of STAT3 prevented the number of premetastatic niches as well as metastasis in mouse models [40]. Clinical studies have also identified a correlation between STAT3 activity and the number of premetastatic niches in the lymph nodes of patients with varying tumor types [41]. Therefore, targeting STAT3 could provide a potential anti-metastatic therapeutic strategy across multiple cancer types.

24.5 Current and Developing Therapies Targeting STAT3

To date, the outcomes associated with pancreatic cancer remain poor. The only current potentially curative treatment for pancreatic ductal adenocarcinoma is the surgical removal of the tumor and its surrounding structures. However, many patients are not candidates for this operation and must instead receive chemotherapy and/or radiation therapy. Additionally, many surgical candidates receive adjuvant or neoadjuvant chemotherapy to enhance their chance of survival. Thus, chemotherapy remains an important treatment modality for patients with PDAC. The standard first-line chemotherapeutic regimen for pancreatic ductal adenocarcinoma is the combination of fluorouracil, leucovorin, irinotecan, and oxaliplatin, often abbreviated as FOLFIRINOX. Alternatively, a gemcitabine-based treatment can be used as first-line chemotherapy in addition to, or independent from, FOLFIRINOX. Unfortunately, many patients have a poor response to standard chemotherapy, and developing improved chemotherapeutic or targeted therapeutic approaches is an area of active research.

Immunotherapy, the stimulation of the body's immune system to target and fight disease, is a rapidly emerging strategy for targeting cancers that are difficult to treat or of an advanced stage. Given the role of STAT3 in suppressing the immune response to tumors, targeting STAT3 presents a promising strategy for sensitizing the immune system to identify and destroy cancer cells, thereby driving immune-mediated termination of the malignancy.

Several therapies currently under development specifically target STAT activity through inhibition of its phosphorylation and its subsequent dimerization. FLLL31 and FLLL32, both derived from curcumin, are novel small molecule therapies that bind to JAK2 and the SH2 domain of STAT3. In vitro, they inhibit STAT phosphorylation, dimerization, and activation (Fig. 24.2). Through this mechanism, they induce apoptosis in pancreatic cell lines. FLLL32 also demonstrated the ability to inhibit vascularity and tumor growth in vivo in chicken embryo xenografts [42]. Stattic and LLL12, also small molecule STAT3 inhibitors, prevent STAT3 phosphorylation and thereby decrease the expression of genes involved in angiogenesis, cell survival, and proliferation known to be upregulated by STAT3 in pancreatic stemlike cancer cell lines (Fig. 24.2). These agents subsequently decreased cell viability in these stemlike cancer cell lines, and LLL12 and FLLL32 diminished tumor sphere formation [43]. HO-3867, another derivative of curcumin, has demonstrated the ability to induce apoptosis in pancreatic cancer cell lines. This potential therapy was found to act via two independent mechanisms. It induces endoplasmic reticulum stress by increasing cellular levels of reactive oxygen species, and it inhibits the phosphorylation of STAT3 (Fig. 24.2) [44].

In addition to inhibition of STAT3 via interruption of dimerization, STAT3 inhibition can occur further down the activation pathway. For example, agents can exert their antineoplastic effects on PDAC by modulating STAT interaction with DNA. IS3-295 is a novel platinum compound that exerts its antineoplastic effect through this mechanism. In vitro, IS3-295 selectively disrupts the binding of STAT3/STAT3 homodimers and STAT3/STAT1 heterodimers to DNA (Fig. 24.2). Aphanin

is a triterpenoid derived from the *Amoora rohituka* plant. It has been shown to inhibit STAT3 in two ways: (1) by preventing its phosphorylation and dimerization and (2) inhibiting its transcription (Fig. 24.2). The effects of this inhibition are consistent with the effects of STAT3 inhibition caused by the abovementioned agents. It caused G0–G1 cell cycle arrest and induced apoptosis in pancreatic cancer HPAF-II (ΔKRASG12D) cells [45].

24.6 Conclusion

In summary, this chapter has discussed the role of STAT3 as a transcription factor in the JAK-STAT signaling pathway and how this signaling is linked to a number of biological outcomes, including proliferation, evasion of apoptosis, tumorigenesis, immune evasion, and metastasis. In detail, we have highlighted the importance of STAT3 as a key signaling molecule that promotes cancers, focusing on pancreatic cancer, and have discussed the potential for developing STAT3 inhibitors as an emerging therapeutic strategy for pancreatic cancer. Moving forward, it will be important to deepen our understanding of the basic mechanistic roles of STAT3 in JAK-STAT signaling and other biological processes. Ultimately, we must discern the efficacy and adverse consequences of targeting STAT3 in in vivo models and in patients with the goal of moving these therapies forward toward clinical use.

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STAT3 in Pancreatic Cancer Metastasis

25

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Abstract

Pancreatic cancer is one of the leading causes of mortality associated with cancer. Signal transducer and activator of transcription (STAT)3 is implicated in metastasis of pancreatic cancer. Various cytokines, growth factors and environmental stimuli have been identified to activate STAT3. Attenuation of malignant transformation susceptibility of different cell types was noted on inhibition of STAT3 activation; thus, STAT3 has been put forward as a possible drug target for pancreatic cancer therapy. Several inhibitors have been proposed to regulate the upstream positive or negative control elements of STAT3 activation and target STAT3 directly. However, it is imperative to explore further for the effective STAT3 inhibitors that could improve the clinical outcome in patients with pancreatic cancer.

Keywords

Pancreatic cancer · Activated STAT3 · STAT3 inhibitors · Cancer targets

The original version of this chapter was revised. The book was inadvertently published without Abstracts and Keywords, which are now included in all the chapters. An erratum to this chapter can be found at https://doi.org/10.1007/978-981-10-6728-0_39

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25.1 Introduction

Pancreatic cancer is extremely aggressive fatal human disease and multifactorial in evolution. Inflammation and repeated acute pancreatic injury are important factors that contribute to the development of pancreatic cancer [10]. This disease is the fourth leading cause of cancer-related mortality, with a 5-year survival rate after diagnosis of less than 5%, and is most commonly found in those of above 65 years of age [35]. The prognosis for patients with later stage of pancreatic cancer is poor with a median survival of only 6 months. Difficulties in the disease prognosis include a limited understanding of the biology of metastasis, weak early diagnosis tools and lack of effective chemotherapeutics [23, 31].

Inflammation has been considered as an important factor of pancreatic cancer [47]. Immune cells at the pancreas secrete some growth factors and cytokines that create a favourable microenvironment for pancreatic cancer [13, 41] by activation of transcription factors such as STAT (signal transducer and activator of transcription)3 which is found to play a key role in causing the inflammation [15]. Constitutively active STAT3 has been noted in majority of human pancreatic carcinoma samples [36]. This chapter attempts to comprehend the role of STAT3 in the biology of pancreatic carcinoma and as a potential molecular target for the cancer therapy.

STAT transcription factors are acute-phase response factors and were discovered for the first time in 1994 [1]. The members of the STAT family (STAT1 to STAT4, STAT5a, STAT5b and STAT6) are conserved in nature [8]. The STAT proteins were found to be latent in the quiescent cell cytoplasm, and the phosphorylation of its specific tyrosine residue leads to activation of the protein [21]. Further, STAT proteins dimerize by reciprocal interaction of src homology (SH)2 phospho-tyrosine residues. The translocation of dimerized STATs into the nucleus leads to initiation of transcription by binding of the proteins to specific enhancer elements [8].

STAT proteins were found to have functional role in various physiological, proliferative, anti-apoptotic and inflammatory pathways. The STAT proteins are of 750–850 amino acids in length and are encoded by genes that are found on 3 chromosomes, chromosome 2 (STAT1 and STAT4), chromosome 17 (STAT2 and STAT6) and chromosome 12 (STAT3, STAT5a and STAT5b) [19, 21]. The conserved regions of STAT proteins were identified to be the NH2-terminal, coiled-coil, DNA binding, linker, SH2, C-terminal and transactivation domains [21]. These domains were found to be helpful in binding to cytokine or growth factor receptor and to further activate the STAT proteins by phosphorylation of specific residues, dimerization and nuclear translocation. In majority of pancreatic tumour tissues, STAT3 was found to be activated followed by STAT5 [21]. Though STAT3 pathway is noted to be inactive during normal pancreatic development [27], an atypical activation of STAT3 was found in pancreatic tumour cells, in association with cell proliferation, survival, invasion, angiogenesis and metastasis [18] (Fig. 25.1). STAT3 has been demonstrated to be obligatory for an early event in pathogenesis of pancreatic cancer, i.e. the acinar-to-ductal metaplasia process that is mediated by Pdx1 transcription factor [30].

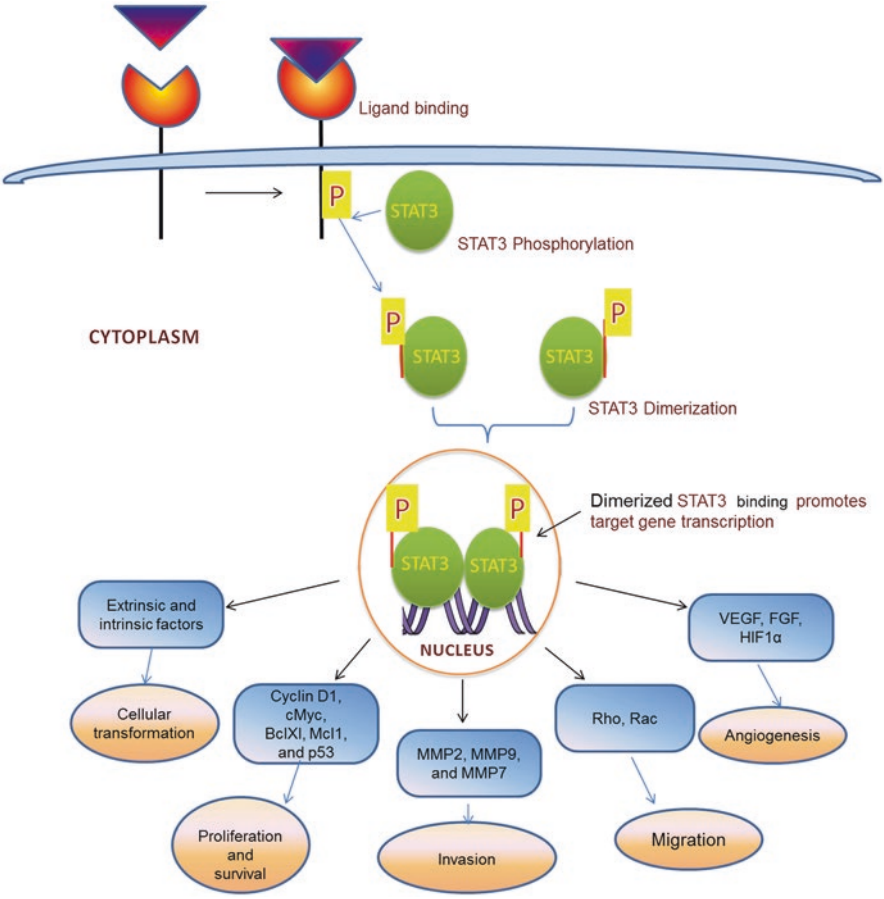

Fig. 25.1 Various ligands (cytokine or growth factor) bind to their cognate cell surface receptors and cause phosphorylation of STAT3 that further dimerize, translocate to the nucleus, and activate target gene transcription by binding to their promoters

25.2 Molecular Mechanism of STAT3 Activation

STAT3 is found to be activated in pancreatic carcinoma by phosphorylation of a tyrosine residue at position 705. The upstream signalling components of STAT3 include cytokines such as interleukin (IL)-6, IL-9, IL-10 and IL-27, tumour necrosis factor (TNF) α and monocyte chemoattractant protein (MCP)-1 and activated tyrosine kinases [35]. The IL-6 secreted by pancreatic myeloid cells was noted to activate STAT3 protein which promotes the progression of pancreatic intra-epithelial neoplasia (PanIN) and further development of pancreatic carcinoma [28, 35]. Various receptors that were found to activate STAT3 are the receptors with intrinsic tyrosine kinase activity, activated cytokine receptors and non-receptor tyrosine

kinases [26, 42]. Several serine kinases have been also noted to activate STAT3 through serine phosphorylation at position 727 [22, 42]. Histone acetyltransferase p300 was found to cause acetylation of STAT3 on a single lysine residue at position 685 which leads to regulation of its transcriptional activity and homodimer stability [42, 51]. Ultraviolet radiation or sunlight, carcinogen, stress, smoke and infection are the other factors that are also known to play a significant role in STAT3 activation [42]. Different STAT3 forms with mutations in their SH2 domain have been detected in the pancreatic tumours [25, 34, 42].

25.3 Role of STAT3 in Metastasis

STAT3 activation has a critical role in each step of the cancer metastasis [21] (Table 25.1) [35] (Fig. 25.1) and is as follows.

25.3.1 Role of STAT3 in Cell Proliferation and Cell Cycle

STAT3 plays an important role in cellular proliferation. Activated STAT3 was noticed to stimulate proliferation of cells and cause cell malignancy through various tyrosine kinases, oncogenes and viruses [3, 21]. The activated STAT3 accelerates cell-cycle (G1/S phase) progression through upregulation of cyclin D1 and c-Myc expression [7]. In addition, the expression of growth-promoting gene pim-1 has been shown to be upregulated by STAT3 [38]. Malignant transformation and tumorigenesis in a number of cell types were noted to be decreased on inhibition of STAT3 activation; thus, STAT3 could be a possible target for therapy of pancreatic cancer [12, 35].

Table 25.1 Critical roles of activated STAT3

Role of STAT3	Facilitate through
Inflammation	Interleukin (IL)-6 and cyclooxygenase 2 enzyme
Cell proliferation	Transcription of cyclin D1 and cyclin B1
Apoptosis inhibition	Expression of B-cell lymphoma (Bcl)-2, Bcl-xL and myeloid leukaemia cell differentiation protein (Mcl)-1
Metabolism	Expression of heat shock protein (Hsp)70, Hsp90 and cell division cycle protein (Cdc)2
Several aspects of tumorigenesis	
Angiogenesis	Transcription of vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and hypoxia-inducible factor (HIF)1α
Invasion and metastasis	Expression of matrix metalloproteinase (MMP)2, MMP9, twist-related protein (TWIST) and intercellular adhesion molecule (ICAM)1

25.3.2 Role of STAT3 in Cellular Invasion and Migration

Invasion of extracellular matrix and cellular migration are vital steps in metastasis formation. Several reports strongly implicate that STAT3 plays a crucial role in this process through regulation of the expression of matrix metalloproteinase (MMP)1 and MMP9 [9, 20, 35]. The role of STAT3 has been also indicated in cellular migration under normal and pathological conditions [35].

25.3.3 Role of STAT3 in Tumour Cell Intravasation and Survival During Circulation

Tumour cells degrade basement membrane, intravasate the lymphatic system, survive in circulation, extravasate to potent new organs and adhere to form metastasis [35]. Various non-specific forces such as mechanical stress, haemodynamic turbulence, cellular cytotoxicity and loss of adhesion-induced cell death influence the tumour cells that enter blood vessels [14, 21]. Consequently, a less percentage of tumour cells survive during circulation which leads to micrometastasis in distant organs. The protection of tumour cells from host immune surveillance during circulation has been noted on STAT3 activation which increases the number of surviving tumour cells [21].

25.3.4 Role of STAT3 in Angiogenesis

Angiogenesis is formation of fresh blood vessels from the vasculature that is preexistent. This step is necessary for tumour development and metastasis. The tumour cells are known to secrete angiogenic molecule, vascular endothelial growth factor (VEGF) [17]. VEGF has been found to bind to transmembrane tyrosine kinase receptors that are present on endothelial cells and effectively contributes to neovascularization. The expression of VEGF has been found in association with pancreatic cancer progression. The presence of STAT3-responsive element on the VEGF promoter may indicate that STAT3 directly enhances VEGF expression and further angiogenesis, growth and metastasis of pancreatic cancer [21]. The coincidence of overexpression of VEGF with constitutive STAT3 activation has been also noted in several human pancreatic cancer specimens [21, 46].

The vessel formation was prohibited by inhibition of STAT3 signalling in endothelial cells [48]. Hypoxia-inducible factor (HIF)1 is one more important mediator of angiogenesis that has also been reported to be regulated by STAT3 [21, 37]. The binding of both STAT3 and HIF1 to the VEGF promoter, during hypoxic conditions, enhances transcription of VEGF gene and subsequently angiogenesis [33].

25.3.5 STAT3 and Tumour Microenvironment

Tumour cells modify their microenvironment during the adaption process. STAT3 signalling in the tumour cells was found to subvert the function of immune cells for the self-benefit [16]. STAT3 causes increase in synthesis of immunosuppressive agents [21] and negatively regulates immune activation signals such as pro-inflammatory cytokines [50]. Dendritic cells are known to have antitumour activity; however, the dendritic cells surrounding the tumour cells were found to be nonfunctional as they lack MHC class II molecules [21, 43]. These partially differentiated dendritic cells are generated as a consequence of the tumour-secreted factors such as VEGF, IL-6 and IL-10 and have reduced antigen-presenting ability [45]. Thus, STAT3 deactivation in tumour cells could cause efficient production of the immune factors that promote the maturation of dendritic cells. Other immune cells such as natural killer cells, regulatory T cells, neutrophils and macrophages also show STAT3 activation and limit their ability of immune surveillance in response to the tumour-secreted factors [21, 49]. The tumour cell secretions that cause infiltration of endothelial progenitor cells also cause the metastatic spread of stromal tumour cells by enhanced upregulation of stromal cell-derived factor (SDF)-1/C-X-C motif chemokine ligand (CXCL) 12 receptors [16, 21]. In view of the above, STAT3 has been considered as an important target to surmount immunosuppression, and the utilization of STAT inhibitors could improve the antitumour ability of immune cells [21].

25.3.6 Role of STAT3 in Apoptosis

STAT3 signalling has been demonstrated to suppress the cancerous cell apoptosis and promote cell proliferation and malignant transformation. The expression of tumour protein p53 gene was found to be negatively regulated by STAT3 and hence induces cellular proliferation and inhibits apoptosis [32]. However, STAT3 has been found to be a pro-apoptotic factor in mammary glands [4].

25.4 Regulation of STAT3 Activity

Negative regulation of STAT3 activation is through numerous mechanisms which involves the following:

25.4.1 Protein Tyrosine Phosphatases (PTPs)

PTPs, low molecular weight tyrosine phosphatases and dual-specificity phosphatases, have been found to play a key role in inactivation of STAT3 [5, 21]. These proteins have a conserved signature motif VHCSXGXGR [T/S] and similar tertiary structure [2, 21].

25.4.2 Protein Inhibitors of Activated STATs (PIAS)

PIAS family proteins (PIAS1, PIAS3, PIASy, PIASxa and PIASxb) with a conserved signature motif (LXXLL) at its amino terminal region have been found to regulate the activity of STAT protein [21, 39]. Zinc-binding domain and acidic domain are the other conserved elements in PIAS proteins. PIAS proteins mediate gene regulation by blocking the binding of transcription factors to DNA, recruiting transcriptional corepressors and promoting the protein sumoylation [21, 39]. PIAS3 has been reported to interact with STAT3 and repress its transcriptional activity.

25.4.3 Suppressors of Cytokine Signalling (SOCS) Proteins

SOCS proteins (SOCS1–SOCS7, cytokine-inducible SH2-containing protein (CIS)) are a family of proteins that have been found to be inducible and inhibit cytokine signalling [21, 40]. The STAT signalling is inhibited by binding of SH2 domain of SOCS1 to Janus kinase (JAKs), CIS and SOCS2 to receptor complex and SOCS3 to receptor cytoplasmic domain [6, 21]. Proteasome degradation pathway has been also found to be induced by SOCS proteins through SOCS box.

25.5 STAT3 Inhibitors

Excessive activation of STAT3 plays a key role in tumorigenesis. The complex STAT signalling pathways may contribute to the resistance to chemotherapy of pancreatic tumour. Aberrant STAT3 activation causes dysregulated cancer cell growth and survival, and hence, STAT3 serves as a potential therapeutic target to inhibit tumorigenesis [35]. In view of the noted association with multidrug resistance protein (MRP) expression in pancreatic tumours, the downregulation of MRPs is essential for restoration of chemosensitivity in pancreatic tumours [24, 29].

Some inhibitors and antisense oligonucleotides are implicated in inhibition of STAT3 (Table 25.2) [21, 35, 42] with further attenuation of proliferation and survival of cancer cells and reversal of malignancy with little or no influence on normal cells [21]. Direct targeting of STAT3 has been proposed as a strategy for antitumour therapy apart from the regulation of upstream positive or negative control elements of STAT3 [11, 21, 44].

Table 25.2 STAT3 inhibitors, identified both in vivo and in vitro

STAT3 inhibitor	Mechanism of inhibition
Curcumin	Inhibition of activation and DNA binding of STAT3
Resveratrol	Inhibition of activation and regulation of signalling pathway of STAT3
Cucurbitacin I	Inhibition of STAT3 binding to DNA and gene transcription
Cucurbitacin B	Inhibition of activation and DNA binding of STAT3
Cucurbitacin E	Inhibition of activation and gene expression of STAT3
Cucurbitacin Q	Inhibition of activation without inhibition of STAT3 in JAK2
Flavopiridol	Disruption of STAT3-DNA interactions and inhibition of STAT3 gene transcription and translation
Deoxytetrangomycin	Abrogates dimerization, nuclear translocation and DNA binding of STAT3
Cyclopentenone derivatives	Inhibits tyrosine phosphorylation and serine phosphorylation of STAT3
N-acyl homoserine lactone	Inhibition of STAT3 activation
Indirubin derivative E804	Inhibition of activation and gene expression of STAT3
6-bromoindirubin-3-oxime	Inhibition of activation and gene expression of STAT3
Tyrphostin/AG490	Inhibition of activation and DNA binding of STAT3
Cisplatin	Dephosphorylation of JAK2-STAT3
Platinum (IV) inhibitor CPA-1	Inhibition of activation and DNA binding of STAT3
Platinum (IV) inhibitor CPA-7	Inhibition of activation and DNA binding of STAT3
Atiprimod	Inhibition of activation of STAT3
Pentoxifylline	Inhibition of activation, nuclear translocation and DNA binding of STAT3

25.6 Conclusions and Future Perspectives

STAT3 plays a pivotal role in normal tissues as well as in the cancer initiation and progression. Pancreatic carcinoma is an aggressive cancer disease due to its asymptomatic and rapid early metastasis nature. Most of the tumour specimens have been found to show persistent activation of STAT3, while its expression is stringently regulated in normal tissues. The abnormal activation of STAT3 provides favourable conditions to the tumorigenesis and metastasis. On the other hand, blocking of STAT3 signalling pathway in tumour cells inhibits tumour growth, invasion, anti-apoptosis, angiogenesis and metastasis. Hence, inhibition of STAT3 has been found to be a useful approach in cancer therapy. High resistance to conventional therapy and aberrant STAT3 activation in pancreatic cancer have led to studies to discover small molecules that effectively inhibit STAT3 signalling. However, it is imperative to use interdisciplinary approaches to explore effective STAT3 inhibitors that improve the clinical outcome in patients with pancreatic cancer in addition to alternative combinational therapies that also include inhibitors of other signalling components that are often upregulated in pancreatic cancer.

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Conflict of Interests We declare that we do not have any conflict of interests.

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HIF1α: A Key Emerging Player in Pancreatic Cancer

26

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Abstract

Pancreatic cancer is the world's fourth leading vulnerable cancerous disease with poor diagnosis and low survival rate. Intratumoral hypoxia is the characteristic feature of pancreatic cancer. Hypoxia-inducible factor 1α (HIF1α) is a well-known transcriptional molecule which is associated with aggravation of the pancreatic cell proliferation, invasion, metastasis, and apoptosis. HIF1α belongs to a family of Per-ARNT-Sim (PAS) with heterodimeric basic helix-loop-helix (bHLH) transcriptional proteins. Under oxygen depletion conditions, catalytic function of prolyl hydroxylases is downregulated, thereby inhibiting the binding to von Hippel-Lindau protein (pVHL); thus HIF1α does not undergo ubiquitin-proteasomal degradation and gets accumulated in the cancerous tissue. It may also stimulate many signaling molecules and lead to lymph node metastasis. HIF1α may induce invadopodia formation through reactive oxygen species mediated by NADPH oxidases involving the stimulation of Notch signaling, thus leading to pancreatic cancer cell invasiveness. HIF1α also stimulates the production of anti-angiogenic factors which might be one of the probable reasons for poor therapeutic response toward pancreatic cancer. Future studies may focus on the development of new therapeutic pathway toward inhibition of hypoxia-induced HIF1α signaling to reduce the risk for the incidence of pancreatic cancer.

The original version of this chapter was revised. The book was inadvertently published without Abstracts and Keywords, which are now included in all the chapters. An erratum to this chapter can be found at https://doi.org/10.1007/978-981-10-6728-0_39

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Keywords
Hypoxia-inducible factor · Pancreatic cancer · Hypoxia · Prolyl hydroxylase · Notch signaling

26.1 Introduction

Pancreatic cancer is one of the noncommunicable lethal diseases with least survival chances in the world population. Its prevalence is increasing at an alarming rate over the recent decades. It has a rapid progression capacity to the surrounding tissues through invasion as well as metastasis [26]. Many types of pancreatic cancerous tumors exist in both the cells of the pancreas, i.e., in exocrine and in endocrine cells, as pancreatic ductal adenocarcinoma (PDAC), adenosquamous carcinomas, signet ring cell carcinomas, undifferentiated carcinomas, squamous cell carcinomas, and neuroendocrine tumors (NETs) also known as islet cell tumors. Of these, pancreatic ductal adenocarcinoma is the most common malignant disease that contributes to the increased mortality (95%) of pancreatic cancer with poor response to various therapies [25]. Although many advanced medical technologies have been introduced to reduce the occurrence of this dreadful disease, the incidence of pancreatic cancer still remains increasing. Probably the reason for the least survival chances of this cancer could be due to poor and late diagnosis and lack of early molecular detection markers. Many risk factors such as modifiable smoking, alcohol consumption, obesity, physical inactivity, age, gender, family history, and inherited genetic mutations have also played a vital role in increasing the susceptibility of this disease. Various transcriptional molecules like NF-κB, STAT-3, HIF1α, SP1, and TWIST modulate the genes responsible for tumor development in the pancreas. Nevertheless, hypoxia-inducible factor 1α (HIF1α) takes the leading role in aggravating the pancreatic cancer [20]. However the underlying and associated molecular mechanisms in the development and progression of PDAC are not well understood. Thus this chapter briefs about the pivotal role of HIF1α, a key regulator in PDAC cell proliferation, invasion, metastasis, and finally apoptosis which may be targeted as an early diagnostic marker and suitable therapy by understanding the deep insight of molecular mechanisms associated with PDAC to cure this aggressive disease.

26.2 Hypoxia Condition in Pancreatic Cancers

A common and prominent feature observed in malignant cell microenvironment is hypoxia, where oxygen tensions are depleted. Many studies on oncology emphasize on intratumoral hypoxia, which is the consequent effect of rapid tumor growth and development, metastasis, and therapeutic resistance resulting in pancreatic cancer [29]. The occurrence of hypoxia was evidenced by avascular appearance through computed tomography and also by intratumoral oxygen tension measurements [17] in pancreatic tumor cells. This was also supported by another study where decreased oxygen tension was reported in the human pancreatic tumors with reference to their

surrounding normal cells [15]. Indeed this was also in line with other studies conducted on experimental animals indicating an intratumoral hypoxia status in PDAC microenvironment. Tissue hypoxia was also associated with epithelial-to-mesenchymal transition (EMT) in cancer cells of the pancreas, where these cells get adapted to hypoxia condition and acquire and promote tumor invasion and metastatic properties with therapeutic resistance. One of the possible mechanisms underlying the depleted tumor oxygenation is the lack of sufficient blood supply to all the sites of tumor cells due to abnormal vasculature. Evidence from the available literature also suggests that angiogenesis promoted by the growing tumor is leaky and ineffective as these sites cannot tolerate depleted O2 levels. PDAC cells also produce anti-angiogenic factors like angiostatin, endostatin, and pigment epithelium-derived factor resulting in reduced O2 delivery and intratumoral hypoxia. The stromal cells of PDAC might be one of the contributing factors to this hypoxia through the amplification of the production of anti-angiogenic substances or physically by the constriction of the capillaries through extracellular matrix deposition in the periacinar spaces [19]. In addition intratumoral hypoxia is an important inducer of pancreatic stellate cells (PSCs), the major fibroblastic cells of the pancreas, which enables the vicious cycle of hypoxia and fibrosis [3], thus the probable reason for the resistance toward anti-angiogenic therapies in pancreatic cancer. Adaptation of tumor cells to hypoxia signaling triggers a large transcriptional program primarily coordinated by hypoxia-inducible genes.

26.3 Role of HIF1α in the Malignancy of Pancreatic Cancer

Hypoxia condition is stimulated by inducible genes accommodated by hypoxia response elements (HREs). These HREs constitute 50 base pairs approximately, thereby combining with hypoxia-stimulating transcriptional proteins [23]. Several of these transcriptional factors have been participated in the tumor progression; however research has been focused much and better studied on hypoxia-inducible factors (HIFs). HIFs are the members of Per-ARNT-Sim (PAS) family which contain heterodimeric basic helix-loop-helix (bHLH) motif [28]. HIFs act as an important target for antineoplastic therapy as they enhance the HREs–HIFs complex formation and regulate the gene expression. This triggers the maintenance of tumor, aggressiveness, as well as angiogenesis. HIFs exist in three isoforms, i.e., HIF 1, HIF 2, and HIF3, and each form is constituted by two subunits such as α-subunit and β-subunit [21]. The regulatory action of these HIFs is stimulated by the dimerization of their respective α-subunit and β-subunit [22]. HIF1 and HIF2 transregulate a wide range of genes participating in the metabolism, angiogenesis, erythropoiesis, tumor cell maintenance and inflammation, pH homeostasis and finally targets the tumor cell to adapt to hypoxia [12]. Although HIF1α and HIF2α form complex with common HREs, they perform differential activity in hypoxic environment. HIFs, in addition to HREs, also form complex with other transcriptional molecules like Notch, p53, and myc11, thereby regulating the consequent signaling pathways [10]. HIF1 was first isolated as nuclear protein factor from Hep3B cells in 1992 by the

stimulation of hypoxic condition. The two subunits of HIF1 have distinct expression in normoxic conditions as HIF1β protein has continuous expression in mammalian cells, whereas HIF1α has unstable expression due to its rapid degradation by ubiquitin-proteasome pathway leaving its half-life for around 5 min [4]. An oxygen-sensitive prolyl hydroxylase plays an important role in the degradation of HIF1α as it catalyzes the hydroxylation of proline molecules which enhances the complex formation to von Hippel-Lindau protein (pVHL), thereby triggering them for proteasome degradation. As the catalytic activity of prolyl hydroxylases is dependent on oxygen status, its activity is reduced in hypoxic state, thereby inhibiting the binding of HIF-α to pVHL, thus accumulating HIF1α [5]. Ample evidence from the literature suggests the association of upregulated expression of HIF1α with intratumoral hypoxic pancreatic cancer. Its expression was significantly induced in regional lymph node metastasis, tumor mass, and advanced TNM condition [13]. Indeed the expression of HIF1α was positively correlated with increased cellular number and microvessel density/neoangiogenesis. It showed positive staining in human PDAC subjects and correlated with increased transition and migration, higher tumor pathologic stage, bad prognosis, and higher AJCC condition associated with higher hepatic metastasis [18]. It indirectly enhances the function of other epithelial-to-mesenchymal transition-promoting transcription factors like TWIST, ZEB, and Snail through TGF-β signaling [24]. Thus HIF1α can be given identity as molecular marker specifically for intratumoral hypoxic condition.

26.4 Molecular Mechanisms Associated with Pancreatic Cancer Through HIF1α

In general cellular invasiveness is the crucial stage of metastatic cascade and involves a series of events such as epithelial-to-mesenchymal transition (EMT) and migration to secondary sites. These events can further be triggered by several cell-to-cell communications along with hypoxia [1]. The cancer cells may also choose either protease-dependent or protease-independent mode of invasion keeping in view of multiple parameters like the cancerous tumor origin, migration, invasive dissemination, and metastatic area. Various molecular mechanisms have been elucidated in the development of pancreatic cancer cells through HIF1α regulation.

Studies on different cancerous tumors indicate that hypoxia induces invadopodia formation through reactive oxygen species (ROS) generated by NADPH oxidases (NOX), which represents an additional mechanism for cancer cell invasiveness [7]. The same scenario was observed in human pancreatic cancer cells showing increased invadopodia formation under hypoxia through HIF1α-dependent stimulation of Notch signaling pathway [8]. Under hypoxic condition, metalloprotease activity is induced through Notch signaling, thereby leading to epidermal growth factor (EGF) receptor ligand heparin-binding (HB) complex release, thus enhancing invadopodia formation [2]. Thus EGF receptor pathway through paracrine signaling could be one of the mechanisms associated with PDAC invasion and metastasis (Fig. 26.1). Similarly hypoxia-stimulated HIF1α was positively

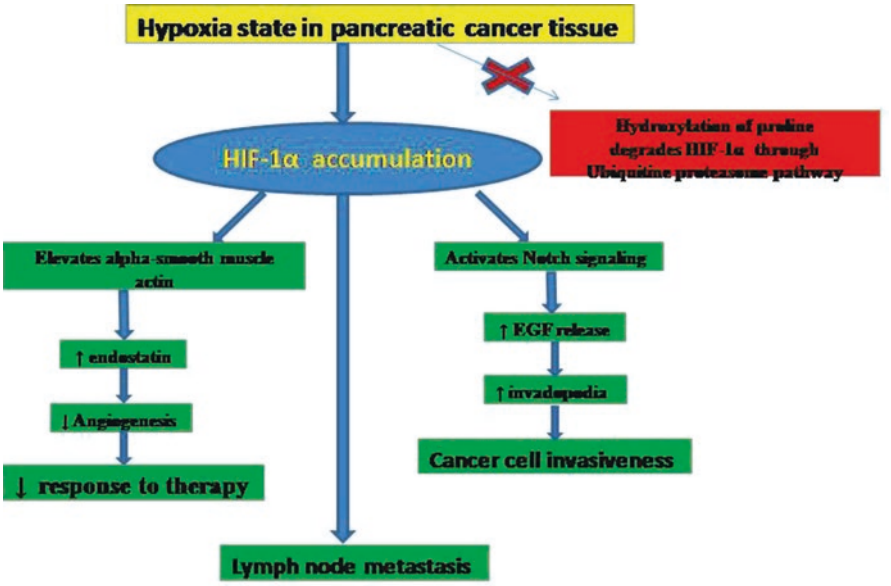

Fig. 26.1 Consequences of HIF1α accumulation under hypoxia in pancreatic cancerous cell

correlated with angioinvasion which is crucial for metastasis. In this mechanism HIF1α enhanced the transcriptional expression of urokinase-type plasminogen activator receptor (uPAR) leading to angioinvasion [6]. HIF1α also stimulated fascin gene overexpression by complex formation of HIF1α-HRE on fascin promoter region. This in turn elevated the expression of matrix metalloproteinase-2 (MMP-2) resulting in the promotion of PDAC [30]. Furthermore, hypoxia-induced pancreatic stellate cells (PSCs) stimulate the production of some proteins such as fibronectin, type I and III collagen, and periostin, resulting in elevated fibrosis and desmoplastic reaction, a prominent feature of pancreatic cancer [9, 16]. Hypoxia-induced pancreatic stellate cells are associated with pancreatic tumor cells to enhance tumor growth and metastasis. Cultural stem cells of pancreas under hypoxia showed increased HIF1α expression, induced their motility [11], upregulated the expression of alpha-smooth muscle actin (SMA), and stimulated the angiostatic factor endostatin, which may reduce angiogenesis (Fig. 26.1). Hypoxia-induced HIF1α molecule extends its role in facilitating lymphatic invasion (Fig. 26.1) which is a characteristic event of lymph node metastasis [27]. In fact studies from human pancreatic cancer cell line reported increased HIF1α activity even at invasive migration to surrounding organs. It was also reported that hypoxic HIF1α regulates its potential action on various organs in PDAC by stimulating high levels of VEGF in the ascites. PDAC also reflects invasion in nervous tissue along with lymphatic nodes, the liver, and lungs [14]. Thus the abovementioned studies focused the action of HIF1α through multiple mechanisms associated with the development of pancreatic tumors.

26.5 Conclusions and Future Perspectives

Several studies from the literature have stressed that HIF1α programming under hypoxia results in pancreatic tumor invasion and metastasis. This signaling enhances the EMT and invadopodia formation enhancing the tumorous cells to overcome the stromal barrier and promote invasive migration. Keeping in view the hypoxia-induced anti-angiogenesis, these cancer cells show poor response to therapy, hence resulting in rapid mortality rate of this malignancy. It can be concluded that hypoxia-induced HIF1α can be recognized as molecular marker for pancreatic malignancy. Considering the importance of hypoxia-induced HIF1α in pancreatic tumor development, future research should focus on identifying alternate pathways and mechanisms associated with hypoxia condition and early diagnostic marker and develop new therapeutic approach toward inhibition of hypoxia-induced HIF1α signaling to reduce or at least delay the onset of the lethal pancreatic malignant disease.

Conflict of Interest The authors reported no potential conflicts of interest.

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Hypoxia-Inducible Factor (HIF)-1α and Its Regulation in Pancreatic Cancer

27

Ganji Purnachandra Nagaraju and Subasini Pattnaik

Abstract

Pancreatic malignancy is a highly metastatic disease with a poor prognosis and extremely low overall survival rates. Despite recent advancements in the traditional chemopreventive approaches for pancreatic cancer (PC), there has been very little improvement in the overall survival rates for patients (Guilford JM, Pezzuto JM, Expert Opin Investig Drugs 17:1341–1352, 2008). Therefore, novel phytochemicals such as curcumin and genistein have emerged as promising targets in developing treatment options for patients with PC. Curcumin is a natural compound found in turmeric (Kunnumakkara AB, Guha S, Krishnan S et al, Cancer Res 67:3853–3861, 2007), and genistein is an isoflavone found in soybeans (Banerjee S, Zhang Y, Ali S et al, Cancer Res 65:9064–9072, 2005). Both compounds are known to possess outstanding anti-inflammatory, antioxidant, and anticancer properties.

Keywords

Hypoxia-inducible factor 1 · Curcumin · Pancreatic cancer

The original version of this chapter was revised. The book was inadvertently published without Abstracts and Keywords, which are now included in all the chapters. An erratum to this chapter can be found at https://doi.org/10.1007/978-981-10-6728-0_39

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27.1 Introduction

Pancreatic cancer (PC) is the 3rd leading cause of cancer-related deaths and the 11th most common malignancy diagnosed in the USA [43]. PC has a very poor prognosis due to common clinical presentations with minor symptoms and the lack of initial detection markers. Hypoxia is a condition of low oxygen availability in tumor cells, which is a vital emerging marker in PC progression since PC tumors are highly hypoxic [1]. Hypoxic conditions activate signaling pathways that directly affect the stimulation of pro-invasive properties of PC cell lines and facilitate the invasiveness via hypoxic environment [24]. Hypoxic conditions in the tumor could arise due to elevated metabolic activities and the consumption of oxygen by rapid tumor cell proliferation [34]. These activities lead to altered pH levels that result in oxidative stress in the tumor microenvironment. Hypoxia-inducible factor 1 (HIF-1), a transcription factor, is the key regulator of hypoxic condition in PC cell lines [19]. HIF-1α is a member of the HIF-1 family of basic helix-loop-helix-Per-Arnt-Sim transcription factors, and it is the most persistently expressed modulator of oxygen homeostasis in PC cell lines.

Regulation of HIF-1α involves the control of mRNA expression, protein stability, and activity [17]. Generally, HIF-1α regulation occurs at the level of protein stability. Under normal conditions, oxygen facilitates posttranslational hydroxylation of two proline residues in the oxygen-dependent degradation (ODD) domain of HIF-1α [57]. Under hypoxic conditions, HIF-1α escapes degradation and enables heterodimerization with HIF-1β, followed by the binding to hypoxic-responsive elements (HREs) within the promoter regions of target genes [9]. A series of such activities lead to elevated levels of HIF-1α protein expression and its messengers under such hypoxic conditions. At the mRNA levels, HIF-1α is regulated via PI3K/AKT signal transduction pathway [7]. Dysregulation of growth factors and their corresponding receptor tyrosine kinases lead to the stimulation of PI3K pathway and modulated tumor cell growth. HIF-1α activity is induced by the activation of PI3K/AKT signaling by growth factors and the stimulation of downstream PI3K/AKT signaling molecules [7]. The inactivation of PTEN, a tumor suppressor gene and an inhibitor of AKT activation by PI3K, is associated with elevated levels of HIF-1α expression [60]. Insulin-like growth factor 1 (IGF-1) is also linked with induced HIF-1α activity via stimulation of the PI3K/AKT signaling pathway [61].

27.2 HIF: Hypoxia-Inducible Factor

HIF is a heterodimeric transcription factor containing an alpha and beta subunit [51, 52], which each contains a Per/Arnt/Sim (PAS) domain and a basic helix-loop-helix (bHLX) domain [51] needed for DNA binding and dimerization. HIF-1α is rapidly degraded under normoxia and undergoes posttranslational regulation. Expression of HIF-1β is constitutive and is not regulated by hypoxia [23]. Once translocated into the nucleus, HIF-1 binds to promoters of hypoxia response element (HRE) genes and thereby enhances their transcription [42].

There are three isoforms of alpha subunits, namely, HIF-1α, HIF-2α, and HIF-3α. HIF-1α plays the main role in cellular response due to hypoxia [23]. The HIF-2α subunit, also known as the HIF-related factor (HRF) or endothelial PAS1, is induced by hypoxia and then binds to HREs [11, 13, 48, 53]. Although HIF-1α and HIF-2α share some functional and structural resemblances, they are induced by different stimuli [5] and have diverse tissue expression patterns [54]. The third isoform, HIF-3α, is also known as inhibitory PAS (IPAS) and is subjected to alternative splicing that can remove the transactivation domain and functions in a dominant negative manner when it binds to HIF-1α in place of HIF-1β.

27.3 HIF-1α Regulation by Hypoxia

The most prominent feature of HIF biology is the induction of the alpha subunit during hypoxia. HIF-1 levels are very low when oxygen conditions are normal and no stimulation occurs by growth factors, due to the continuous proteasomal degradation in the presence of oxygen. The process of degradation is very rapid during normoxia, and the half-life is predicted to be less than 5 min [22, 51]. As the oxygen level lowers, proteasomal degradation ceases and thereby HIF-1 protein is accumulated. In this way, HIF-1 efficiently responds to fluctuations in oxygen concentrations without depending on changes in transcription and translation which are lengthy processes.

HIF-1 degradation is mediated by an oncogenic suppressor such as VHL (von Hippel-Lindau). It was demonstrated that VHL binds to HIF-1 in a region called the oxygen-dependent degradation (ODD) domain, thereby arbitrating its ubiquitination and proteasomal degradation. Moreover, the interaction of VHL and HIF-1 occurred in the presence of oxygen and was disrupted in hypoxia conditions or by treatment with CoCl2 (iron chelators). Delineating the oxygen-sensing mechanism became possible with the discovery of the interaction between VHL and HIF-1 that only occurred when conserved proline molecules in the ODD known as residues 402 and 564 existed in a hydroxylated form [26, 38, 56]. The enzymes mediating proline hydroxylation belong to the prolyl hydroxylase domain (PHD) family. Three non-heme-containing PHD enzymes were found to hydroxylate HIF-1α [35]. When the levels of oxygen decrease, the hydroxylation of proline also reduces, and the interaction of VHL and HIF is attenuated. In such a way, the degradation rate of HIF-1α is controlled by PHD enzymes in response to changes in oxygen availability.

HIF-1 transcriptional activity was reported to be elevated under hypoxia conditions [27, 39]. Transcriptional activity is dependent on the hydroxylation state of asparagine residue 803 in the HIF-1α transcription domain. In normal oxygen conditions, the asparagine residue is hydroxylated by asparaginyl hydroxylase, thereby disrupting the interaction of CBP/p300 (transcriptional coactivator) with HIF-1α [3, 10, 21, 30, 31]. Under hypoxia conditions, asparaginyl hydroxylation is disrupted, and the interaction of HIF-1α with CBP/p300 is enhanced. In such a way, the expression levels of HIF-1α protein and its messengers are increased under hypoxia conditions.

27.4 HIF-1α Regulation by Growth Signaling

In addition to its regulation by variations in the level of oxygen, HIF-1α is also regulated by the activity of receptor and non-receptor tyrosine kinases. Growth factors, cytokines, and hormones that can induce HIF-1α include IGF-1 [59], insulin, PDGF [45, 49, 59], IGF-2 [12], TNFα [20], HGF [46], EGF [60], IL-β [20, 45, 47], thrombin [16, 58], and angiotensin-2 [58]. HIF-1α stabilization occurs during conditions like hypoxia. Growth signals induce HIF-1α by increasing the amount of HIF-1α synthesis, as shown by experiments using cycloheximide (inhibitor of translation) and pulse labeling [14, 32, 49]. These studies have shown that the kinase cascade involving PI3K, AKT (protein kinase B), and FRAP (also called mTOR) is crucial for the increased rate of synthesis. The role of these kinases was determined using dominant negative versions and through the use of chemical inhibitors [14, 45, 49, 60].

The ability of the AKT/FRAP/PI3K kinase cascade to enhance HIF-1α synthesis is mainly determined by the ability of FRAP kinase to phosphorylate two molecules of the translational machinery, i.e., p70 S6 kinase (S6K) and eIF-4E-binding protein [4]. Subsequently, 4E-BP1 inhibits eIF-4E, leading to increased 40S ribosomal subunit recruitment to the 5′ mRNA (11). FRAP phosphorylation activates S6K, which facilitates the activation of the 40S ribosomal S6 protein and leads to increased mRNA transcription with 5′polypyrimidine tract [15, 50]. In addition to the regulation of HIF-1 by the AKT/PI3K signaling pathway, MAPK signaling is also reported to be involved in receptor-facilitated HIF-1 activation [14]. Even though its role in HIF-1α regulation is not well studied, it is likely that p42/p44 MAPK activation results in HIF-1 transcriptional activation [37, 40, 44]. Furthermore, studies have shown that in many cell lines, the activation of MAPK was observed in response to hypoxia [8, 36, 37].

27.5 Phytochemicals

The involvement of phytochemicals in the treatment of PC has increased tremendously in order to understand the beneficial properties of these compounds in the inhibition of tumor progression. Increasing evidence suggests that natural compounds such as curcumin and genistein possess the ability to prevent pancreatic tumor progression by modulating cellular signaling, miRNAs, and epigenetics [25]. Curcumin is a naturally occurring polyphenolic compound in turmeric and possesses key antitumorous properties. It is known to inhibit the expression of HIF-1α transcription, COX-2, EGFR, NF-κB, ERK1/2, LOX, and iNOS, preventing the progression of PC cell lines [28]. Curcumin is known to inhibit the survival pathway activation, which leads to increased apoptosis and inhibition of PC cell growth (Fig. 27.1) and angiogenesis (low VEGF secretion Fig. 27.2). Genistein is an isoflavone found in soy and upregulates the expression levels of tumor suppressor genes and miRNAs by controlling DNA methylation and chromatin configuration, which ultimately results in the inhibition of PC cell growth (Fig. 27.1) and metastasis [6]. The anti-angiogenic property of genistein is known to be controlled by the inhibition of HIF-1α, an important modulator of VEGF protein homeostasis under mainly

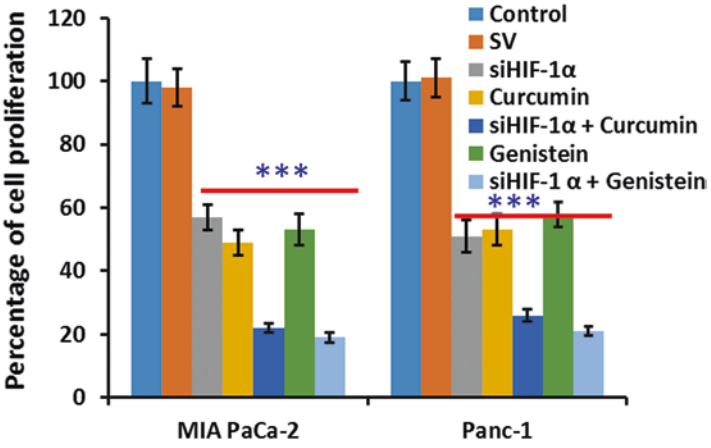

Fig. 27.1 Knockdown of HIF-1α sensitizes to curcumin and genistein and potentiates the inhibition of proliferation in pancreatic cancer cell lines

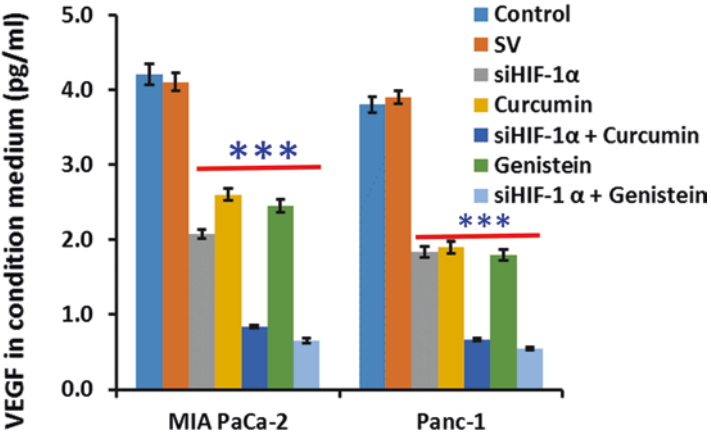

Fig. 27.2 Knockdown of HIF-1α sensitizes to curcumin and genistein and potentiates the inhibition of VEGF secretion in conditioned medium contained pancreatic cancer cell lines

hypoxic conditions (Fig. 27.2) [55]. Therefore, this makes genistein an important compound in the treatment of patients with pancreatic tumors. Genistein has also been known to guard the β-cells at specific concentrations via epigenetic regulation of cAMP/PKA pathways, which reveal the inhibitory properties of genistein on PC cell lines by epigenetic modulation [41]. Moreover, genistein inhibits the activities of EGFR, AKT_2, DNA ligase III, and NF-κB in PC patients [33]. It represses cellular growth, induces apoptosis, and inhibits the invasion of PC cell lines via inhibition of many survival pathways. Since these phytochemicals reveal excellent

anticarcinogenic properties via the regulation of cell signaling, miRNAs, and epigenetics, these compounds could serve as important agents for the prevention or combination treatment of pancreatic malignancies.

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Role of E2F1 in Pancreatic Cancer

28

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Abstract

Pancreatic cancer is the deadliest of human cancers to date. The hostile nature of pancreatic cancer, mostly due to its tendency for early local and distant spread, is in due course responsible for poor diagnosis and reduced survival. Most of the pancreatic cancers are originated in exocrine glands. About 95% of these exocrine cancers are adenocarcinomas that affect the pancreatic ducts. Other types of pancreatic cancers include neuroendocrine cancers that arise in endocrine cells. But these provide extensive capillary networks for metastasis of tumor cells. Several computational approaches are employed to couple the gene expression measurements with a network of known relationships between gene products, like the NetRank algorithm similar to Google's PageRank algorithm, to determine the marker genes that are better involved in clinical outcome prediction. One such marker genes are those that encode transcription factors. One important group is E2F transcription factor family. These regulate a varied range of cellular functions, which include cell differentiation, cell proliferation, and cell death. The current chapter focuses on the E2F1 transcription factor, its mechanism of regulating the cell cycle, and its role in apoptosis and metastasis in context with the devastating pancreatic cancer. The insights into the molecular mechanisms with further investigation and research may provide improved diagnostic and treatment options for this type of cancer.

Keywords

Pancreatic cancer · E2F1 · Cell proliferation · Cell cycle regulation

The original version of this chapter was revised. The book was inadvertently published without Abstracts and Keywords, which are now included in all the chapters. An erratum to this chapter can be found at https://doi.org/10.1007/978-981-10-6728-0_39

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28.1 Introduction

The pancreas is an organ positioned behind the stomach and comprises of two main cell types: exocrine and endocrine cells. Majority of cells are exocrine that form exocrine glands and ducts whose secretions are released into tiny ducts that merge to form large pancreatic duct. This duct along with common bile duct empties its contents into the duodenum at ampulla of Vater. Small percentage of cells in the pancreas are endocrine in nature that form small clusters called islets of Langerhans. These groups of cells make hormones such as insulin and glucagon that regulate blood sugar levels in our body. These contents are directly released into the blood. When this organ gets damaged, it may lead to cancer, which is the uncontrolled proliferation of cells that topples the normal functions of the organ. Pancreatic cancer is the deadliest among human cancers to date. The hostile nature of pancreatic cancer, mostly due to its tendency for early local and distant spread, is in due course responsible for poor diagnosis and reduced survival. According to global scenario in 2012, pancreatic cancer was the seventh most frequent origin of death from cancer, constituting 4% of all cancers diagnosed (www.cdc.gov/cancer/international/statistics.htm). [1] About 80% of patients have fatal disease at the time of diagnosis, and the larger part live for less than 12 months [2]. Up to 10% of pancreatic cancer cases are known to be transmitted by autosomal dominant pattern of inheritance [3]. Most of the pancreatic cancers are originated in exocrine glands. About 95% of these exocrine cancers are adenocarcinomas that affect the pancreatic ducts. Other types of pancreatic cancer include neuroendocrine cancers that arise in endocrine cells. But these provide extensive capillary networks for metastasis of tumor cells.

Genome-wide association studies of pancreatic cancer are being carried out worldwide, giving insights into the cancer susceptibility loci on chromosomes. Several computational approaches are employed to couple the gene expression measurements with a network of known relationships between gene products, like the NetRank algorithm similar to Google's PageRank algorithm, to determine the marker genes that are better involved in clinical outcome prediction. One such marker genes are those that encode transcription factors. *Transcription factors* are proteins that exclusively bind at specific DNA sequences, regulating the transcription of genes and thereby the expression of the genome. One important group is E2F family of transcription factors, initially named due to their ability to bind to the adenovirus E2 gene promoter [4]. These regulate a diverse range of cellular functions that includes cell proliferation, differentiation, and death. As depicted in Fig. 28.1, E2F includes activators (E2F1, E2F2, E2F3a, and E2F3b) and repressors (E2F4, E2F5, E2F6, E2F7, and E2F8) [5, 6]. These factors control the expression of key cell cycle regulators, thereby deciding as to whether a cell should divide or not. E2F activation is adequate to irrevocably committing the cells to DNA replication; therefore E2Fs play a major role in the control of cell proliferation both in normal and tumor cells. The E2F activity is influenced by interactions with members of the retinoblastoma (Rb) protein family, primarily involved in inhibition of transcription activation and active repression of E2F-responsive genes. The activation of signal transduction pathways that drive cell cycle converges on E2F/Rb molecular switch.

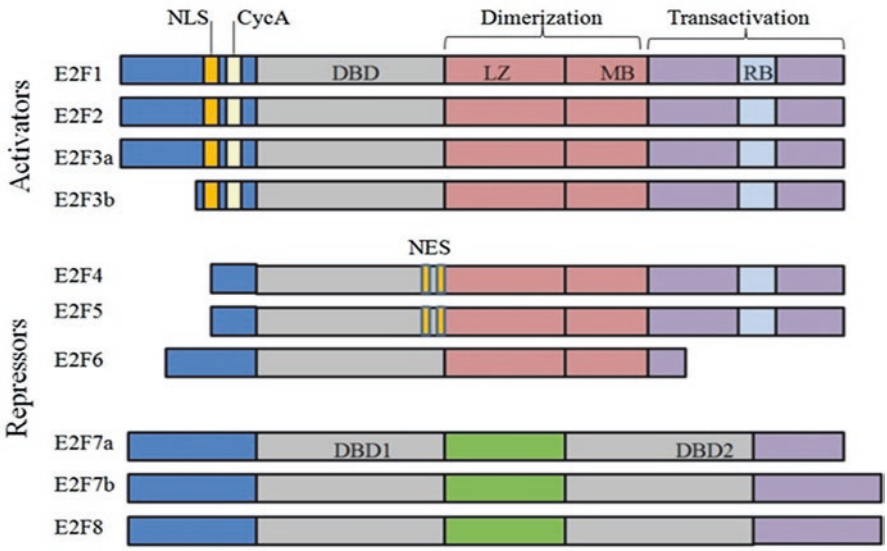

Fig. 28.1 E2F transcription factor family in mammals

This chapter focuses on the E2F1 transcription factor, its mechanism of regulating the cell cycle, and its importance in devastating pancreatic cancer. The insights into the molecular mechanisms with further investigation and research may provide improved diagnostic and treatment options for this type of cancer. One study showed that the analysis of different proteins expressed in patients with pancreatic ductal adenocarcinoma (PDAC) provides clues for personalized medicine. The proteins detected in short-term surviving patients (STS, survival <14 months) were markedly different from those in very long-term surviving patients (VLTS, survival ≥10 years). In STS-associated patients, the cytoskeletal proteins which are dynamic structures involved in cell movement, cell division, and endocytosis were most significantly enriched, whereas in case of VLTS, copine proteins involved in membrane trafficking were enhanced [7]. Tissue culture and animal experiments have indicated the role of E2F1 overexpression in several types of cancer, making it a potential anticancer therapeutic.

A marked feature of this family is the presence of DNA-binding domains (DBD). The E2F family members (E2F1–E2F6) form heterodimers with dimerization partner (DP) proteins (TFDP1–TFDP3) which are mediated by leucine zipper (LZ) and marked box (MB) domains. Transcriptionally active forms of the heterodimers are localized into the nucleus by amino terminal nuclear localization sequence (NLS), located adjacent to cyclin A (Cyc A) binding site [8]. The binding of Rb protein family takes place at transactivation domain (RB) of E2F1–E2F3. The export of E2F4 and E2F5 is mediated by their bipartite nuclear export signals (NES) [9]. E2F3a and E2F3b are two highly related isoforms whose expression is driven by alternative promoters at E2F3 locus [10]. E2F7a and E2F7b are isoforms generated by alternate splicing of the primary transcript [11].

28.2 Role of E2F1 in Cell Proliferation

Cell proliferation is an essential phenomenon during development, as the formation of a tissue and organ requires cells to divide exponentially so as to form large number of cells. After attaining maturity, the proliferative ability is utilized less frequently. The external signals combined with intracellular signaling molecules involving the cell cycle machinery decide the proliferative potential of a cell. The cell cycle machinery consisting of diverse group of molecular components controls the progression through the cell cycle. Disruption of this network leads to spreading out of cancer cells. Understanding how this machinery works in connection with the signal transduction pathways at the molecular level will give insights into the mechanism of development and progression of pancreatic cancer. Cells have the ability to divide only for a definite number of times after which they enter into replicative senescence, where growth is terminally arrested. For transformation of cells, it is essential for them to become resistant to both apoptosis and senescence instigation. The activation of tumor suppressor p53 leads to direct induction of its transcriptional target p21, leading to inhibition of CDK2/cyclin E complex and RB-mediated E2F1 inhibition [12]. p21 plays a pivotal role in senescence induction even in p53-defective cancer cell lines [13]. There exists a positive feedback loop between E2F1 and its target gene encoding CIP2A (human oncoprotein). CIP2A inhibit phosphatase activity of phosphatase complex PP2A [14]. This leads to the stability of E2F1 in phosphorylated form at Ser 364 and prevents its downregulation by p53-mediated regulators, enhancing proliferation. More than a dozen of human cancer types have been implicated with high levels of CIP2A levels envisaging poor survival rate, as it plays a crucial role in senescence resistance.

Cell cycle is a theoretical construct based on experimental approaches which describes the stages through which a cell passes in a sequential fashion to generate an accurate copy of itself. The cell cycle consists of two basic phases comprising interphase and M phase (mitosis phase). The interphase further comprises of G1, S, and G2 phases. The G1 and G2 phases part DNA replication (in S phase) from chromosome segregation (in M phase). Restriction point or commitment point is a clear transition in G1 phase, wherein cells take the decision as to divide or withdraw from the cell cycle. The progression through this point is highly regulated by signaling cascades providing a novel link between E2F1 activity and Ras/Raf/MEK/ERK signaling pathway. Once the restriction point is crossed, the cells are committed to divide and undertake proliferative cycle (Figs. 28.2 and 28.3).

Studies carried out on human diploid fibroblasts demonstrate a cross talk between Ras/Raf/MEK/ERK pathway and E2F1. It was seen that PDGF-induced phosphorylation of ERK reduced considerably with reduced E2F1 protein expression. When E2F1 expression is enhanced, ERK phosphorylation was increased. Even at low levels of growth factor, presence of E2F1 at notable levels induced ERK phosphorylation. E2F1 carries out phosphorylation of ERK comparable with MEK. But blocking MEK activity diminishes ERK phosphorylation even in the presence of E2F, further providing clues for E2F1 transcriptional targets of MEK/ERK pathway to be acting upstream to MEK [15].

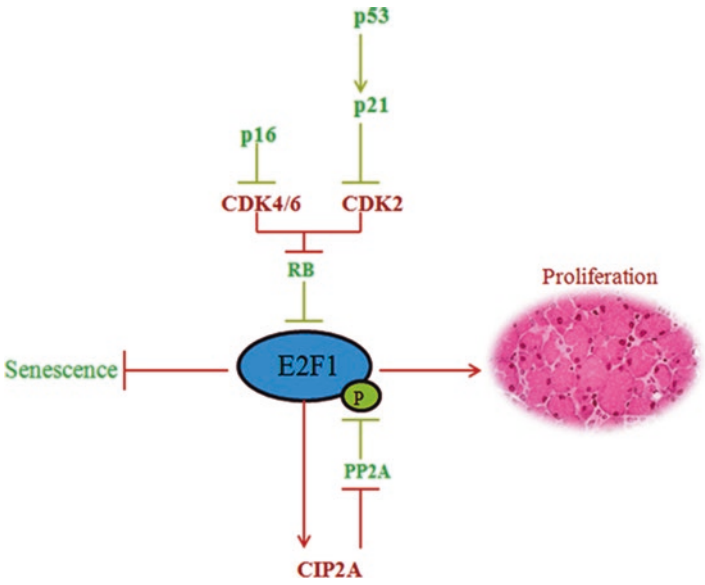

Fig. 28.2 Mechanism regulating senescence sensitivity in human cancer cells. The components and mechanisms regulating senescence in normal cells are shown in green color and those in tumor cells are shown in brown

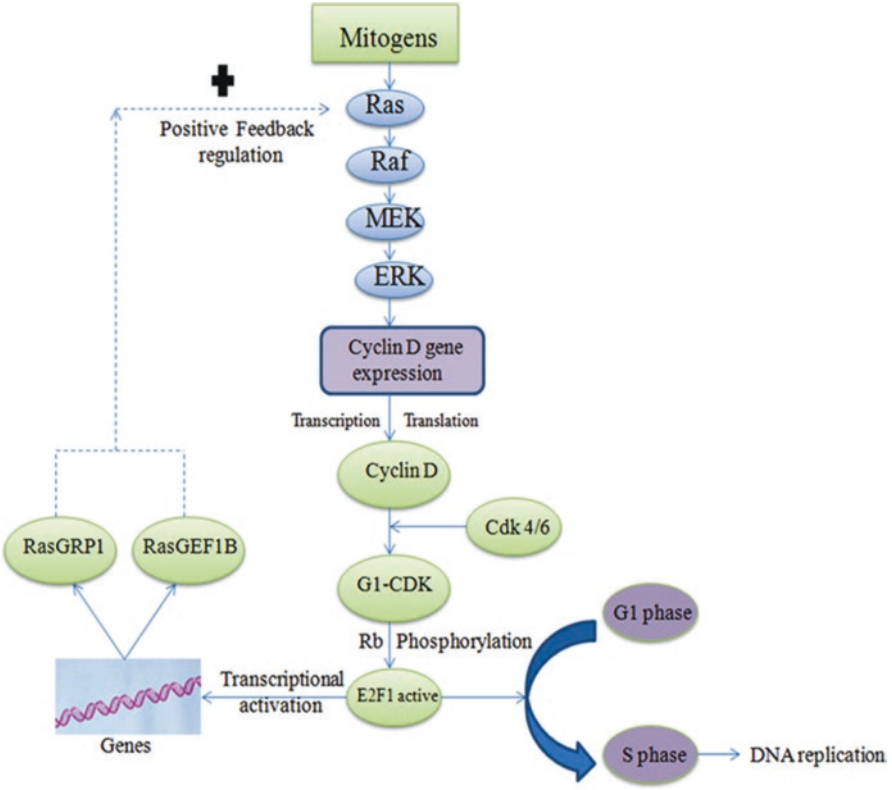

Fig. 28.3 Bidirectional crosstalk between Ras/Raf/MEK/ERK pathway and E2F1

Several research groups have confirmed K-Ras mutations to be majorly involved in about 90% of pancreatic cancer cases [16–18]. Ras is a small GTPase protein recruited to the membrane by Grb2-SOS complex, on activation of receptor tyrosine kinases by mitogens. This complex activates the GTPase family member Ras by converting into GTP-bound active form that further activates downstream MAP kinases, regulating gene expression and modulating cell growth, differentiation, and survival. The activation of G1-CDK causes phosphorylation of retinoblastoma protein family and activates E2F1. The active E2F1 now drives the expression of genes that leads to succession from G1 phase into S phase of cell cycle. E2F1 directly regulates two target genes: RasGEF1B (Ras guanine exchange factor 1B) and RasGRP1(Ras guanyl-releasing protein 1) [15]. RasGRP1 drives conversion of inactive Ras-GDP into active Ras-GTP, subsequently affecting activation of ERK. Levels of RasGEF1B are also elevated on activation of E2F1. Hence there is a two-way interaction between activation of E2F1 by MAP kinase pathway and positive feedback regulation of E2F1 in activating ERK. This mechanism indicates E2F1 to be playing a pivotal role, thereby being a possible drug target in the presence of Ras mutations.

28.3 Role of E2F1 in Pancreatic Cancer Metastasis

E2F transcription factor and its implications in vivo vary in different organs; it acts as tumor promoter in pancreatic cancer although it has been shown to be a tumor suppressor in other organs. Although studies on E2F family members show complex involvement of E2F in activation of cancer-promoting and cancer-regulating genes, extensive studies on E2F in cancer prognosis are needed to draw precise conclusion. In several pancreatic cancers, *src* (protein tyrosine kinase) is shown to be overexpressed. Immediate downstream regulator of *src* is E2F1. Overexpression of ribonucleotide reductase small subunit 2 (RRM2), a procarcinogenic enzyme, leads to proliferation and metastasis of cells. E2F1 acts as a promoter for this enzyme [19]. Duxbury et al. in 2004 found that in pancreatic adenocarcinoma ($PANC1^{GemRes}$) cells, overexpression of src directly enhanced RRM2 and metastasis of the cells [20]. The role of E2F1 in angiogenesis increasing adhesion properties of pancreatic carcinoma cells is not reported; however it directs toward a need to study these carcinogenic properties in depth. In vivo significance of study in order to properly understand the role of E2F1 is also lagging behind in this field.

28.4 Role of E2F1 in Apoptosis

Multiple pathways are involved in E2F1-induced apoptosis (Fig. 28.4). The pro-apoptotic activity of E2F1 indicates that its deregulation prevents tumor development by targeting premalignant cells to undergo apoptosis. Several studies have demonstrated a role of E2F1 in promoting apoptosis in various systems independent

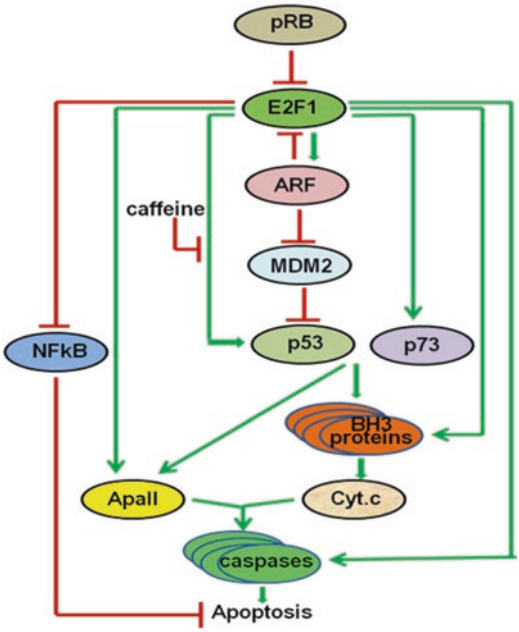

Fig. 28.4 Pathways of E2F1-induced apoptosis
E2F1-induced apoptosis is mediated by both p53-dependent [23–25] and p53-independent [26, 27] pathways. A number of pathways are further involved in p53-dependent apoptosis induced by E2F1, one of which involves activation of ARF, another is caffeine-sensitive, while yet another utilizes physical interaction with p53. Transcriptional activity of E2F is modulated via multiple mechanisms; negative regulation by pRb, a product of Rb tumor suppressor gene; and direct activation of p73 transcription by E2F1 resulting in activation of p53-responsive target genes and apoptosis [28] and triggering of apoptosis and sensitizing the cells to pro-apoptotic stimuli via disruption of NF-κB signaling by deregulated E2F1 [29, 30]

of the endogenous p53 status [21, 22]. This suggests E2F1 to be a potent tumor suppressor engaging in apoptotic pathways, thereby protecting organisms from tumor development.

In case of PDAC, E2F1 might serve as a tumor promoter. Histological analysis by Yamazaki et al. demonstrated a direct correlation of E2F1 expression with cell proliferation index and an inverse relationship between immunopositivity of E2F1 and disease-associated survival as well as histological grade [31]. Pancreatic cancer has antineoplastic resistance to apoptosis, which is partially associated to the absence of functional p53. Decreased pRb expression and E2F1 overexpression were found to increase chemotherapy-induced apoptosis in pancreatic cancer [32]. In addition, increased gemcitabine-, etoposide-, or roscovitine-induced apoptosis in pancreatic cancer cell lines has been reported using E2F1-expressing adenoviral vector infection [33, 34].

28.5 Treatment Strategies Based E2F1

Chemotherapy is the only therapeutic option for patients with metastatic cancer and may fail frequently due to change in E2F1's function from a tumor suppressor to an oncogene, promoting survival. E2F1 has a dichotomous role. Drugs can be designed effectively by repressing E2F1-based survival pathways and reactivation of those involved in apoptosis. E2F1 overexpression sensitizes various cell types to apoptosis upon treatment with ionizing radiation or chemotherapeutic drugs such as etoposide and adriamycin, topoisomerase II inhibitors [35, 36]. Gene therapy studies have demonstrated that overexpression of E2F1 effectively induces apoptosis in various cancers both in vitro and in vivo [37–42]. Apoptosis and cell cycle arrest can be triggered by arresting DNA replication in the presence of E2F1 by utilizing its regulatory role in a specific S-phase checkpoint [43–45]. This theory is additionally supported by studies that have demonstrated that exposure to DNA-damaging agents initiates E2F1 upregulation which in turn arrests the cell cycle and induces apoptosis subsequently [46, 47]. These studies suggest that E2F1 "chemogen" therapy might be a promising treatment strategy. Moreover, E2F1 gene therapy has a significant potential advantage due to the fact that E2F1-mediated apoptosis can proceed independent of the pRb or p53 status of tumors [37–40]. Elliot et al. have shown that adenoviral vector system-mediated E2F1 overexpression effectively induces apoptosis in pancreatic cancer cells expressing mutant p53 [33]. This proteolytic caspase cascade has been implicated as a mediator of E2F1-induced apoptotic cell death in a number of cell lines [37–42].

Recently, a novel miR-34a delivery-based therapeutic strategy has shown promising success in PDAC. This strategy comprises of nanocomplexes consisting of CC9 peptide which acts as a bifunctional molecule, tumor targeting and tumor penetrating. Treatment with these nanocomplexes elevated the levels of miR-34a, thereby downregulating its target genes, viz., Bcl-2, E2F3, cyclin D1, and c-MYC, which ultimately brings about the cell cycle arrest, migration suppression, and apoptosis. Hu et al. have further shown that nanocomplexes when used in vivo significantly repressed tumor growth and prompted apoptosis of cancer cells [48].

28.6 Future Perspectives

E2F transcription factor family plays crucial role in cell differentiation, cell cycle progression, apoptosis, and stress responses. It has the ability to control numerous signaling pathways directly or indirectly through its interactions and crosstalk between these pathways. This property can be utilized in development of treatment strategies involving E2F agonists that would promote apoptosis and E2F antagonists that would suppress cell proliferation. The use of apoptotic E2F1 targets as molecular therapeutics alone or in combination with established chemotherapeutic agents needs consideration as a potential treatment for cancer.

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The Expanding Role of Sp1 in Pancreatic Cancer: Tumorigenic and Clinical Perspectives

29

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Abstract

It is increasingly being recognized that in gastrointestinal malignancies, including pancreatic cancer, transcription factors get activated and alter the gene expression in the tumor cells to metastasize and develop. Pancreatic cancer is associated with a relatively very poor prognosis and survival outcomes. Currently, there are significant barriers to effective therapeutic interventions for this disease; hence timely diagnosis and clinical decision-making leading to appropriate treatment strategies for patients with metastatic pancreatic cancer are essential. This chapter summarizes some of the selected peer-reviewed translational and clinical research findings in the area of a transcription factor [specificity protein 1 (Sp1)] as it relates to the tumorigenesis and mechanistic role(s) for the potential of improving therapeutic targets/responses in pancreatic cancer. Particular emphasis has been given on the mechanistic roles of Sp1 in the cell cycle, metastasis (cellular adhesion, invasion, migration, angiogenesis), and apoptosis. Based on the available information and several ongoing clinical studies, it is implicated

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that Sp1 can well serve as an important prognostic marker for pancreatic tumor. Several drugs targeting Sp1 for pancreatic tumor therapy have also been studied and discussed in this chapter. These drugs work by downregulating the expression and activity of Sp1 in tumor cells in pancreatic cancer. Taken together, these molecular and cellular mechanisms/targets can have a very significant impact on the prognosis and overall quality-of-life and clinical outcomes for patients with pancreatic tumor.

Keywords
Pancreatic cancer · Specificity proteins · Sp1 · Transcription factors · Cellular and molecular mechanisms · Tumorigenesis · Anticancer drugs · Clinical outcomes

29.1 Introduction

Gastrointestinal malignancies are broadly covered with the colon, esophagus, gastric, liver, and pancreatic cancers. Pancreatic tumor continues to be a leading cause of carcinoma-related deaths globally. Pancreatic ductal adenocarcinoma (PDAC) is the fourth most lethal cancer in the USA with an estimated 53,670 new cases and 43,090 deaths in the year 2017 [25]. It has a very poor prognosis and an increasing impact on cancer-related mortality with a 5-year survival rate of less than 5%, and its median survival is about 6 months [25]. In fact, one of the recent estimates suggests that by the year 2030, the disease may become the second leading cause of cancer-related deaths in the USA [20]. The risk factors for pancreatic cancer include smoking, obesity, diabetes, heavy alcohol use, and chronic pancreatitis. Preclinical and clinical outcome studies have demonstrated that PDAC is a systematic disease wherein its clinical course is generally aggressive leading to a remarkable decrease in the patients' quality of life (QOL) and/or survival. Most recently, the American Society of Clinical Oncology (ASCO) has updated potentially curable pancreatic cancer guideline [16].

In this chapter, we summarize some of the experimental (translational) and clinical research findings in the investigational area of a key transcription factor [i.e., specificity protein 1 (Sp1)] as it relates to the tumorigenesis and signaling mechanistic role(s) in pancreatic cancer.

29.2 Tumorigenesis and Clinical Scenario in Pancreatic Cancer

Tumor microenvironment in pancreatic cancer plays important role(s) in the cancer cell progression with a relatively poor therapeutic response to the cytotoxic agents. Currently, the clinical efficacy of anticancer pharmacotherapies is rather limited due to the fibrotic stromal component of pancreatic cancer, which forms a mechanical barrier around the tumor. Plasma circulating tumor DNA (ctDNA), also known as

"cell-free DNA," are now being proposed as one of the potential biomarkers in advanced pancreatic cancer [18]. There is a greater interplay between the tumor stroma and pancreatic cancer; hence for the practicing oncologists, it is critical to have a better understanding of the impacts of stromal microenvironment on tumor progression as it relates to the drug delivery and drug resistance in this setting.

In patients with pancreatic cancer, the main symptoms often include abdominal pain and loss of appetite, weight, and functional activities. Additionally, there could be other related symptoms, which include biliary tract obstruction issues and pancreatic insufficiency that may also affect the nutritional depletion. The development of PDAC is primarily influenced by the genetic mutations at some key genes (such as the V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, popularly known as the *KRAS* gene). This gene accounts for about 90% of the PDAC cases, which defines their malignant growth, invasiveness, lymphatic spread, as well as therapeutic resistance.

Recently, the US Food and Drug Administration (FDA) has approved phase I clinical trial of MVT-1075 (a novel, fully human antibody radio-immunotherapy), as a therapeutic treatment for pancreatic cancer. There has been evolving trend in the standard of care for resected pancreatic cancer, especially the clinical trials in which the adjuvant therapies with chemotherapy or chemoradiation have prolonged patients' survival postoperatively [27]. A recent elegant report on the therapeutic implications of molecular subtyping for pancreatic cancer has reviewed seminal articles which evaluated the molecular architecture of the disease thereby providing a better understanding of the therapies that may potentially improve the patient outcomes [19].

Despite the enormous literature available worldwide about the pathobiology and clinical management of patients with pancreatic cancer, the disease remains one of the leading causes of cancer-related deaths. Resistance to chemotherapy and/or radiation therapy contributes to a relatively higher recurrence rate with this disease. Also, serum biomarkers for the diagnostic and curative treatment of pancreatic cancer are generally good for early-stage disease only, which often lack assay standardization [6]. Therefore, it is important for developing effective therapeutic strategies for treating and managing pancreatic cancer at a relatively early stage of the disease.

29.3 Targeting Specificity Proteins as Transcription Factors

Transcription factors are now increasingly being recognized as one of the novel targets for the developments of anticancer drugs. Specificity protein families (mainly Sp1, Sp3, and Sp4) are "sequence-specific" transcription factors that are associated with the poor prognosis and survival of patients with different types of cancers, including pancreatic cancer [21]. These agents regulate a number of genes involved in the critical cellular processes required for the tumor cell growth and progression (i.e., cell cycle, proliferation, differentiation, and/or apoptosis).

These key sequence-specific Sp transcription factors bind to GC-rich promoter sites (a sequence of contiguous guanine, guanine, guanine, cytosine, and guanine, in

that order, along with a DNA strand) and regulate a set of genes associated with different cancers. Importantly, Sp1 expression correlates with the aggressive disease and poor prognosis. Sp proteins also regulate vascular endothelial growth. While multiple candidates are involved in the aggressive disease and poor prognosis, Sp1 and Sp3 transcription factors mediate the expression of both c-Met and survivin. Preclinical studies have demonstrated that both c-Met and survivin along with specific Sp proteins (e.g., Sp1 and Sp3) mediate their expression in different malignancies [5, 7, 17, 22, 29]. Understanding the key players associated with the aggressive disease and inducing resistance to therapy is critical in treating pancreatic cancer.

29.4 Role of Sp1 as Transcription Factor

The Sp1 transcription factor is a DNA binding protein belonging to the Krüppel-like factor family that is important for the transcription of many regulatory genes that contain GC boxes in their promoters. It is a sequence-specific zinc finger transcription factor. The zinc fingers in the C-terminal domains bind to the GC/GT box (GGGGTGGGG) of target genes. The genes whose transcription is regulated by Sp1 in turn regulate tumor cell growth, differentiation, survival, angiogenesis, invasion, and metastasis [9]. The abnormal activation and expression of this transcription factor hence result in the development and progression of many tumors including pancreatic cancer. Sp1 overexpression is related to the increase in development, growth, angiogenesis, survival, and metastasis and eventually to a relatively poor prognosis of pancreatic cancer [14]. The target genes on which Sp1 transcription factor acts are involved in cell cycle regulation, progression, and angiogenesis. The effect of Sp1 on pancreatic cancer is due to the regulation of the expression of these target genes by Sp1.

29.5 Role of Sp1 in Cell Cycle

There are genes that encode proteins, which play major roles in the cell cycle. The abnormal expression of these genes relates to the cancer development and progression. The proteins encoded by these genes regulate cell cycle at various stages. Evidence suggest that Sp1 increases expression of some of these genes [23]. The promoters of these genes have enrichment sites that are specific binding sites for Sp1 transcription factor. This suggests that the Sp1 transcription factor regulates the expression of these genes that are involved in cell cycle and increased expression of these is the cause of pancreatic cancer development.

Some of these genes are *LMNB1* (lamin B1), *CENPF* (centromere protein F), *DDIT3* (DNA damage-inducible transcript 3), *MYB* (MYB proto-oncogene), *SKP2* (S-phase kinase-associated protein 2), *KIF20A* (kinesin family member 20A), and *CCNE2* (cyclin E2) [23]. Below we summarize some of the key functional properties of these genes:

LMNB1 is a component of the nuclear lamina and plays a key role in nuclear structural integrity by providing a framework for the nuclear envelope and chromosomal stability through its interaction with chromatin. *CENPF* helps in chromosome segregation during mitosis. In the G2 phase of interphase, the nuclear matrix is composed of many proteins, and *CENPF* is one of its components. Its expression is also correlated with poor prognosis in prostate cancer patients. *DDIT3* is activated by the endoplasmic reticulum stress and mediates apoptosis by causing cell cycle arrest. *MYB* is a transcriptional regulator and is involved in the tumorigenesis. *SKP2* forms a complex with the cyclin A-CDK2 S-phase kinase, and its expression correlates with the metastasis and poor outcomes in pancreatic cancer patients. *KIF20A* is a microtubulin-associated mitotic kinesin required for chromosome passenger complex (CPC)-mediated cytokinesis. It is involved in the migration and invasion of pancreatic cancer cells. *CCNE2* encodes a protein which is a cyclin and plays a central role in G1/S transition, its expression peaking at this phase of the cell cycle. It has been found that the expression of this gene is upregulated in the tumor-derived cells.

The promoters of the above-noted genes have binding sites for various transcription factors including Sp1. Sp1 binds to its binding sites on promoters of these genes and regulates its expression thus leading to their increased activity and cancer.

29.6 Role of Sp1 in Pancreatic Cancer Metastasis

Metastasis is a complex process that consists of many steps/pathways. It is a major factor in determining the morbidity and mortality of patients with pancreatic cancer. Metastasis basically involves various sequential steps that include primary tumor cells invading the local tissue, transport of tumor cells in blood or lymphatic vessels to target tissues, and proliferation of tumor cells at the secondary sites. Sp1 transcription factor is involved in the regulation of each step of the metastasis of pancreatic cancer by different mechanisms – and some of the critically important ones are described below.

29.6.1 Role of Sp1 in Invasion

Sp1 has been positively correlated with increased vascular invasion by pancreatic tumor cells [12]. The molecular mechanisms involved are not absolutely clear, but it has been suggested that Sp1 collaborates somehow synergistically with an enzyme phospholipase D1 (PLD1) and increases the invasion of pancreatic tumor cells. Abnormal metabolism is a factor in tumor initiation and growth, and PLD1 is a key enzyme that hydrolyzes phosphatidylcholine and is involved in lipid metabolism. PLD1 is elevated in pancreatic tumors and is positively correlated with Sp1. Combined overexpression of both Sp1 and PLD1 promotes pancreatic tumor

invasion, and their simultaneous expression is considered as an independent factor in pancreatic tumor invasion, metastasis, and poor prognosis [12].

There has been some evidence to support that Sp1 also regulates the expression of proteolysis-related genes [13]. The synthesis and secretion of proteases formed by the expression of these genes are involved in the degradation of basement membrane components and extracellular matrix, which plays a major role in pancreatic cancer invasion and metastasis.

29.6.2 Role of Sp1 in Migration

Epithelial-mesenchymal transition (EMT) is an important step in the metastasis of tumor cells. It is a biological process in which sessile epithelial cell phenotype undergoes a developmental switch in a migrating mesenchymal phenotype by biochemical changes with increased migratory capacity, invasiveness, resistance to apoptosis, dissolution of tight epithelial junctions, loss of cell adhesion, downregulated expression of some epithelial markers, and increased production of the extracellular matrix components. Pancreatic cancer cell migration and invasion depend on epithelial-mesenchymal transition.

Sp1 regulates metastasis of pancreatic cancer cells as it is required for transforming growth factor-beta (TGF-ß)-induced EMT and migration of pancreatic cancer cells [15]. TGF-ß itself induces vimentin expression, a type III intermediate filament. Its transcriptional induction of vimentin is carried out through the increased transcription of vimentin from its proximal promoter site. Vimentin is important in cell migration and subsequent metastasis. The prevention of transcriptional induction of vimentin by Sp1 through TGF-ß results in a relatively decreased pancreatic carcinoma cell migration.

29.6.3 Role of Sp1 in Angiogenesis

In the initial phases of pancreatic cancer metastasis due to the increased metabolic activity and increased nutritional demands of cancer, angiogenesis is required to supply nutrients to the tumor cells. Spread and proliferation of the tumor cells in the target organs also require angiogenesis. Thus, angiogenesis is important for the pancreatic cancer metastasis. Many direct and indirect angiogenic factors play key role(s) during angiogenesis. Some of the angiogenic factors with direct actions are endothelial growth factor, placental growth factor, and angiopoietins. Whereas, angiogenic factors with indirect actions are: fibroblast growth factor (FGF), platelet-derived endothelial growth factor (PDEGF), and interleukins (ILs). Transcription factors regulate expression of these direct and indirect angiogenic factors. These angiogenic factors affect the angiogenic phenotype. Sp1 is one of the central transcription factors in that it regulates angiogenic gene expression and subsequently regulates the tumor angiogenesis.

Vascular endothelial growth factor (VEGF) is one of the direct angiogenic factors that plays central role(s) in angiogenesis, growth, and metastasis of tumors [24]. Sp1 promotes angiogenesis and metastasis by transcriptional regulation of VEGF [2]. It induces VEGF expression by directly binding to its promoter site. Overexpression of Sp1 causes increased VEGF expression and tumor microvessel formation in the pancreas [2]. The importance of this mechanism can be described by the fact that the tumor suppression genes like *p53*, *p75*, and *von Hippel-Lindau* suppress the expression of VEGF by binding and forming the complexes with Sp1, which inhibits binding of Sp1 to the promoter region of VEGF and hence interferes with its transcription.

Vascular endothelial growth factor receptor 2 (VEGFR2), also known as kinase insert domain receptor (KDR), has also been found to be involved in the pancreatic tumor angiogenesis. Sp1 also binds the promoter region of the VEGFR2/KDR and increases its expression, hence mediating the angiogenesis process in pancreatic tumors [10].

29.6.4 Metastasis to Lymph Nodes

Sp1 overexpression is directly related to the lymph node metastasis in tumors. Increased gene expression and posttranslational modification of Sp1 protein leading to the enhanced Sp1 action have a 100% specificity for the presence of lymph node metastasis [14].

29.7 Role of Sp1 in the Decreased Apoptosis of Tumor Cells

Pancreatic tumor cells have a great ability to evade cell death. This ability is due to the survival proteins. Heat shock proteins (HSP) are a class of survival proteins that are important in pancreatic tumor cell survival. HSP1 and HSP70 are the two such key heat shock proteins that are regulated by the Sp1 transcription factor [3].

Posttranscriptional modification of Sp1 is important in the regulation of transcriptional activity of Sp1. Hexosamine biosynthetic pathway (HBP) is a shunt pathway that plays major role(s) in the cellular signaling cascades and regulation of the transcription factors involved in cancers. Sp1 transcription factor is also modified through HBP pathway through O-GlcNAcylation at Ser-484. O-GlcNAc transferase (OGT) is the enzyme that catalyzes this reaction. It modifies many cellular proteins and is overexpressed in a number of cancers. This glycosylation helps in the translocation of the Sp1 transcription factor to the nucleus and subsequently to its binding with its promoters on their target genes. These target genes include *NFk-b* and *HSP1*. Eventually, the increased expression of heat shock survival proteins results in the increased ability of pancreatic tumor cell survival (Fig. 29.1).

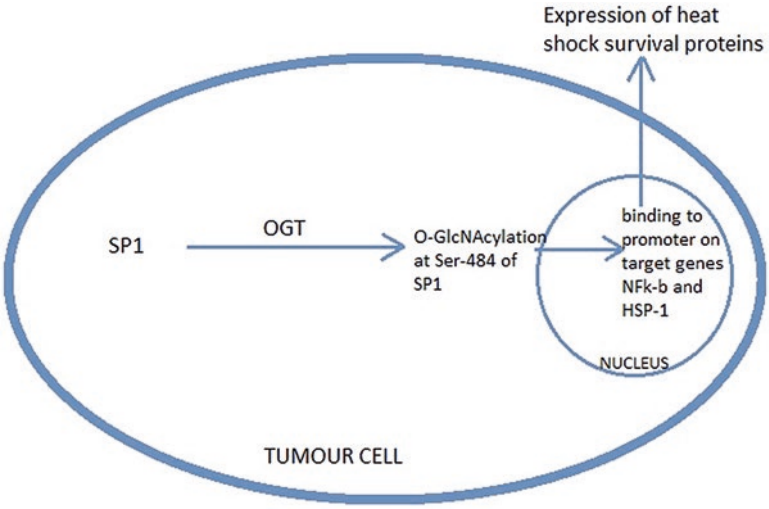

Fig. 29.1 Schematic representation of the cross talk between the Sp1 transcription factor and O-GlcNAc transferase (OGT) in the tumor cell/gene responses through the expression of heat shock survival proteins

29.8 Anticancer Drugs Affecting Sp1

In recent years, several drugs have been shown to inhibit the proliferation of pancreatic cancer cells and tumors. The mechanisms of anticancer activity of these drugs have also been associated with the decreased activity of Sp1 transcription factor. Some of the main drugs that affect the Sp1 transcription factor are detailed below:

Triptolide is a diterpenoid epoxide obtained from the plant *Tripterygium wilfordii*. It inhibits the glycosylation of Sp1 by decreased expression and activity of OGT, hence leading to the decreased survival proteins and cell death.

Minnelide, a water-soluble prodrug of triptolide, is effective against pancreatic cancer at doses which have been shown to be safe in phase I clinical trial. Oral Minnelide has great potential to emerge as a novel therapy for pancreatic cancer [4].

Tolfenamic acid is a novel small molecule nonsteroidal anti-inflammatory drug (NSAID), which belongs to the class "fenamates," and has been implicated in several cancer models [22]. This agent has long been used for treating migraine headaches in many parts of the world. Its therapy causes apoptosis and cell cycle arrest in pancreatic tumor cells mainly by the degradation of Sp1. The degradation of Sp1 leads to decreased expression of VEGF and other downstream targets like survivin and c-Met, which eventually leads to the decreased growth and metastasis of pancreatic tumors [1, 23].

Cyclooxygenase-2 (COX-2) inhibitors are correlated with the increased angiogenesis in the pancreatic tumor in a process which is dependent on Sp1 and VEGF. It is overexpressed in many tumors of the body including pancreatic tumors. Its expression is correlated with a poor prognosis in PDAC [8]. The overexpression of COX-2 is correlated with an increased Sp1 activity [26]. Sp1 binds to COX-2 promoter sequence position around −25/−240 and causes the increased transcription of COX-2. Sp1 also can transcriptionally activate COX-2 expression through another mechanism. Evidence suggest that in pancreatic tumors, there is an alteration of activated epidermal growth factor receptor (EGFR) and its downstream p38 mitogen-activated protein kinase (p38-MAPK). This EGFR/p38-MAPK signaling pathway could be used by Sp1 to transcriptionally activate COX-2 expression. Overactive EGFR phosphorylates Sp1 via a p38-MAPK signaling pathway [11]. The highly activated Sp1 subsequently activates COX-2 transcription, leading to the elevated levels of COX-2 expression, which finally promotes the secretion of VEGF (Figs. 29.2 and 29.3). Thus, besides expression of VEGF by Sp1 directly, Sp1 can also upregulate the expression of VEGF by increasing the expression of COX-2.

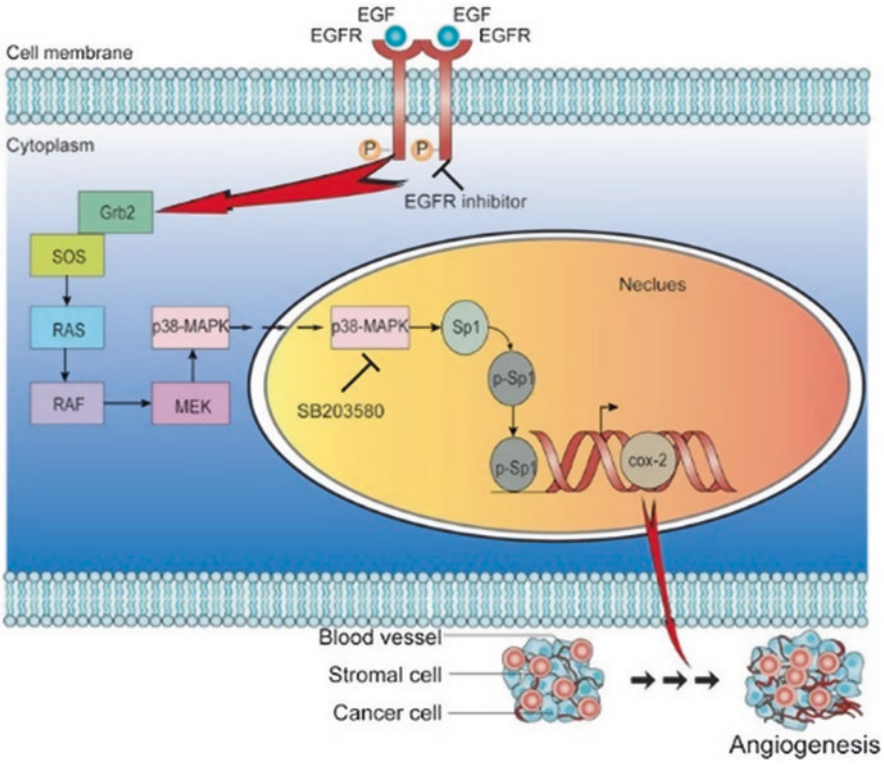

Fig. 29.2 Schematic representation of some of the key mechanisms (pathways) of anticancer activity of the drugs that have been associated with the decreased activity of Sp1 transcription factor

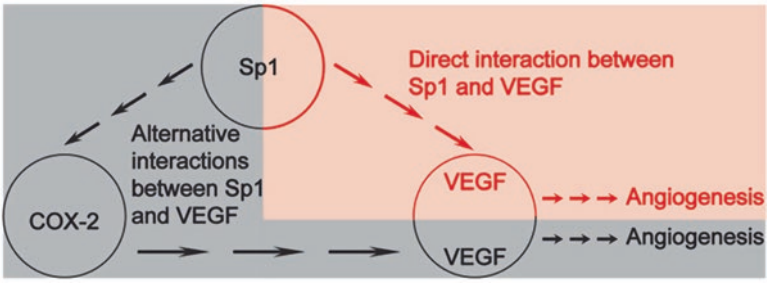

Fig. 29.3 Schematic representation of some of the key interactions of Sp1 transcription factor with cycylooxygenase-2 (COX-2) and/or vascular endothelial growth factor (VEGF) during the angiogenesis process

Celecoxib is a selective COX-2 inhibitor that inhibits tumor growth and metastasis, mainly by inhibiting the angiogenesis and tumor microvessel formation. It suppresses the VEGF expression at the mRNA and protein levels by reducing the Sp1 DNA binding activity and trans-activating activity. This decreased activity of Sp1 is due to the reduced Sp1 phosphorylation. Phosphorylation of Sp1, which occurs during the posttranslational modification of Sp1, increases its DNA binding activity.

Mithramycin, an antibiotic, decreases the expression of Sp1 and its downstream molecules such as VEGF, EDF, and PDGF. It inhibits Sp1 by binding to the genes with GC-rich promoter sequences, thus regulating the transcription of these genes [28].

29.9 Conclusions and Future Prospectives

Pancreatic tumors are a leading cause of carcinoma-related deaths globally. Given the relatively poor outcomes after surgery (i.e., survival rate) of patients with pancreatic cancer, molecular markers/targets can potentially serve as therapeutic targets for this lethal disease. Identifying Sp1 as an important molecular marker for pancreatic tumors may have a very significant prognostic value. Surgery is the main treatment of pancreatic cancer as chemotherapy and/or radiotherapy have rather limited role(s) in the disease treatment and management. Early recurrence and metastasis are quite common as pancreatic cancer has a high propensity for early distant metastasis. As overexpression of Sp1 correlates with poor prognosis, advanced stage of the tumor, lymph node metastasis, decreased survival, and aggressiveness of the pancreatic cancer, therapeutic strategy directed at Sp1 can be very significant for controlling pancreatic tumors. Also, Sp1 can serve as an important prognostic marker for pancreatic cancer. Several drugs targeting Sp1 for pancreatic tumor therapy have been studied. Most of these drugs work by downregulating the expression and activity of Sp1 in tumor cells. This can have a very significant effect on the overall clinical outcomes and prognosis of patients with pancreatic cancer.

Conflict of Interest None of the authors has any potential financial or commercial conflict of interest associated with this research manuscript.

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Targeting Arachidonic Acid Pathway-Associated NF-κB in Pancreatic Cancer

30

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Abstract

Pancreatic cancer is one of the foremost causes of cancer-related death in the United States and worldwide. Nuclear factor-kappa B (NF-κB) is a transcription factor which plays a pivotal involvement in pancreatic cancer owing to its connection at the downstream stage of many signaling cascades including arachidonic acid (AA) pathway. AA cascade is an upstream pathway and regulator of NF-κB pathway. Moreover, NF-κB can bind to the cis-acting elements in the

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promoter of phospholipase A_2s, cyclooxygenases, and lipoxygenases, and these pathways are upregulated in a variety of cancers. Several investigators have proved that AA pathway-associated NF-κB has key role in pathophysiology of pancreatic cancer and has been considered as a vital therapeutic target. Several phytochemicals have been explored as novel therapeutic and preventive agents by targeting AA cascade-associated NF-κB pathway. In this chapter, the role of AA cascade-associated NF-κB pathway in pancreatic cancer has been reviewed and discussed about plant-derived inhibitors of this pathway for the prevention and the therapy of pancreatic cancer.

Keywords
NF-κB · Arachidonic acid pathway · Pancreatic cancer · Natural products

30.1 Introduction

Pancreatic cancer is the fourth-leading cause of cancer-related morbidity, with 39,590 deaths and 46,420 estimated new cases in 2014 in the United States [1]. Mutations are considered to be the genetic event occurring at the beginning of the development process of pancreatic cancer, leading to cellular proliferation due to

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constitutive activation of intracellular pathways [2]. The latter is related to numerous alterations in growth factors and their receptors, involved in the control of growth and differentiation during transduction pathways [2]. There are several molecular signaling pathways that play a vital role in cell survival, proliferation, angiogenesis, metastasis, and promotion of pancreatic cancer, as well as in chemoresistance, including epidermal growth factor receptor (EGFR), nuclear factor-kappa B (NF-κB), signal transducer and activator of transcription factor 3 (STAT3), and arachidonic acid (AA) pathway [3].

30.2 Arachidonic Acid Pathway-Associated NF-κB Signaling in Pancreatic Cancer

The transcription factor NF-κB plays a critical role in pancreatic cancer as involving at the downstream stage of many signaling cascades including AA pathway. AA is an upstream mediator and regulator of NF-κB pathway. NF-κB and activation protein 1 (AP-1) can bind to the cis-acting elements in the promoter of phospholipase A2 (PLA2), cyclooxygenase (COX-2), and lipoxygenases (LOXs), and these pathways are upregulated in several cancers. The active form of PLA_2 stimulates the proliferation of MIAPaCa-2 pancreatic cancer cells by the activation of mitogen-activated protein kinases (MAPKs)/NF-κB [4]. PLA_2s gene knockout and transgenic studies demonstrated the pro-tumorigenic role of PLA_2 in pancreatic cancer [5].

A chemical compound bromoenol lactone (BEL) caused decrease in agonist-induced activation of EGFR in pancreatic cancer cells [6]. The NF-κB inhibition has become a major target in natural drug discovery, and it is necessary for survival and immunity of cell because it regulates the immune, inflammatory, and carcinogenic responses [7]. Compared to normal pancreatic tissues, NF-κB, which is linked with COX-2, is constitutively expressed in 67% of human pancreatic cancers. Zhang et al. [5] reported the acute pancreatitis induced by PLA2 by the activation of transcription factor NF-κB. Gong et al. [8] reported combinatorial target of COX-2/NF-κB/STAT3/prostaglandin receptor E-prostanoid 4 pathway for effective treatment of pancreatic cancer [8, 9] reported the TLR4-/NF-κB-/COX-2-mediated inflammatory pathway involved in tumorigenesis of pancreatic cancer.

Although COX-2 expression along with NF-κB is incremented in several types of malignancies including pancreatic, esophagus, colon, and breast cancers [10–12], the molecular mechanism is still unclear. It has been reported that COX-2 has inherent role in pancreatic ductal adenocarcinomas initiation and progression by the activation of the PI3K/AKT pathway [13]. In cancer, prostaglandin E2 (PGE2) the metabolite of COX-2 is overexpressed in the majority of epithelial malignancies [14, 15]. Approximately 80% of human pancreatic ductal adenocarcinomas (PDA) overexpress the tumor form of mucin 1 (tMUC1), a heavily glycosylated membrane-tethered glycoprotein generally expressed on glandular epithelial cells [16]. tMUC1 is overexpressed and aberrantly hypoglycosylated in malignant cells [16].

In a study, it has been shown that, in addition to the overexpression of tMUC1, the tumors have high levels of COX-2 and PGE2 [17]. Mucin 1 (PDA.MUC1) mice are highly resistant to celecoxib (a COX-2 inhibitor) and gemcitabine when each drug is administered independently; however, a clinical relevant antitumor response was observed after treatment with a blend of MUC1 vaccine, celecoxib, and gemcitabine [18]. Previous reports suggested that the transmembrane mucin glycoprotein mucin 1 (MUC1) is overexpressed in pancreatic ductal adenocarcinomas along with COX-2. Molecular studies by Nath et al. [17] demonstrated the MUC1 associated with the same gene locus where NF-κB/p65 binds with the COX-2 promoter. The study suggests that MUC1 regulates pancreatic ductal adenocarcinomas through COX-2/NF-κB pathway [17]. Animal LOXs were classified into five different types—5-LOX, 8-LOX, 11-LOX, 12-LOX, and 15-LOX [19]. LOXs also involved in various human cancers including colon, pancreatic, lung, breast, and prostate [10]; however, comparatively diminutive efforts have been made to explicate its role in cancer development.

Recently, it has been shown that treatment with omega-6 fatty acids increases leukotrienes B4 (a LOX metabolite) levels in human pancreatic ductal epithelial (HPDE and HPDE-Kras) and cancer (AsPC1 and Panc1) cells, in vitro [20]. Compared to EL-Kras/5LO+/+ mice, EL-Kras mice lacking 5LO (EL-Kras/5LO−/−) had decreased mast cell infiltration and developed fewer pancreatic lesions [20]. These results suggest the LOXs involvement in pancreatic cancer. Previously, the progression of human pancreatic cancer cells by leukotrienes B4 (LTB4) via MAPK and PI-3 kinase pathways was reported, although it did not affect the activity of JNK/SAPK [21]. In fact, different LOXs exhibit tumor response in a tissue-specific manner either pro-tumorigenic or antitumorigenic activities [22]. Inhibitors of lipoxygenases have been found to be very effective in the suppression of pancreatic cancer cell lines as well as pancreatic adenocarcinoma. In a study, the inhibition of 5-lipoxygenase by zileuton (5-LOX inhibitor) in pancreatic cancer cells by inducing apoptosis, SW1990, has been demonstrated [23].

30.3 Targeting Arachidonic Acid Pathway-Associated NF-κB by Phytochemical Compounds for Prevention and Therapy of Pancreatic Cancer

Phytochemical agents and their derivatives act as anticancer drugs by inhibiting the migration, invasion, growth, survival, and metastasis of cancer cell during the carcinogenesis process by multiple pathways [24]. Cannabinoid (1), lupeol (2), plumbagin (3), quercetin (4), sulforaphane (5), triptolide (6), γ-tocotrienol (7), boswellic acid (8), curcumin (9), and garcinol (10) are different phytochemicals exhibiting anticancer activities against various cancers, including pancreatic, by targeting AA pathway and NF-κB-mediated signaling pathways (Table 30.1).

Moreover, paclitaxel, etoposide and teniposide, vinblastine and vincristine, and camptothecin derivatives are reported anticancer agents [25, 26]. These compounds act by inhibiting the expression of NF-κB and associated genes by modulating

Table 30.1 Phytochemicals targeting AA pathway associated transcriptional factros for cancer prevention and therapy

Phytochemical	Mechanism	References
Cannabinoid (1)	Upregulation of CB2 receptor	[37]
	Upregulation of p8, ATF-4, and TRB3 genes	
Lupeol (2)	Reduced activation of NF-κB	[38]
	Reduced protein expression of PKCa/ODC, PI3K/Akt, and MAPK pathways	
Plumbagin (3)	Decrease in NF-κB/p65 phosphorylation	[39]
	Inhibition of STAT3 phosphorylation	
	Upregulates the expression of IL-6	
	Inhibited expression of Cdc25A, cyclin D1, and MMP9	
	Decreased expression of proliferative markers such as PCNA and ki67	
Quercetin (4)	Diminished ALDH1	[40]
	Inhibition of NF-κB	
	Reduced proliferation, angiogenesis, cancer stem cell-marker expression	
	Induction of apoptosis	
Sulforaphane (5)	Diminished NF-κB binding, induction of apoptosis	[41]
	Blockage of tumor growth and angiogenesis	
Triptolide (6)	Downregulates NF-κB	[42]
	Inhibition of HSF1 and HSP70	
	Inhibition of glycosylation of Sp1	
	Inhibition of hexosamine biosynthesis	
	Inhibition of *O*-GlcNAc transferase	
ɣ-Tocotrienol (7)	Inhibition of NF-κB activity	[43]
	Inhibition of cell growth and cell survival	
	Decreased p65 (ReIA) binding	
	Inhibition of proliferation, angiogenesis, invasion	
Boswellic acid (8)	Downregulate the expression of COX-2, MMP-9, CXCR4, and VEGF and inhibit the expression level of c-Myc	[44]
Curcumin (9)	Downregulation of iNOS, COX-2, and 5-LOX expression	[45]
	Upregulation of p21 expression	
Garcinol (10)	Reduction in PGE2 expression	[46]
	Downregulation of NF-κB and COX-2	
	O2, nitric oxide (NO), iNOS, and COX2	

various signal transduction pathways [25, 26]. Researchers reported that individual phytochemicals and in combination with chemotherapeutics can prevent pancreatic cancer by inhibiting several signaling pathways [27]. Phenolics can inhibit the promotion and progression of cancerous cell by modulating the activities of NF-κB and AP-1 [28]. Curcumin downregulates the expression of NF-κB, COX2, and phosphorylated STAT3 in peripheral blood mononuclear cells in some pancreatic cancer patients [29] (Fig. 30.1). Lev-Ari et al. [30] reported that combination of curcumin and gemcitabine enhanced cytotoxic effect against pancreatic adenocarcinoma in vitro, decreasing the COX-2 and p-ERK1/2 levels.

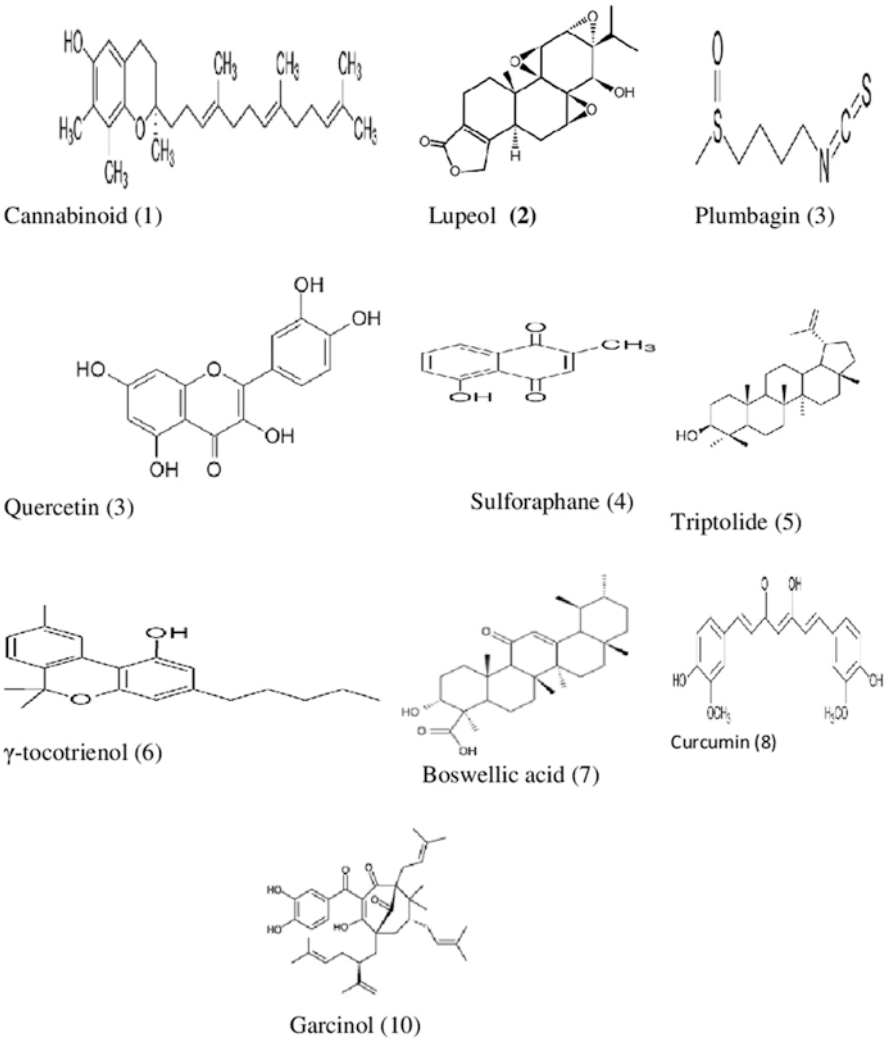

Fig. 30.1 Chemical structures of some of the important anticancer phytochemicals by targeting AA cascade-associated NF-κB pathway

Cascinu et al. [31] conducted a clinical trial in pancreatic cancer, by using celecoxib (COX-2 inhibitor) associated to oxaliplatin and gemcitabine. The expression of COX-2 was found in 30 tumors as well as NF-κB expression was found in 16 tumors. Authors reveal that COX-2 inhibitor does not enhance the efficacy of gemcitabine/oxaliplatin. Nafamostat mesilate inhibits induced apoptosis in pancreatic cancer cells, but its maximum concentration (1.8×10^{-7} M) is not effective to inhibit NF-κB [33]. The recommended dose of regional arterial infusion of nafamostat mesilate (1.8×10^{-6} M), used to target gemcitabine-induced NF-κB activation,

in combination with gemcitabine is found to be safe in pancreatic cancer patients [32]. The inhibition of gemcitabine-induced NF-κB activation enhances the antitumor activity of gemcitabine against pancreatic cancer [34].

Pristimerin, a quinonemethide triterpenoid, exhibited antiproliferative and proapoptotic activities in pancreatic cancer cells by inhibiting multiple signaling pathways NF-κB/COX-2 [35]. Nordihydroguaiaretic acid (NDGA), a 5-LOX inhibitor, prevented the progression of acute pancreatitis by inhibiting multiple pathways, including NF-κB [36]. Nexrutine®, an anti-inflammatory nutraceutical, inhibited growth of pancreatic cancer cells and reduced levels and activity of NF-κB, expression of COX-2, and subsequent decreased levels of PGE2 and PGF2 [8]. 6-Shogaol (a phenol extracted from ginger) alone and in combination with gemcitabine suppressed the growth of pancreatic tumors, by suppressing the TLR4-/NF-κB-/COX-2-mediated pathway [9].

30.4 Conclusions and Future Directions

NF-κB is an important transcription factor and has a crucial role in pancreatic cancer as it is involved at the downstream stage of many signaling cascades including AA pathway. Metabolic enzymes and products of AA pathway are also involved in activation of NF-κB. Cross talk between eicosanoids and NF-κB plays a key role in pathophysiology of pancreatic cancer. Therefore, researchers have considered AA cascade-associated NF-κB pathway as novel therapeutic target in pancreatic cancer. Moreover, several AA cascade-associated NF-κB pathways are targeted by natural products. Therefore, these compounds have been suggested as novel preventive and therapeutic agents against pancreatic cancer.

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Pancreatic Cancer: Role of STAT-3 and Intervention of STAT-3 by Genistein

31

Gangishetti Umesh and Sudarshan Malla

Abstract

Pancreatic cancer (PC) is a cancer that is initiated in the pancreas and spreads rapidly to other organs. Pancreatic cancer is the third most leading cause of cancer-related deaths in the United States (US) and is often regarded as one of the deadliest cancers with 95% mortality within 5 years of detection, which is the highest compared to the rest of any cancer-related deaths. Pancreatic cancer is tough to diagnose at early stages, and by the time the patient is hospitalized and detected with pancreatic cancer, it is already in advanced stages. Apart from surgical procedures, inhibition of many key proteins that are tumorigenic pathways has been tested in several model systems including mouse and are further corroborated in various clinical studies. Included in that group of several tumorigenic proteins is the signal transducer and activator of transcription 3 protein, namely, STAT-3. STATs were identified in the year 1994 that traffic signals from the activated cell surface receptors internally to the nucleus and act as potent transcription factor (TF) that regulates several key aspects of intracellular functioning, namely, cell proliferation, cell differentiation, apoptotic cell death, and angiogenesis. In normal cells, STATs are in non-phosphorylated, inactive form, but upon external stimuli, they are phosphorylated and activated that leads to translocation to the nucleus and act as transcription factors. In the STAT family, STAT-3 functions are most important as STAT-3 knockout are lethal and mice are reported to die at day 7.5. STAT-3 inhibition by RNAi or chemical compound

The original version of this chapter was revised. The book was inadvertently published without Abstracts and Keywords, which are now included in all the chapters. An erratum to this chapter can be found at https://doi.org/10.1007/978-981-10-6728-0_39

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in vivo in model systems such as mice led to a block in ductal adenocarcinomas and PanIN formation. Mouse models expressing endogenous mutant Kras mutation, STAT-3 found to phosphorylated and significantly activated. Due to STAT-3 established role in angiogenesis, several chemical and natural compounds have been developed and tested over the last two decades. STAT-3 inhibitors such as nexrutine, crizotinib, and miR-216a overexpression have shown to inhibit pancreatic cancer growth by reducing the STAT-3 activity levels. Chemical compounds such as thiosemicarbazone treatment in vitro and in vivo (model systems) inhibited interleukin-6 (IL-6)-induced activation of STAT-3 by the decrease in phosphorylation at Tyr705. Metformin along with aspirin doses significantly reduced the phosphorylation of STAT-3 and mechanistic target of rapamycin (mTOR). Apart from several chemicals mentioned above, there are various natural products such as genistein derivatives used as STAT-3 inhibitors. Genistein was used and tested in treatment of pancreatic, breast, and prostate cancer (PC). Genistein is shown to prevent p-STAT-3 binding to DNA in a concentration-dependent manner. Pretreatment of human pancreatic cancer cell lines such as COLO 357 and L3.6pl, by genistein for 24 h followed by gemcitabine, resulted in inhibition of growth up to 80% compared to only 30% in gemcitabine alone condition. In 2016, promising results were published from the clinical study conducted at Karolinska where patients who received genistein lived 6 months longer than their counterparts. A crystalline formulation of genistein, namely, AXP107-11, has been shown to have improved physiochemical properties and oral bioavailability in comparison to other universal forms of genistein. Pancreatic cancer (PC) patients treated with AXP107-11 along with gemcitabine resulted in improved survival. Forty-four percent of the total 16 patients in the study survived longer than 6 months, and half of them even survived longer than a year.

31.1 Overview of Pancreatic Cancer (PC)

Pancreatic cancer [1] is a widespread disease that initiates in the pancreatic tissue and spreads rapidly to nearby organs. Pancreatic cancer is difficult to treat with 95% mortality rate within the first 5 years of detection [2, 3]; in rare cases it is detected in its early stages, hence making it difficult to treat patients longitudinally admitted to the hospital; and thereby disease prognosis starts mostly in the advanced stages of cancer.

Pancreatic cancers are the third most leading human cancer death cause in the United States (US) and have surpassed breast cancer in European (EU) population in 2016 [4]. It is currently estimated that in 2016 alone, 53,070 Americans are diagnosed with pancreatic cancer and 41,780 will die from pancreatic cancers and furthermore 71% of the patients will die in the first year of diagnosis [5]. In comparison African-Americans have the highest incidence rate of pancreatic cancer, i.e., between 28% and 59% higher than other racial/ethnic groups.

With advanced surgical procedure and with the help of latest medical intervention techniques, the overall general cancer appearance and death rates are decreasing; on

the contrary the prevalence and death rates for pancreatic and lung cancer (LC) are exponentially increasing. A 5-year relative survival is estimated as 18% for lung cancer (LC) and 8% for pancreatic cancer, thus making pancreatic cancer the most fatal form of cancer. Although accurate early diagnosis of pancreatic cancer is underway, the tendency is higher for the people with pancreatic cysts or familial cases of pancreatic cancer. Hence, one way to increase the pancreatic cancer early diagnosis is robust increase in cancer screening steps that might help in detection of early-stage pancreatic cancer. It is previously observed that diabetic patients experiencing weight loss, jaundice, and pain in the upper abdominal region that spreads to the back are positive indications of pancreatic cancers. The pancreatic cancers are mainly distinguished into two major tumor types, namely, adenocarcinoma constituting about 85% of pancreatic cancer cases and the pancreatic endocrine tumor (PET) that has a prevalence rate less than 5% of all pancreatic cancer cases [6]. According to recent study published by Torre et al., death incidents caused by pancreatic cancer are expected to surpass colorectal cancer and become the second leading cause of cancer-related death in the United States around 2020 [7].

Pancreatic carcinoma (PC) is broadly classified into "exocrine and endocrine pancreatic neoplasms" [8]. Exocrine pancreatic neoplasms (EPN) are the most prevalent form of pancreatic cancers that comprise up to 95% of pancreatic cancer. Exocrine pancreatic neoplasms (EPN) comprise tumors that originate at several pancreatic regions, namely, the pancreatic duct, acinar cells, and related pancreatic somatic stem cells, while on the other side, endocrine pancreatic neoplasms occur at pancreatic ductal adenocarcinoma, intraductal papillary mucinous tumors, and mucinous cystic tumors. Apart from these abovementioned adenocarcinomas, 5% of the neoplasms are from the islet cell tumors. Among all the adenocarcinomas, pancreatic ductal adenocarcinoma is the most prevalent and destructive type of pancreatic cancers. Previous, studies have identified three different ductal precursor lesions that lead to invasive pancreatic ductal adenocarcinoma: pancreatic intraepithelial neoplasia (PIN), intraductal papillary mucinous neoplasia (PMN), and mucinous cystic neoplasia (MCN).

31.2 Signal Transducer and Activator of Transcription (STAT) in Cellular Metabolism

The signal transducer and activator of transcription (STAT) is a family of seven members (namely, STAT-1 to STAT-6, including two forms of STAT-5A and STAT-5B) first identified in 1994 transducing signals from activated cell surface receptors through intracellular kinases to the nucleus and acts as transcription factors that regulate variety of cellular processes such as cell proliferation, cellular differentiation, apoptotic cell death and the inflammatory immune reaction, and angiogenesis [9]. At a molecular level, these STAT proteins have conserved structural features and have molecular weights ranging from 750 to 847 amino acids (Fig. 31.1).

Each STAT transcription factor (TF) is composed of an N-terminal dimerization domain involved in activation upon dimerization: a coiled-coil domain which assists

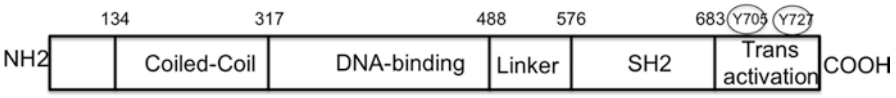

Fig. 31.1 STAT3 structural domains and phosphorylation sites

in interaction with other proteins and a DNA-binding domain that specifically guides to bind DNA double helix. Apart from these domains, it consists of a linker domain called Src homology 2 that enables in receptor binding and dimerization and a conserved phosphorylation site in C-terminal region of the protein that upon phosphorylation acts as transcription activation domain (Fig. 31.1).

In normal cells the STATs are in non-phosphorylated form which upon phosphorylation through cytokine binding or through external stimuli transmigrates into the nucleus. STAT proteins specifically (STAT-2, STAT-4, and STAT-6) each play crucial and defined role in the T cell response and in IFN gamma (interferon gamma) signaling and are activated by some certain cytokines. STAT-3 and STAT-5 are most often implicated in human cancer progression, while STAT-1, STAT-3, and STAT-5 are activated in various tissue types.

31.3 STAT-3 Activation and Signaling Pathway

Of all the proteins in STAT family, STAT-3 function is very essential and holds key in several developmental processes. STAT-3 knockout (KO) mice are lethal and are reported to die at day 7.5 [10]. Although STAT-3 was identified as mediator of IL-6 pathway activated by IL-6-type cytokines such as IL-6, IL-10, and IL-11, it is a converging point for several other pathways such as epidermal growth receptor pathway [11] (Fig. 31.2). Upon activation by IL-6, vascular endothelial growth factor receptor (VEGF-R), receptor tyrosine kinases (RTKs), and epidermal growth factors, the STAT-3 transcription factor exhibits its pro-transcription effects. For example, when IL-6 binds to its receptor, IL-6 receptor leads to Janus kinase (JAK) activation via complex formation involving GP130. In turn the activated JAKs are phosphorylated and activate STAT-3 by phosphorylation of a tyrosine residue (RTKs) within the Src homology 2 domain at Tyr705 (Fig. 31.2). In the end the phosphorylation of STAT-3 promotes homodimerization of the STAT-3 itself and thus migrates into the nucleus, and there it interacts with other coactivators that guide the STAT-3 protein to specifically bind to enhancer DNA elements in the promoter regions of the target genes, thus regulating gene transcription. Phosphorylation on the serine S-727 in the C-terminal domain of STAT-3 promotes association of STAT-3 with other transcription coactivators including p300/CBP and specifically activates particular target genes. This specific STAT-3 gene activation response activates genes that are required for cancer growth and metastasis, sustaining cell survival, cell proliferation/replication, angiogenesis, and immune evasion. STAT-3 also activates transcription regulator of a variety of

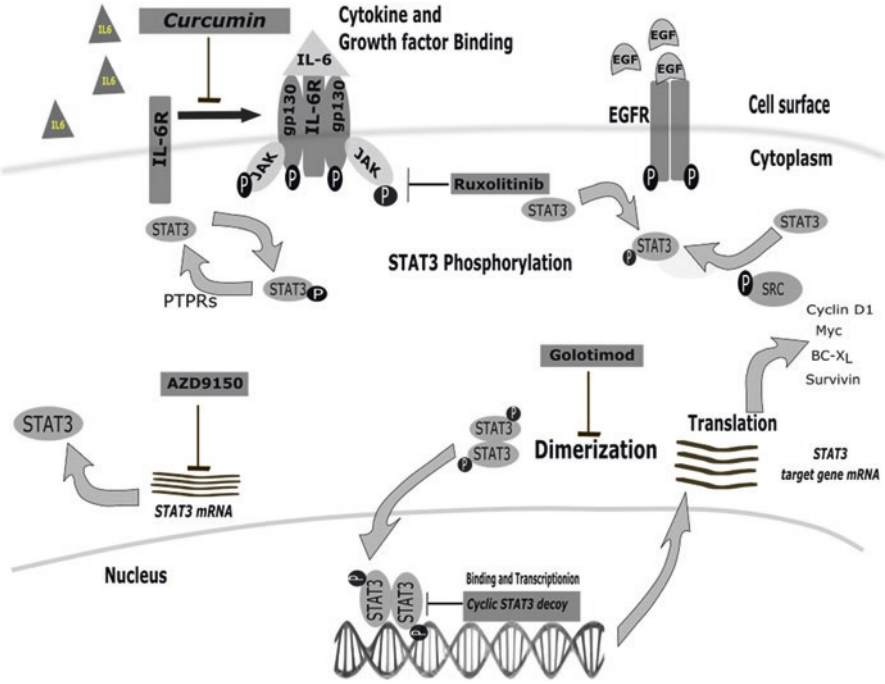

Fig. 31.2 Schematic diagram of STAT3 pathway: STAT3 are activated as a downstream effect of external stimuli such as IL6 and EGF to their respective IL6, EGF receptors. IL6 receptor forms a complex with gp130 and phospho JAK. This in turn phosphorylates STAT3, and phosphorylated STAT3 forms dimers and translocated into the nucleus. Once STAT3 is phosphorylated and forms a dimer then STAT3 is in an active state and acts as transcription factor for several genes that are involved in tumor progression and metastasis (Adapted from Geiger JL et al., Oral Oncology 56 (2016) 84–92

tumor-promoting gene targets like cyclin D1, cyclin D2, c-Myc, and p53 that are involved in cell growth and tumor progression. Additionally, STAT-3 acts on B-cell lymphoma-extra large (Bcl-xL), B-cell lymphoma 2 (Bcl-2), and myeloid leukemia cell differentiation protein (Mcl-1) (Fig. 31.3). STAT-3 is known to be involved in tumor development and progression [12–14]. Activated STAT-3 that is phosphorylated at Tyr705 has been reported up to 30–70% gastric cancer, where it has been shown to be transmigrated and localized in the nucleus [15]. The extent of phosphorylation of STAT-3 transcription factor is known to be directly co-related with differentiation, stage of the disease, and poor cell survival [16, 17].

31.4 STAT-3 Inhibitors

Over the years, several chemical compounds have been developed, screened, and tested for the use of cancer treatment [18]. The mechanism of action varies among them. They either block phosphorylation, dimerization, or nuclear translocation or

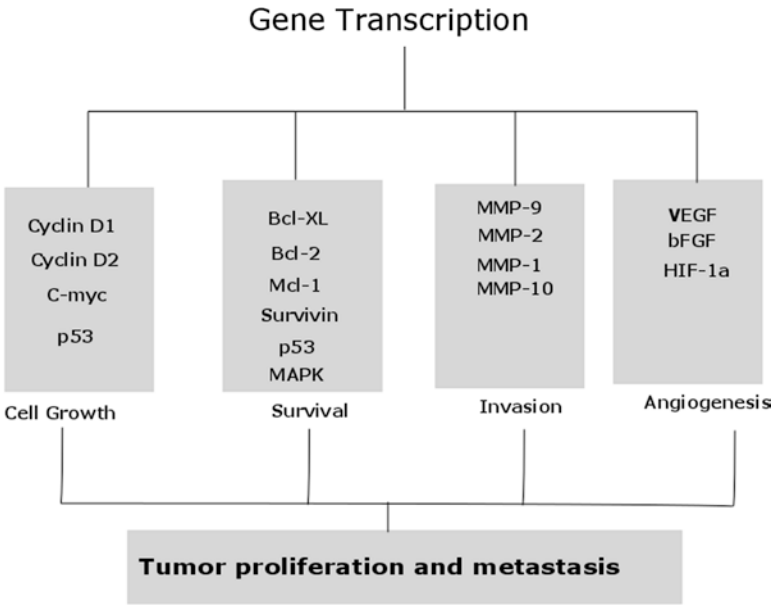

Fig. 31.3 Activated STAT3 involved in up-regulation of several proteins by acting as a transcription factor. These proteins are involved variety of cellular processes that are part of tumor proliferation and metastasis. Cycin D1, D2, c-myc involved in cell growth, Bcl-XL, Bcl-2, Mcl-1, Survivin, p53, MAPK involved in survival, MMP-1,2,9 and 10 involved invasion and VEGF, HIF-1alpha involved in angiogenesis

DNA-binding ability of the STAT proteins [19]. Inhibitors such as Stattic, STA-21, LLL-3, and LLL-12 selectively inhibit the STAT-3 dimerization (IC_{50} = 5.1) (Fig. 31.4). Stattic selectively inhibits STAT-3 over other STAT-s in the family. It also prevents STAT-3 from translocation in to the nucleus [20]. STA-21 is identified as STAT-3 SH2 domain inhibitor, demonstrated to block STAT-3 translocation. LLL-3 and LLL-12 are improvised derivatives of STA-21. Both LLL-3 and LLL-12 are demonstrated to decrease tumor cell survival. These compounds were successfully tested in breast, pancreatic, and glioblastoma cell lines [21, 22]. S31-201 is another potent compound that has three times more affinity to STAT-3 over STAT-1 and thus blocked the formation of homodimers and inhibits proliferation of breast and hepatocellular cancers in mice model system [23]. S31-1757 is another analogue of S31-201 that is shown as equally potent inhibitor of STAT-3. Although most of these drugs targeted the Src homology 2 (SH-2) domain of the STAT-3, the others were developed to target the DNA-binding domain (DBD), thereby reducing the STAT-3 DNA binding and its transcriptional functional ability. One such example is the platinum compounds known to form DNA adducts and therefore can disrupt the ability of STAT-3 to bind to DNA [24]. Platinum compounds proposed as STAT-3 inhibitors are classified as platinum (IV) complexes and differ from chemotherapeutics such as cisplatin, a platinum (II) that shows no inhibitory effect on STAT-3. The most widely known platinum-based STAT-3 inhibitors are CPA-1, CPA-7, and

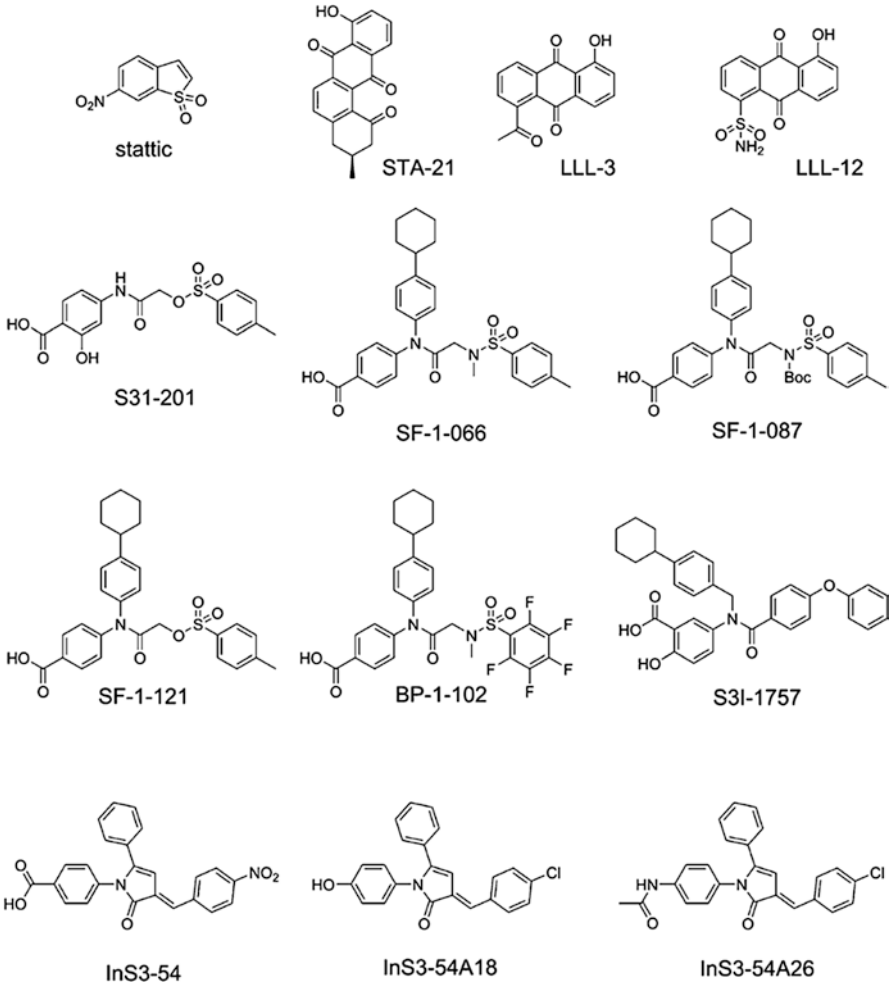

Fig. 31.4 Chemical compounds used as STAT3 inhibitors

platinum (IV) tetrachloride. S3-54 displayed selective inhibition of activity of STAT-3 over STAT-1 and induced apoptosis in both breast and lung cancer cell lines [25]. InS3-54A18 and InS3-54A26 are improvised compound that is highly specific to STAT-3 compared to its parent compound, S3-54 [26], but due to their poor solubility, further optimization is needed.

31.5 Potential Inhibitors of STAT-3 and Pancreatic Cancer

STAT-3 signaling is activated due to loss of SMAD-4. This loss of SMAD-4 switches TGF-alpha to tumor-promoting pathway from tumor suppressor pathway in pancreatic cancer [27]. Thus inhibitors of TGF-alpha and SMAD-4 could target STAT-3

signaling and are actively considered as good therapeutic intervention to suppress the progression of pancreatic tumor. TGF-beta/SMAD4 regulates various cellular functions like embryonic development and immune function in normal cells, and thus SMAD-4/TGF-beta is inactivated in approximately 60% of the pancreatic cancer [1, 28]. About 50% pancreatic adenocarcinomas is known to have genomic deletions and single point mutations of the tumor suppressor gene SMAD-4 [29]. SMAD-4 restoration by gene therapy reported to inverse the invasive phenotype and attenuated proliferation in pancreatic cancer cells [30, 31]. Whenever SMAD-4/TGF-beta signaling is altered, STAT-3 signaling is activated. Hence, targeting STAT-3 inhibition will help to restore SMAD-4 pathway.

Even though several chemicals were tested, only few of them such as dasatinib, LLC, STA-21, and Stattic have shown promising results in cell culture experiments and preclinical trials. STAT-3 has shown to be activated by several important pathways such as mitogenic signaling, as well as tyrosine kinase receptors such as Src, implying the importance of targeting these pathways to inhibit pancreatic cancer. Previous studies have established p60-Src as an oncogene and overexpression of Src tyrosine kinase has been reported in human pancreatic adenocarcinomas.

Transcription factor STAT-3 represents a key signaling point in pancreatic ductal adenocarcinoma (PDAC) [32]. Pancreatitis induction in mouse models expressing endogenous mutant Kras is reported, and activated STAT-3 was significantly increased. On the other hand, RNAi and chemical inhibition of STAT-3 in the mice resulted in blocking of acinar-ductal metaplasia and PanIN formation which resulted in reduced infiltration and IL-6 expression.

There are a number of drugs tested in pancreatic cancer that target JAK/ STAT-3 pathway. It is reported that pancreatic cancer has high Src activity, hence targeting Src activity by its inhibition by chemical compounds has been widely considered, one such chemical is dasatinib which showed to inhibit Src activity and promised to a key chemical compound in treating pancreatic cancer [33]. Dasatinib has also been shown to transiently inhibit STAT-3. Currently, a combination of dasatinib and another Src inhibitor, AZD0530, is underway in clinical trial for treatment of advanced PC.

There are several studies used targeting STAT-3 pathway which have been published that reported to treat pancreatic cancer. Guggulsterone, an inhibitor of STAT-3 pathway, in cell culture experiments has shown to decrease mucin expression in Capan1 and CD18/HPAF cell via transcription regulation by binding to JAK/STAT-3 pathway [34]. Not only chemicals but also microRNA treatment found to be effective in treating pancreatic cancer targeting STAT-3 such as MiR-216a overexpression markedly inhibited the JAK/STAT-3 signaling and xenograft tumor growth [35]. Crizotinib and nexrutine suppressed the growth of pancreatic cells in a dose-dependent manner by reduced activity of STAT-3 and NF-κB [36, 37]. Treatment with thiosemicarbazones in both in vivo and in vitro inhibited IL-6-induced activation of STAT-3 by decreasing phosphorylation at Tyr705 [38]. Some drugs were tested in combination such as metformin with aspirin which was found to significantly decrease the phosphorylation of STAT-3 [39]. Apart from several chemicals mentioned above, there are various natural products such as genistein derivatives used as STAT-3 inhibitors.

31.6 Structure of Genistein and Natural Sources

Genistein is a soy-derived isoflavanoid compound found to be a potent agent in treatment of cancer [40–42]. Soy-derived phytoestrogen compounds have been part of traditional Chinese cuisine known to have beneficial health properties. Although it is highly concentrated (0.2–1 mg/g of total weight) and widely isolated from soybeans nowadays, genistein was first isolated in 1899, from the dyer's broom, *Genista tinctoria*, hence, the chemical name derived from the source plant. Genistein is abundantly found in leguminous plant foods such as chickpeas, and it is moderately found in barley meal, broccoli, sunflower, cauliflower, clover sprouts, and clover seeds. It structurally resembles human endogenous estrogens with a diphenol structure (Fig. 31.5).

Intestinal cells, and not the liver cells, are the major site of metabolism for genistein [43]. In Intestine, genistein is conjugated with glucuronic acid and to a lesser extent to the sulfate [44, 45]. These genistein conjugates especially their glycosides are carried to the target tissue and are associated with lower cardiovascular diseases [46], hormone-dependent breast and prostate cancer [47], and colon cancer [48]. In 1999, the US Food and Drug Administration (FDA) approved a health claim for the cholesterol-lowering effect of soy protein, based on clinical trials that reported significant decrease in total low-density lipoprotein (LDL) cholesterol with soy protein intake (25 g/day) compared to same amount of animal protein consumption [49]. It has been shown that people consuming large amounts of genistein-rich soy products are less likely to be affected by breast or prostate cancer [50].

31.7 Genistein Inhibits the DNA Binding of Pro-tumorigenic Transcription Factors

Studies have demonstrated that genistein inhibited the activation of NF-κB in multiple cell lines such as LNCaP and PN-3 prostate cancer cell lines [51] [52]. Genistein at 30 μM for 24 h pretreatment abrogated the activation of NF-κB in PC-3 cells by the decrease in DNA-binding activity shown by EMSA [53].

Genistein inhibited NF-κB DNA binding, thus preventing nuclear translocation of the NF-κB. Genistein has been shown to increase p-STAT-3 and prevented DNA-binding activity at 72 h of reperfusion in a dose-dependent manner, but didn't

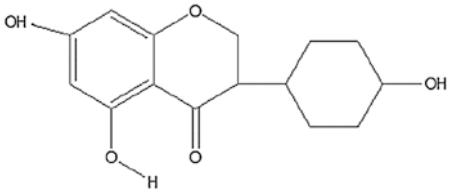

Fig. 31.5 Chemical structure of genistein

change the expression of STAT-3 [54]. Not only in prostate cancer cell lines, but the effect of genistein was also tested in pancreatic cancer cell lines.

Pretreatment of genistein for 24 h in human pancreatic cancer cell lines such as COLO 357 and L3.6pl followed by gemcitabine resulted in inhibition of growth up to 80% compared to only 30% in gemcitabine alone condition. In these pancreatic cell lines also, the study revealed that NF-κB activity was less [55]. Most interesting data shown by a study conducted at Karolinska Institute reported that patients who received genistein lived 6 months longer than their counterparts. Researchers at Karolinska developed genistein in a crystalline form with other components known as AXP107-11, shown to have better oral availability compared to its natural form of genistein and also have an improved physiochemical property. When patients were treated with gemcitabine in combination with AXP107-11, it resulted in a favorable pharmacokinetics and maximum tolerance dose. Forty-four percent of the total 16 patients in the study survived longer than 6 months, and half of them even survived longer than a year [56].

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Curcumin and Genistein Role in Regulation of STAT-3 in Pancreatic Cancer

32

Balney Rajitha and Ganji Purnachandra Nagaraju

Abstract

Pancreatic cancer (PC) is one of the most fatal malignant tumors across the world. STAT-3 is involved in PC growth and metastasis. STAT-3 is a transcription factor that regulates many oncogenic transduction pathways. In the current chapter, we discuss the importance of STAT-3 in hypoxia and hypoxia-inducible genes. Further, we also explore the inhibition of STAT-3 by phytochemicals such as curcumin and genistein.

Keywords

Signal transducer and activator of transcription · Pancreatic cancer · Curcumin · Genistein

32.1 Introduction

32.1.1 STAT (Signal Transducer and Activator of Transcription)

STATs are set of transcription factors that exist in unphosphorylated forms in the cytoplasm (during latent periods) [1]. STATs undergo tyrosine phosphorylation both by receptor and non-receptor tyrosine kinases. The function of Src and Abl leads to STAT phosphorylation.

The STAT family includes STAT-1 through STAT-6 proteins. Among these, STAT-1, 3, and 5 are more often activated in various cancers [2]. STAT-1 is basically growth suppressive and is not implicated in oncogenesis [3]. Data demonstrates the role of

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STAT-3 and STAT-5 in tumor progression. STAT-5 is more often activated in leukemia [4]. STAT-3 is triggered in head and neck cancer [5], multiple myeloma [6], prostate cancer [7], and breast cancer [8, 9]. STAT-3 activation is required for the transformation of several oncogenic proteins. Furthermore, cytokines (PDGF, EGF, and IL-6) and growth factors are capable of inducing STAT-3 [5–7, 10]. Together, these data demonstrate that STAT-3 is a mediator for several oncogenic proteins.

32.2 Is STAT-3 Involved in Angiogenesis?

STAT-3 is a key mediator of tumor angiogenesis. In many cancer cell lines including pancreatic cancer cell lines, STAT-3 activation is linked with the production of cytokine VEGF [11, 12]. Furthermore, the growth and oncogenic signaling pathways that enhance VEGF production are associated with STAT-3 activation. For example, results show that the activation of Src and EGFR signaling pathways lead to enhanced production of VEGF [8, 13–17]. This evidence shows that there is an interaction between the STAT-3 activation and VEGF expression in many cancers.

It was showed that STAT-3 is important for the production of VEGF due to tyrosine kinases and growth signals. In v-Src transformed cells, an elevated level of STAT-3 activity and VEGF expression was observed, and blocking STAT-3 considerably lowers VEGF levels [11]. The induction of VEGF by glycoprotein (gp) 130 or IL-6 relies on STAT-3 signaling [18, 19].

STAT-3 has also been shown to act as a mediator of angiogenesis through several other mechanisms. Recently, STAT-3 was demonstrated to control the expression of the angiogenesis and metastasis promoters MMP-2 and MMP-9 [20–22]. STAT-3 is required for the mitogenic role of PDGF [10]. Further, STAT-3 is also necessary for endothelial metastatic properties like cell migration and angiogenesis [23]. These results suggest that STAT-3 is a promoter of metastasis.

32.3 STAT-3 Is Necessary for Induction of HIF-1α by Hypoxia

HIF-1α induction by hypoxia is well characterized. Under normoxia, rapid degradation of HIF-1α takes place. When there is limited oxygen, HIF-1α is stabilized leading to rapid accumulation of protein. This mechanism appears to be independent of the increased rate of synthesis that occurs due to activation of the AKT/PI3K pathway. Decreased degradation and increased synthesis can coordinately enhance the levels of HIF-1α. Contrary reports exist regarding the necessity for the PI3K/AKT pathway in HIF-1α induction by hypoxia. Studies indicate that PI3K inhibition by dominant negative forms or by using chemical inhibitors could prevent the hypoxic induction of HIF1-α [24–26]. However, other reports indicate that hypoxic induction of HIF1-α is not blocked by chemical inhibitors or by inhibiting the PI3K/AKT pathway [2, 27–29]. Chemical inhibition of PI3K prevented the activation of AKT, concurrently with no effect on HIF-1α induction [2]. Hypoxia induces AKT and activates phosphorylation of GSK-3 in PC12 cell lines. These studies indicate that

AKT is not necessary for the hypoxic stimulation of HIF-1α. When STAT-3 was silenced, expression of AKT was decreased. Reports indicate that decreased HIF-1α expression is observed following induction with growth signals or IL-6. Thus, if STAT-3 silencing also prevents the hypoxic stimulation of HIF-1α, it is possible that other mechanism may be involved.

Although the importance of STAT-3 in HIF-1α induction by hypoxia was observed in cell lines expressing v-Src or activated c-Src, this was not examined in other cell lines. Recent studies have indicated that both HIF-1α and STAT-3 are important for the expression of VEGF in response to hypoxia or Src activation [30]. This study suggests that STAT-3, HIF-1α, and Src supportively stimulate VEGF expression in hypoxic environment. These results are not new, since the hypoxia-induced expression of both VEGF [31] and HIF-1α [30] requires Src. Overexpression of v-Src enhances HIF-1α levels [32, 33] and activates STAT-3 [17]. This data indicates the role of Src in response to hypoxia and that Src cooperates with STAT-3 and HIF1-α to enable that process.

32.4 STAT-3 Is Important for Inducing Multiple Hypoxia-Inducible Genes

To define the function of STAT-3 in mediating angiogenesis, researchers determined if STAT-3 was important for inducing hypoxia-inducible genes. Studies show that STAT-3 is required for VEGF oncogenic expression [11, 19]. STAT-3 silencing prevents the induction of VEGF by hypoxia. STAT-3 is important for the induction of MMP-2 and MMP-9 [20, 22]. MMP-2 and MMP-9 play a key role in degrading the extracellular matrix and are induced by hypoxia [34]. MMP-2 expression levels were decreased in dominant negative STAT-3D cells. Hypoxia also stimulated MMP-2 in MSCV control cells but is eliminated in STAT-3D-expressing cell lines. STAT-3 is necessary for the induction stimulation of MMP-2 and VEGF by hypoxia. STAT-3 regulates both genes at the transcriptional level, although both genes are under transcriptional control by HIF-1α.

32.5 STAT-3 Is Activated by Hypoxia

When oxygen levels are high, HIF-1 hydroxylation is mediated by PHD enzymes. Binding of VHL to the hydroxylated HIF-1α initiates degradation. During low levels of oxygen, HIF-1α cannot be hydroxylated. Interaction of VHL with HIF-1α is disrupted and deprivation is terminated.

HIF-1α stimulation by hypoxia is well-known, but the initiation of growth signaling due to hypoxia is not well defined. Reports indicate PI3K/AKT pathway activation was found in a few cell lines [2, 28, 35–37]. However, AKT activation by hypoxia is not common as HIF-1α and has not been detected in other cell lines including prostate and breast cancers [2, 26, 38].

Researchers have investigated the hypoxic activation of STAT-3 in detail. In prostate cell line, there was considerable STAT-3 stimulation after 24-h culture in hypoxic conditions. The exact concentration of O_2 cannot be measured in the hypoxia chamber, and as a substitute the hypoxia imitator $CoCl_2$ was used. In fibroblasts (BALB/c), there was considerable STAT-3 stimulation in 1 h, and levels remained elevated up to 4 h. In prostate cancer cells (DU145), STAT-3 activation was perceptible within 2 h and was high at 24 h. STAT-3 activation ceased at 48 h, accompanied with substantial cell death. Whole and nuclear cell lysates were examined by Western blotting to assess the expression of VEGF and HIF-1α. In distinction to the stimulation of STAT-3, accretion of HIF-1α was very fast with protein measurable at the early time frame of 30 min. Levels of HIF-1α were improved up to 24 h. STAT-3 signaling ceased and levels of HIF-1α remained high at 48 h. These data show the correlation between STAT-3 activation and VEGF expression. However hypoxic STAT-3 activation was observed in many cancer cells and was not found in melanoma cells. So, activation of STAT-3 by hypoxia is likely to be cell-type specific.

32.6 STAT-3 Is Necessary for Induction of HIF-1α by Oncogenic/Growth Signals

IL-6 stimulation has been shown to enhance HIF-1α expression. Even though induction of HIF-1α by IL-6 is limited in comparison to the hypoxia induction, it is elevated when compared with reported growth signals [39]. Elevated levels of HIF-1α observed after IL-6 treatment did not result from enhanced transcription. Thus, IL-6 is also capable of enhancing the expression of HIF-1α during normal conditions.

Because STAT-3 is the main effector for IL-6 signaling, researchers assessed whether it is also needed for HIF-1α induction. To accomplish this, STAT-3 knockout cancer cell lines were treated with IL-6 and examined by Western blotting. Compared with the control cells, the cells expressing STAT-3 siRNA showed limited HIF-1α induction in response to IL-6. Moreover, HIF-1α induction in DU145 cells (STAT-3 antisense oilgonucleotide treated) due to EGF was prevented. Evidence indicates that STAT-3 is important for the initiation of HIF-1α by various growth signals.

32.7 Phytochemicals

Treatment of pancreatic malignancies through modern drug-targeted therapies has certainly improved the overall survival rate among patients suffering from all stages of PC. However, the treatment of advanced metastasized PC remains untreatable. Therefore, there is a constant need to search for safer and more effective alternative chemoprevention and treatment options for PC patients. PC chemoprevention using bioactive components from natural plants or phytochemical compounds is an emerging strategy to prevent and cure numerous metastatic cancers including PC. Two most widespread and highly researched phytochemicals used in the treatment of PC are curcumin and genistein due to their low toxicity and increased efficacy.

Curcumin is a polyphenol that is resultant from the plant *Curcuma longa*. Curcumin is known to decrease the expression levels of STAT-3-regulated cyclin D1, BCL-2, and Bcl-xL [40]. STAT-3 regulates essential biological progressions such as proliferation, survival, and development, and its activity is vital for malignant transformation of cultured cells, which influence multiple oncogenic pathways. Survivin/BIRC5 are downstream targets of STAT-3 [41], and their elevated expression level is associated with apoptosis in PC cell lines as well in the advancement of pancreatic tumors [42]. Higher levels of STAT-3 and subsequent expression of survivin/BIRC5 are considered to be the most important factors in the promotion and survival of pancreatic tumor cells. Activated STAT-3 expression in tumor cells aids in modulating gene expression and is involved in regulating cell cycle progression, angiogenesis, as well as apoptosis. Therefore, inhibiting the STAT-3 signaling pathway with antisense oligonucleotides also inhibits the activity of survivin/BIRC5. In addition, blocking the activity of STAT-3 also inhibits cell proliferation (Fig. 32.1) and induces apoptosis in PC cell lines. This indicates the importance of STAT-3-mediated survivin/BIRC5 expression in the survival of PC cell lines [43]. Many investigations have revealed that the activation of STAT-3 and increased expression levels of survivin/BIRC5 take place simultaneously in PC cell lines. Therefore, developing strategies that could downregulate STAT-3 pathways as well as survivin/BIRC5 expression in PC cell lines could serve as significant breakthroughs in cancer treatment by inducing apoptosis and sensitizing tumor cells to chemo- and radiotherapies [44]. Phosphorylation of STAT-3 plays an important role in modification and proliferation of pancreatic tumor cells [45]. Phytochemical such as curcumin suppresses the phosphorylation of STAT-3 and downregulates the expression levels of antiapoptotic gene survivin/BIRC5 and leads to the apoptosis of PC cell lines [46]. Curcumin eliminates the constitutively phosphorylated form of STAT-3^{tyr705} in a concentration-dependent manner, which contributes toward pancreatic

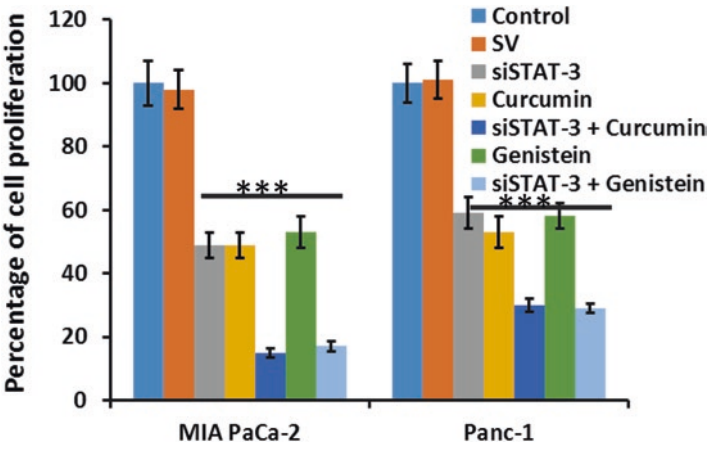

Fig. 32.1 Knockdown of STAT-3 sensitizes to curcumin and genistein and potentiates the inhibition of proliferation in pancreatic cancer cell lines

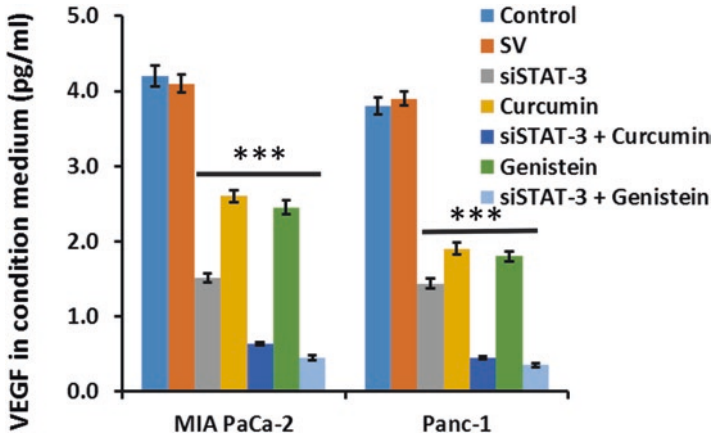

Fig. 32.2 Knockdown of STAT-3 sensitizes to curcumin and genistein and potentiates the inhibition of VEGF secretion in condition medium contained pancreatic cancer cell lines

oncogenesis by shielding tumor cells from apoptosis. Inactivation of STAT-3 in PC cell lines is shown to inhibit tumor proliferation (Fig. 32.1) and VEGF secretion (Fig. 32.2) [47]. Curcumin is known to increase tumor apoptosis through the cytotoxic properties of the natural killer (NK) cells and also block the phosphorylation STAT-3 [48]. These properties make curcumin a key therapeutic agent in facilitating apoptosis. In contrast to tumor cells, normal cells are relatively tolerant to interruption of the STAT-3 signaling, which makes STAT-3 an excellent target for molecular therapy of PC [49, 50].

Genistein, another important phytochemical that is an isoflavonoid found in soy products, is known to inhibit the activity of many tyrosine kinases including those that are responsible for phosphorylating STAT-3 [51]. Genistein also activates the physiological inhibitors, which are known to downregulate STAT-3 activation either directly or indirectly such as suppressors of cytokine signaling, STAT-induced STAT inhibitor, JAK-binding protein, and STAT-3-interacting protein [52]. Inhibition of STAT-3 signaling pathway via genistein is known to induce antiangiogenesis and apoptosis [53]. Genistein can significantly increase the EGFR inhibitor erlotinib-induced growth inhibition and apoptosis in PC cell lines [54]. Genistein also induces the antiangiogenic properties through significantly inhibiting the STAT-3 activity and VEGF secretion (Fig. 32.2). Genistein initiates the antitumor effect of 5-FU by inducing apoptotic and autophagic cell death in human PC cell lines by downregulating bcl-2 and upregulating beclin-1 expression levels [55]. These studies indicate the potential of curcumin and genistein as a supplementary therapeutic agent. Future studies should focus on outlining the molecular events following tumor exposure to low toxicity and natural agents like curcumin and genistein in combination with traditional cancer therapies.

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Role of Curcumin: A Suppressor of NF-κB Activity in Hepatocellular Carcinoma

33

Saipriya Lammata, Mundla Srilatha, and Ganji Purnachandra Nagaraju

Abstract

Leading as the third cause of cancer-related deaths in the world, hepatocellular carcinoma (HCC) is an aggressive cancer that offers little to no treatment for patients in the advanced stages due to the frequency of recurrence. Moreover, the upregulation of the nuclear factor-kappaB (NF-κB) signaling pathway leads to uncontrolled cell growth, metastasis, and resistance in HCC. Curcumin, a polyphenol derived from turmeric, has been found to inhibit NF-κB activity in HCC and to present other antitumor properties such as anti-proliferation, anti-inflammation, and anti-angiogenic properties. Furthermore, curcumin also acts as a collaborative agent with available chemotherapy and radiotherapy. Working against the drawbacks of poor bioavailability and rapid metabolism, researchers are discovering new ways of encapsulating curcumin in order to exhibit its full efficacy against HCC metastasis.

Keywords

Curcumin · NF-κB pathway · Hepatocellular carcinoma · Angiogenesis · Chemotherapy

The original version of this chapter was revised. The book was inadvertently published without Abstracts and Keywords, which are now included in all the chapters. An erratum to this chapter can be found at https://doi.org/10.1007/978-981-10-6728-0_39

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Abbreviations

Akt	Protein kinase B
bFGF	Basic fibroblast growth factors
COX-2	Cyclooxygenase-2
FLHCC	Fibrolamellar hepatocellular carcinoma
HBx	Protein X of the hepatitis B virus
HCC	Hepatocellular Carcinoma
HHC	Hexahydrocurcumin
IκBs	Inhibitors of NF-κB
IKK	IκB kinase
NF-κB	Nuclear Factor-kappaB
PCD	Programmed cell death
PEI	Percutaneous ethanol injection
PPAR-γ	Peroxisome proliferator-activated receptor-γ
THC	Tetrahydrocurcumin
VEGF	Vascular endothelial growth factor

33.1 Introduction

Caused by either hepatitis B or C, hepatocellular carcinoma (HCC) is a type of liver cancer that stands as the third most common cause of cancer-related deaths in the world [32]. Most cases of HCC range from less than ten patients per 100,000 people in North America and Europe to between 50 and 150 cases per 100,000 people in Asia and Africa [9]. When it comes to the treatment of HCC, surgical resection or liver transplant still stands as the primary approach for most optimal cases of patients in the initial stages of liver cancer [11]. Both resection and transplantation allow patients in the early phases of HCC to preserve any remaining liver functionality. Nonsurgical methods such as percutaneous ethanol injection (PEI), hormonal therapy, and systemic chemotherapy also serve as treatment options [33]. These treatment approaches seek to prevent the progression and any metastatic migration of HCC. Despite the aforementioned treatment methods, HCC patients who are diagnosed at later or advanced stages of the cancer have no hope for impactful treatment [10].

HCC progression and metastasis can occur due to the several signaling pathways at the molecular level. For instance, the nuclear factor-kappaB (NF-κB) pathway (a set of transcription factors) has been known to induce tumor promotion in hepatocytes [26]. In this pathway, NF-κB proteins—p50, p52, c-Rel, RelB, and p65—are retained in the cytoplasm of the cell by inhibitors of NF-κB (IκBs) until the pathway receives an appropriate stimulus, which triggers the ubiquitination of the IκBs and induces the translocation of these proteins into the nucleus [6]. When these proteins accumulate in the nucleus of a hepatocyte, the cell undergoes specific expression of certain genes. These genes that are involved in cell growth control, immune, and inflammatory responses eventually lead to oncogenesis [6]. When NF-κB was

inactivated in later stages of cancer, it was found that tumor growth was suppressed; therefore, drugs that target signaling pathways such as NF-κB could serve as efficient therapeutic approaches to the treatment of HCC [29]. Inhibition of the NF-κB pathway should be the focus of research as targeting molecular pathways can prompt the emergence of treatment that serves patients in the advanced stages of HCC.

Many investigations have chosen to look toward more natural agents in order to prevent the progression of tumors in several cancer malignancies. Inhibitors of NF-κB include the natural agent, curcumin, which exhibits anti-inflammatory, anti-necrotic, and anti-cancerous properties [26]. Curcumin is the primary polyphenol found in turmeric, an Asian herb that has been used for medicinal purposes since ancient times [31]. Throughout the decades, curcumin has been utilized by numerous civilizations as it exhibits pro-health qualities against diseases and tumorous characteristics. For instance, in the field of cancer, curcumin works with several genes and proteins—vascular endothelial growth factor (VEGF), cyclin D1, NF-κB, cyclooxygenase-2 (COX-2), and more—in order to reduce the progression of tumor growth in carcinogenesis [31]. Despite its salubrious nature, curcumin in the human body retains a low bioavailability and poor absorption [18]. Thus, many clinical trials involving curcumin are still undergoing as higher dosages of the compound are contemplated and researched.

33.2 Structure of Curcumin

Turmeric comprises of three primary curcuminoids in the following order from most to least: curcumin, demethoxycurcumin, and bisdemethoxycurcumin [39]. The structure of curcumin consists of the combination of methoxy groups on the phenyl rings [31]. Although this specific structure allows curcumin to exhibit anti-inflammatory and antioxidant qualities, it also prevents the stability of the compound in aqueous solutions, such as water, except for highly acidic solvents [7, 31]. In addition to highly acidic conditions, curcumin was also found to have dissolved in organic solutions under the presence of light, resulting in the photodegradation of the compound [40]. Lowering the pH of the solvent retained the chemical stability of curcumin. The compound's instability can be specifically attributed to a B-diketone moiety in the molecule's diene structure, and deletion of this group can lead to a potential stabilized structure of curcumin [23, 43]. Therefore, modification of the structure of curcumin permits researchers to incorporate the molecule into treatment of the HCC via regular physiological conditions of the human body.

Not only does curcumin exhibit chemical instability in neutral or physiological conditions, but the natural agent also presents poor bioavailability. The human body absorbs curcumin at a low rate due to several factors: the placement of bodily tissues, a decrease in plasma, and an elevation in metabolism [36]. Experiments containing high dosages of curcumin—about 400 mg—only demonstrated 60% absorption in a rat model, and researchers found a large portion of curcumin exiting the body via feces, aligning with the discovery of most of the curcumin in the large intestine [36]. In addition to the large intestine, metabolized curcumin was also

found in the liver [30]. Fortunately, heating curcumin increased its solubility in neutral aqueous solutions and did not destroy its biological identity [21]. Furthermore, the administration of curcumin is also significant in the resulting level of bioavailability; for instance, nasal and intravenous administration of curcumin was followed by higher levels of absorption in rat-based model as compared to that of an oral administration of the natural compound [30]. A low concentration of curcumin in the body prevents its full efficacy in the treatment of cancers such as HCC, often pushing research to implement either greater doses, ways to increase solubility, or modified versions of the golden compound.

A significant factor behind its poor bioavailability is curcumin's rapid metabolism. Research has found curcumin to remain in the body after ingestion, primarily in three locations: the liver, intestine, and kidney [17, 41]. These tissues have a high rate of metabolism, and this aspect coupled with the body's eradication of the natural compound results in low concentrations of curcumin in the body after treatment [41]. The amount of curcumin used in treatment—whether a high or low dosage—did not carry much significance because the compound quickly metabolized after ingestion [41]. In the body, however, curcumin metabolites include curcumin glucuronide, curcumin sulfate, tetrahydrocurcumin (THC), and hexahydrocurcumin (HHC) via catalysis by enzymes located in the cytosol and microsomes [17]. Several investigations have found traces of the aforementioned metabolites in the liver and intestine. Some metabolites were found to have the same salubrious properties as curcumin itself [17]. Although curcumin and its metabolites exhibit antitumor properties, the delivery as a treatment approach has pushed researchers to investigate other forms of modification of the natural agent such as curcumin nanoformulation, which has shown a decrease in the necessary dosage and in increase in the efficacy of the treatment [44]. Since the rapid metabolism of curcumin prevents its higher bioavailability in the body, further research is required into investigating the uses of curcumin metabolites and potential modifications—such as nanoparticles and other forms of encapsulation—so that the full efficiency of curcumin can be utilized in HCC treatment.

33.3 The Nuclear Factor-kappaB Pathway in Hepatocellular Carcinoma

The NF-κB pathway, also known as a set of transcription factors, plays an important and progressive role in many cancers, including HCC. The NF-κB pathway drives the metastasis of cancer cells [14]. As stated before, this signaling pathway consists of the following five proteins known as p50, p52, c-Rel, RelB, and p65, and these proteins remain inactive in the cytoplasm [14]. What keeps these proteins is the cytoplasm are molecules known as IκBs, another set of proteins that latches onto the domains of the aforementioned NF-κB proteins [14]. When activated by stimuli or signal cascades, the IκBs result in ubiquitination and start to degrade [45]. Stimuli that active the pathway include stress promoters and cytokines associated with an inflammation response [6]. When the IκBs start to degrade due to the activation of the NF-κB pathway, the translocation of the NF-κB proteins, which have dimerized

at this point, into the nucleus results in gene expression and responses: inflammation, cell growth, and ultimately the progression of cancer [27, 45]. The activation of the NF-κB pathway ends in the binding of the DNA in the nucleus and the upregulation of genes associated with inflammatory responses and apoptotic properties, which aid in the progression and metastasis of cancer cells [6]. The goal of suspending and preventing further metastasis and progression of HCC can give researchers a specific pathway to target in hopes of creating a therapeutic approach with a drug that inactivates NF-κB.

Not only does the activation of the NF-κB pathway result in gene expression of cancerous properties, but it also results in the stimulation of other NF-κB subunits [6]. Within the NF-κB signaling pathway exists two specific pathways: canonical and noncanonical [27]; the IκB kinase (IKK) and its two components, IKKalpha and IKKbeta, comprise the canonical NF-κB pathway as these molecular aspects of the pathway result in the ubiquitination and degradation of the IκBs [35]; on the other hand, the phosphorylation of p100 results in the translocation of the p52/RelB complex into the nucleus [38]. Despite either the canonical or noncanonical pathway, the NF-κB signaling cascade nonetheless plays a stimulating role in the progression of HCC tumorigenesis. For instance, Wang et al. [42] found that protein X of the hepatitis B virus (HBx) plays a potential role in the activation of the NF-κB pathway, leading to the promotion of tumor growth in cases of HCC. Furthermore, this investigation went on to find that a positive correlation existed between the amount of HBx and the amount of a NF-κB transcription factor known as p65, showing that HBx may indeed influence the activation of the NF-κB pathway [42]. Another investigation involving the technique of immunostaining and fibrolamellar hepatocellular carcinoma (FLHCC) tissue samples revealed that there were amounts of the NF-κB protein p65 found in FLHCC tissues as compared to little or none of the protein's presence in normal tissue [22]. A sharp contrast exists between the expression levels of NF-κB proteins in tumorous liver tissue as compared to the lack of the NF-κB pathway's influence in normal tissue; this shows that the NF-κB signaling pathway definitely plays a role in the progression of HCC tumorigenesis as its presence marks an identification between cancerous and noncancerous tissue.

In tumorous tissue, NF-κB tends to express levels of inflammation and promotes cell growth to advance HCC tumorigenesis. The NF-κB pathway was found to have a heavy influence in the control of apoptosis or an amplified form of programmed cell death (PCD), which stands as an essential factor in the promotion of tumorigenesis in HCC [12]. High levels of the activation of the NF-κB pathway were found in carcinoma tissues as the pathway promoted cell proliferation in vitro by preventing apoptotic expression [19]. Although the NF-κB signaling pathway may play other beneficial roles in the immune system, the translocation of NF-κB proteins into the nucleus leads to a dysregulation that advances tumorigenesis [19]. The versatile uses of the NF-κB pathway have led researchers to determine a way to prevent the promotion of tumorigenesis but retain the continuation of other immune responses for the benefit of other bodily systems [19]. The NF-κB pathway has several functions—advantageous and disadvantageous—in the human body's immune system; this objective leads to the development of an exclusive drug in HCC treatment with

the goal to regulate a multifarious signaling cascade by preventing the HCC cell proliferation and metastasis.

33.4 Curcumin and the Nuclear Factor-kappaB Pathway in Hepatocellular Carcinoma

Research has found the natural golden agent of curcumin as a mechanism of the NF-κB pathway's inactivation. The NF-κB signaling pathway is present in numerous cancers as the pathway silences apoptotic activity [20]. Curcumin has been found to suppress the NF-κB pathway and its subunits and to increase apoptotic results in HCC cell lines by depending on the mediation of caspase-3, caspase-8, caspase-9, or other molecules [28]. In addition to the suppression of the NF-κB pathway, curcumin also suppressed the expression levels of COX-2, which appears in other cancers and diseases; furthermore, curcumin also reduced the expression of cytokines associated with an inflammatory response [18]. Liver inflammation has been designated as a marker of HCC progression in the initial stages, and the use of curcumin has reduced expression levels of HCC indicators, such as the VEGFs and oxidative stress [1]. Not only does curcumin have apoptotic and anti-inflammatory qualities, but the natural compound has also led to the designation of markers in HCC progression by identifying the receptors that promote the progression of HCC.

In addition to apoptotic and anti-inflammatory action, curcumin also decreases levels of cell proliferation. For instance, by decreasing the expression levels of cyclin D1 and the Wnt/beta-catenin signaling pathway, curcumin has restrained the growth of HCC tumors and cell proliferation [20]. The beta-catenin signaling pathway regulates the expression of cell proliferation in numerous cancers and targets the protein known as cyclin D1 of which an increase can induce cell multiplication due to an uncontrolled cell cycle [37, 46]. An addition, however, must be made to the aforementioned statements; not only does curcumin exhibit anti-proliferative properties by inhibiting the Wnt/beta-catenin signaling pathway and cyclin D1, but the natural agent does the same job by activating a molecule known as peroxisome proliferator-activated receptor-γ (PPAR-γ). Through deactivation of the NF-κB pathway, the PPAR-γ molecule reduces inflammation and cell proliferation; in vivo models revealed that an increase in the expression levels of PPAR-γ occurred in the presence of curcumin [24]. The reduction of anti-apoptotic genes also decreased cell proliferation in several cancer cell lines, and curcumin had an impactful influence in this decline [34]. Overall, curcumin's targets are molecules or pathways that increase cancer cell amplification; therefore, by allowing curcumin to enter the body and inhibit these proliferative molecular structures, cancer cell proliferation ceases to continue.

Another factor that promotes tumorigenesis is the formation of new blood vessels, known as angiogenesis, which can be combatted by curcumin as this natural agent retains anti-angiogenic properties in addition to its aforementioned qualities: anti-inflammatory, anti-proliferative, and anti-apoptotic. Angiogenesis is the growth of new blood vessels from existing channels, and this formation aids in the

promotion of tumors [25]. Angiogenesis is one of the primary factors of tumor metastasis because it provides two functions: (1) providing nutrients to growing tumors and (2) allowing cancer cells to enter the body circulation [13]. Any dysregulation in oncogenes or tumor suppressor genes can result in the development of angiogenesis and the advancement of tumorigenesis [13]. By reducing the expression levels of proangiogenic receptors such as VEGF, correlated genes, and basic fibroblast growth factors (bFGF), curcumin exhibited anti-angiogenic expression and could prevent the progression of tumor growth [25]. In vivo study revealed a dichotomy of purpose in curcumin: first, the natural compound suppressed the proangiogenic markers to inhibit tumor growth, but curcumin could also inhibit angiogenesis in tumors that already expressed escalated levels of these proangiogenic indicators [5]. The upregulation of such proangiogenic markers—VEGFs, bFGFs, and oncogenes—is essential for tumor growth, yet curcumin suppresses these markers and even inhibits NF-κB activation; furthermore, the natural yellow compound also decreases the expression of cell adhesion molecules, which aid in the migration and metastasis of HCC tumors [8]. By inhibiting angiogenesis in HCC metastasis through the use of curcumin, researchers have been able to prevent the progression of tumorigenesis into advanced stages, where little to no treatment improves the patient's survival rate; rather, the utilization of curcumin offers protection from the advancement of HCC metastasis.

Although curcumin retains properties associated with anti-inflammation, apoptosis, and anti-proliferation, researchers have taken the natural agent found in turmeric and have combined it with the current chemotherapy available to discover new treatment approaches for HCC. For example, a chemo drug known as paclitaxel has shown anti-proliferative effects in the treatment of cancers such as breast and lung; its combination with curcumin, however, shows promising results in HCC treatment [47]. When exhibited on HCC cell lines, the combination of curcumin and paclitaxel showed that the addition of the natural yellow compound enhanced the antitumor properties of the chemo drug, reducing cell proliferation and promoting cell apoptosis [47]. Furthermore, paclitaxel is known to induce other signaling pathways such as the NF-κB and protein kinase B (Akt) pathways, which curcumin suppresses; in this way, the combination of paclitaxel and curcumin allows chemotherapy to continue without any detrimental activations that could impel tumorigenesis [3]. Paclitaxel, however, is not the only chemo drug that has been intertwined with curcumin in HCC treatment; cisplatin and doxorubicin, additional drugs in chemotherapy, have also displayed several benefits when used in combination with curcumin. The integration of curcumin and cisplatin as a treatment approach showed that cells were sensitized to just cisplatin, whereas curcumin and doxorubicin revealed supplementary effects on the cells [28]. Overall, incorporating curcumin with either cisplatin or doxorubicin revealed that NF-κB expression levels were lower than those of a single chemo drug at work [28]. As an instrument of sensitization and efficiency, curcumin enhances the effects of its complementary chemo drug, thus inhibiting the migration and invasion of HCC cells; this combination of curcumin and chemotherapy opens a new door of treatment options that offer a way to terminate, or potentially reduce the uncontrolled growth, HCC metastasis.

Researchers have not only experimented with curcumin and chemotherapy, but they have also taken note of the effects of curcumin on radiotherapy. Radiotherapy is imperfect in HCC treatment because the frequent recurrence of the cancer calls for higher doses of chemo, but this escalation in chemotherapy prevents the patient from fully undergoing irradiation [16]. Curcumin, however, sensitizes targeted cells to radiation and even to the effects of other chemo drugs in vitro and in vivo [4, 16]. In more specificity, curcumin was found to enhance the antitumor effects of radiotherapy by decreasing the expression levels of any NF-κB activity that radiation promoted initially; in this way, curcumin enhances the effects of radiation on the tumors while diminishing the activity of a signaling pathway that promotes tumorous agents [16]. In vivo study reveals that curcumin not only increased the anticancer effects of radiation, but the natural compound also enhanced the antitumor effects of gemcitabine, a known chemo drug, when put into use as a treatment approach [15]. Hatcher et al. [15] also revealed that the activation of the NF-κB pathway occurs due to the radiotherapy, but curcumin inactivates this signaling pathway known for cell growth and other NF-κB subunits. In fact, radiative toxicity relates to an abnormal regulation of the NF-κB signaling pathway, but curcumin protects cells from this damage—known as radioprotection—by inhibiting the proteins that stimulate this toxicity [2]. From its role in fighting cancer, researchers can see that not only does curcumin have anticancer properties, but the natural agent is a collaborative factor; curcumin works in several combinations with radiation and chemotherapy in order to enhance antitumor characteristics and lessen factors of tumor promotion.

33.5 Conclusion and Future Prospectus

HCC is an aggressive cancer that metastasizes very quickly; unfortunately, little to no treatment is available for patients undergoing advanced stages of this form of liver cancer. With the discovery and studies done on curcumin, however, research has allowed the possibility of offering treatment to patients at both initial and advanced stages of HCC. Curcumin, a natural golden compound found in turmeric, promises several salubrious effects: anti-inflammatory, anti-proliferative, and antioxidant. Thus, this yellow agent has made its debut in HCC treatment and leads to a promising future with chemotherapy, radiotherapy, and possibly targeted molecular therapy.

Although curcumin promises many pro-health qualities, the natural agent does have its drawbacks in deliverance. For instance, curcumin exhibits chemical instability in physiological conditions; the solubility of the yellow compound is only limited to highly acidic or organic solvents. Furthermore, curcumin displays poor bioavailability due to its rapid metabolism; in fact, the compound's fast metabolism has revealed that curcumin's metabolites exhibit the same properties as the original agent itself. Faulty deliverance of this natural agent as a therapeutic target is what prevented curcumin from exhibiting its full efficacy in treating HCC tumorigenesis. Thus, further research—such as the ongoing investigations on curcumin

nanoformulation—is required so that proper modifications can be made to condone curcumin's full efficiency.

Curcumin inhibits the NF-κB signaling pathway, also known as a set of transcription factors, in HCC. The translocation of these NF-κB proteins into the nucleus leads to gene expression of inflammation and cell growth of which both exhibit escalated levels in tumorous tissue. By deactivating the NF-κB pathway, curcumin enhances levels of apoptosis and anti-inflammation. Furthermore, the yellow compound also inhibits cyclin D1 and the beta-catenin pathway in accordance to enhancing expression of the PPAR-γ molecule in order to decrease cell proliferation and HCC metastasis. Curcumin does not stop at its anti-proliferative quality for the compound also reveals anti-angiogenic qualities: terminating formation of new blood vessels and inhibiting proangiogenic markers in developed HCC tumors. Lastly, curcumin pairs with chemotherapy and radiotherapy in order to inhibit the induced NF-κB activity and to sensitize cancer cells to the full exposure of the chemo drugs and radiation, which stand as complementary factors to the effects of curcumin. Alas, curcumin allows research to inhibit, or at least hinder, the aggressive nature of HCC metastasis as this golden compound welcomes the scientific community to a range of new treatment approaches that apply to patients at all stages of HCC.

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Exosomes Potentiate NF-κB Signaling, Tumor Progression, and Metastasis in Hepatocellular Carcinoma

34

Kishore Kumar Jella and Zhentian Li

Abstract

Worldwide, HCC is considered as one of the major cancer-related deaths. Tumor-derived exosomes play a potential role in HCC by mediating intracellular communication, immune responses, and antigen presentation. Exosomes communicate between the cells using proteins, mRNA, miRNA, lipids, and DNA present in their cargo. Increased understanding of exosomes and their role in cancer could lead to a powerful strategy for the treatment of HCC. In this chapter, we summarize the role of exosomes in cancer initiation, progression, and metastasis and in NF-κB through its miRNA. MiRNA derived from exosomes of HCC cells can enhance and modulate TAK1 and downstream signaling in recipient cells. Exosomes have a greater potential in the near future making it as prognostic biomarkers; they can serve in anticancer drug resistance and immunotherapy in the near future.

Keywords

HCC · Exosomes · miRNA · Proteins · NF-κB · TNF-α · TGF-β

Abbreviations

ACS	American Cancer Society
CAFs	Cancer-associated fibroblasts
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
EPCAM	Epithelial cell adhesion molecule

The original version of this chapter was revised. The book was inadvertently published without Abstracts and Keywords, which are now included in all the chapters. An erratum to this chapter can be found at https://doi.org/10.1007/978-981-10-6728-0_39

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ESCRT	Endosomal sorting complex required for transport
FAP	Fibroblast activation protein
Flt-3	FMS-like tyrosine kinase-3
GPC1	Glypican I
GTPase	Guanosine triphosphate
HCC	Hepatocellular carcinoma
HCV	Hepatocellular virus
HIF	Hypoxia-inducing factor
IL-1	Interleukin 1
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MMPs	Matrix metalloproteinases
NF-κB	Nuclear factor kappa B
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDK1	Phosphoinositide-dependent kinase I
TAK	TGF-β-activated kinase
TGF	Tumor growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor

34.1 Introduction

In adult men, liver cancer is considered the fifth most frequent cancer, and it is the second leading cause of death related to cancer [1]. Among adult women, liver cancer is considered as the seventh most common diagnosed cancer and sixth in causing cancer-related deaths. Nearly 70,000 people are diagnosed with liver cancer every year around the globe. It is considered as a leading cause of death among cancer patients that account for 6,000,000 every year [1]. The ACS estimates that in the United States, there are about 40,710 new cases of liver cancer (29,200 in men and 11,510 in women) that will be diagnosed and around 28,920 people will die of this cancer [1]. Among the population, liver cancer has occurred more than three times since 1980. The death rates of liver cancer patients have been increased by 3% per year since 2000. It is most commonly seen among women. It is most commonly observed in countries such as sub-Saharan Africa and Southeast Asia [1].

In the liver, stellate cells, hepatocyte, and immune cells secrete exosomes, and they play a vital role in liver homeostasis by mediating communication between cells. Exosomes are cell-derived vesicles with lipid bilayer, sizing from 30 to 150 nm in diameter, with a cup-shaped morphology; the conserved lipid layer matches with the parental cells. They are secreted by a wide variety of mammalian cells and presented in many biological fluids [2–5]. The vesicle density ranges from

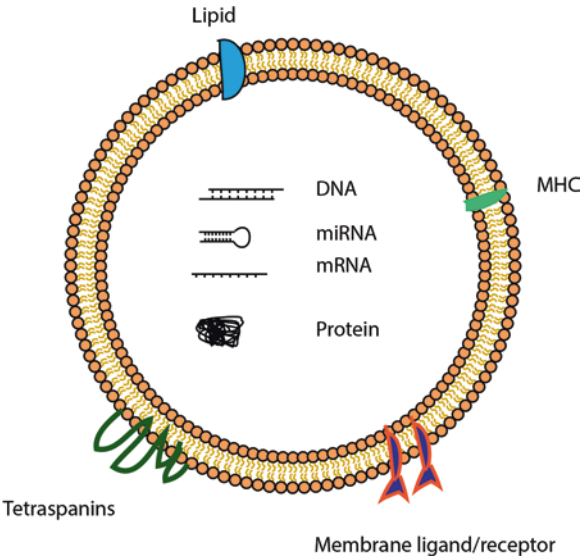

Fig. 34.1 Illustrate the basic structure of exosomes

1.12 to 1.19 g/ml reported by density gradient separation studies [6, 7]. Exosomes are formed through the endolysosomal pathway; they are released upon fusion of the multivesicular bodies (MVBs) with plasma membrane to the outside of the cells [8]. The function of exosomes were initially considered as "garbage bags" to release the molecular fragments of cells to its extracellular environment in the 1990s, during when exosomes were found to play a role in the presentation of B lymphocyte antigens; thus these exosomes were thought to relate with the functions of the immune system [9]. After that, immunology became a focus of exosomes research but mainly in its physiological roles, such as interaction between tumor and immune cells. After the year 2010, miRNA, mRNA, and DNA were discovered in exosomal vesicles by several research groups [10–16] reviewed in [17]. Now, exosomes are considered as messengers to communicate between cells and transfer information between them. The basic exosomal structure is depicted in Fig. 34.1.

Exosomes were reported in various pathophysiological processes such as tumorigenesis, inflammation, and drug resistance [17]. The lipid structure is important in cell-cell communication; exosomes regulate various signaling pathways that play a crucial role for any organism in both normal and disease states. To date, 92,897 proteins, 27,642 mRNAs, 4934 miRNAs, and 584 lipids have been reported from a wide variety of tissues. The exosomal RNA was found to shuttle to distant sites and regulate the function of remote cells as a signaling messenger [19]. It was also found that exosomes can circulate in the blood and interact with platelets and endothelial cells in vivo [20]. Most importantly, exosomes can affect the biological behavior of remote cells and educate the target cells to respond accordingly, which play a major role in the development of disease [21, 22].

34.1.1 Exosomal Communication

It has been confirmed that exosomes can interact and communicate with the recipient cells by several studies [23, 24], but the direct interaction mechanism is largely unknown. Based on in vitro studies and some other evidence, mechanisms of uptake of exosomes have been proposed: [1] exosomes bind to the surface of recipient cells through adhesion molecules, [2] fusion of extracellular vesicles after adhesion with plasma membrane of target cells, or [3] through receptor-mediated endocytosis process or phagocytosis by internalizing the exosomes into endocytic compartments. The interaction between target cells and exosomes occurs through transfer of membrane receptors, growth factors that are expressed on the surface of vesicles, or delivery of specific proteins to target cells. Once exosomes are taken up by the recipient cells, exchange of internal genetic material could be the next level of communication through these vesicles [25]. Importantly, a well-known mechanism of intercellular communication is through signaling molecules such as proteins to interact with the receptors that are present on the surface of target cells. Exosomes can transfer a wide range of molecules such as proteins, RNA, DNA, and lipids and regulate various pathways in recipient cells at various sites. To a little extent, the exosomal secretion of cells may be considered as endocrine secretion and regulation of an organism.

34.1.2 Exosome Biogenesis

Exosomes contain a distinct set of proteins, which are endosomal/lysosomal related, without any protein of nuclear, mitochondrial, or endoplasmic reticulum origin. Exosomes, different from microvesicles which originated from the plasma membrane [26], are derived from endosome origin. The initial step of exosome formation is when endocytic vesicles fuse together to form early endosomes, followed by maturation into late endosomes [3, 27, 28]. *ILVs* are then formed by invagination of the endosomal membrane, in which the vesicles are formed on the inner membrane of the endosome and internalized [3, 27, 28]. Endosomes containing multiple ILVs are referred as multivesicular bodies [28]. Spontaneous fusion of MVBs with the plasma membrane leads to the release of ILVs. The released small vesicles are termed as exosomes (Fig. 34.1). In addition to be released as exosomes, ILVs could also be degraded by fusion of MVBs with lysosome; they could also contribute to the biogenesis of other lysosome-related organelles, such as melanosomes, azurophilic granules, etc. [29]. Though the mechanisms of biogenesis and secretion of exosomes are still unknown, recent reports revealed that syndecan, heparan sulfate proteoglycans, and syntenin regulate the formation of exosomes [30, 31]. The secretion of exosomes is regulated by Rab GTPases pathway [32, 33]. The delivery and secretion of exosomes to their recipient cells are regulated by ESCRTs, Ca^{+2} channels, and cellular pH [34–39]. The recipient cells take up exosomes either by receptor-mediated endocytosis or receptor-ligand fusion or fusion process [40]. Exosomal cross talk between donor cells (secreting cells) and recipient cells is clearly shown in Fig. 34.2.

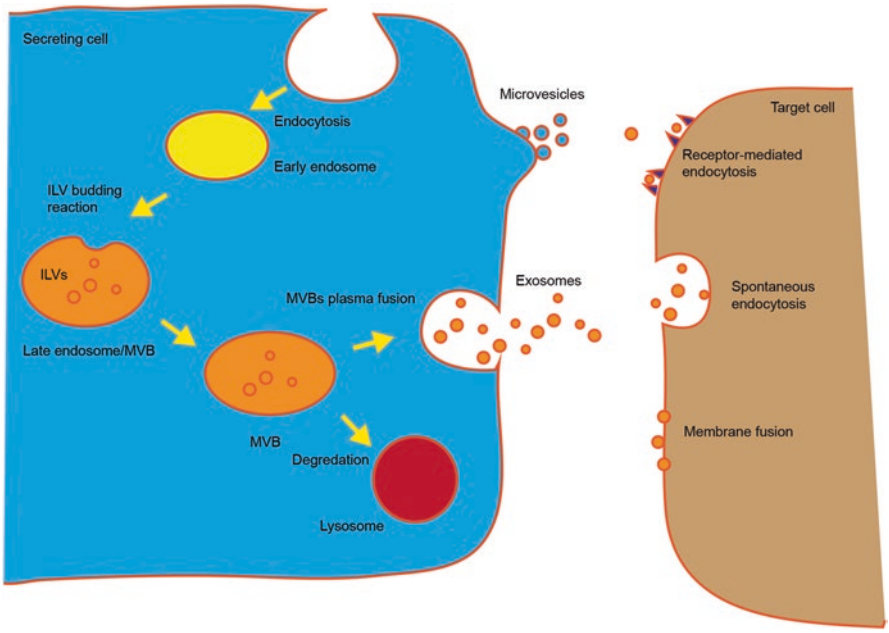

Fig. 34.2 The secretion of exosomes by donor cells and uptake by recipient target cells

34.1.3 Exosomes in Cancer Initiation and Progression

The tumor occurs with the incidence of one or multiple mutations within the cell(s), leading to the transformation of cells and other hallmarks of cancer [41]. During this initiation and progression of the tumor, intercellular communication occurs between cancer cells and neighboring cells, resulting in the substantial changes in proteomic and transcriptomic profiles of affected cells, while the exact cell-cell communication pathway is still unclear. The role of exosomes in tumor progression has gained enormous interest in the recent years. Tumor exosomes are thought to be taken up by neighboring cells and alter their physiological properties by transferring signals from donor cells. Exosomes are shown to play a role in immunosuppression, angiogenesis, cancer initiation, and progression. It has been shown that exosomes change the microenvironment and help in the occurrence of a niche for cancer initiation and progression [42, 43]. Multiple studies indicated that exosomes released by tumor cells are taken up by various types of surrounding cells in the microenvironment, including epithelial cells, endothelial cells, fibroblasts, and immune cells. Stimulation of cancer initiation and progression was observed [44–51]. Exosomes produced from stromal cells were also found taken up by the tumor cells resulting influence in both directions [52, 53]. Transformed tumor cells have been reported to carry oncoproteins such as EGFR and K-Ras to their neighboring cells lacking their expression and results in achieving unrestricted proliferation, which promotes cancer progression [54, 55]. Oncogenic viral proteins were also carried by exosomes,

resulting in the transformation of normal epithelial cells to neoplastic cells [56]. Since exosomes carry materials from tumor cells, it could potentially serve as biomarkers for early cancer diagnosis; it was reported that exosomes harboring GPC1 proteins might serve as a tool for early diagnosis of pancreatic cancer [57].

Invasion of surrounding stromal tissue by cancer cells is an important pathological step for malignancy. The transition of EMT, which is a phenomenon being studied extensively, supports this key step [58]. Exosomes derived from tumor cells have shown to carry pro-EMT proteins that are comprised of TNF-α, TGF-β, PDGF, and other MMPs (matrix metalloproteases) that plays vital role in remodeling of stoma adjacent to tumor [50, 56, 59]. This results in the transformation of stromal fibroblasts to CAFs that play an essential role in agonistic tumor progression [44, 47, 60–62]. It has been shown that tumor exosomes can enhance TGF-β/Smad signaling pathway that initiates differentiation of mesenchymal stem cells, normal stromal fibroblasts, and myeloid-derived suppressor cells to pro-angiogenic myofibroblasts [47, 48, 63, 64]. Cytosolic redox state is also very important for exosomal signaling in tumor progression; exosomes released from tumor cells in hypoxic conditions are functionally different from the exosomes produced from the same tumor cells growing in normoxic conditions. Exosomes released from tumor cells in hypoxic conditions regulate the host angiogenesis which leads to the formation of new blood vessels for the tumor. Tumor exosomes release growth factors that stimulate pericytes through the activation of AKT pathway and affect surrounding endothelial cells [65].

34.2 Exosomes Cross Talk with Transcription Factors

The occurrence of transcription factors in the contents of exosomes derived from neoplastic cells may transfer signal to distant cell and results in cell alteration including transcription; it results in the transfer of signals from normal to cancer cells and vice versa. Transfer of transcription factors from cancer cells to distant normal cells may mediate malignant signaling pathways to transfer progression of the disease. Such type of examples mainly exists in nutrient deprived cancer microenvironment, here the cancerous cells involves in upregulation of HIF1A, EGFR and VEGF that can transfer signals to nearby cells via exosomes. The signaling of transcription factors via exosomes will prime proximal cells for microenvironment toxicity.

34.2.1 Exosomal miRNA in Hepatocellular Carcinoma

Secretions of exosomes by cancer cells were explored by various scientific methods [66–69]. Among them, differential ultracentrifugation approach is considered as the best method of choice for purification of exosomes from cell culture supernatants. The cargo of exosomes containing miRNA plays a wide role in transmitting signal from donor to recipient cells. The content of RNA in Hep3b and PLC/PRF/5 cell

exosomes was investigated [70]. Interestingly, the miRNA size was found to be less than 200 bps in size with less fraction of internal control RNA [70]. In Hep3B cell-derived exosomes, the expression of 11 miRNAs was detected (miR-584, miR-451, miR-517c, miR-378, miR-520f, miR-142-5p, miR-376a, miR-133b, and miR-367). Identification of these miRNA predicts the selective enrichment of miRNA in exosomes derived from HCC [70]. With interesting facts, other miRNAs such as miR451 seem to enter into exosomes preferentially in various cell types [71].

Transfer of exosomes mediated miRNA is involved in the modulation of growth and progress of HCC [70]. Upon internalization, these exosomes release their miRNA into recipient cells to mediate the transfer of functional transgenes and modulate their cell activities. The transfer involves the regulation of gene expression, cell behavior, and signaling and the transformation of recipient cells. An analysis of 108 genes in exosomes identified that they are involved in the TAK1 pathway, which could be a potential candidate pathway for these targeted miRNA [70]. The TAK1 pathway was found to associate with the activation of signaling cascade mediated by TNF-α, TGF-β, and IL-1 [72]. TAK1 pathway is an upstream member of MAPK pathway, which is an important component of cell homeostasis, tumorigenesis, and intercellular communication in the liver. Downregulation or complete loss of TAK1 expression in liver hepatocytes has considerable links for the progression of HCC [73]. Modulation of TAK1 expression and its association with signaling pathways in cells that uptake exosomal miRNA could represent tumor progression. Exosomes derived from HCC can transfer their miRNA into recipient cells and inhibit the continuous expression of TAK1 and affect its downstream signaling pathways that are involved in the development of metastasis. Hep3B-derived exosomes are capable of inducing anchorage-independent growth of transformed cells and decreasing cell viability in recipient cells [70].

Exosomal miRNA and long noncoding RNA (lncRNAs) are capable of HCC progression and lead to the failure of its treatment. It was reported that increased expression of miR-429 in liver tissue resulted in hypomethylation that can be used as a predictive factor in patients suffering from HCC [74]. HCC cells that are enriched with miR-429, results in $(EPCAM)^{+}$- T-ICs can results in shedding of exosomes carrying miR-429, thus resulting in tumor formation. miR-429, which is released by exosomes into surrounding target cells, targets retinoblastoma binding protein 4 expression and results in increased transcriptional activity of E2F1, leading to increased expression of POU class 5 homeobox 1 proteins in target cells [74]. Due to this reason, exosomes play a significant role in self-renewal of hepatocytes, malignant proliferation, tumorigenicity, resistance to chemotherapy, and progression of the disease. Exosomal transfer of linc-RoR may result in the failure of anticancer therapy toward HCC [75]. In normal hepatocytes, if the levels of linc-RoR decrease, tumor progression and cell proliferation are inhibited by increasing the stability of mRNA that encodes c-myc [76]. There is a decreased expression of miR-145 with increased expression of linc-RoR in HCC; therefore, it increases the expression of HIF-1α and PDK1 expression and thus improves overall function of mitochondria in hypoxic conditions [78]. TUC339 is a highly expressed lncRNA in exosomes derived from HCC. There is a significant reduction of HCC

cell proliferation and adhesion with suppression of TUC339 using siRNA; these results suggested the role of TUC339 in HCC growth and metastasis [77].

Liver-specific miRNA miR-122 plays an important role in physiological functions of the liver and promotes HCV replication [78]. MiR-122 can be transferred between Huh7 and HepG2 cell lines via exosomes and reduces cell proliferation and growth of recipient cells [79]. Exosomes can also involve the transfer of miRNA between different cells; exosomes derived from adipose-derived mesenchymal stem cells can transfer miR-122 to the other HepG2 cells [80]. Exosomes derived from colorectal cancer cells carry miR-221, miR-192, and miR-21 to HepG2 cells and A549 cells [81]. All these findings suggest that exosomal miR can shuttle between different cells and promote HCC invasion and progression. Exosomal miRNA transfer between the cells regulates gene expression. miR-122 that are effectively packaged into exosomes of HCC cells, resulted in the expression of their target genes such as insulin growth factor receptor 1 and cyclin G1 in hepatoma cells [80].

34.2.2 NF-κB and Its Regulation Through miRNA

Activating NF-κB signaling pathway via TLRs/NLRs following infections is a common response in a wide variety of epithelial cells. The transcription factor NF-κB contains five members: p50, p52, RelA (p65), RelB, and c-Rel. In many cells, the transcription factor NF-κB exists in the cytoplasm as latent state and is bound to inhibitory kBs which mask its nuclear localization signal. The activation of NF-κB helps to move into nucleus and results in the regulation of gene expression including miRNAs [82]. For positive gene regulation, the transcription domain of NF-κB is necessary, and it is present only in p65, RelB, and c-Rel; thus promoter binding is involved in activation of various gene expressions. As p50 and p52 lack transcription activation domain, they are mainly involved in transcription repression [70, 83, 84].

In THP-1 monocytes, TLR signaling is activated in NF-κB-dependent manner for the transcription of miR-146a gene [85]. Several studies were carried in non-epithelial cells and malignant cells to identify the transcription of miR genes in NF-κB-dependent manner [86]. MiR-155 that plays an important role in inflammation was found to be activated by NF-κB signaling pathway in several cell types in response to LMP1 and LPS [87, 88]. In a similar way, both miR-146a and miR-155 genes are to be induced via NF-κB-dependent manner to several immune mediators such as LMP1, LPS, IL-1β, and TNF-α. NF-κB pathway also regulates miR-16 and miR-21 in gastric cancer cell line. NF-κB also involves the activation of miR-301a in several pancreatic cancer cells [89]. In human biliary epithelial cells, a subset of miRNA genes are expressed via NF-κB activation under the response of LPS stimuli [90]. A protozoan parasite, *Cryptosporidium parvum* upon infecting biliary epithelial cells results in the activation of a TLR4/NF-κB signaling pathway, also shown the expression of miRNA gene [91]. Inhibition of NF-κB pathway using IKK2 inhibitor (SC-514) blocked the increased expression of pri-miRNAs, including pri-miR-125b-1, pri-miR-30b, pri-miR-21, and pri-miR-23b-27b-24-1 [90, 91]. In relation to NF-κB pathway, miRNA is involved in both upregulation and downregulation.

In biliary epithelial cells, transcription of *let-7i* genes in response to stimulation of *C. parvum* and LPS has been suppressed by binding of p50 subunit of NF-κB pathway [92]. Along with that, NF-κB pathway negatively regulates the transcription of miR-29b by interacting with another transcription factor in various cell lines [93, 94].

34.3 Treatment of HCC

Suitable markers for the identification and treatment of HCC are still lacking; presently resection, liver transplant, treatment with radiation, and chemoembolization remain to be major choices for the treatment of HCC. It has a high risk of recurrence, and the metastasis of HCC is strongly influenced by various environmental factors. Radiation is the major treatment for cancer treatment. The contributions of nontargeted effects of radiation for the initiation and development of secondary cancer are far less clear [95–101]. Recent studies on exosomes revealed that they are a very good source for identifying potential biomarkers for the treatment of HCC. Cancer cells are surrounded by numerous fibroblasts and inflammatory cells that release various exosomes, growth factors and cytokines, or enzymes related to matrix remodeling. In such environment, cancerous cells gain the ability to proliferate, invade, and grow; this made many researches to work on tumor microenvironment [102]. Scientists follow different strategies to target tumor microenvironment by targeting extracellular matrix components or by blocking cross-talk signals between cancer or cancer and epithelial cells through their stroma [103, 104]. Treatment options for HCC are clearly shown in Table 34.1. Treatment of HCC has two major components such as targeting components of angiogenesis and inflammatory pathways. Many drugs have been designed to target these pathways [105]. One of the most efficacious drugs, sorafenib (a multi-kinase inhibitor), is capable of targeting VEGFR, Raf-kinase, and PDGFR and suppressing both angiogenesis and cell proliferation. Sorafenib successfully completed phase III trials to confirm its tolerability and safety [106]. Drugs such as brivanib (targets VEGFR and FGFR), sunitinib (VEGFR, PDGFR, FLT-3, C-KIT), linifanib (VEGFR, PDGFR), bevacizumab

Table 34.1 Treatment of HCC with various drugs

Agent	Target	Clinical phase stage
Sibrotuzumab	FAPs	I [118]
Galunisertib	TGF-β	I [117]
PI-88	HPR	II [109]
Bevacizumab	VEGF	II [119]
Erlotinib	EGFR	III [120, 121]
Cetuximab	EGFR	II [122]
Selumetinib	MEK	II [123]
Axitinib	VEGFR	II [124]
Linifanib	VEGFR	III [124]
Sorafenib	VEGFR, PDGFR	III [106]
Brivanib	VEGFR, FGFR	III [125]

(targets VEGF), erlotinib (targets EGFR), axitinib (targets VEGFR), and cetuximab (targets EGFR) are presently being used in various clinical trials [107]. The PI-88 suppresses HCC metastasis through inhibition of sulfatase and heparanase enzymes. This formulation can also negatively inhibit angiogenesis in tumor cells [108]. In a phase II clinical trial, a dose of 160 mg/day of PI-88 is considered safe for patients who have undergone surgery [109]. Inhibition of stromal cells that are actively involved in the development of neoplastic niche, among these cells, TAMs, HSCs, and CAFs are considered as good candidates. The drug sibrotuzumab actively targets HSCs with specificity [110]. Both HSCs and myofibroblasts express serine proteases on their membranes and act as FAP [111]. FAPs belong to prolyloligopeptidase gene family that plays an important role in the field of tumor biology, and they are well expressed on several solid cancers such as gastric breast [112], and HCC [113]. FAPs function as ECM remodeling enzymes that are capable in targeting collagen that are actively produced for the tumor invasion and growth [114]. Targeting TGF-β in HCC is another approach of targeting central signaling pathway. TGF-β is mainly produced by HSCs, and it is involved in the synthesis of ECM and helps in remodeling of cell proliferation and migration. Galunisertib is a well-known TGF-β inhibitor that specifically reduces neoangiogenesis, desmoplastic reaction, and intrastation in HCC [115, 116]. This drug is in the phase II stage of clinical trials for patients who failed response toward sorafenib therapy [117].

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Role of Hypoxia-Inducible Factor (HIF) in Liver Cancer

35

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Abstract

Liver cancer is one of the major causes of cancer-related deaths in the United States, accounting for 4.5% of the total estimated cancer deaths in 2016 and standing as the second leading cause of cancer-related deaths in men worldwide in 2012. There are two major types of primary liver cancers, including hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA). The most common type of primary liver cancer is HCC, which begins in hepatocytes and accounts for approximately 75% of all liver cancers. Hypoxia, a condition of oxygen deprivation in the tissue, is a common feature of the cancer microenvironment due to increased cell proliferation and limited blood supply. Hypoxia-inducible factor-1 (HIF-1) was the first transcription factor discovered to regulate a wide range of target genes involved in many cellular processes in response to low oxygen levels. HIF-1 is a heterodimeric protein complex composed of two different subunits, α and β. During a condition of hypoxia, HIF-1 heterodimer activates target genes that contain a hypoxia response element (HRE) in the promoter region. The overexpression of HIF-1 is frequently observed in many human solid tumors, including liver cancer, and is associated with tumor development, poor prognosis,

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and resistance to chemotherapy, suggesting that HIF-1 is a new therapeutic target in liver cancer treatment. In this chapter, we define the molecular mechanism that controls HIF-1 and how it maintains a variation of biological processes in hypoxic environments.

Keywords
Liver cancer · Hepatocellular carcinoma · Hypoxia-inducible factor · Metastasis · Cell cycle

35.1 Introduction

The liver is a central organ that has many functions, including the metabolic change of excessive nutrients, the detoxification of toxic metabolites, the supply of energy-producing substrates, etc., all divided into eight functionally independent segments according to French surgeon Claude Couinaud's classification system [1]. These eight segments are numbered in Roman numeral fashion (segment I to VIII) in a clockwise manner. Segment I is located posteriorly and is not visible anteriorly. Furthermore, according to Bismuth [2, 3], segment IV can diverge into segment IVa and IVb.

Liver cancer is one of the major causes of cancer-related deaths in the United States, accounting for 4.5% of the total estimated cancer deaths in 2016 and standing as the second leading cause of cancer deaths in men globally during 2012 [4, 5]. In cases of males aged over 55 years in the United States, the incidence rate of liver cancer has increased in contrast to the stable trends for incidences driven by the three major cancers such as lung, prostate, and colon cancer [4]. There are two major types of primary liver cancers, including hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA). The most common type of primary liver cancer is HCC, also known as hepatoma, which begins in the hepatocytes and accounts for approximately 75% of all liver cancers. Fortunately, well-recognized indicators in HCC give notification to populations that need screen testing and monitoring [6]. The risk factors associated with HCC development are chronic hepatitis B virus (HBV) or the hepatitis C virus (HCV) along with aflatoxin B1 exposure, chronic alcohol consumption, cirrhosis, or diabetes [7–10]. HBV and HCV infect approximately 2 billion and 170 million individuals worldwide, respectively [11, 12]. Bile duct cancer (CCA) accounts for 10–20% of all liver cancers [13]. Located in the small bile ducts of the liver, CCAs are best classified according to their anatomical location as intrahepatic (iCCA), perihilar (pCCA), or distal (dCCA) [14]. Overall survival rate for liver and intrahepatic bile duct in the United States was 18% from 2005 to 2011 [4]. Other types of liver cancer such as hepatoblastoma originated from immature liver precursor cells and are much less common [15]. Common liver cancer symptoms include weight loss, decrease in appetite, nausea, vomiting, fatigue, enlarged liver (hepatomegaly), enlarged spleen, abdominal swelling (ascites), or jaundice.

Hypoxic regions in HCC are marked by the indicators of increased cell proliferation and limited blood supply. Furthermore, hypoxic conditions can promote HCC tumorigenesis by promoting angiogenesis [6]. Hypoxia-inducible factor-1 (HIF-1),

a transcriptional factor responsible for regulating a wide range of target genes involved in many cellular processes such as cell proliferation, cell cycle, metastasis, angiogenesis, apoptosis, cell survival, energy metabolism, and more stands as an essential component in a hypoxic state [16–18]. At the current point in research, approximately 60 genes have been identified to be transcriptionally activated by HIF-1 in which the promoter regions of its target genes consist of a *cis*-acting transcriptional regulatory sequence known as a hypoxia response element (HRE) [19]. The regulation of various genes and their transcription occurs through the binding of HIF-1 to the DNA regions of HRE [20].

HIF-1, first discovered as a transcription factor, regulates erythropoietin expression in response to low oxygen (O_2) levels in the blood via de novo protein synthesis [21]. Erythropoietin stimulates bone marrow to produce more red blood cells (i.e., erythrocyte), resulting in the increase of the oxygen-carrying capacity in blood.

As a heterodimeric protein complex, HIF-1 is comprised of both subunits α and β. Both HIF-1α and HIF-1β subunits comprise of the basic helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS) domains [22–24], which allow the heterodimerization to occur. DNA binding on HRE with the according sequence (5′-G/ACGTG-3′) in the promoter regions of target genes occurs due to the presence of the bHLH domain [19]. Aryl hydrocarbon receptor nuclear translocator (ARNT), also known as HIF-1β, is constitutively expressed and insensitive to O_2; under normoxic conditions, HIF-1α is sensitive to O_2 and is degraded through the ubiquitin-proteasome pathway [25, 26].

HIF-1α in normoxic conditions gets hydroxylated on proline residues at the oxygen-dependent degradation domain (ODDD) by prolyl hydroxylase domain enzymes (PHDs), which promote the binding to von Hippel-Lindau (VLH) proteins in the E3 ubiquitin ligase complex for ubiquitination and proteasome-mediated degradation [27, 28]. During hypoxia, PHD activity is a frequent characteristic of the cancer microenvironment (generally at pO_2 levels <2%) and leads to the unhydroxylation of HIF-1α even under limitations of O_2 availability. HIF-1α then translocates into the nucleus and binds with subunit HIF-1β. The HIF-1 heterodimer forms a complex with CREB-binding protein (CBP)/p300. Hypoxia prevents the hydroxylation of Asn803 and promotes the activity of CBP/p300 in the carboxyl-terminal transactivation domain of HIF-1α [29].

Many human cancers—liver, breast, pancreatic, etc.—contain the elevated expression levels of HIF-1 [30–34]. This elevation in HIF-1 expression in HCC cell lines is correlated with the advancement, poor diagnosis, and resistance of HCC cell lines, indicating that HIF-1 stands as a molecular target in HCC treatment [35–37]. Researchers also found HIF-1 as an indicator of HCC progression as higher levels of HIF-1 were found in the sera of HCC patients as compared to the lower levels in cases of benign disease [38, 39].

35.2 Role of HIF-1α in Cell Proliferation and Apoptosis

Hypoxia induces expression of several growth factors including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor B (PDGFB), transforming growth factor (TGF)-α or TGF-β, insulin-like

growth factor-2 (IGF-2), endothelin-1 (ET-1), and erythropoietin (EPO), which are known to promote cell proliferation in different cancer cells [40–43]. Cell proliferation stands as the primary concern in maintaining cell survival in the condition of hypoxia. An upregulation of cell survival genes including NK-κB, Mcl-1, Bcl-XL, and Bcl2 and the downregulation of pro-apoptotic genes—Bax and Bid—occur through the activation of HIF-1α in hypoxic conditions [44, 45]. Furthermore, the upregulation of Bcl2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3) and BNIP3-like (BNIP3L) proteins in hypoxic conditions has been known to prevent malignant cells from cell death [46].

After cell proliferation, the malignant cells get involved in metastatic processes such as cell migration, adhesion, and invasion under low oxygen conditions. Extracellular growth factors prompt the p42/p44 mitogen-activated protein kinase pathway to phosphorylate HIF-1α, activate HIF-1α target genes transcription, and regulate cell proliferation. A strong correlation exists between elevated levels of HIF-1 and HCC proliferation and apoptosis; the role of HIF-1, however, presents a controversy even though several investigations indicate HIF-1 as an anti-apoptotic agent.

Xia et al. [47] reported that the transcription factor, Forkhead box M1 (FoxM1), induces HCC cell proliferation and resistance to apoptosis and its promoter binds to HIF-1α by the initiation of tumor necrosis factor-α (TNF-α). This observation revealed that TNF-α/HIF-1α stimulated the expression of FoxM1 [48]. Xu et al. [49] reported that HIF-1 stimulated cell cycle progression and cell proliferation by enhancing the expression of cyclins A and D in HCC. HIF-1 also decreases the apoptosis of HCC by increasing the levels of survivin and Bcl2 to prevent mitochondrial Omi/HtrA2 expression [49, 50]. Through several independent and HIF-1-dependent pathways, hypoxia induces the expression of downstream molecule VEGF, resulting in the increase of Bax/Bcl2 levels that leads to the survival of HCC [51]. Furthermore, hypoxia stimulates the levels of cAMP-responsive element-binding protein and is activated by extracellular signals [52].

Apoptosis, coined by Currie and colleagues in 1972, originated from Greek and means “to fall away from.” Stimulated by signaling pathways that result in the activation of caspase cascades and cell death, the apoptosis of a cell depolymerizes the cytoskeleton, condenses chromatin, and translocates nuclear fragments and phosphatidylserine to the surface of the cell. The increase in apoptotic activity stands as an indicator of several diseases, including AIDS, neurodegenerative disorders, insulin-dependent diabetes, myocardial infarction, and atherosclerosis. Two major pathways of apoptosis are responsible for processing stress signals and executing cellular demolition. Because of the importance and lethal nature of apoptosis, it is highly regulated by B-cell CLL/Lymphoma 2 (Bcl2) family proteins. Bcl2 proteins share one or more of four conserved Bcl2 homology (BH) domains (i.e., BH1, BH2, BH3, and BH4). Of these four domains, BH3 domain is found in all Bcl2 proteins, which interact with each other through the BH3 domain.

The Bcl2 protein family has either pro-apoptotic or anti-apoptotic activities. For example, Bcl2, B-cell lymphoma-extra large (Bcl-XL), and myeloid cell leukemia sequence 1 (Mcl-1) have anti-apoptotic activities, whereas Bcl2-associated x protein (Bax), Bcl2 antagonist/killer 1 (Bak), Bcl2-related ovarian killer protein (Bok),

and BH3-only proteins have pro-apoptotic functions. Among the BH3-only proteins, activators such as Bax- and Bcl2-interacting mediator of cell death known as Bim interact with Bax and Bak to induce the mitochondrial outer membrane permeabilization (MOMP), whereas depressors neutralize the functions of anti-apoptotic proteins in response to various apoptotic stimuli such as hypoxia, ionizing radiation, cytotoxic agent, DNA damage, and growth factor withdrawal.

35.3 Role of HIF-1α in Cell Cycle

The inactivation of enzymes responsible for nucleotide synthesis causes hypoxically induced cell cycle arrest, and this arrest inhibits DNA replication [53, 54]. The inactivation of the nucleotide synthesis, however, only occurs under the condition of severe hypoxia known as anoxia, which is 0.01% oxygen [55]. On the other hand, relative changes to normoxia occur under moderate hypoxia, which is associated with the hypophosphorylation of retinoblastoma protein (Rb) and the inhibition of the cell cycle [55, 56]. While hypoxia may induce angiogenesis and glycolysis necessary for cell growth, it can also lead to cell cycle arrest and apoptotic activity. Furthermore, HIF-1α upregulates genes under low oxygen tension and has been indicated as a required mechanism for hypoxia-induced growth arrest and for *p21*^{cip1}, a key cyclin-dependent kinase inhibitor in control of the cell cycle; the specificity of the exact mechanism, however, still remains unclear. HIF-1α acts against the activation of Myc through the downregulation of Myc-activated genes—*hTERT* and *BR*—and, thus, induces cell cycle arrest, which leads to the suppression of *p21*^{cip1} activity. Thus, Myc is an essential part of the HIF-1α pathway, which regulates Myc genes in hypoxic conditions. Hence, researchers have uncovered a divergent role for HIF-1α as it is not required for cell cycle arrest.

Several investigations have revealed that hypoxia-stimulated cell cycle arrest is associated with the downregulation of cyclin-dependent kinase (CDK) activity and the expression of retinoblastoma (Rb). Although the induction of cyclin G2, a negative regulator of cell cycle progression, was reported under hypoxia via HIF-1 activation, very little information is available to clarify the role of HIF-1 in the regulation of cell cycle machinery [57]. Recent studies have demonstrated the critical role of HIF-1 in the regulation of cell cycle progression under hypoxia by showing that the activation of HIF-1 impedes G1/S transition via two diverse mechanisms [58]. The expression of two CDK inhibitors (CKIs), p21^{Cip1} and p27^{Kip1}, was increased in a HIF-1-dependent manner. Conserved expression of these CKIs was not observed in HIF-1α null cells and suppressed cyclin/CDK2 activity, leading to the reduction of the ratio of phosphorylated/dephosphorylated Rb protein, resulting in cell cycle arrest at G1/S [58].

35.4 Role of HIF-1α in Metastasis

Tumor metastasis is the multistage process that includes the dissociation, arrest, adhesion, and extravasation. It is also one of the primary causes of poor HCC diagnosis [59]. Adhesive molecules on tumor cells including intercellular adhesive

molecule-1 (ICAM-1) and vascular cellular adhesive molecule-1 (VCAM-1) consequently rest the cells entirely and then extravasate from the blood vessels into other tissues or organs where metastatic tumor is formed. Throughout the metastatic signaling cascade, cancer cells communicate with endothelial cells, platelets, lymphocytes, and the homotypic as well as the heterotypic cell clusters from a multicellular emboliform nucleus [60, 61]. The potential of metastasis is associated with the activation of surface adhesive fragments including selectin, ICAM-1, or VCAM-1 [61]. Recently, it was reported that a key role is played by ICAM-1 in the metastatic process. Intra- and extrahepatic metastasis can cause poor prognosis and HCC, and the invasion of such metastases can also consist of epithelial-mesenchymal transition (EMT), which refers to the attainment of motility by tumor cells. Further, EMT requires the loss of E-cadherin, which is a major factor in the maintenance of epithelial polarity [62].

Hypoxia is clinically related to metastasis and poor prognosis [63]. Hypoxic stress speeds up the invasion of liver cancer cells by upregulating ETS-1 as well as the family of matrix metalloproteinases through HIF-1α-independent pathway [64]. The activity of HIF-1α is associated with VEGF [65]. The metastasis of HCC is inhibited by rapamycin and vitexin by downregulating the expression levels of VEGF and HIF-1α [66, 67]. The VEGF expression level in the plasma is elevated after transcatheter arterial chemoembolization (TACE) in patients with varied uptake of venous thrombosis. A 6-month follow-up revealed metastatic foci in almost 70% of the patients with elevated levels of plasma VEGF although patients with reduced plasma VEGF levels did not develop metastasis at all [66]. Consequently, in HCC, improved plasma VEGF level could be correlated with the advancement of metastasis after TACE. Furthermore, amplified plasma insulin-like growth factor II (IGF II) expression levels after TACE appear to be related to metastasis [66, 67].

35.4.1 Adhesion

Hypoxic conditions include the regulation of the transcription factor known as hypoxia-inducible factor 1α (HIF-1α), which is a master regulator that has pro-angiogenic activities such as the regulation of vascular endothelial growth factor (VEGF). Hypoxia-induced angiogenesis prompts researchers to investigate the multistep process by analyzing aortic and coronary artery smooth muscle cells with the additional treatment of cobalt chloride. Results show that HIF-1α activation reduced migration of smooth muscle cells and adhesion to the extracellular matrix. The presence of HIF-1α and cobalt chloride reduces the expression levels of tyrosine phosphorylation of focal adhesion kinase (FAK). Through FAK activation, HIF-1α acts as a suppressor of adhesion and migration of these smooth muscle cells by, but HIF-1α expression can also expand vessel growth by allowing smooth muscle cells to migrate and detach from the basement membrane and endothelial cells [68].

35.4.2 Invasion

Invasion consists of multiple steps including initiation. Epithelial-mesenchymal transition (EMT) offers tumor cells with motility to initiate the invasion. EMT includes the loss of E-cadherin, which influences on the adhesion junctions, which aids in maintaining the epithelial polarity [69]. HIF-1 may act as a leading modulator of EMT through upregulating the expression of various transcription repressors including transcription factor 3 (TCF3), E-cadherin, Snail, Twist1, Zfhx1a, and Zfhx1b [70]. It has been elucidated that HIF-1 enhances invasion as well as metastasis of HCC by inducing EMT during hypoxic conditions. HIF-1 possibly communicates with two HREs in the promoter region of Snail and also upregulates its activities in order to indirectly influence the expression levels of vimentin, E-cadherin, and N-cadherin [71].

35.4.3 Migration

Caused potentially by hypoxic conditions, pathological characteristics such as invasion and vascular proliferation can stand as indicators of malignant gliomas. Hypoxia-inducible factor-1 (HIF-1), which is a transcription factor derived from the HIF-1α subunit, forms the cellular response in hypoxic conditions. Though hypoxia has been correlated to the progression of angiogenesis, HIF-1 does not have a clear role in malignant gliomas. Therefore, investigations of the role of HIF-1α in the migration and invasion of human glioma cells in hypoxic conditions have been undertaken. Cell migration plays an essential role in expanding the breadth and growth of various cellular responses such as embryogenesis, inflammatory responses, and tumor metastasis [72].

35.4.4 Angiogenesis

Cells tend to undergo many biological and physiological responses to low oxygen levels known as hypoxia. One of the most studied responses to hypoxia is the expression of pro-angiogenic growth factors that activate their receptors and result in new blood vessel formation known as angiogenesis [73, 74]. Angiogenesis is an essential component of tumor cell migration and formation [75, 76]. HIF-1α upregulates the expression level of VEGF, an important pro-angiogenic factor [40, 77, 78]. Growth factors activated by HIF-1α regulate endothelial cell proliferation and blood vessel formation. HIF-1α activates the transcription of VEGF, VEGF receptor 1, adrenomedullin, COX-2, angiopoietin-2, angiopoietin receptor Tie-2, endothelin-1, endothelin-2, monocyte chemotactic protein-1, fibroblast growth factor-3, osteopontin, hepatocyte growth factor, transforming growth factor (TFG)-α, histone deacetylase, placental endothelial factor, nitric oxide synthase, TGF-β1, and TFG-β2. VEGF is one of the most critical angiogenic factors, which is secreted by normal as well as oncogenic cells in response to hypoxic conditions, and the

receptors are mainly expressed on the endothelial cells. Angiogenesis that is induced via hypoxia is inhibited by agents blocking RAS, EGFR, and the receptor tyrosine kinase ERBB2 indicating that carcinogenic and hypoxia response signaling pathways are overlapped. HIF-1 stimulation can also reduce the expression levels of anti-angiogenic genes such as thrombospondin-1 and thrombospondin-2.

Angiogenesis is essential for tumor growth and progression by supplying oxygen and nutrients through the newly created blood vessels. It is already proven that the effective way to treat HCC is through anti-angiogenic therapy [79]. Under hypoxic conditions, HIF-1 acts as a direct transcriptional activator of the VEGF pathway, which promotes the migration and proliferation of vascular endothelial cells. HIF-1 directly activates the BEGF transcriptional pathway during hypoxic conditions as well. Sorafenib, an approved multi-kinase inhibitor and approved drug for advanced HCC patients, contains mechanisms that inhibit the expression levels of VEGF and HIF-1 proteins in order to result in the reduction of the expression of HCC vascularization [80]. A study done by Wang et al. [81] reports that a rat model experienced elevated levels of HIF-1 and VEGF after 20 weeks of hepatocarcinogenesis induction, revealing pro-angiogenic roles. Other pro-angiogenic markers include angiopoietin-2 (ANGPT2), stromal-derived factor 1 (SDF1), platelet-derived growth factor-B (PDGF-B), placental growth factor (PGF), and stem cell factor (SCF) [82]. Further research is required in order to clarify the pro-angiogenic role of HIF-1 in hypoxic conditions and possibly placing HIF-1 as a therapeutic target in HCC treatment.

35.5 Molecular Mechanism of HIF-1α

Cells respond to decreased oxygen levels through HIF-1 that is a heterodimer composed of the hypoxia response factor known as HIF-1α and the aryl hydrocarbon receptor nuclear translocator (ARNT) also identified as the HIF-1β. During the absence of oxygen, HIF-1 and hypoxia response elements (HREs) bind to each other in the promoter regions of the hypoxia response genes and as a result activate the expression of a number of genes such as VEGF, which is a pro-angiogenic growth factor. The redox active apurinic/apyrimidinic endonuclease-1 (APE1) has been shown to allow HIF-1α to function transcriptionally in a reduced state.

Under normoxic conditions, posttranslational HIF-1α is rapidly degraded by the proteasome. Prolyl hydroxylases (PHDs) hydroxylate the proline residues (402 and 564) at the ODDD of HIF-1α (Fig. 35.1). Hydroxylation of these residues facilitates the binding of the von Hippel-Lindau tumor suppressor gene (pVHL), which is a key component recognized by the E3 ubiquitin ligase complex in targeting HIF-1α for ubiquitination and degradation by the 26S proteasome [83]. In this degradation process, three PHDs (PHD1, PHD2, and PHD3) are the oxygen sensors controlling HIF-1α. These homologs are recently identified in mammals that have an immense potential to hydroxylate HIF-1α in normoxia [22]. The expression of PHD2 is controlled through the concentration of oxygen in the cell, and the conversion of hydroxyproline from proline involves ascorbate, iron, and 2-oxoglutarate. Under hypoxic environment, the inactivation of PHDs releases HIF-1α from hydroxylation

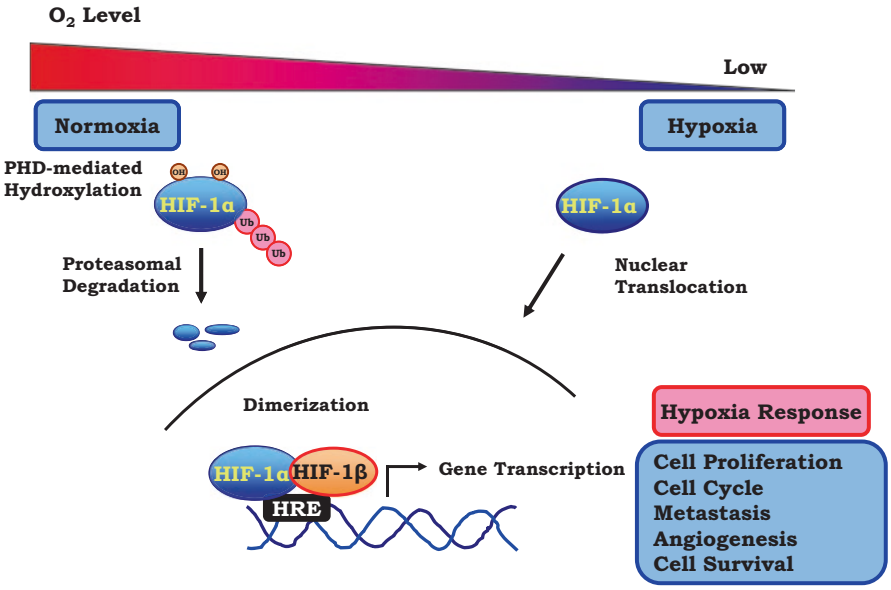

Fig. 35.1 Molecular mechanism of HIF-1α. HIF-1 consists of HIF-1α and HIF-1β. In hypoxia, HIF-1, a heterodimer, binds to DNA promoter regions of HRE and thereby activates the expression of numerous hypoxia response genes involved in cell proliferation, cell cycle, metastasis, angiogenesis, and cell survival. In contrast, HIF-1α is hydroxylated by PHDs and rapidly degraded by the proteasome under normoxic conditions. *HIF* indicates hypoxia-inducible factor; *HRE* indicates hypoxia response element; *PHD* indicates prolyl hydroxylase domain enzyme

by inhibiting pVHL from binding to proline residues and leading to the stabilization of HIF-1α in the cytoplasm.

In reference to the oxygen-dependent pVHL pathway, arrest defective 1 (ARD1) acetylates HIF-1α in order to induce HIF-1α degradation by acetylation of Lys532, resulting in an enhanced interaction of HIF-1α with pVHL [84].

Due to hypoxic conditions, certain miRNAs become responsible for the regulation of the HIF-α expression [85–87]. The miRNAs miR-424, miR-200b, and miR-429 stabilize HIF-α expression, while other miRNAs such as miR-199a and miR-22 reduce HIF-α activity. Endothelial cells in hypoxic conditions experience HIF-1α accumulation in the nucleus. Hypoxic conditions drive the upregulation of miR-424, miR-200b, and miR-429 expression, which stabilizes HIF-1α isoforms through the inhibition of the pVHL scaffold protein Cullin 2 (CUL2) or PHD activation [86]. Further, miR-199a downregulates the HIF-1α activity of cardiomyocytes [87]. Hypoxic conditions decrease miR-199a activity but enhance HIF-1α expression.

EMT is characterized by the loss of cell-cell adhesion and apical-basal polarity and important to tumor development as it promotes invasion and metastasis. During EMT, epithelial cells lose E-cadherin expression, a hallmark of EMT, and obtain mesenchymal markers such as vimentin and fibronectin. There are three types of

EMT: type 1 appears in embryogenesis and organ development, type 2 is essential for tissue regeneration and organ fibrosis, and type 3 is associated with cancer stem cell properties. The EMT process involves master regulators, including SNAIL, TWIST, and ZEB transcription factors. By being activated earlier in the EMT process, these transcription factors play a central role in the development, fibrosis, and cancer and depend on the type of cell or tissue involved in the initiation of the EMT process.

Hypoxia in the tumor environment can promote EMT through HIF-1α expression, which activates TWIST, SNAIL, and other EMT inducers. In epithelial cells undergoing EMT, the mammalian TOR complex 1 (mTORC1) and mTORC2 are activated due to the presence of the AKT activity. TWIST is a direct transcriptional target of HIF-1α, whereas SNAIL is regulated by hypoxia at the posttranscriptional level. The upregulation of TWIST activity, stemness of cancer cells through the TWIST-BMI1 axis, members of the LOX/LOXL2 family, and other EMT inducers such as ZEB1/2 are crucial for the progression of the metastasis in a hypoxic environment. The hypoxic environment allows for the stability of HIF-1α, which leads to increased stemness and EMT of cancer cells and the activation of the following pathways: Wnt, Notch, and TGF-β. Furthermore, the activity of HIF-2α induces more stemness by increasing the expression levels of Oct-4. Under severe hypoxic conditions (0.1% O_2), PERK, ATF4, and ATF6 potentiate the EMT of cancer cells.

35.6 Conclusions and Future Perspective

Hypoxia in the liver regulates gene expression in physiological conditions and diseases such as cirrhosis and cancer. Hypoxic conditions are known to promote the tumorigenic factors of proliferation, angiogenesis, invasion, and even resistance (chemo and radio) in the progression of HCC. Understanding hypoxic conditions in HCC can allow researchers to further investigate the progression of HCC malignancies. However, only HCC patients suited to palliative treatment can benefit from therapeutic methods that induce hypoxic conditions. Both treatment methods TACE and TAE induce hypoxia and, thus, promote to HCC angiogenesis; thus combining the benefits of TACE and TAE along with anti-angiogenic targeted therapy can introduce new approaches for HCC treatment. Unfortunately, hypoxic conditions prevent HCC cells from the combination therapy featuring the benefits from TACE, TAE, and anti-angiogenic factors. As angiogenesis becomes a major factor in the frequency of HCC recurrence, anti-angiogenic therapy can stand as the solution for future prevention and even benefit those who have surgical resections. By favoring a therapeutic approach of agent-inducing hypoxia with agent-targeting hypoxia factors, researchers can provide new treatment approaches for HCC patients.

In conclusion, compelling evidence reveals that HIF-1 activity promotes HCC proliferation, invasion, and metastasis. Along with the promotion of angiogenesis, HIF-1 activity also increases resistance of HCC cells to both chemotherapy and radiotherapy. Furthermore, clinical data supports the correlation between an increase

in HIF-1 activity and the HCC's poor prognosis. Thus, researchers have considered HIF-1 as targeted molecule in targeted therapy in HCC treatment, including inhibition of the HIF-1 pathway via small molecular targets. However, these molecular targets exhibited some disadvantages: detrimental side effects, low specificity, and the lack of evaluation in clinical trials. Therefore, further research is required to understand how to utilize the full efficacy of the therapeutic methods targeting HIF-1 in HCC treatment.

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Role of STAT3 in Liver Cancer

36

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Abstract

Hepatocellular carcinoma (HCC) is a malignant cancer that shows heterogeneous etiology and the third leading of cancer-related deaths in the world. In spite of its severity, there is only one systemic chemotherapy drug known as sorafenib showing a valuable effect on a small number of HCC patients. Signal transducers and activators of transcription (STATs), a family of transcription factors, consist of seven members in mammalian cells: STATs 1, 2, 3, 4, 5a, 5b, and 6. STAT transcription factors have a characteristic Src homology 2 (SH2) domain whose function is indispensable to the activation of STAT proteins triggered by Janus kinase (JAK) signaling. Among the STAT transcription factor family, STAT3 performs a vital function in advancement and tumorigenesis because it participates in replicating various proteins that are responsible for angiogenesis, proliferation, invasion, metastasis, and apoptosis. Although the activation of STAT3 is usually transient in normal cells, cancer cells show a positive feed-forward loop that results in the persistent activation of STAT3. The expression and activity of STAT3 are increased in a wide range of malignancies. Although several studies have reported the oncogenic functions, the antitumor effects of STAT3 should be considered carefully in the development of therapeutic methods

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that target STAT3 signaling. We believe that STAT3 signaling will be a promising target of treatment for HCC patients who need further research of adverse effects or personalized treatment via the modulation of STAT3 signaling.

Keywords
Hepatocellular carcinoma · Signal transducers and activators of transcription · Cell proliferation · Cell cycle · Metastasis

36.1 Introduction

Hepatocellular carcinoma (HCC) is a malignant cancer that shows heterogeneous etiology [82], and HCC is one of the most common cancers and stands as the third leading cause of cancer-related deaths in the world [85]. In spite of its severity, only a small portion of HCC patients (about 30%) can be treated with appropriate therapies such as liver resection, transplantation, or local ablation, and there is only one systemic chemotherapy drug known as sorafenib that shows a valuable effect [56]. Moreover, not all HCC patients experience the optimal therapeutic effects of sorafenib. The overall survival prolongation period of patients in the SHARP (Sorafenib HCC Assessment Randomized Protocol) trial was only 2.8 months, and an objective tumor response was observed only in a small number of patients (0.6–2%) [13]. Therefore, urgency arises to identify a molecular signaling specific for HCC in order to develop suitable chemotherapies.

Signal transducers and activators of transcription (STATs) are a family of transcription factors, which were first discovered in the signaling context of interferon-α (INF-α), interferon-γ (INF-γ), and interleukin-6 (IL-6) in 1994 [19]. There are seven members in the STATs family in mammalian cells: STATs 1, 2, 3, 4, 5a, 5b, and 6 [1]. STAT proteins have a characteristic Src homology 2 (SH2) domain whose function is indispensable to the activation of STAT proteins [29]. The activation of STAT proteins is triggered by Janus kinases (JAKs) signaling [37, 69, 79]. After activated by JAKs, phosphorylated STAT dimers are able to shuttle into the nucleus and bind to IFN-γ-activated site (GAS; 5′-TTN4–6AA-3′) [72]. Among the STAT transcription factor family, STAT3 performs a vital function in advancement and tumorigenesis because it participates in replicating various proteins that are responsible for angiogenesis, proliferation, invasion, metastasis, and apoptosis [96]. Actually, the functions of STAT3 signaling in oncogenesis have been demonstrated in various tumor models and types [82]. Moreover, it has been reported that the expression and activity of STAT3 are increased in a wide range of malignancies [63]. Although the activation of STAT3 is usually transient in normal cells even in the presence of stimuli such as cytokines, cancer cells show a positive feed-forward loop that results in the persistent activation of STAT3 [103]. Here we review the STAT3 signaling pathway and highlight its significance in liver cancer.

36.2 Role of STAT3 in Cell Proliferation and Apoptosis

STAT3 plays a key role in cellular proliferation, survival, and cell transformation. Accumulating evidence indicates that constitutive STAT3 signal is associated with the upregulation of transcriptional factors such as c-Fos, c-Myc, and Sox2, which are required for the regulation of the cell cycle [20, 26, 47]. STAT3 has also been shown to upregulate the expression of regulators such as Bcl-2, Bcl-xL, and cyclin-D1 that are responsible for apoptosis or proliferation [10, 15, 52]. In addition, STAT3 downregulates the expression of p53, the most common inhibitor of cellular proliferation and inducer of apoptosis [62]. It was reported that STAT3 can also act as an apoptotic factor, especially during post-lactation regression where a leukemia inhibitory factor (LIF) plays as the only activator of STAT3 in order to cause apoptosis in mammary glands [11]. In addition to the pro-apoptotic function of STAT3, it was reported that the loss of STAT3 promotes cellular proliferation and transformation [20].

36.2.1 Oncogenic Function of STAT3

STAT3 is usually considered an oncogene in HCC [82]. NSC 74859, a STAT3-specific inhibitor, promotes apoptosis and impairs proliferation of several hepatocellular cancers in vitro and in transplantation models [55]. Moreover, the nuclear Tyr^{705} phosphorylation, the activation marker of STAT3, was observed in 60% of HCC cases without the relation of tumor etiology [36]. A recent study showed that STAT3 activity in monocytes of the HCC tumor stroma accelerates cancer progression [94]. This investigation showed that IL-6/JAK/STAT3 signals in monocytes promote the proliferation of hepatocellular carcinoma cells, which indicates that the activation of STAT3 in the stromal part is essential for HCC progression. Interestingly, no naturally occurring mutations that produce constitutive activation of STAT3 have yet been reported despite the activated STAT3 that was detected in the majority of human cancers and tumor-derived cell lines [51]. Mutations in gp130 or IL-6, however, were reported to activate STAT3 signaling in the absence of ligands, which are considered to drive oncogenesis [71].

The oncogenic function of STAT3 in HCC has been demonstrated in various studies, showing the pro-oncogenic function of STAT3 driven by either cytokine IL-6- or IL-22. It was reported that the expression of IL-6 is elevated in human patients with liver disease and HCC [66]. It was also reported that HCC development triggered by obesity is associated with the increased production of cytokines IL-6 and TNF-α, which is dependent on the activation of STAT3 [65]. The second major cytokine activating STAT3 expression in the liver is IL-22. It was reported that the expression of IL-22 is elevated in tumor-infiltrating lymphocytes of HCCs due to high expression of IL-22 receptors [45]. This study showed that HCC formation in the animal model was reduced in IL-22-deficient mice, which implicates the oncogenic function of STAT3 in HCC.

The function of STAT3 was directly estimated in diethylnitrosamine (DEN)-treated mice with the conditional inactivation of STAT3 in hepatocytes, which showed controversial results suggesting oncogenic [90] and anti-oncogenic activity [4]. The study showing the anti-oncogenic function of STAT3, however, also demonstrated an oncogenic aspect of STAT3, depending on a negative regulator of STAT3 activity known as tyrosine phosphatase SHP2 (PTPN11). The tyrosine phosphatases SHP1/SHP2 may regulate STAT3 activity in HCCs [82]. The IKKβ-deficient hepatocytes showed an increase in the formation of HCCs, which were initially implausible because IKKβ deficiency downregulated the activity of NF-κB. However, IKKβ deficiency also increased the production of reactive oxygen species and downregulated the activity of SHP1/SHP2, resulting in the upregulation of STAT3 and the formation of HCC [36]. In addition, the deletion of SHP2 in hepatocytes showed persistent STAT3 activation, resulting in the promotion of HCC by DEN [4]. Other regulators of STAT3 except SHP proteins have been identified as tumor suppressors in HCC, such as SOCS3 whose deficiency led to the activation of STAT3 and promoted formation of DEN-induced HCCs [73]. STAT3 promotes carcinogenesis in the liver through epigenetic regulation, which is involved with microRNAs (miRs) [35]. HNF4α, a hepatocyte differentiation factor and suppressor of HCC formation, was downregulated by miR-24 and miR-629, which are induced by IL-6/STAT3 signaling. Moreover, the expression of miR-124, another miR regulated by HNF4α, is decreased. In consideration of negative regulation of IL-6R by miR-124, this epigenetic regulation induces the permanent suppression of HNF4α through an amplifying feedback mechanism of IL-6/STAT3/miR-24/miR-629 [77].

36.2.2 Anti-oncogenic Function of STAT3

On the contrary to the aforementioned oncogenic role of STAT3, there are several reports indicating the anti-oncogenic function of STAT3 in HCC formation. The study using hepatocytes genetically ablated in p19(ARF) gene clearly indicated the tumor-suppressive function of STAT3 [76]. STAT3-deficient hepatocytes, immortalized by additional deletion of p19(ARF), the cyclin-dependent kinase inhibitor and mouse homolog of human p14(ARF), and transformed by Ras, showed increased tumor formation in xenografts, suggesting the anti-oncogenic function of STAT3. However, STAT3 itself induced the development of HCC in the presence of p19(ARF), indicating that p19(ARF) is a critical regulator of oncogenic and anti-oncogenic functions of the STAT3 protein in HCC. Furthermore, a hypothetical ARF-X, a binding partner of p19(ARF)/p14(ARF), controls oncogenic or anti-oncogenic activity of STAT3 [76]. NF-κB was also found to interfere with the activation of STAT3 in HCC cells [36], but p19(ARF) is a negative regulator of NF-κB [74]. Therefore, downregulation of p19(ARF) might induce the increase of NF-κB activity, resulting in the downregulation of STAT3 activity. This molecular mechanism could diminish the anti-oncogenic activity of activated STAT3 in p19(ARF)-deficient HCCs [8]. However, the inactivation of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor, promoted hepatocarcinogenesis through increasing cytokine secretion and

STAT3 activation [33]. This study indicates that the loss of a distinct cyclin-dependent kinase inhibitor promotes anti-oncogenic activity of STAT3, whereas the loss of another promotes oncogenic activity [82]. The accurate function of STAT3 in liver tumorigenesis may be determined by the etiology because the oncogenic function of STAT3 was demonstrated in DEN-induced HCCs, whereas the anti-oncogenic activity was shown in HCCs developed by chronic damage and fibrosis through CCl4 induction [90]. Feng summarized conflicting roles of molecules—including STAT3—in hepatocarcinogenesis and proposed that excessive compensatory reaction to the loss of a proliferative signal may also contribute to the unexpected tumor-promoting effect observed upon removal of a pro-tumorigenic molecule [25]. Overall, these reports reveal the anti-oncogenic function of STAT3 in HCC, depending on the expressional profiles of tumor suppressor genes and etiology.

36.3 Role of STAT3 in Metastasis

Metastasis of tumor cells is a multistep process in which cells invade the surrounding tissue and basal membrane, resulting in the entrance into blood vasculature. During this process, tumor cells move through vasculature, adhere into distant organs, and induce angiogenesis to form a secondary tumor [46]. Cumulative evidence has indicated that STAT3 activation is critical for metastasis factors such as proliferation, invasion, migration, and angiogenesis.

36.3.1 STAT3 in Transformation

It was reported that the malignant transformation of cells can be mediated through the activation of STAT3, which is triggered by the activation of protein tyrosine kinases, the expression of oncogenes, and the presence of viral infection [7]. The activation of STAT3 is required for IL-6-induced transformation in tumor-promotion sensitive mouse skin epithelial cells [102]. Moreover, the activation of STAT3 is induced by Src signaling, which may contribute to cell transformation by preventing apoptosis, increasing cell numbers [87], and being triggered by an oncogene known as TRK [58]. Similarly, the transformation of fibroblasts was induced by the activation of RET/PTC tyrosine kinase, which was mediated through STAT3 activation [41]. Transformation triggered by viral infection is also induced through the activation of STAT3. For example, oncogenic proteins from the hepatitis C virus, the simian virus 40, and the herpes virus play key roles in cell transformation mediated by the activation of STAT3 [17, 89, 100]. Recent studies suggest that the inhibition of STAT3 activation may exert beneficial effects both as a direct and an indirect mechanism [27]. For example, rituximab, a monoclonal therapeutic antibody against CD20, decreases STAT3 activation in B cell non-Hodgkin's lymphoma [2]. In addition, bacterial quorum sensing molecules of the N-acylhomoserine lactone class can induce apoptosis in breast carcinoma cells, which is correlated with the downregulation of STAT3 signaling [53]. Furthermore, the synthetic triterpenoid

CDDO-imidazolide can induce growth arrest and apoptosis of myeloma and lung cancer cells by inhibiting the phosphorylation of STATs, including STAT3 [54]. Therefore, these reports emphasize the role of STAT3 in malignant transformation and as a target for anticancer therapy.

36.3.2 STAT3 in Invasion

Cumulative evidence suggests that STAT3 signaling plays an important role in cancer cell invasion. There was a report showing that the overexpression of phosphorylated STAT3 is associated with the invasion as well as metastasis of cutaneous squamous cell carcinoma [81]. In contrast, RNAi-mediated STAT3 gene silencing inhibited invasion and metastasis of pancreatic cancer cells [68]. STAT3 activation also regulates the expression of matrix metalloproteinase (MMP)-2, MMP-9, and MMP-1 and tumor invasion and metastasis [22, 43, 95]. Moreover, STAT3 knockdown reduced pancreatic cancer cell invasiveness and MMP-7 expression in nude mice [23]. Invasion of cancer cells into the extracellular matrix is a key step in tumor metastasis and is able to be processed by regulating MMPs [46]. All these studies implicate that STAT3 plays a key role in the complex and multistep process of cell invasion by regulating MMPs.

36.3.3 STAT3 in Migration

The function of STAT3 activation in cell migration was first identified in keratinocytes [75] and then confirmed in ovarian cancer cells using siRNA [80]. It was reported that STAT3 can regulate microtubules by antagonizing the depolymerization activity of an oncoprotein 18 known as stathmin that binds to α/β tubulin, resulting in the modulation of cell migration [59]. The role of STAT3 in cell migration was also indicated in the regulation of Rho GTPase [21]. Accordingly, constitutively activated STAT3 induces cell motility of prostate epithelial cells through integrin β6 [3]. Recently, it was reported that STAT3 promotes directional cell migration by regulating Rac1 activity via its activator βPIX [84]. Therefore, these investigations implicate that activation of STAT3 plays a critical role in cellular migration under normal as well as pathological conditions.

36.3.4 STAT3 in Intravasation

Intravasation, the invading technique of entering into the blood or the lymphatic vessel, is an essential stage in the progression and metastasis of tumor cells [14]. Tumor cells enter the circulatory or lymphatic system (intravasation) after degradation of basal membrane and move out of the blood vessel into new distant organs [46]. In this process, tumor cells might get eliminated by the frictional force of the

blood (vascular wall shear stress) and the immune system, or it can get blocked in a distant capillary bed, where they might endure extravasation, the process of moving out of the blood vessel into the tissue during metastasis, and form a secondary cancer cell [6]. Actually, the metastatic progression is extremely incompetent because only a small number of circulating tumor cells (about 0.01%) are required to form cancers at the secondary site. However, it is very critical to study the procedure of intravasation since it is the rate-limiting step in metastasis because tumor cells that have access to the peripheral blood circulation could control the effectiveness of metastasis [14]. There are several reports indicating the key role of STAT3 activation in protecting the tumor cells from the immune system during their travel in vasculature. It was reported that colon carcinoma cells can be tethering, rolling, and forming firm adhesion under the dynamic flow condition by immobilizing platelets [57]. Moreover, the activation of STAT3 signaling in tumor cells or in inflammatory immune cells can regulate the secretion of various inflammatory cytokines such as IL-6 or TNF-α, which can affect the survival of tumor cells [60]. In addition, STAT3 activation can reduce the activation of NK cells, which enhance the survival of tumor cells during circulation [91]. Therefore, STAT3 activation may increase the probability of tumor cell survival and intravasation into distant organs.

36.3.5 STAT3 in Angiogenesis

In tumor growth and metastasis, the neovascularization from existing blood vessels is an essential process, and vascular endothelial growth factor (VEGF) is known as tone of the most potent angiogenic factor [14, 32]. VEGF inhibition through a neutralizing antibody is known to decrease microvessel density and the intravasation process of prostate carcinoma cells [18]. In addition, vascular permeability and intravasation process of breast cancer cells were stimulated by the secretion of VEGF from macrophages, which were inhibited by an anti-VEGF neutralizing antibody [34]. STAT3 is considered as one of the critical transcription activators in angiogenesis, especially for activating the VEGF gene [12]. It was found that VEGF expression in melanoma cells is enhanced by constitutive STAT3 activation, resulting in the upregulation of tumor angiogenesis [61]. In addition, it was reported that STAT3 activation regulates the expression of VEGF and human pancreatic cancer angiogenesis [93]. Nuclear translocation of the phosphorylated form is the process of STAT3 activation and is essential for VEGF-induced human dermal microvascular endothelial cell migration and tube formation [98]. Moreover, the activation of STAT3 plays a crucial role in endothelial VEGF receptor signaling [5]. Interestingly, targeting STAT3 results in the downregulation of both hypoxia-inducible factor-1 (HIF-1) and VEGF expression [97]. On the other hand, however, it was reported that STAT3 can induce the expression of HIF-1α, an important angiogenic factor [78]. Recently, the angiogenic activity of HIF-1α was elucidated in human hepatoma cells. HIF-1α enhanced the expression of haptoglobin, which plays a role in angiogenesis by improving the binding of STAT3 to the promoter [64].

36.4 Molecular Mechanism of STAT3 Activation

STAT3 is activated by the phosphorylation of its tyrosine and serine residues, especially Tyr at 705th and Ser at 727th via signaling from upstream regulators [48, 70]. There are various tyrosine kinases catalyzing the phosphorylation including receptors by means of fundamental tyrosine kinase activities like the vascular endothelial growth factor receptor (VEGFR), epidermal growth factor receptor (EGFR), and platelet-derived growth factor receptor (PDGFR) [28, 46, 50]. This phosphorylation is also catalyzed by cytokine receptors such as IL-6R associated with JAKs as well as non-receptor tyrosine kinases such as Src or P190 (ABL) [42, 101]. Serine kinases such as MAPK [16, 86], PKCδ [44], mTOR [99], and NLK [49] catalyze the phosphorylation along with tyrosine kinases, resulting in the activation of STAT3. In addition, STAT3 dimerization can be regulated by reversible acetylation of a single Lys residue at 685, which is catalyzed by a histone acetyltransferase known as p300 [104].

36.5 JAK/STAT Signal

The Janus kinase (JAK)/STAT pathway was discovered in the context of IFNα-, IFNγ-, and IL-6-mediated downstream signaling in T cells [19, 83, 103]. The JAK/STAT3 signaling pathway has been known to be stimulated by numerous cytokines and growth factors and is one of the most well-studied intracellular signaling pathways [9]. Among them, IL-6 is one of the most widely researched STAT3 activators [10, 105]. The effects of IL-6 are mediated by its receptor (IL-6Rα), which induces conformational change of the receptor and results in the development of the hexameric signal complex that consists of an IL-6Rβ (or gp130) homodimer and two IL-6-IL-6Rα heterodimers [38]. These processes trigger the stimulation of JAKs, which are fundamentally linked with a proline-rich membrane-proximal and cytoplasmic domain of gp130. After the activation of JAKs, these processes mediate the gp130 phosphorylation, which leads to the stimulation of cytosolic STAT3 that gets translocated to the nucleus (Fig. 36.1) [103]. Other members of the IL-6 domain such as IL-11, IL-31, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), and oncostatin M (OSM) are also able to trigger receptor-transducing signals via JAK/STAT3 [31, 67]. Recently, Toll-like receptors (TLRs) like TLR4, TLR2, and TLR9 have been identified as key modulators of the JAK/STAT3 pathway [24, 40, 88]. Furthermore, TLR9 and STAT3 formulate a feed-forward loop that is critical for maintaining glioma stem cell, which means that STAT3 can upregulate the expression of TLR9 and thereby promote tumor progression [39]. In addition to its role in the nucleus, STAT3 is also observed in mitochondria and regulates the activity of electron transport system [92]. Moreover, mitochondrial STAT3 supports the malignant alteration of mouse embryonic fibroblasts through activating RAS [30]. Thus, mitochondrial STAT3 contributes to carcinogenesis, and future therapeutic trials that aim at blocking STAT3 might work as potential cancer therapy through modulating the activities of the mitochondrial STAT3 [103].

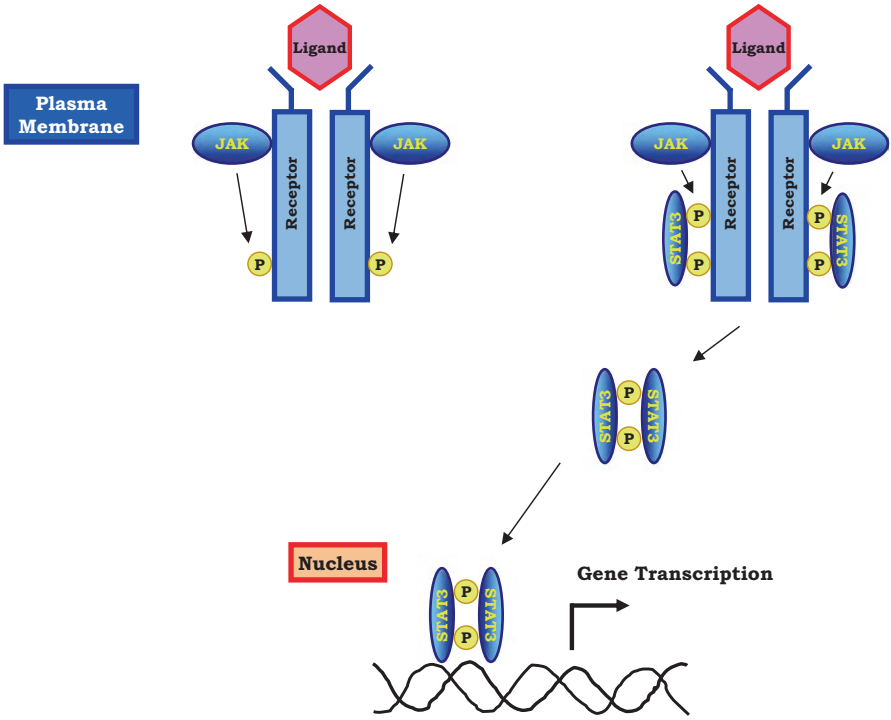

Fig. 36.1 Molecular mechanism of STAT3 activation through JAK/STAT3 signaling. Ligands (e.g., IL-6) activate cytokine receptor tyrosine kinase (e.g., IL-6 receptor) and cytokine receptor-associated JAK. STAT3 in the cytoplasm is recruited to phosphotyrosine motif within receptors and then activated by the phosphorylation on a tyrosine residue by JAK. Phosphorylated Stat3 dimerizes and translocates to the nucleus, where Stat3 homodimer binds to promoter of target genes and regulates gene transcription that promotes various cellular processes. *JAK* indicates Janus kinase; *STATs* indicates signal transducers and activators of transcription

36.6 Conclusions and Future Perspectives

Because of the heterogeneity and complexity of molecular signals in HCC, systemic anticancer therapy is still unsatisfactory. Actually, we have only one therapeutic reagent, sorafenib (Nexavar™), that inhibits tyrosine protein kinases, such as VEGFR, PDGFR, and Raf family kinases. Unfortunately, however, the overall survival prolongation of HCC patients is about 3 months by sorafenib treatment. Therefore, accumulative studies have identified molecular signals or oncogenes specific for HCC development and have also tried to find out the reagents blocking these molecular addictions. The persistent activation of STAT3 has been confirmed in the majority of tumor cells, despite the necessity of its regulated expression for normal cellular function. This persistent activation of STAT3 provides beneficial

conditions to tumor cells in order to perform metastasis processes (proliferation, invasion, migration, and angiogenesis). Although several studies have reported the oncogenic functions of STAT3 and the anticancer effects of its inhibition, implications of the oncogenic mechanisms of STAT3 in the cellular protective function in the liver still remain to be clarified. Furthermore, the antitumor effects of STAT3 should be considered carefully in the development of therapeutic methods that target STAT3 signaling. Therefore, further studies should be scrutinized to make the modulation of STAT3 signaling more effective for HCC treatment. We believe that STAT3 signaling will be a promising target of treatment for HCC patients who need further research of adverse effects or personalized treatment via the modulation of STAT3 signaling.

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Role of Sp1 in Liver Cancer

37

Himanshu Tillu and Pallaval Veera Bramhachari

Abstract

Liver cancer is one of the most serious public health concerns in the world with unhealthy lifestyle and infection with oncogenic viruses contributing to tumorigenesis. Hepatocellular carcinoma (HCC) which originates from the hepatocytes is the most ubiquitous form of liver cancer and is the sixth most prevalent cancer globally. The specificity protein 1 (Sp1) transcription factor occupies an important functional niche in varied cellular processes including cell division, differentiation, cell adhesion, immune response, apoptosis, chromatin remodeling, and DNA damage response. Considering the central position of Sp1 in the life cycle of cells, it is conceivable that Sp1 could be intimately associated with the process of transformation. Some of the pharmacological agents exert their antineoplastic effects through the inhibition of Sp1 response, thereby underlining the importance of Sp1 in the process of carcinogenesis. Over the years, evidence has built up implicating the role of Sp1 in various features of tumorigenesis including proliferation of cancerous cells, cell cycle regulation, invasion and metastasis, angiogenesis, and apoptosis. This review evaluates the contributions of Sp1 to various aspects of HCC.

Keywords

Hepatocellular carcinoma (HCC) · Specificity protein (Sp1) · Hepatitis C virus (HCV) · Liver cancer · Hepatitis B virus (HBV)

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37.1 Introduction

Cancer is one of the prominent causes of morbidity and mortality globally. Various types of cancers have amounted to 8.8 million global deaths in the year 2015; out of which liver cancer has accounted for 788,000 deaths (WHO fact sheet 2017). There are different types of liver cancer depending upon the type of cell it originates from, namely, hepatocellular carcinoma (HCC) which originates from hepatocytes, cholangiocarcinoma or the bile duct cancer, liver angiosarcoma which is the cancer of liver-supplying blood vessels, and hepatoblastoma. HCC is the most ubiquitous form of liver cancer and is the sixth most prevalent cancer in the world [8]. Infection by some hepatotropic viruses such as hepatitis C virus (HCV) and hepatitis B virus (HBV) significantly increases the predilection for liver cancers. Other predisposing factors for liver cancer include lifestyle disorders such as diabetes, obesity, chronic alcoholism, liver cirrhosis, hemochromatosis, and consumption of foods contaminated by fungal aflatoxin (CDC 2016).

Specificity protein 1 or Sp1 is a transcription factor which is the founding member of Sp transcription family and is equipped with a zinc finger motif and binds to the GC-rich regions of several promoters ([22], 1989) and CACCC box (GT box) [23]. Sp1 regulates the growth, differentiation and apoptosis of cells, chromatin remodeling, DNA damage response, and immune response [6].

37.2 Role of Sp1 in Cell Proliferation

The first indications implicating Sp1 in cell proliferation were obtained when Sp1 was shown to mediate the effects of serum stimulation at the rep3a promoter of quiescent cells in murine fibroblasts [72], at the dihydrofolate reductase (DHFR) promoter of the hamster cells [30, 54], and at the ornithine decarboxylase promoter in Rat2 fibroblasts [37]. Sp1 has also been shown to contribute to the growth-inducing actions of the genes coding for vascular endothelial growth factor (VEGF) [61], insulin-like growth factor (IGF)-binding protein 2 [39], serum response factor [78], and thymidine kinase [77]. The c-Myc gene [4, 76], which is an extremely potent oncogene, stimulating cell growth, proliferation, and oncogenesis, is a target of Sp1 [3, 56]. This indicates the potential of Sp1 to mediate tumorigenesis.

Sp1 was found to favor the growth of HCC. The liver-specific and liver-non-specific enzymes methionine adenosyltransferase (MAT) catalyze S-adenosylmethionine synthesis. Sp1 was implicated in a transcriptional switch from MAT1A to MAT2A in human hepatocellular carcinoma (HCC) and facilitated the growth of transformed cells [95]. Improved prognosis in HCC cases is associated with the expression of RING1- and YY1-binding protein (RYBP) [90]. Sp1 was found to suppress the expression of RYBP transcriptionally; the reduced expression was associated with larger tumors, poorer differentiation status of tumors, and an increased propensity to metastasis [102]. Sp1 was also found to be necessary for the expression of the long

noncoding RNA (lncRNA) highly upregulated in liver cancer (HULC) which is instrumental in the liver cancer cell proliferation in three transformed liver cell lines SK-Hep-1, SNU-449, and HepG2. Knockdown of Sp1 inhibited cell proliferation, induced apoptosis, and decreased cellular migration and invasion [20]. Sp1 also activated the transcription of another lncRNA ANRIL which is associated with larger tumor size and Barcelona Clinic Liver Cancer (BCLC) stage [48].

37.3 Role of Sp1 in Cell Cycle

In non-transformed eukaryotic cells, cell division is an ordered, tightly regulated cyclic process commonly termed as the cell cycle. Numerous checkpoints verify the extracellular signals, size, and genomic integrity of the dividing cells before allowing the cell cycle progression. The cell cycle comprises of four phases: G1, S, G2, and M. DNA replication occurs in the S phase and mitosis in the M phase. The G1 and G2 phases represent the gaps between S and M phases. During the G1 phase, the cells prepare for DNA replication, while during G2 phase, the cells prepare for mitosis. The G0 phase is used to represent quiescent non-cycling cells. Cyclins and their corresponding cyclin-dependent kinases (CdKs) regulate cell cycle progression. The cyclin-CdK complexes and their corresponding inhibitors control the action of proteins working at the G1-S, S-G2, and G2-M checkpoints. Abnormalities at these checkpoints lead to failure of cell cycle arrest and may lead to cells acquiring a malignant phenotype [71].

Sp1 induces the transcription of D-type cyclins, cyclin E, Cdk2, E2F-1, and c-Myc, which are important intermediates required for regulating progression through G1 phase and entry into the S phase. Sp1 can be expected to promote G1/S transition since cyclin D/Cdk4 and cyclin E/Cdk2 cooperate to induce entry into the S phase [73, 74] and because c-Myc and E2F-1 are the only known transcription factors capable of inducing S-phase entry of quiescent cells [14, 56]. Accordingly, Sp1 overexpression leads to increased fraction of cells in the S phase, while Sp1 knockdown decreased the S-phase cell population [1, 67]. A dominant-negative Sp1 mutant was found to mediate G1 arrest of HeLa cervix carcinoma cells, thereby confirming a requirement of Sp1 for S-phase entry [24]. The role of Sp1 in cell cycle regulation is complex. Sp1 also activates the cyclin-dependent inhibitors of cell cycle, namely, $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, $p19^{INK4D}$, $p21^{WAF1/CIP1}$, $p27^{KIP1}$, and $p57^{KIP}$. Thus, Sp1 also plays a role in inducing cell cycle arrest [93].

Sp1 was involved in dysregulated cell cycle progression in HCC. Aberrant Sp1-mediated regulation of cell cycle by histone deacetylase 2 (HDAC-2) contributed to development of HCC [55]. Poly (ADP-ribose) polymerase 1 (PARP-1) was found to inhibit Sp1 signaling pathway to promote cell proliferation. PARP inhibitors or PARP-1 knockdown induced G0/G1 cell cycle arrest leading to inhibition of proliferation of hepatoma cells and elevated the expression levels of checkpoint proteins p21 and p27 [94].

37.4 Role of Sp1 in Adhesion, Invasion, and Metastasis of Liver Cancer

Cell adhesion molecules (CAMs) are crucial for maintaining tissue integrity. CAMs also mediate interaction of the cells with their microenvironment. Cell adhesion molecules mainly belong to four families—immunoglobulin superfamily, the integrins, the cadherins, and the selectins. In a normal tissue, the expression of the CAMs is under stringent regulation. Aberrant expression of CAMs warps normal cell-matrix and cell-cell interactions, thereby freeing the cells from their constraints ultimately leading to metastasis [88]. The extracellular matrix (ECM) is a term applied to a set of varied molecules secreted by cells that provide structural scaffold and biochemical assistance to the cells. Cellular adhesion and differentiation and cell-to-cell communication are the principal roles of the ECM [2]. Matrix metalloproteinases (MMPs) are members of zinc-dependent family of endopeptidases which play an important role in tumorigenesis metastasis, by mediating proteolytic degradation of ECM, modulating the cell-ECM and cell-cell interactions by inducing angiogenesis. Overexpression of certain MMPs such as MMP-2, MMP-9, MMP-13, and MMP-14 was shown to induce epithelial-mesenchymal transition [21]. Sp1 was also essential to maintain the mesenchymal phenotype of the HCC cells [20].

Sp1 is known to induce MMP-2 secretion by itself [82] and also by interacting with Sp3 [63], Brg1 [47], and Src [38]. The interaction of Sp1 with MMP-2 promoter is inhibited by the tumor suppressor p16 [91]. The interaction of Sp1 with MMP-2 promoter was shown to induce cell invasion and correlated with poor prognosis in human glioma cases [25]. Thus, in the context of HCC, Sp1 induced MMP-2 may lead to metastasis. S-phase kinase-associated protein 2 (SKp2), one of the components of E3 ubiquitin ligase, has been shown to mediate degradation of p21, the Sp1-MMP-2 promoter-binding inhibitor [7, 81], and enhance cell proliferation. As might be expected, SKp2 was also able to induce MMP-2 and MMP-9 expression in Sp1-dependent manner leading to increased invasiveness of tumor cells [28]. In addition, the expression of MMP-9 has been found to associate with vascular invasion and poor prognosis in the context of HCC [53]. Inhibitors of matrix metalloproteinase activity such as reversion-inducing cysteine-rich protein with kazal motifs (RECK) can inhibit tumor metastasis. RECK promoter has binding sites for Sp1 [69]. An oncogene Ras was shown to induce the binding of histone deacetylase to Sp1 binding sites in RECK promoter to repress the expression of RECK [10]. In another study, the oncogene CD340 was found to inhibit RECK expression by inducing the binding of Sp1 as well as HDAC1 to RECK promoter, thereby promoting metastasis [27]. RECK expression has been detected at mRNA level in HCC tissues and is higher as compared to noncancerous tissue. Moreover, higher mRNA levels of RECK were correlated with better survival in HCC cases [19].

Studies on several HCC cell lines have implicated Sp1 in inducing the expression of several molecules such as CD151 [89] and Coxsackie and adenovirus receptor (CAR) [13] that can promote metastasis. Studies on HBV-induced hepatocellular carcinoma cells (SW398), gastric cancer cells (SW638), and human embryonic

kidney cells (HEK293E) revealed that zinc finger E-box-binding homeobox 2 (ZEB2) can cooperate with Sp1 to induce the secretion of integrinα5 to facilitate metastasis. ZEB2 is also known to repress antimetastatic E-cadherin [52]. The neuron-specific protein T-cell lymphoma invasion and metastasis 2 (TIAM2) is found to be ectopically expressed in hepatocellular carcinoma (HCC). The short form of TIAM2 (TIAM2S) plays a role of an oncogene in the context of hepatic tumorigenesis. Sp1 was reported to bind to the GC box of the TIAM2S core promoter. Sp1 overexpression in HepaRG cells enhanced expression of TIAM2S, and Sp1 knockdown caused considerable reduction in TIAM2S mRNA and protein levels in the HCC cell lines, HepG2, and PLC/PRF/5 [12]. Basigin-2 is an extracellular matrix metalloproteinase inducer. Binding sites of Sp1 are located in basigin-2 promoter [43]. Promoter hypomethylation was observed in basigin-2 promoter in HCC cells which led to enhanced binding of Sp1. This resulted in higher expression of basigin-2 and poor outcome in HCC cases [34, 35]. However, the situation with basigin-2 was found to be complicated with the discovery that other isoforms of basigin, basigin-3 and basigin-2, in conjunction could inhibit invasion and proliferation of HCC cells [43]. Thus, the role of basigin-2 in HCC needs to be investigated further. Lin et al. have recently reported elevated Sp1 expression in HCC as compared to normal tissues. Moreover, higher Sp1 expression correlated with tumor differentiation, vascular invasion, and metastasis. Sp1 was also associated with poor survival rates [44].

37.5 Role of Sp1 in Angiogenesis

Tumors require nutrients and oxygen for their growth and maintenance for which angiogenic pathways are activated. Vascular endothelial growth factor (VEGF) is a renowned positive regulator of angiogenesis [31, 75]. VEGF promoter has several Sp1 binding sites, which enhance the expression of VEGF [57, 85]. Binding of Sp1 to these sites was shown to induce VEGF expression in response to platelet-derived growth factor [18]. Furthermore, Sp1 interacts with several other factors such as hepatocyte growth factor (HGF) [66], glucose [15], androgen receptor [16], estrogen [79], and relaxin [36] to upregulate the expression of VEGF. The intracellular signaling cascades such as ERK, RAS, RAF, and MEK mediate Sp1-dependent upregulation of VEGF [70]. Several signaling intermediates which are the components of these signaling pathways such as protein kinase B, phosphoinositide 3-kinase [61], and protein kinase C zeta [58] cooperate with Sp1 to enhance VEGF synthesis. The role of Sp1 in angiogenesis in the liver has not yet been elucidated. However, VEGF expression correlates with HCC in HCV-induced liver cirrhosis [50] and has been shown to be associated with metastasis [96] and pain [101] in liver cancer patients. Moreover, HCC is a highly angiogenic tumor [97]. Thus, it is conceivable that Sp1-induced VEGF may contribute to angiogenesis in liver cancer. The pro-angiogenic role of Sp1 in other gastric cancers has been well studied. Sp1 expression was associated with microvascular density and the expression level of VEGF in human pancreatic cancer specimens [99]. Sp1 was expressed at lower levels in early stages of gastric cancer and increased as the stage of cancer advanced.

High Sp1 expression was accompanied by elevated VEGF expression in almost all the cases [98]. Expression levels of Sp1 were positively associated with microvessel density. Strong Sp1 expression also was found to correlate with poor survival in cases of gastric cancer [92]. One of the mechanisms suggested for stimulation of angiogenesis is that Sp1 in conjunction with human telomerase reverse transcriptase (hTERT) stimulates VEGF expression [46]. Sp1 thus appears to mediate angiogenesis in tumors through the induction of VEGF.

37.6 Role of Sp1 in Apoptosis

Apoptosis or programmed cell death is a vital aspect of normal cell turnover and is critical for the survival of an organism. Ability to evade apoptosis is a hallmark of cancerous cells [26]. The targets of Sp1 include both pro- and anti-apoptotic factors. In vitro studies hepatic cancer cells have shown TIGAR (TP53-induced glycolysis and apoptosis regulator) promoter as a target of Sp1 in. TIGAR is induced by p53 and is involved in reduction of glycolysis as well as intracellular reactive oxygen species (ROS) levels and evasion of apoptosis. While Sp1 has been shown to be necessary for basal level transcription from TIGAR promoter, the conditions under which the expression of TIGAR is upregulated remain to be elucidated [104]. Estrogen receptor α (ESRα) while expressed in normal liver, chronic hepatitis, and benign liver tumors are downregulated in HCC. Overexpression of ER led to apoptosis in Hep3B, a human hepatoma cell line; apoptosis increased when estrogen plus ER treatment was given. Western blot analysis revealed high tumor necrosis factor α (TNF-α) expression and active caspase 3 in Hep3B cells. The authors further showed that ESRα induced Sp1-dependent transcription of TNF-α. The upregulation of TNF-α would lead to activation of caspase 3 and consequently to apoptosis [87]. In case of cell death induced by radiation, Sp1 has a different role to play. Sp1 appeared to be negatively regulated by miR1284, a micro-RNA that is a known mediator of radioresistance [100].

Majority of the evidence concerning the mechanisms by which the tumor cells manipulate Sp1 to evade apoptosis arose from the investigations on antitumor actions of pharmacological agents. One such example is mithramycin. Mithramycin is an antineoplastic antibiotic secreted by *Streptomyces plicatus*. It is DNA and RNA polymerase inhibitor which also suppresses Sp1 expression [99]. Acyclic retinoid (ACR), a synthetic retinoid, is known to prevent the recurrence of HCC in patients who have primary tumors surgically excised, by inducing cell death in HCC cells by apoptosis [51]. The mechanism of induction of apoptosis was revealed to be transglutaminase 2-mediated cross-linking and inactivation of Sp1 and activation of caspase 3 simultaneously. Sp1 inactivation also decreased epidermal growth factor expression in HCC cells thereby contributing to apoptosis. The results were also recapitulated in nude mice treated with ACR and transplanted with HCC cells and also in hepatocarcinogenesis model induced by N-diethylnitrosamine treatment in ACR-treated rats [84].

A combined treatment of quercetin (3, 3', 4', 5, 7-pentahydroxyflavone), a flavonoid contained in several vegetables and fruits, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was found to rapidly induce apoptosis in HCC cells which are resistant to TRAIL. The combined treatment was unaffected by Bcl-xl overexpression which affords resistance to several chemotherapeutic agents. TRAIL-mediated proteolytic processing of procaspase-3 was found to be inhibited partially in HCC cells. Simultaneous treatment with quercetin was able to efficiently recover TRAIL-induced caspase activation. Sp1 induced the upregulation of DR5, a death receptor of TRAIL upon quercetin treatment of HCC cells. Moreover, quercetin treatment led to proteasomal degradation of c-FLIP, an inhibitor of caspase-8 [32]. Capsaicin is an anticancer agent which is known to cause cell death of TRAIL-induced death-resistant cells. The mechanism of apoptosis induction of Capsaicin is similar to that of quercetin. Capsaicin also induces upregulation of DR5 through Ca^{2+} influx-dependent Sp1 activation, thereby sensitizing the HCC cells to TRAIL-mediated apoptosis [49].

Some pharmacological agents exert their antitumor actions through upregulation of inhibitory microRNAs. One such example is a berberine. It is an alkaloid widely distributed in medical plants used in traditional Chinese prescriptions [80]. Berberine treatment was found to upregulate microRNA-22-3p (miR-22-3p) which directly targeted Sp1 to suppress the proliferation of HepG2 cells [11]. Physicon, an active ingredient in several traditional medicinal plants, upregulated miR-370 through AMPK/Sp1/DNMT1 signaling pathway leading to apoptosis of HCC cell lines, SMMC7721 and HepG2 [59].

37.7 Interactions of Sp1 with HBV and HCV

Chronic HBV/HCV infection greatly increases the risk of HCC development. Sp1 is a transcriptional regulator of some of the genes of HBV. HBV core promoter has two binding sites for Sp1, while the upstream ENII enhancer has one. While the upstream Sp1 site in the core promoter represses the transcription of HBV X and S genes, the binding site for Sp1 in the ENII enhancer activated the transcription of the same genes [42]. The promoter of HBV major surface antigen has four binding sites for Sp1 [65], and the synthesis of large surface antigen could be activated by exogenously expressed Sp1 [64]. Sp1 is directly involved in carcinogenic action of these viruses. The protein X of hepatitis B virus enhances insulin-like growth factor-II (IGF-II) transcription in Sp1-dependent fashion. IGF-II is implicated in tumorigenesis of liver cancer [41]. Elevated telomerase activity has been strongly associated with several types of cancers [33]. Hepatitis B virus X protein (HBx) increased Sp1 binding to the hTERT promoter and was found to mediate the activation of telomerase and increase the expression of human telomerase reverse transcriptase (hTERT) in HBx-transfected HepG2 cells and also in human HCC cases positive for HBx [45]. Sp1 is also implicated in the metastatic action of Hbx. Dickkopf-1 protein was reported to enhance HCC invasion and metastasis [83]. Hbx protein of HBV was found to upregulate the expression of DKK1 through Sp1 [60].

In the case of HBV, core protein of HCV mediates transactivation of IGF-II in Sp1- and Erg1-dependent manner [40]. HCV, through Sp1, enhances the expression of the 3β-hydroxysterol δ24-reductase which impairs p53-mediated cellular response and contributes to tumorigenesis [68, 86]. HCV-induced Sp1 binds to the promoter of TGF-β1 and activates TGF-β1 expression through the involvement of Src, p38, JNK, MAPK, and MEK1/2 kinases. Secreted TGF-β1 is implicated in proliferation, invasion, and activation of hepatic stellate cells (HSCs) [62]. Osteopontin (OPN), a secreted phosphoprotein, is strongly correlated with tumor metastasis in numerous cases including HCC in which it is highly expressed. Sp1 was reported to bind to OPN promoter and mediate its upregulation synergistically with AP-1 [29].

37.8 Conclusions and Future Perspectives

Similar to most of the transcription factors, Sp1 regulates the transcription of numerous genes which have an impact on cell proliferation, cell cycle regulation, angiogenesis, metastasis, and overall survival. Most of the studies indicate that a higher Sp1 response favors a detrimental outcome in case of HCC (Fig. 37.1). There are a host of unanswered questions regarding the role Sp1 plays in the context of liver cancer. There is scarcity of data regarding the impact of Sp1 signaling in the context of other liver cancers excluding HCC, i.e., cholangiocarcinoma, liver angiosarcoma, and hepatoblastoma. Moreover, most of the studies have utilized transformed liver cell lines for the investigations and if the observations will hold true in vivo remains to be seen. One of the major hurdles in the study of HCC is the dynamic hepatic microenvironment, which the in vitro cell line systems cannot recapitulate. A significant advancement in the said direction would be the development of improved mice models and organoid systems. Organoid systems present several advantages. It is a near physiological system which can be propagated for years without significant genomic alterations. Organoids can be generated to include various types of tissues and are an excellent model system for diseases which have poor animal models [17]. Several mice models were developed to assess various facets of liver tumors; HBV/HCV transgenic mice to study the impact of HBV/HCV infection on hepatic tumorigenesis, hepatic oncomice to profile individual genes, Mdr2 and Aox mice to understand metabolism of liver cancer cells, and tissue-specific and mosaic genetically engineered mice to assess the influence of genetics and tumor microenvironment on tumorigenesis to name a few. As is suggested previously, none of the currently existing mouse models can take into account all the complexities of HCC biology [5]. In addition, there are numerous lacunae in the literature regarding how Sp1 contributes to various hallmarks of cancer, especially aberrant cell cycle regulation, angiogenesis, and evasion of apoptosis. Filling these gaps would further our understanding of HCC biology and greatly aid us to develop new strategies of treatment.

Since Sp1 is upregulated in a majority of HCC cases, it could be an attractive candidate biomarker as a predictor of prognosis. Further clinical studies on biopsy samples of HCC could perhaps reveal a cutoff of expression levels. An expression

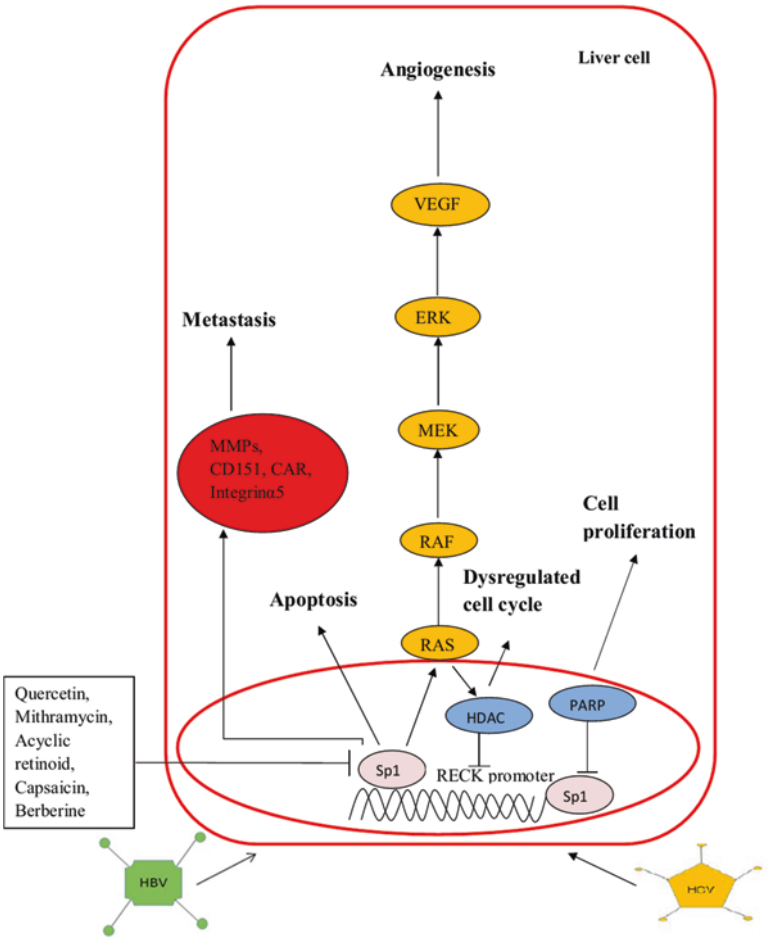

Fig. 37.1 Sp1 is a critical mediator of tumorigenesis in hepatocytes. Infection by HBV/HCV predisposes hepatocytes to undergo transformation. Sp1 mediates angiogenesis via the RAS/RAF/MEK/ERK pathway. The MMPs, CAR, and integrinα5 mediate metastasis by manipulating Sp1 activity. PARP and HDAC play an important role in dysregulating cell cycle to favor tumor growth

higher than the cutoff could perhaps predict a predilection for poor prognosis. The knowledge gleaned from these studies could then be translated to treatments. The main drug which is used to treat HCC is sorafenib tosylate which is a kinase inhibitor. Several pharmacological agents have been shown to be able to suppress the expression of Sp1 and induce apoptosis of cancerous cells. The drugs targeting Sp1 could be an attractive strategy for chemotherapy in addition to sorafenib (Fig. 37.2). Thus, Sp1 is a very attractive candidate for research, diagnostics, and therapy in the context of liver cancer with a wealth of information waiting to be tapped.

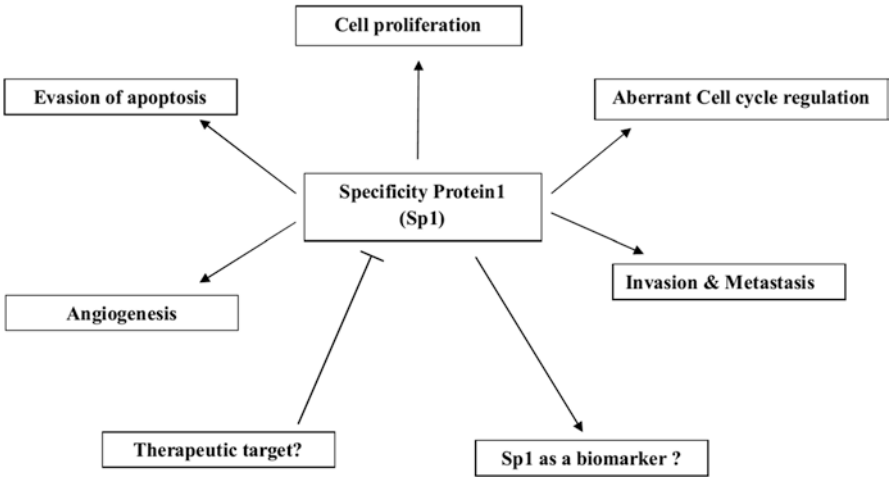

Fig. 37.2 Sp1 is an attractive target for therapy and diagnostics. The detrimental role played by Sp1 in liver cancer implies that monitoring the Sp1 expression in HCC could be invaluable in predicting prognosis as well as pharmacological agents which target Sp1 could prove to be extremely potent

Conflict of Interest Authors declare that there is no conflict of interest.

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Targeting Transcriptional Factors in Gastrointestinal Cancers and Future Prospective

38

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Abstract

Transcription factors (TFs) are deregulated in the majority of human cancers and play a major role in tumor progression and metastasis. Targeting TFs could prove to be highly effective in the treatment of gastrointestinal (GI) malignancies, as highlighted by the clinical efficiency of target molecules aiming at the nuclear hormone receptors. In this chapter, we summarize the role of different TFs discussed in the previous chapters with a focus on the emerging chemical as well as phytochemical approaches to control their functions. The outstanding diversity and efficacy of TFs as the driving force of cell transformation demands a continued search of TFs as novel and therapeutic agents for anti-GI treatments.

Keywords

Transcription factors · Gastrointestinal malignancies · NF-κB · AP-1 · HIF-1α · STAT-3 · YY1 · KLF4 · LEF-TCF · E2F-1

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38.1 Introduction

Gastrointestinal (GI) cancers are the most common and fatal malignancies around the western world, with a mortality rate ranking second in the United States [1]. GI malignancies are characterized by a stepwise growth of various epigenetic and genetic modifications upon transformation of normal cells into tumor cells [2]. Treatment options for patients with GI malignancies are very limited since these tumors are diagnosed at an advanced stage; therefore, the surgical method is not normally recommended [3]. Moreover, results achieved through alternate treatments like radio and chemotherapy are very uncertain [4]. GI tumors tend to respond poorly to chemotherapy due to various factors such as alteration of the molecular target of the anticancer drug, overexpression of the target molecule, or simultaneous activation of parallel signaling pathways. GI malignancies exhibit aberrant gene expressions, and transcription factors (TFs) serve as a junction for oncogenic signaling [5]. Upregulation of TFs such as NF-κB, AP-1, HIF-1α, STAT-3, YY1, KLF4, LEF-TCF, and E2F-1 promote the expression of oncogenes and induce tumor progression. Therefore, targeting transcription factors would serve as an important hallmark for GI cancer therapy via both direct antitumor effects as well as immunomodulatory activities.

38.2 Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells (NF-κB)

NF-κB is a TF that plays an important role in inflammation and GI cancer. The NF-κB family includes proteins like NF-κB 1, NF-κB 2, c-Rel, p65, and RelB; all of these proteins share a Rel homology domain and also form active TFs via homo- and heterodimerization [6]. Factors such as stress, cytokines, and pathogen-related molecular patterns result in the IkB degradation and eventually the NF-κB translocation into the nucleus, where it controls the protein transcription and promotes tumor cell proliferation, survival, invasion, and metastasis [7]. NF-κB also transcriptionally modulates the pro-inflammatory protein and the monocyte chemotactic protein-1 (MCP-1) [8]. GI cancer exhibits continuous activation of NF-κB, which is known to stimulate the tumor progression by triggering an inflammatory response [9]. Therefore, blocking the NF-κB signaling pathway could exhibit therapeutic benefits in GI malignancies. An important strategy for targeting the NF-κB signaling pathway is the direct inhibition of NF-κB DNA binding [10]. One such class of molecules is the Pyrrole–imidazole polyamides that specifically bind to the minor grooves of DNA at the NF-κB sites and block the activity of NF-κB in both in vitro and in vivo [11]. Phytochemicals such as curcumin inhibit GI cancer cell invasion through AMPK activation and consequent inhibition of NF-κB [12]. Curcumin downregulates the expression levels of several NF-κB-regulated genes such as B-cell lymphoma 2, COX-2, TNF, and adhesion molecules [13]. Curcumin also improves the effect of 5-FU in downregulating the NF-κB pathway and its downstream genes [14]. Another natural agent in GI malignancies is genistein,

which induces apoptosis in GI cancer cell lines via inhibition of NF-κB signaling pathway and through the downregulation of Bcl-2 and the upregulation of Bax [15].

38.3 Signal Transducer and Activator of Transcription-3 (STAT-3)

STAT-3 is a member of the signal transducers and activators of transcription family, which exists in the cytoplasm until stimulated by tyrosine phosphorylation [16]. Upon activation, STAT-3 forms dimers that translocate into the nucleus and modulate tumor growth, proliferation, and survival [17]. GI cancer cell lines have persistent STAT-3 activation either from the overexpression of tyrosine kinases or the loss of function—also known as hyperactivation—of negative modulators. Since STAT-3 drives the protein expressions that are involved in tumor proliferation and survival, blocking STAT-3 activity could serve as an effective antitumor therapy in the treatment of GI malignancies [18]. One such strategy that inhibits the activity of STAT-3 in GI cell lines is decreasing its expression through RNA interference (RNAi), which reduces the expression of genes that are specific to STAT-3 and also suppresses GI cancer cell growth in vivo [19]. Another efficient strategy of STAT-3 inhibition is blocking its DNA binding by using oligodeoxynucleotide (ODN) decoys [20]. MicroRNAs that mediate protein silencing through translational repression or via target degradation exemplify another important strategy for overcoming STAT-3-related immune resistance in GI cancer patients [21]. Curcumin in combination with 5-FU shows a synergistic inhibition of STAT3 and survivin expression levels, leading to increased cell death in GI malignancies [22]. Furthermore, genistein also causes tumor cell death in GI cancers [23].

38.4 Hypoxia-Inducible Factor-1α (HIF-1α)

Along with the expression levels of NF-κB and STAT-3, hypoxia-inducible factor-1α (HIF-1α) also exists in the cytoplasm until it is affected by an appropriate stimulus [24]. Under hypoxic situations, HIF-1α is not hydroxylated and is released from the von Hippel–Lindau protein and then translocated into the nucleus [25]. In the nucleus, HIF-1α associates with HIF-1β in order to form the HIF-1 dimer, which then binds to the DNA [26]. The activity of HIF-1α is also controlled by growth factors that regulate proliferation and survival in GI cell lines [27]. HIF-1α aids in tumorigenesis by initiating angiogenesis and modulating cell metabolism in order to enable cancer cells to survive hypoxic conditions as well as nutrient deprivation [28]. Small molecule and protein therapeutic strategies that target HIF-1α have been successfully able to inhibit the tumor development and metastasis in GI cancer patients. Curcumin aids in sensitizing gastrointestinal cell linings by targeting HIF-1α through RNAi or siRNA, thereby reducing chemoresistance [29, 30]. Genistein acts as an antiangiogenic agent by inhibiting the HIF-1α activities particularly under hypoxic conditions [31].

38.5 Activating Protein-1 (AP-1)

The activating protein-1 (AP-1) family of TFs involves multiple Jun and Fos members in which Jun is the predominant partner to form the A P-1 complex; Jun members either homodimerize with Jun itself or heterodimerize with various Fos members, and Fos members heterodimerize only with Jun members [32]. The AP-1 complex converges several growth signals at the transcriptional level, which makes the AP-1 complex an important connecting node required by many signal transduction pathways [33]. AP-1 controls tumor proliferation, variation, apoptosis, and invasion in GI malignancies. Proliferation of GI cancer cell lines requires growth factor signals such as estrogen, EGF, TGFα, heregulin, and IGFs, which activate AP-1 signaling [34]. Thus, blocking the AP-1 signaling complex might arrest several growth signals that are important for GI cancer cell proliferation and metastasis. Curcumin reduces the variation agent-dependent increase in AP-1 expression level and DNA binding [35]. It is also known to effectively inhibit gastric activation of ERK1/2 and JNK MAPK pathways, AP-1 genes, and EMT modifications as shown in in vivo studies [36]. Genistein is known to promote anti-invasive and anti-metastatic properties in HCC cell lines via the downregulation of MMP-9 and EGFR activities and the consequent suppression of AP-1 activation through the inhibition of MAPK, IκB, and PI3K/Akt pathways [37].

38.6 Yin Yang-1 (YY-1)

Yin Yang (YY)-1 is an abundant and multifaceted zinc finger TF member of the Polycomb group gene family, which acts as an activator or repressor of transcriptional activity [38]. It can bind to DNA directly or indirectly via association with other TFs [39]. It can also control the epigenetic regulation through its association with genes that regulate chromatin organization [40]. YY1 changes its structure and DNA-binding activity according to temporal redox modifications in the cell. YY1 plays a key role as a suppressor mechanism and in the resistance of GI tumors to Fas-associated apoptosis. YY1 controls many GI cancer-related proteins such as c-Myc, c-Fos, and p53 [41]. According to a study, YY1 is shown to serve as a potential biomarker for the sensitivity of hepatocellular carcinoma (HCC) cell lines to HDAC inhibitors [42]. YY1 also reduces the properties of HDAC inhibition on HCC carcinogenesis in vitro and in vivo [42]. These properties indicate the potential role of YY1 and HDAC1 in the clinical prognosis and treatment of HCC patients. Another study revealed that the knockdown of YY1 in gastric cancer cell lines suppressed tumor proliferation by inhibiting the Wnt/β-catenin signaling pathway, while YY1 overexpression exerted oncogenic properties via initiation of the Wnt/β-catenin signaling pathway [43]. This study reveals that downregulated expression levels of YY1 via siYY1 would reduce its oncogenic properties through tumor growth inhibition, elevated G1 phase accumulation, and apoptosis.

38.7 Krüppel-Like Factor 4 (KLF4)

Krüppel-like factor 4 (KLF4) is a TF with three carboxyl C_2H_2 zinc fingers. KLF4 is primarily expressed in epithelial cells of the GI tract, vascular endothelial cells, and the thymus. KLF4 is a key regulator of tumor proliferation [44]. This TF also inhibits amplification of redox-sensitive development and promotes the expression levels of numerous negative regulatory proteins such as p21, p27, p53, and retinoblastoma [45]. These results indicate that KLF4 is a negative regulator of cell proliferation. KLF4 has been known to serve as an important regulator in tissue differentiation [45]. Downregulated KLF4 expression in GI malignancies could possibly contribute to the transformation of normal cells to malignant cells and cellular hyper proliferation [46]. These outcomes are consistent with KLF4's role in tumor growth inhibition and cell cycle arrest. A study revealed that KLF4 gene expression was significantly reduced in gastric cancer samples as compared to normal tissues [47]. Another study revealed low expression levels of KLF4 as an independent negative prognostic factor [48]. It also suggested that KLF4 might exert a suppressive effect on the tumor proliferation and metastasis in GI cancers. Moreover, the expression and activity of KLF4 in GI malignancies could serve as important biomarker in GI cancer research in the future. KLF4 and curcumin reveal synergistic effects in promoting apoptosis as well as inhibiting tumor proliferation and invasion in gastric cancer cell lines via the inhibition of the PI3K/Akt and JNK/MAPK signaling pathways [49].

38.8 T-Cell Factor/Lymphoid Enhancer-Binding Factor (TCF/LEF)

T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) proteins are the primary downstream effectors of the Wnt pathway [50]. TCF/LEFs are multifunctional genes, which use their sequence-specific DNA-binding and context-dependent interactions to identify which proteins will be modulated by Wnts. β-Catenin is known to control the transcriptional regulation through the formation of a complex with LEF/TCF TFs, resulting in the stimulation of downstream target genes [51]. A study revealed that retinoic acid (RA) reduces the expression of beta-catenin-LEF/TCF signaling pathway [52], thereby inhibiting GI tumorigenesis. β-Catenin/TCF–LEF increased the expression levels of AKT1 and revealed the link between abnormal Wnt/β-catenin signaling and resistance to apoptosis [53]. This shows that β-catenin/TCF controls the transcriptional regulation of the AKT1 protein.

38.9 E2F-1

E2F-1 is a member of the E2F family of TFs. E2F-1 is linked specifically with pRb and its transcriptional activities, which are negatively regulated by pRb gene. The growth suppressive action of pRb relies on its capability of interacting with E2F

[54]. Cyclin-cdk-dependent phosphorylation of pRb eliminates this inhibition and releases E2F-1 transcriptional activity [55]. As a TF, E2F-1 stimulates many genes that aid in the synthesis of DNA, including repair, cell-cycle regulation, and apoptosis [56]. Moreover, E2F-1 also attracts many upstream signals in development of the cell via the cell cycle or death through apoptosis [57]. E2F-1 promotes cell proliferation by activating many genes that stimulate the transition from the G1 phase to the S phase [58]. E2F-1 increases the proliferation of cells in GI malignancies [59], and the overexpression of E2F-1 encourages inactive cells to enter into the S phase [60]. All these suggest that E2F-1 plays a key role in controlling cell growth. Moreover, E2F-1 overexpression is known to increase apoptosis in numerous cell types, indicating its critical role in synchronizing programmed cell death [61]. Recent in vivo studies have suggested that E2F-1 also functions as a tumor suppressor [62]. Dysregulation of the E2F TFs is common in most human cancers, including GI malignancies, and plays a critical role in cell growth survival. These properties make E2F-1 a novel target for new GI cancer treatments that are aimed at controlling the E2F-1 activity.

38.10 Conclusion

Although several potential molecular targets for the treatment of GI malignancies have been developed, TFs are particularly attractive. In the process of transducing a signal from the cell surface to the genome, TFs lay at the hinge. Moreover, TFs are controlled by several converging pathways, and they control multiple genes that could contribute toward tumor progression in return. Preclinical and clinical investigations have supported the idea of targeting TFs. Further research, however, must focus on more similar studies in order to develop a novel and potent anti-GI malignancy treatment.

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