Ganji Purnachandra Nagaraju *Editor*

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This book is dedicated to my family,
my teachers, and my friends

Preface

Enzymes are essential for live cells to maintain homeostasis and to generate energy for survival. In cancer cells, enzymes may behave aberrantly and activate transduction pathways. These transduction pathways are implicated in cancer cell proliferation, migration, and other metastatic behaviors. Recent findings of biochemical and genetic approaches in gastrointestinal (GI) malignancies have amended the knowledge of the intricate signal transduction cascades as well as their associated enzymes in GI cancer cells. Research during the past few decades has revealed the significance of biochemical aspects and transduction pathways in the progression of GI malignancies. Almost all GI cancers are highly malignant and aggressive; therefore, finding vulnerable targets is important for GI cancer therapy. Preclinical and clinical investigations have shown the efficacy of the inhibitors of specific enzymes including tyrosine kinases. Although several investigations have evaluated the involvement of these enzymes in GI malignancies, these studies are still not comprehensive. Therefore, this book provides the current knowledge available on the relevance of the aforementioned enzymes and their inhibitors in the therapeutic uses in GI cancers.

This book has sixteen chapters that covers different GI malignancies (esophagus cancer, gastric cancer, pancreatic cancer, liver cancer, and colorectal cancer) associated with tyrosine kinases and their inhibitors. In most of the chapters the clinical significance of tyrosine kinase inhibitors has been elaborated. It is my pleasure to present this book to the scientific community for a better understanding of GI malignancies. I hope this will help spark new ideas and innovative research for the benefit of scores of patients affected with this deadly disease.

Atlanta, GA, USA — Ganji Purnachandra Nagaraju

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Dr. Ganji Purnachandra Nagaraju is a faculty member in the Department of Hematology and Medical Oncology at Emory University School of Medicine. He obtained his MSc and PhD, both in biotechnology, from Sri Venkateswara University in Tirupati, Andhra Pradesh, India. His present research focuses on translational projects related to gastrointestinal malignancies. He has published over 60 research papers in highly reputed international journals and has presented more than 50 abstracts at various national and international conferences. He serves as editorial board member of several internationally recognized academic journals. *Dr. Nagaraju* is an associate member of the Discovery and Developmental Therapeutics research program at Winship Cancer Institute. He has received several international awards. He also holds memberships with the Association of Scientists of Indian Origin in America (ASIOA), the Society for Integrative and Comparative Biology (SICB), the Science Advisory Board, the RNA Society, and the American Association of Cancer Research (AACR).

1 HGFR and FGR2: Their Roles in Progression and Metastasis of Esophageal Cancer

Ranjeet Kumar, Akriti Gupta Jain, Mamoon Ur Rashid, Saeed Ali, Neelam Khetpal, Ishtiaq Hussain, and Sarfraz Ahmad

Abstract

Esophageal cancer (EC) is the sixth leading cause of malignancy-related death in the world. The disease is characterized by two types of histologies: esophageal squamous cell cancer (ESCC) and esophageal adenocarcinoma (EAC), which are the most common in the Western world. While alcohol has proven to lead to ESCC, it has not been associated with EAC. Progressive dysphagia (first with solids, followed by liquids) and rapid involuntary weight loss are the two most common symptoms, which make most patients seek medical attention. Most patients have a long period of symptoms before they seek care. At diagnosis, ~50% of the patients already have metastasis. The treatment of gastroesophageal cancers continues to pose significant clinical challenges for various defined reasons. The majority of patients fail intensive and toxic multimodality therapy for locoregional disease, and systemic chemotherapy for metastatic carcinoma gives short-term benefits only. Our understanding of the molecular pathology of gastroesophageal cancers has considerably increased during the recent years, leading to the development of novel targeted therapeutic agents that have proven to be promising in improving the patients' survival with minimal adverse events. Receptor tyrosine kinases (RTKs) play pivotal role(s) in the formation, maintenance, growth, and differentiation of the malignant cells encompassing both his-

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tological types of EC. Malignancies treated with chemotherapy/radiation therapy face the challenge of developing resistance and increasing the aggressive nature of cancerous cells leading to undesirable recurrence. In peer-reviewed literature, an array of RTKs have been described in ESCC, and more recently, they are being assessed for their therapeutic utility. Notably, structures of hepatocyte growth factor receptor (HGFR) and fibroblast growth factors receptor 2 (FGR2) are two of the many prominent RTKs studies thus far. In this chapter, we thoroughly discuss the clinical characteristics of the disease and structure-functional aspects of various RTKs with focus on HGFR and FGR2 as it relates to the translational and clinical outcomes of EC.

Keywords
Esophageal cancer · Receptor tyrosine kinases · Hepatocyte growth factor receptor · Fibroblast growth factors receptor 2 · Metastasis

1.1 Introduction/Background

Esophageal cancer (EC) is diagnosed in nearly 500,000 patients and is the world's sixth leading cause of malignancy-related death after lung, liver, gastric, colorectal, and breast cancers [1]. EC is characterized by two types of histology, squamous cell cancer (ESCC) and adenocarcinoma (EAC). While the ESCC is endemic in East Asia, the EAC is the most common histology found in the Western countries [2]. The ESCC and EAC not only differ in epidemiology but also in the risk factors and treatment approaches [3]. While EAC arises from a premalignant lesion, Barrett's esophagus, which stems from increasing incidence of gastroesophageal reflux disease (GERD) and obesity, ESCC is known to occur with a synergistic effect of alcohol and tobacco with some contributions from the environmental factors such as nutritional deficiencies, limited consumption of fruits and vegetables, and intake of hot beverages [4]. In a prospective cohort study on more than 120,000 people with 16 years' follow-up, ESCC was shown to be associated with alcohol consumption with a 4.6-fold increased risk, and combined exposure with smoking increased the risk more than 8-fold [5].

While alcohol has proven to lead to ESCC, it has not been associated with EAC. In a survey-based cohort study, Freedmen et al. showed that people who consumed more than three alcoholic drinks a day in comparison to one drink a day had an increased risk of ESCC but not EAC [6]. Some other risk factors for ESCC include lye ingestion, esophageal stricture, radiation exposure, head and neck cancer, achalasia, smoked opiates, Plummer-Vinson syndrome, tylosis, chronic ingestion of extremely hot tea, and zinc, molybdenum, and vitamin A deficiency (Table 1.1). While the ESCC most commonly occurs in the upper two-thirds, the EAC arises in the distal third of the esophagus. Overall EC most commonly starts in the lower third of the esophagus (75%), followed by the middle third (20%), and finally upper third (5%).

At diagnosis, 50% of the patients already have metastasis [2]. In the early 1970s, only 5% of the patients with EC remained alive at 5 years after the diagnosis, while in the recent decades, the number of survivors has increased to 20% [7, 8], but for

Table 1.1 Common risk factors for esophageal cancer depending on the histopathology

Histology	Risk factors
ESCC	Alcohol
	Tobacco
	Nutritional deficiencies (vitamin A)
	Limited intake of fruits and vegetables
	Intake of hot beverages
EAC	Obesity
	GERD, Barrett's esophagus

Abbreviations: *ESCC* esophageal squamous cell cancer, *EAC* esophageal adenocarcinoma, *GERD* gastroesophageal reflux disease

advanced-stage cancer, the number drops to 0.9% [4]. The incidence of ESCC has declined from 2.8 to 1.2 per 100,000 cases in the USA and Europe, but the incidence of EAC has increased from 0.4 to 2.8 per 100,000 people from the mid-1970s to 2012 [9]. This change is presumably stemming from the basic etiologic difference between the ESCC and EAC, as obesity is becoming rampant with almost 40% of the population of the USA being obese, and with the increasing GERD, the EAC incidence is also increasing. In ESCC, several molecular markers are being identified, and thus, newer molecularly targeted therapies are being developed in addition to the preoperative chemo-irradiation, surgical interventions, and postoperative chemotherapy [10, 11] as prognosis remains poor in patients with ESCC who undergo esophagectomy and lymph node dissections. Identification and understanding of these molecular markers are essential for the discovery of novel therapeutic targets and hence seeking new strategies for the EC treatment.

1.2 Clinical Characteristics of Esophageal Cancer

Progressive dysphagia (first with solids, followed by liquids) and rapid involuntary weight loss are the two most common symptoms, which make most patients seek medical attention. Most patients have a long period of symptoms before they seek care. Both ESCC and EAC are more common in older men (aged >60 years). The EAC and ESCC differ in the prevalence based on gender with a mean male to female ratio of 6:1 and 3:1, respectively [12]. The race differences have been shown with EAC being more common among the Whites, whereas Blacks and African-Americans are rather more at risk for ESCC.

Other clinical presentations based on the clinical stage and spread of the disease include chest pain due to mediastinal spread, odynophagia, hoarseness (from recurrent laryngeal nerve involvement), hypercalcemia (paraneoplastic; related to the release of parathyroid hormone-related peptide), pulmonary aspiration (tracheoesophageal fistula from necrosis and extension, obstruction), and infrequent bleeding. An examination is often benign.

1.3 Structures of Hepatocyte Growth Factor Receptor (HGFR) and Fibroblast Growth Factor Receptor 2 (FGR2)

The role of tyrosine phosphorylation was first reported by Ogawa et al. in 1989. They discovered enhancement in the levels of tyrosine phosphorylation in different cancer including EC by using a monoclonal antibody against O-phosphotyrosine (PTYR) [13]. Over the years, an array of different receptor tyrosine kinases (RTKs) have been described in ESCC, and more recently, they are being assessed for their therapeutic utility. FGR2 and HGFR are two of the many prominent RTKs studied thus far.

FGFR is a member of the RTK family, and the transmembrane RTKs are encoded by the FGFRs which interact with FGFs and are involved in signaling [14]. This signaling pathway plays an imperative role in the normal human growth, and aberrations in the genetics of FGFRs enhance downstream signaling, hence impairing the regulation of cell proliferation, differentiation, and migration [15–17]. The FGFR2 gene encodes *KGFR* (keratinocyte growth factor receptor) and *K-sam* and is located on chromosome 10q26 [14].

Hepatocyte growth factor (HGF) is a ligand that binds to HGFR. It is also known as MET (mesenchymal-epithelial transition) factor and is located on chromosome 7 (7q21–q31), spanning more than 120 kb in length, and consists of 21 exons separated by 20 introns [18]. The *MET* gene encodes a transmembrane receptor, which has intrinsic tyrosine kinase activity. Usually, HGF activation of Met is securely controlled by the machinery including paracrine ligand delivery and ligand-activated receptor internalization and degradation. This HGF/Met signaling plays part in the progression and metastasis of several human cancers including gastroesophageal, colorectal, lung, breast, and renal among many more [19, 20].

1.4 Expression and Roles of HGFR and FGFR2 in EC

MET amplification has been reported with a prevalence of 1.5–30.5% in gastroesophageal cancers [21–27]. The prevalence varies depending on the method used for the detection and the cutoff set for the assay. The presence of MET amplification is associated with poor prognosis, higher grade and stage of the tumor, and shorter median survival time [21, 22]. Interestingly MET expression was correlated with PD-L1 expression [28]. An interesting finding in non-small cell lung cancer that can possibly be extended to EC while deciding treatment is that MET amplification has been suggested to be involved in resistance to EGFR tyrosine kinase inhibitors [29, 30].

1.5 Signaling Pathways Related to HGFR and FGR2 in EC

1.5.1 Structure and Functions of HGF and c-Met Receptors

The RTKs are the cellular surface receptors for numerous polypeptide growth factors, which play vital roles in the physiologic regulation of cell growth, differentiation, and survival. Same physiologic features are also required for various other

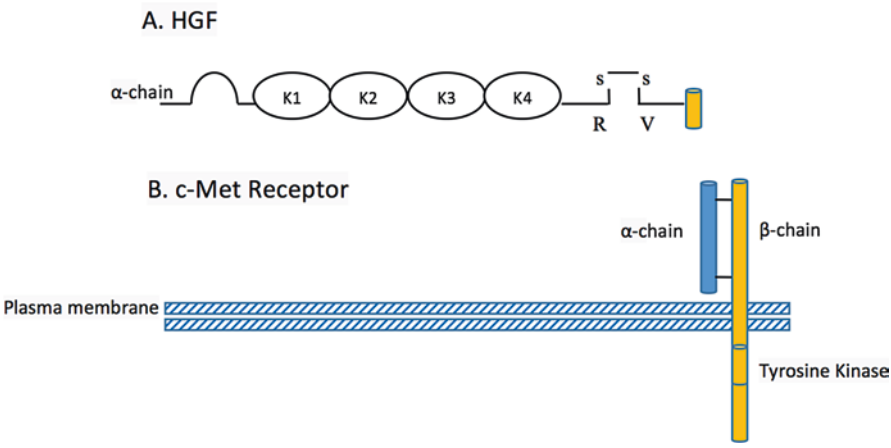

Fig. 1.1 Schematic representation of the structure of hepatocyte growth factor (HGF) and c-Met (mesenchymal-epithelial transition). (**a**) Alpha-chain with a hairpin loop at the N-terminal end followed by kringle domains and beta-chain at the other end connected by disulfide bonds. (**b**) Alpha and beta subunits of the c-Met receptor. The intracellular component of the beta subunit of the c-Met receptor has tyrosine kinase activity

cancers as well. Cancers acquire their astute nature by the dysregulation of RTKs in order to proliferate exponentially and survive longer by altering the apoptotic mechanisms. One of such mechanisms utilized by the tumors is overexpression of the c-MET and its ligand HGF.

The location of c-*MET* proto-oncogene is on chromosome 7, and its protein product is c-MET tyrosine kinase which is expressed in numerous organs, such as the liver, pancreas, prostate, kidney, muscle, and bone marrow [31]. Weidner et al. studied and identified the resemblance of HGF with scatter factor (SF) because both of which are involved in the cellular movement and growth during the physiologic or pathologic regeneration, and therefore, HGF has been observed to play shielding role(s) in numerous diseases such as cirrhosis of the liver and fibrosing lung and kidney diseases [32–35].

The structure of HGF consists of α- and β-chains. The α- and β-chains are linked with each other by a disulfide bond. The α-chain contains a hairpin loop at the N-terminal and subsequently followed by four kringle domains as shown in Fig. 1.1.

1.5.2 HGF-c-Met Signaling Mechanisms

In the normal cells, signaling mechanisms are controlled by the discrete signaling cascades that translate extra- and intracellular processes into specific output responses. These signaling pathways are triggered after the binding of ligand to the extracellular domain of a receptor, followed by recruitment of the adaptor proteins or kinases that activate an intracellular cascading network of protein and lipid intermediaries that produce a cellular response.

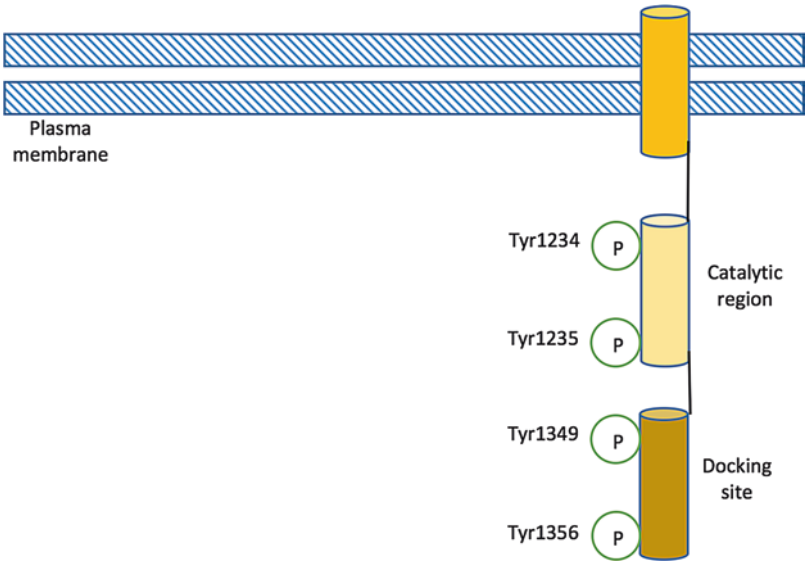

Fig. 1.2 Schematic demonstration of the downstream phosphorylation of various tyrosine kinases after hepatocyte growth factor (HGF) binds to c-Met (mesenchymal-epithelial transition)

The binding of HGF to c-Met receptor results in the phosphorylation of two intracellular tyrosine kinases, viz., Tyr1234 and Tyr1235. Subsequently, Tyr1349 and Tyr1356 are phosphorylated, and these tyrosine kinases then act as "degenerate motif" for plethora of downstream activation pathways such as PI3K/AKT (phosphoinositide 3-kinase-Akt) [36] and extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathways that play important role(s) in the tumor growth, survival, angiogenesis, and metastasis [37] (Fig. 1.2).

1.5.3 Role of MET Signaling in Physiologic and Pathologic Growth

As noted above, the HGF-c-Met pathway has been linked to the cellular proliferation, growth, and differentiation – and these functions are essentially required during the normal embryonic development as well as the disease development. In embryonic hepatocytes and trophoblastic cells of placenta, HGF and MET are found to carry growth and survival signals, so, in experimental HGF or c-Met knockout embryos, significant reductions in the size of the liver is noticed [38, 39]. The disturbances in HGF-MET pathway also disrupt the proper nervous system connections, leading to the abnormal growth and often death of the sensory and motor nerves [40–42]. In tumors, the transcription of MET is induced by the presence of hypoxia, inflammatory cytokines, and various angiogenic factors that are abundant in the life of tumors.

1.5.4 Structure, Functions, and Signaling of FGR2

Virtually all tissues of our body express FGFs where they serve essential purpose during the embryonic development as well as in the adult for the maintenance of tissue repair, regeneration, and metabolism. FGFs partake essential role(s) in the regulation of cellular proliferation, migration, and differentiation. FGFs have also been found to be cardioprotective following the ischemic heart injury due to its role in tissue repair [43].

FGF binding to its receptor FGFR2 activates the tyrosine kinase and causes receptor dimerization and autophosphorylation of the kinase domain. The activated FGFR2 phosphorylates FRS2 (FGFR substrate 2) on several sites and causes recruitment of surplus adaptor proteins. FGFR2 is linked with four other major pathways, including RAS-MAPK, PLCγ (phospholipase C-γ), PI3K, and JAK/STAT [44].

1.5.5 Role of FGFR2 in Esophageal Cancer

In a study by Kato et al., FGFR2 was found to be amplified in gastric cancer cell lines and endometrial carcinomas. The authors also demonstrated the FGFR2 overexpression in esophageal squamous cell carcinoma through FISH (fluorescence in situ hybridization) analysis [45]. Yet in another study, overexpression of FGFR2 was linked with the progression of Barrett's esophagus to early esophageal adenocarcinoma [46]. These findings suggest that like many other cancers, FGFR2 can also be a potential for targeted therapies in esophageal cancers.

1.6 Molecular-Targeted Therapy of the HGFR and FGFR2 in EC

Esophageal and gastric cancers are among the most common gastrointestinal cancers and leading causes of morbidity and mortality. For gastric cancer, although incidentally more common than the esophageal cancer, the mortality rate of esophageal cancer labels it as the sixth most common cause of cancer-related deaths globally [47]. Though relatively early detection of esophageal and gastric cancers has now become possible with the help of advanced surveillance and improved community awareness, still the bulk of patients present with clinical evidence of advanced-stage disease.

With the traditionally aggressive surgical resection, chemotherapy, and radiotherapy, only 20% of the patients are able to achieve 5-year survival rate [48]. Because of this and the fact that our understanding of molecular biology has much advanced, now the therapy is trending more toward the targeted molecular and immunotherapies. Table 1.2 outlines the targeted therapies with regard to their receptor targets found in the upper gastrointestinal malignancies.

Table 1.2 Target receptors and targeted agents currently in the use and under trials for the upper gastrointestinal malignancies

Target receptors	Targeted therapies
HER-2	Trastuzumab, lapatinib, pertuzumab
EGFR	Cetuximab, panitumumab
VEGF and VEGFR	Ramucirumab, ziv-aflibercept, bevacizumab, apatinib
c-MET	AMG 337, onartuzumab, rilotumumab, foretinib
FGFR (various kinds)	Brivanib, cediranib, nintedanib, lenvatinib, sulfatinib, dovitinib, ponatinib, and lucitanib (nonselective multi-kinase inhibitors) and AZD4547, BGJ398, LY2874455, and JNJ-42756493 (selective inhibitors)
mTOR	Everolimus
PD-1	Pembrolizumab, nivolumab

Abbreviations: *HER-2* human epidermal growth factor receptor 2, *EGFR* epidermal growth factor receptor, *VEGF* vascular endothelial growth factor, *VEGFR* vascular endothelial growth factor receptors, *c-MET* mesenchymal-epithelial transition, *FGFR* fibroblast growth factor receptor, *mTOR* mammalian target of rapamycin, *PD-1* programmed cell death protein-1

1.6.1 c-Met-Targeted Therapies, Adverse Reactions, and Relevant Clinical Trials

c-Met overexpression has been documented in various malignancies including lung cancer, breast cancer, gastric cancer, colorectal cancer, bladder cancer, and esophageal adenocarcinoma. The rate of overexpression is comparatively higher in esophageal cancer than gastric cancer, and c-MET overexpression in esophageal cancer is also associated with the poorer prognosis [49]. AMG-337, rilotumumab, and onartuzumab are a few prominent Met inhibitors that are currently being tested in various clinical trials. Foretinib is another multi-kinase inhibitor predominantly active against the c-Met and VEGFR.

AMG 337 is a selective MET inhibitor; therefore, it blocks the MET kinase activity in the tumors that show *MET* amplification [50]. Most common adverse reactions in one phase I study of AMG 337 were headaches, nausea, and drug-related hepatic disorder [51]. NCT02016534 is a phase II, multicenter clinical trial evaluating the role of AMG-337, a c-MET inhibitor, in patients with gastric and gastroesophageal cancers exhibiting MET overexpression.

Rilotumumab is a humanized monoclonal antibody (mAb) that interfere with the interaction between HGF and c-Met and, thus, effectively block c-Met phosphorylation leading to the inhibition of c-Met-activated downstream signaling pathways. Early phase II clinical study data reported fatigue, nausea, peripheral edema, and constipation as some of the main treatment-related side effects. Other rare serious adverse reactions reported include edema, deep vein thrombosis, pulmonary embolism, and diarrhea [52]. Rilotumumab was tested in RILOMET-1 study, but because the study primary end points were not met and the risk of death was also relatively higher, the study was ceased prematurely [53].

Onartuzumab is an antibody tested against various MET-amplified neoplasms. It was assessed in phase III clinical trial (METGastric) for advanced gastroesophageal

cancers in combination with mFOLFOX6 – but this study was also sacked after the study failed to meet its primary end points and also revealed that the drug is associated with serious adverse reactions such as neutropenia [54].

1.6.2 FGFR-Targeted Therapies, Their Adverse Reactions, and Relevant Clinical Trials

Various drugs such as brivanib, cediranib, nintedanib, lenvatinib, sulfatinib, dovitinib, ponatinib, and lucitanib are developed as nonselective multi-kinase inhibitors. On the other hand, the selective FGFR inhibitors including AZD4547, BGJ398, LY2874455, and JNJ-42756493 are also developed. Studies have shown FGFR2 amplification in 3–6% of gastroesophageal neoplasms, and its overexpression is also associated with a relatively poorer prognosis [55].

Dovitinib, a multi-kinase nonselective inhibitor, was evaluated in a few clinical trials of gastric neoplasms overexpressing FGFR2. In a study (NCT01719549), the efficacy and safety of dovitinib monotherapy failed to control the growth of gastric cancers with FGFR2 amplification. In another phase II study (NCT01921673), the combined effects of docetaxel with dovitinib in patients with gastric neoplasms are currently being evaluated. Common adverse reactions associated with dovitinib are gastrointestinal such as nausea, diarrhea, vomiting, decreased appetite, and fatigue. Less common adverse events related to dovitinib includes hypertension, hypertriglyceridemia, non-cardiac chest pain, pulmonary embolism, and neutropenia [56].

AZD4547 is a selective and potent FGFR 1–3 inhibitor used in patients with gastric and gastroesophageal cancers. The SHINE study, a randomized open-label phase IIa trial, assessed the efficacy and safety of AZD4547 in patients with advanced gastric and gastroesophageal cancers and compared the results with paclitaxel treatment. The results of the study showed that the monotherapy of AZD4547 was better than paclitaxel (NCT01457846) [57]. In another study, the most common adverse effects associated with AZD4547 were dysgeusia, diarrhea, and stomatitis [58].

1.7 Economic/Financial Issues for Patients, Professionals, and Hospitals

Several studies have claimed that generally the White patients with EC have better survival outcomes than the African-American/Black patients [59–64]. Because the racial disparities are also associated with socioeconomic status (SES), newer studies have questioned whether these differences are more of a function of the SES differences [65, 66]. The SES has previously been shown to have a considerable influence on the overall health and outcomes of malignancies. More confounding factors including smoking status, worse eating habits, exercise and presence or absence of health insurance, and exposure to the environmental hazards lead to a difference in cancer outcomes in different economic strata [67]. However, Loretta et al. reported in their study by suggesting that race is not substantially related to the overall

survival after adjusting for other prognostic variables; nonetheless, globally the SES continues to be one of the key factors toward the patients' overall survival [68].

1.8 Conclusions and Future Perspectives

The treatment of gastroesophageal cancers continues to pose significant clinical challenges for various defined reasons. Majority of patients fail intensive and toxic multimodality therapy for locoregional disease, and systemic chemotherapy for metastatic carcinoma gives short-term benefits only. Our understanding of the molecular pathology of gastroesophageal cancers has considerably increased during the recent decades, leading to the development of novel targeted therapeutic agents that are being proven to be promising in improving the patients' survival with minimal adverse events. RTKs play pivotal role(s) in the formation, maintenance, growth, and differentiation of the malignant cells encompassing both histological varieties of EC, ESCC, and EAC. Malignancies treated with chemotherapy and/or radiotherapy face the challenge of developing resistance and increasing aggressive nature of cancerous cells leading to the undesirable recurrence. Keeping this scenario in mind, it is even more imperative to be vigilant for newer sites and signaling pathways that develop because of one being blocked.

Hence, more trials, including an integration of the genomics, transcriptomics, metabolomics, and proteomics profiling with biomarker-matched targeted therapy alone (or in combination with the emerging immunotherapies), are needed to overcome this lethal disease and for improving the progression-free survival in ESCC. Another aspect that needs to be explored is co-expression of RTKs in ESCC, hence considering the different combinations of tyrosine kinase inhibitors for such malignancies. This requires a higher understanding of tumor pathophysiology and biology at genomic and bioinformatic levels [3].

Conflict of Interest None of the authors has any potential financial or commercial conflict of interest associated with this research manuscript (chapter).

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Role of Src and VEGFR Tyrosine Kinases in Esophageal Cancer

2

P. S. Sushma

Abstract

Esophageal cancer is considered as highly aggressive and potentially serious malignancy with higher incidence in developing countries. Squamous cell carcinomas are frequent types of esophageal neoplasms. In spite of significant advances in treatment techniques, the prognosis patterns of esophageal cancer remain dismal. Novel strategies are required in order to detect the esophageal cancer in the initial stages and to improve the current style of therapy. Tyrosine kinases are the preliminary mediators of signaling pathways. The oncogene activation being plugged by specific tyrosine kinase inhibitors may be a promising approach for genome-based therapeutics. Src family kinases (SFKs) are expected to accomplish exceptional role in directing signal transduction within cellular environment. The stimulation of these Src tyrosine kinases by interacting with various growth factors leads to the development of esophageal cancer. Growth factor receptors are the important components of cellular transformation in cancers among which VEGF and its receptors are foremost. Emerging advances in the use of growth factor receptors in targeted therapy are expected to promise the esophageal cancer patients survival rate. This review outlines the role of Src and VEGFR tyrosine kinases in esophageal cancer progression.

Keywords

Esophageal cancer · Tyrosine kinases · Src · VEGFR · Growth factors

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2.1 Introduction

Esophageal cancer accounts globally 2 lakh deaths per year being the sixth most common malignancies with the highest prevalence reported in China [1]. There are two categories of esophageal cancers, i.e., squamous cell carcinoma that is more frequent in African Americans and among people who consume tobacco products and the other esophageal adenocarcinoma (EA). The deficiency of trace elements and genetic susceptibility were suspected to contribute to esophageal carcinogenesis. Despite the significant advances in research on esophageal cancer, the accurate pathogenesis of the disease is unclear [2]. Stratified squamous epithelium covers the mucosal surface of the esophagus and maintains cell growth and terminates the process of differentiation [3]. Monitoring the proliferation rate of esophageal epithelium may be the finest approach in treatment of esophageal diseases [4].

2.1.1 Src and Esophageal Cancer

SRC gene in humans that encodes c-Src which is a tyrosine kinase protein phosphorylates tyrosine residues in other proteins. Src tyrosine kinase promotes other signals when elevated and is suggested to be involved in multiple cancer progression. The protein levels of Src and their protein kinase functions are higher in human cancers in comparison with corresponding noncancerous tissues. In addition the levels increased with clinical staging. An increase in activity of protein kinase is noticed in several studies conducted on human cancer cell lines. The mechanisms behind the elevated Src levels is yet to be elucidated despite several studies noticed that the mechanism is dependent on several factors [5, 6].

Although these SFKs (Src family kinases) originated century ago, their activities as biological signals and mediators are a novel understanding. Steven Martin studies on avian retroviruses as well as RSV developed the cellular transformation concept and identified v-Src (RSV transforming gene) along with protein products; these findings resulted in cellular Src discovery. The family of Src tyrosine kinases comprise of nine types of non-receptor TKs (tyrosine kinases) with almost homogenous structures, which include Fyn, Yes, Lck, Hck, Lyn, Yrk, Fgr, Blk, and Src. Src TK is made up of N-myristoyl sequence followed by SH2 and SH3 domains and kinase and C regulatory domain as well [7]. N-myristoyl plays a prominent role in placing Src tyrosine kinase inside the plasma membrane within the cell. The SH2 domain contains around 100 amino acids which bind to short peptide of phosphotyrosine (PT). The SH3 domain consists of 50 amino acids that unite target protein via hydrophobic amino acids and proline. The kinase and C regulatory domains consist of negative regulatory autophosphorylation site Y416 and positive regulatory autophosphorylation Y527 site, respectively [8]. The role of SH3 domain is very dominant in combining the cytoskeleton as well as translocation of Src protein, whereas SH2 and kinases supervise signal transduction process [9]. Generally cells undergo phosphorylation at carboxyl terminal anchored by Src kinase (CSK). The phosphorylated Y527 and SH2 domain combinations result in inhibition of SFK protein

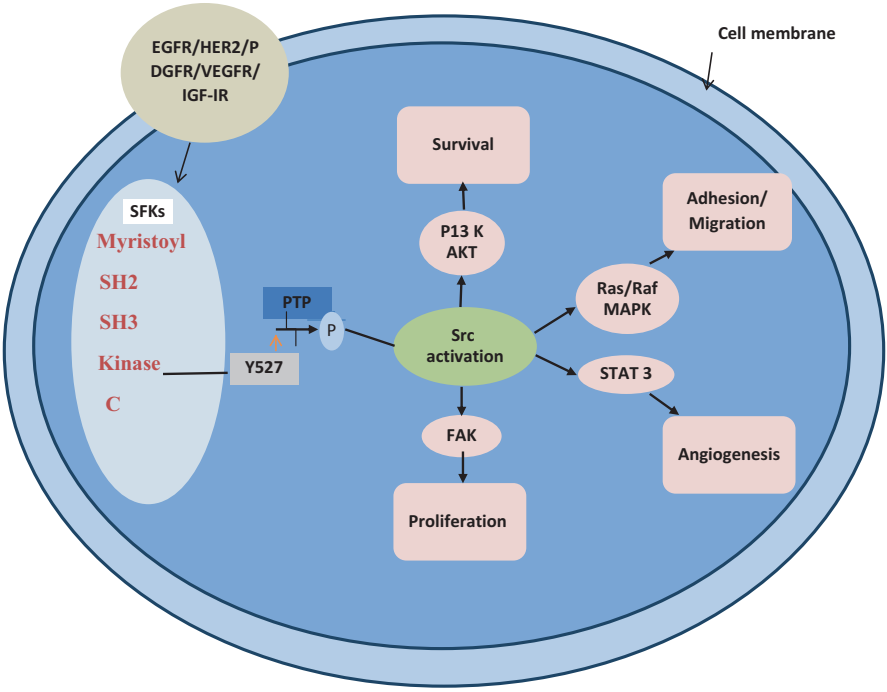

Fig. 2.1 Dephosphorylation at Y527 site of kinase domain leads to Src activation resulting in cell survival, adhesion, angiogenesis, and proliferation. Src activation induced by SFK pathway and growth receptors

with a curling tail and head. However cancer cells or cells that are in mitotic phase, the SFK protein directs Y527 phosphorylation decomposition and Y416 self-phosphorylation so that Src gets activated [10, 11]. After activation Src TK may participate in several activities in vivo which include proliferation, differentiation, migration, as well as angiogenesis through signaling pathways (Fig. 2.1).

Campone and co-workers reported that overexpression of Src delays cancer progression [12]. Zheng reported in human malignancies, Src and its activation due to mutation are very rare [13]. Briefly due to limitations in mutation activity and Src kinase deficiency, further studies discussing the effects of tyrosine kinases in progression of human cancers were hampered. Recent study outlined that the wild Src overexpression contributes to the activities of signaling pathways and molecules [14, 15]. Generally Src reacts with proteins like EGFR/HER2/PDGFR/VEGFR/IGF-IR [16] and other factors like signal transduction, transcription activation factors, as well as focal adhesion kinases (FAKs) [17]. Tyrosine kinase Fyn a member of Src family assembles antigen-presenting cells and is activated by proteins belonging to lymphocyte activation molecular family via SH3 structural domain [18]. Recent studies demonstrated the association between the activation of Src tyrosine kinase and various cancers and their progression [19]. Studies correlated Src TK activation and its association with incidence of colon carcinoma and found the higher

expression levels of Src tyrosine kinases in patients with colon carcinoma metastasis [20]. Another study proposed that the levels of activated Src TK were almost twice higher in colon carcinoma tissues in comparison with corresponding non-tumor tissue, and their study was positively correlated with clinical grading [21].

The Src overexpression and effects of its activation look pleiotropic. Several substrates of Src are found to be phosphorylated within cancer cells containing Src-triggered forms, and these substrates are found to be associated with carcinogenicity [22]. Additionally these variations during the process of signal transduction may contribute to the several transcriptional events like alteration in tumor cell invasivity, angiogenesis, tumor growth, as well as apoptosis, resulting in the contribution of metastatic phenotype development. The activated Src and its role in oncogenesis make it an attractive target for drug discovery. The increase in Src activity was reported since the past three decades in different cancers. Higher levels of Src protein were identified in multiple tumors; however their protein levels did not represent activity of particular protein kinase. Potential kinase experiments were developed for determining the specific protein activity.

Experiments conducted on v-Src proteins demonstrated its role as oncogene in inducing cellular transformation. Peyton Rous discovered that v-Src along with RSV (*Rous sarcoma virus*) induced chicken tumors [23]. Tyrosine kinase is a protein product of v-Src. These tyrosine kinases are found in normal cells along with the cellular homolog and are supposed to function as proto-oncogenes [24]. Despite their homogenous protein structure, carboxyl terminus of protein v-Src is shortened leading to the alterations in amino acid sequences throughout the protein that enhance the activation mechanisms [25]. They execute similar operations; the activity of v-Src kinase and its transformation are considerably in rise in comparison with c-Src. Additionally the process of transformation of v-Src at cellular level can develop a ten times rise in PPT (protein phosphotyrosine) than c-Src. V-Src in spite of its moderate expression levels exhibits cellular transformation at high range significantly by alterations in cellular morphology, proliferation in low level of serum, cytoskeletal reorganization, as well as anchorage-independent growth. The in vitro changes may interpret their results in vivo. V-Src phosphorylates cellular substrates that are present on tyrosine kinase, thus leading to cellular transformation. These events are supposed to influence the proteins in regulation of cell growth as well as differentiation. Some studies concluded that members of signal transduction cascades, transcription factors, as well as growth factor receptors also may be affected by the v-Src phosphorylation [22, 26].

In some conditions oncogenes denote fetal genes that are improperly evidenced in adult tissues. The role of Src in oncogenesis was suggested by different studies conducted on fetal tissues and their differentiation, especially in neurons [27]. Bielfman and co-workers concluded that vertebrate neurons while differentiating may express c-Src in higher levels with elevated activity of kinase. Similar events reported within brain tumors [27]. Elevated levels of c-Src activity transforming minimally represent that exalted Src results in progression of cancer than carcinogenesis [28]. There are no evidences confirming Src functioning as human oncogene. Avian v-Src and chicken c-Src in their mutant forms were composed, but they

are not recognized in humans. Few experiments revealed activating mutations within colon carcinomas in humans with high Src levels hypothesizes the oncogenic prospectives of Src.

Several studies outlined alteration in gene expression, structure, and activity of Src in various stages of esophageal carcinoma [29–31]. BE (Barrett's esophagus) which increases the probability of esophageal cancer is often considered as premalignant condition leading to esophageal adenocarcinoma. According to the studies of Cartwright and his colleagues, the activation of Src in Barrett's epithelia (BE) was due to dephosphorylation of Tyr 527 [32, 33]. The activities of Src-specific kinase are found elevated in premalignant as well as in BE in comparison with duodenum and normal esophagus. An increase in activity of Src kinase was reported at initial premalignant condition. The activity of Src differs between different regions of BE.

BM (Barrett's mucosa) is similar to duodenal mucosa that contains rudimentary villi, surface epithelium, and lamina propria [34]. It is defined from keratinized ESE (esophageal squamous epithelium). Lamina propria consists of plasma cells and lymphocytes, although there are no studies reporting the role of lymphocytes and plasma cells in Src expression). Additionally there is no significant difference within the cellular population of lamina propria of BM and duodenum; thus the differences between the activities of Src between these two epithelia cannot be explained. Probably Src kinase activity in BE may be due to acute neutrophilic cell infiltration. However, Src kinase activity in the neutrophils is unknown, and the BE samples could not detect any infiltration in neutrophils. In EA the Src kinase activity may be directly correlated with stromal fibroblasts activity in tumor cells. Low levels of Src kinase are detected in fibroblasts, which are not transformed [36]. Activation of Src in EA and BE is much the same as ulcerative colitis and colon cancers showing association between the activation of Src, dysplasia, and progression of adenocarcinoma. The Src activation in premalignant and malignant epithelium of the esophagus may be due to elevated specific kinase activity and may not be due to increased Src protein levels.

Src kinases can be activated due to several reasons like alteration in Src regulation through dephosphorylation or phosphorylation, due to shifts in localization of Src at subcellular levels. Genetic mutations and its association with various cellular proteins may result in activation of Src kinase. Phosphates are potential group of proteins which activate Src by dephosphorylation. To address the mechanism behind Src kinase activation in BE, some studies assessed the distribution of Src in between TX100-insoluble and TX100-soluble fractions of tissues. As few cases reported, active kinase forms are associated with insoluble fractions of cytoskeleton [37–41], and we can assume similarly in the case of BE. Anyway the results suggested that the activity of Src in BE may be analogous to soluble fraction designating the activation in BE may not be due to Src subcellular shift within cytoskeleton. This background resulted in hypothesis stating an increase in Src-specific activity in BE may be due to altered tyrosine phosphorylation. This data suggest Tyr 527 is

dephosphorylated on Src suggesting this dephosphorylation is the key mechanism behind Src activation in BE.

The previous works on BE represented notable heterogeneity at molecular level within complex epithelium [42], like expression of actin-binding protein villi at variable levels within BE surface [42], where the same heterogeneity of expression was observed. Heterogeneous expression observed in BE patients without dysplasia was correlated at structural level with microvilli [42] .This concept added new dimension to spatial and anatomic relationships within intestinal dysplasia, metaplasia, and cancer in BE [43]. The above observation confirms the Barrett's epithelia's complexity and shows microheterogeneity without dysplasia with regard to the activity of Src kinase. The consistent nature of activation of Src in premalignant and malignant Barrett's epithelia correlates with high Src function in cellular transformation, suggesting Src activation in initiation of esophageal metaplasia. Research aiming at Src substrate recognition where phosphorylation altered, which dephosphorylate Src, provides striking data concerning the process of metaplasia.

2.1.2 VEGFR (Vascular Endothelial Growth Factor) and Esophageal Cancer

VEGF signaling protein executes prominent function in the process of vasculogenesis as well as angiogenesis. The activity of VEGF is confined to vascular endothelial cells, and there are no studies outlining their effect on other cell types. Few in vitro studies reported that VEGF can enhance cell mitogenesis as well as migration. By stimulating microvascular permeability, VEGF is notable for vascular permeability.

Receptors that correspond to VEGF are called VEGF receptors. The cellular responses of VEGF family are stimulated by holding to the receptors of tyrosine kinase at cellular surface so that they are dimerized and activated. These receptors contain extracellular portion in which 7 immunoglobulin kind of domains, and a transmembrane region. Intracellular portion consists of a split TK domain. VEGF-A binds to VEGFR-1 as well as VEGFR-2. VEGFR-1 functions in angiogenesis monocyte migration and is often known to modulate the signaling of VEGFR-2. It sometimes acts as dummy receptor that sequesters binding of VEGF from VEGFR-2, an important aspect in embryo vasculogenesis. VEGFR-2 mediates cellular responses like cell survival, vascular permeability, and cell proliferation. VEGFR-3 is known to mediate lymph angiogenesis with VEGF-C and VEGF-D (Fig. 2.2).

Overexpression of VEGF was found in majority of esophageal cancer cases, and multiple studies outlined the high expression of VEGF and their correlation with poor patient survival rate [44, 45]. There were no clear evidences of prognostic role of VEGF in presurgical chemoradiotherapy cases. Clinical investigations conducted on ESCC as well as EA never demonstrated a significant correlation between expression of VEGF and treatment response [46, 47]. This condition may be due to VEGF induction and angiogenic activity during preoperative chemoradiotherapy delivery. Antitumoral therapy induces the development of resistant

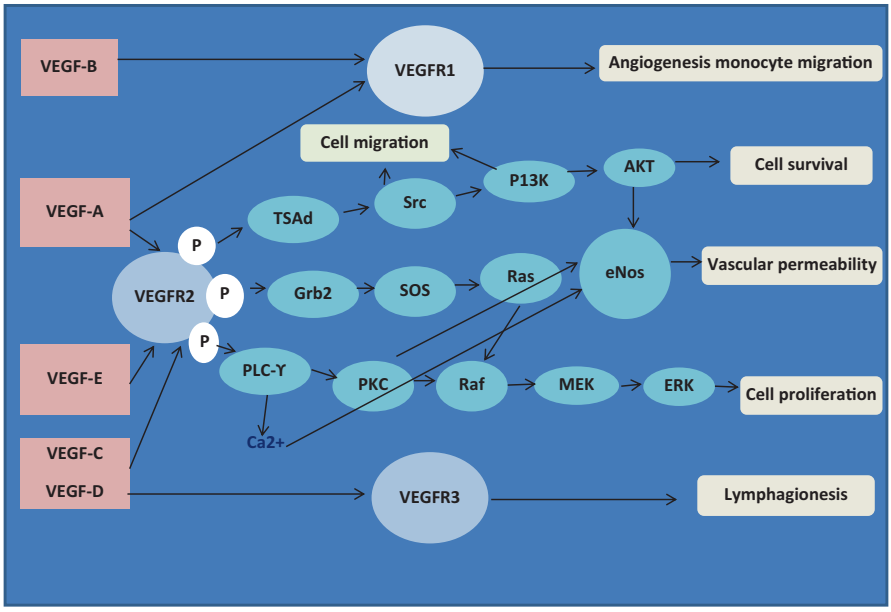

Fig. 2.2 VEGF family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor. VEGF functions by activating members of VEGFR tyrosine kinases. VEGFR1 mediates angiogenesis monocyte vibration; VEGFR2 mediates cell survival, vascular permeability, and cell proliferation. VEGFR3 mediates lymphangiogenesis

tumor phenotype which reduces the interconnection between VEGF levels during pretreatment and treatment response [48, 49], whereas few researchers suggested elevated VEGF levels and poor prognosis among preoperative chemoradiotherapy patients [50–52].

Lymph node metastasis (LNM) as well as micrometastasis (LMM) appears to be the chief factors in ESCC prognosis, and VEGF-C is well known to play a prominent role in lymphangiogenesis [53, 54]. Few observations revealed that the high levels of VEGF-C were significantly associated with cancer and lymphatic invasion as well as LNM. In addition, elevated microvessel density levels correlated significantly with metastasis and lymphatic invasion. Their studies showed overexpression of VEGF-C may be a possible risk factor during lymph node metastasis among ESCC with submucosal invasion, including LMM. Another study reported the association of VEGF expression with angiolymphatic invasion and lymph node metastasis, which is correlated with shorter survival rate among patients with adenocarcinoma [55].

The production of VEGF-C and VEGF-D was noticed in both ESCC and EA, possibly supporting their role in tumorigenesis during premalignant condition [56]. This was reported by Kitadai et al. [57], who demonstrated an increase in VEGF expression along with vessel density and PD-ECGF which is an additional angiogenic factor in ESCC. Auvinen et al. [58] found that VEGF-A is secreted by Barrett's

glandular epithelium, along with sialomucin as well as sulfated mucins. VEGF-A receptor is potentially manifested on angiogenic blood vessels in BE. These conclusions support an interplay within angiogenic glandular epithelium and neovascularization of newly invaded blood vessels of Barrett's epithelium. Studies indicated the impact of neovascularization on Barrett's esophageal carcinoma patient survival, but VEGF may not be the factor stimulating the process among patients with Barrett's esophageal carcinoma [59]. Significantly higher level of VEGF expression and fibroblast growth factor among adenocarcinoma cases was identified than either normal EM or BE [60].

VEGF blockade may prevent tumorigenesis in ESCC. SU6668, an antiangiogenic agent, may act as an inhibitor of TK for VEGFR. Nakamura et al. [61] and colleagues treated xenografted A-431cells, a cell line of human cancers with SU6668, and observed a significantly less number of vessels and tumor volume in study group than in control group, indicating its use for treating ESCC patients. Sunitinib and sorafenib are the two multitargeted tyrosine kinase inhibitors approved for treating metastatic RCC [62]. VEGF 165 antisense RNA with low microvessel density and small tumor volume inhibits tumorigenesis as well as angiogenesis in ESCC patients [63]. Bevacizumab which is a recombinant mAb may bind to the VEGF or its isoforms in humans and blocks VEGF binding to its receptors [64]. Multiple investigations on bevacizumab in humans were conducted at various centers designing bevacizumab as a sole therapeutic option or with radiotherapy, chemotherapy, targeted agents, or antiangiogenic agents. Particularly bevacizumab and irinotecan combination was approved by the USFDA which resulted in overall longer survival time, in comparison with placebo in patients diagnosed with colon cancer [65].

Bevacizumab is under clinical trials at various centers for ESCC. In some lung cancer patients, bevacizumab leads to a life-threatening hemoptysis, so the trials were restricted to EA. In esophageal cancer patients after chemoradiation, there can be a probability of VEGF blockade. In addition to esophageal adenocarcinoma, the effects of VEGF antibody were studied in various xenograft models [49]. The phase II clinical studies conducted on drugs like cisplatin, irinotecan, and bevacizumab reported encouraging results among gastric and GE junction adenocarcinoma patients manifesting around 8 months' time for progression and improvement on controls by 75% [66].

Since years, VEGFR-1 and its role in biology were studied. Fong and his coworkers revealed that *flt-1*-null mutant mice die around E8.5 because of vascular endothelial cellular overgrowth along with blood vessel disorganization [67] suggesting the prominence of VEGFR-1. A mutant mouse strain lacking TK of VEGFR-1 was developed in order to study the -ve regulation of this particular receptor; surprisingly the mouse was found to be healthy with regular circulatory system representing the -ve function of VEGFR-1 and is unconventional of TK function but dependable on ligand-binding domains [68]. This mouse may be useful in representing the VEGFR-1 signal's role and importance in cancer progression. Mutant mice model along with other models like *flt-1 TK* exhibited slow tumor growth low rates of metastasis in lung cancer model and mild inflammation in rheumatoid arthritis model in comparison with wild-type mice [69, 70]. Other studies

reported that slow tumor growth was exhibited in wild-type mice with *flt-1 TK* bone marrow [69, 70]. Kaplan and colleagues reported that anti-VEGFR-1 bone marrow cells might be prominent in premetastatic niche development, which can promote tumor metastasis [71]. These studies outline the prominence of VEGFR-1 signaling in tumor progression in vivo through VEGFR-1-positive cells which are derived from the bone marrow. Additionally human carcinomas may express VEGFR-1 and may utilize signaling phenomena for cancer growth directly [72].VEGFR-3 has specific TK when stimulated with VEGF-C; Ras pathway and t PKC pathway were triggered for lymphangiogenesis. Anyway clarification is required regarding which autophosphorylation sites in VEGFR-3 are prompting these pathways and subsequently leading to lymphangiogenesis.

The VEGF-VEGFR consists of restricted molecules which enhance angiogenesis. The VEGF-A utilizes both tyrosine kinase receptors VEGFR-1 and VEGFR-2 and neuropilin-1 as co-receptor. Ligands like PlGF, VEGF-C, VEGF-D, and VEGFR-3 are involved in pathological angiogenesis like tumor vasculature, whereas tumor metastasis to lymph nodes expresses elevated VEGF-C/VEGF-D reporting VEGF-C/VEGF-D and VEGFR-3 combination takes part in lymph vessel-dependent malignant cellular migration within lymph nodes. Anti-VEGF-VEGFR drugs and TK inhibitors were developed, and bevacizumab, a humanized monoclonal antibody, was approved for cancer therapy [73, 74]. Multikinase inhibitors like sunitinib and sorafenib are approved for hepatic and renal cancer patients. Other drugs which target VEGF-VEGFR combinations are anti-VEGFR-1- or anti-VEGFR-2-neutralizing antibody and soluble VEGFR-3, VEGFR-1 or VEGFR-2 peptide vaccine therapy [75], and anti-PlGF antibody [76, 77] that were developed and are under clinical trials.

Kim and co-workers revealed that antihuman VEGF-A-neutralizing antibody can suppress cancer growth in the immune-deficient mice [78]. Here the Ab suppresses human VEGF-A (tumor derived), secreted from cells derived from the bone marrow of mouse and tumor-associated fibroblasts. In addition, antibody treatment can suppress tumor growth without combinatorial chemotherapy indicating the blocking of blood vessels by anti-VEGF-A antibody within cancer tissues, thereby suppressing the existing cancer vasculature by promoting apoptosis of endothelial cells. As per clinical trials, anti-VEGF-A antibody treatment may not suppress the cancer growth except renal malignancies.

A hypothesis on anti-VEGF antibody efficacy and anti-VEGFR TKI on cancer development is called "vascular normalization," where the retention of VEGF-A produces a more stable vascular structure covered with pericytes with low permeability [79], which results in low tissue pressure in cancer cells having good diffusion of anticancer drugs. Vascular normalization as well as new tumor angiogenesis suppression may occur in cancer patients after anti-VEGF-VEGFR therapy.

Side effects like kidney malfunction, hypertension, bleeding, arrhythmia, proteinuria, and thrombosis were observed after anti-VEGF-VEGFR therapy [65, 80], among which proteinuria and hypertension are higher indicating a direct relationship with VEGF-A blockage in malignant tissues. Decreased VEGF-A levels in

kidney may damage vascular endothelial cells within glomeruli resulting in glomerular microvascular dysfunction leading to proteinuria. The VEGF-VEGFR blockage at molecular level has to be cleared. The resistance acquired by cancer cells after antiangiogenic therapy is a point to be addressed. The clinical trials did not report consistent results with regard to the efficacy of anti-VEGF-VEGFR therapy and survival time. Survival time decreased among patients during the treatment, whereas efficacy decreased after long period, indicating tumor resistance to this therapy. Several experimental models studied resistance. According to Casanovas and colleagues' reports, gene expression among angiogenic factors like FGF causes resistance against anti-VEGF therapy in mice [81], and it was hypothesized that on long-term antiangiogenic treatment, cancer cells receive hypoxia and malnutrition. An in vitro model was developed with malignant cells that were cultured under double stresses. After ten cycles, cancer cells exhibited upregulation of phospho-Akt, as well as increased survival rate along with high invasiveness [82]. So DDS under antiangiogenic treatment may induce a malignant phenotype of tumors. New strategies are needed to address the possible malignant phenotype after antiangiogenic treatment.

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The Clinical and Biological Significance of Tyrosine Kinases in Gastric Cancer

3

Neelam Khetpal, Saeed Ali, Ranjeet Kumar, Mamoon Ur Rashid, and Sarfraz Ahmad

Abstract

Gastric cancer (GC) is the leading cause of cancer-related mortality, and its deadly nature can be secondary to its presentation in advanced stages. Unavailability of any gold-standard treatment and also the lack of unanimous classification schemes that can lead to inter-observer variability, lead to difficulties in the clinical reproducibility. Several classification systems have been proposed for GC. The most used classification system is Lauren classification, which classifies it into "intestinal," "diffuse," and "mixed" subtypes.

This chapter summarizes the characterization of GC with genomic and molecular analysis to stratify the heterogeneous disease, role of tyrosine kinase (TK), receptor tyrosine kinases (RTK) and tyrosine kinase inhibitors (TKI), targeted therapies and ongoing clinical trials, toxicities associated with various commonly used agents/regimens in the disease, and future perspective of TKI in GC.

Advances in the genomic technologies have facilitated the study of key genetic alterations in GC including gene expression, epigenetic disturbances, chromosomal alterations, and transcriptional changes, which therefore can stratify GC at the molecular levels. The characterization of GC at molecular levels has led in developing new therapeutic targets that would potentially provide personalized prognosis and treatment. The RTKs are membrane-bound proteins that play significant role(s) in the pathogenesis of many cancers including GC. Of many RTKs, epidermal growth factor (EGF), vascular endothelial growth factor

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(VEGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) have been found with higher frequencies in metastatic growth and progression of GC, which serve as potential target(s) for targeted therapies in GC. Human epidermal growth factor receptor 2 (HER-2) that promotes cell proliferation, adhesion, migration, and differentiation is overexpressed in 15–30% of GC cases. The phase III ToGA (trastuzumab for gastric cancer) study evaluated the role of adding trastuzumab, an anti-HER-2 monoclonal antibody, to the chemotherapy regimen in the first line of treatment in patients with HER-2-positive advanced-stage GC.

VEGF is seen to be overexpressed in up to 58% of GC cases. The REGARD study and the phase III study have shown an improved overall survival benefit with ramucirumab, a monoclonal VEGFR2 antibody. Cetuximab is used when EGFR is overexpressed in gastric tumors, and dovinitib decreased phosphorylation of FGFR2.

Common toxicities of trastuzumab include fever, chills, hypotension, dyspnea, bronchospasm, and respiratory distress, but life-threatening side effects such as cardiotoxicity and congestive heart failure can also occur. In the RAINBOW trial, ramucirumab plus paclitaxel showed higher rates of neutropenia, and other toxicities related to ramucirumab included hypertension, thromboembolic disease, and hemorrhage. Cetuximab use can lead to skin disorders such as dry skin, dermatitis acneiform/rash, and paronychia.

Taken together, these molecular and cellular mechanisms/targets and supporting clinical trials outcomes have a significant impact on the overall quality of life and the prognosis of patients with gastric cancer.

Keywords

Gastric cancer · Tyrosine kinase · Tyrosine kinase inhibitors · Receptor tyrosine kinase · Biological functions · Tumorigenesis · Clinical trials · Anticancer drugs · Clinical outcomes

3.1 Introduction/Background

Gastric cancer (GC) is the fifth leading cause of cancer after lung, breast, colorectal, and prostate cancers and third most common cause of cancer-related deaths in both genders (Table 3.1). The highest mortality rates are reported in East Asia, whereas the lowest were reported in the North America [1]. Despite its gradually decreasing incidence, ~75% mortality rate makes it one of the highest worldwide [2]. In most countries, its 5-year survival rate is between 10% and 30%, whereas in the United States, it ranges between 20% and 30%. The highest 5-year survival rate is found in Japan, where it ranges from 50% to 70% for both genders [3].

Common risk factors for GC are infection by *Helicobacter pylori*, smoking, pickled vegetables, and obesity. GC is generally divided into epithelial and non-epithelial neoplasms. Most of the GCs are of epithelial origin. Non-epithelial GCs

Table 3.1 Prevalence of the top five types of cancers in men and women [4]

Rank	1	2	3	4	5
Cancer in men	Lung	Prostate	Colorectal	Stomach (gastric)	Liver
Cancer in women	Breast	Colorectal	Lung	Cervix	Stomach (gastric)

predominantly includes lymphomas and mesenchymal tumors. Most cases of GC are sporadic, whereas 10% of the cases run within the families, and between 1% and 3% of the cases are due to genetic syndromes (for instance, hereditary diffuse gastric cancer and gastric adenocarcinoma and proximal polyposis of the stomach). Furthermore, gastric cancer can also develop in the setting of various other hereditary cancer syndromes [4].

As depicted in Fig. 3.1, gastric carcinogenesis is a complex, multifactorial, and multistep process that progresses from normal mucosa through chronic gastritis, atrophic gastritis, and intestinal metaplasia to dysplasia and carcinoma. This sequence of events may take several years to complete. *H. pylori* has been considered as one of the most common environmental agent causing an increased risk of gastric cancer. Per the International Agency for Research on Cancer (IARC), *H. pylori* is a group 1 carcinogen for gastric cancer [5]. Two virulence factors responsible for the pathogenicity of *H. pylori* include CagA (cytotoxin-associated gene A) in the Cag pathogenicity island and the vacuolating cytotoxin (vacA) [6]. Besides *H. pylori*, numerous dietary habits have been studied and reported that alter the risk of gastric cancer (Table 3.2).

Symptoms of GC are non-specific such as abdominal pain and dyspepsia, which are often mistaken for indigestion or peptic ulcer disease. Patients may also present with nausea or early satiety from the tumor mass or in cases of an aggressive form of diffuse-type gastric cancer (linitis plastica) from poor distensibility of the stomach. GC involving Auerbach's (myenteric) plexus may present with dysphagia, and this variety of dysphagia is termed as pseudoachalasia. Therefore, GC should be considered in the differential diagnosis for older patients presenting with dysphagia [7].

The threshold for suspicion of the advanced disease should be low if dyspepsia is concurrently present with alarm symptoms like dysphagia, weight loss, gastrointestinal bleeding, and a palpable abdominal mass. Accumulation of alarm symptoms in GC is associated with a higher risk of death [8]. Like many other cancers, it is not uncommon for gastric cancer to present in the late phases after the disease has already reached an advanced stage. It is only possible in the early stages where a total surgical resection of the tumor can lead to a complete cure. However, most tumors in the early stages are often asymptomatic and even after surgical resection, tumors can recur resulting in a relatively shorter survival times. This innate ability of GC to present in the late stages and the lack of effective therapy for the advanced stages unfortunately is associated with the higher recurrence and mortality rates.

Advanced-stage cases of GC manifest according to the type of tissue or organ involvement such as jaundice, ascites, or gastrointestinal tract obstruction. Peritoneal implant in the pelvis (Blumer's shelf) can lead to peritoneal fluid accumulation or colorectal obstruction. Blumer's shelf or cul-de-sac can be felt on rectal or vaginal

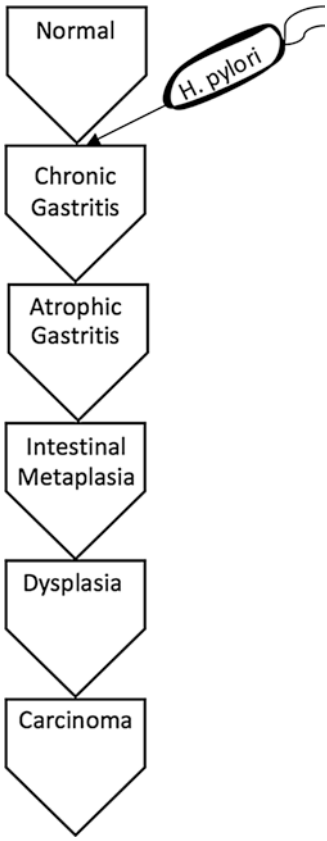

Fig. 3.1 Schematic presentation of the key stages of gastric carcinogenesis

Table 3.2 Effects of key dietary factors on the risk of gastric cancer

Factors that increase the risk of gastric cancer	Factors that decrease the risk of gastric cancer
Helicobacter pylori	Mediterranean diet (high consumption of fruit, vegetables, cereals, legumes, nuts and seeds, and seafood, with olive oil)
Pickled vegetables	
Smoked food	
Lack of fruits and vegetables in diet	Moderate alcohol consumption (particularly red wine)
Red meat	
Processed meat	Fresh fruits and vegetables
Tobacco[a]	

[a]Smoking potentiates the carcinogenic effect CagA positive *H. pylori*

examination, which may indicate that a tumor has metastasized to the pouch of Douglas. The GC involvement of the lymph nodes can easily be appreciated on the physical examination such as supraclavicular lymph nodes (Virchow's node) and protuberant nodules around the umbilicus (Sister Mary Joseph nodule).

Clinically, the gastric neoplasm is classified as an early or advanced stage and histologically into various subtypes (on the basis of major morphologic components). The World Health Organization (WHO) has recognized five major histologic types of gastric carcinoma: tubular, papillary, mucinous, poorly cohesive (with or without signet ring cells), and mixed [9].

3.2 Characterization of Gastric Cancer with Genomic and Molecular Analyses to Stratify the Heterogeneous Disease

Different classification systems exist to characterize gastric cancer. Most popular classification systems for GC include the Lauren classification and the WHO classification (based on the predominant histological pattern) [10]. Lauren classification further classifies it into two subtypes, viz., intestinal type and diffuse type. The two variants exhibit marked molecular and clinical heterogeneity. The intestinal type most commonly occurs in the elderly male patients and affects gastric antrum, which is frequently associated with intestinal metaplasia. Tumor cells exhibit adhesion and lesions are scattered in the distant positions. In contrast, the diffuse type commonly affects the relatively younger population; cells lack adhesion and forms non-cohesive scattered tumor cell population. It frequently involves gastric body and predominantly involves females. The diffuse type has a worse prognosis as compared to the intestinal type and has a high propensity for intraperitoneal metastasis and CDH1 (cell-cell adhesion receptor gene E-cadherin) silencing [11].

Etiologically, the intestinal type of GC is commonly caused by environmental factors such as *H. pylori*, whereas the diffuse type is more genetic in etiology [12]. The carcinogenesis of intestinal GC entails *H. pylori* along with the diet and other environmental factors and is a multistep process involving atrophic gastritis, intestinal metaplasia, dysplasia, and ultimately cancer. The diffuse type develops directly from the chronic active gastritis bypassing the atrophic gastritis and intestinal metaplasia. The 2010 WHO classification divides gastric cancer into four major histological subtypes, viz., tubular, papillary, mucinous, and poorly cohesive (including signet ring cell carcinoma) [13].

Clinically, gastric cancer can be classified into early and advanced-stage disease. The early GC is limited to mucosa and submucosa with and without lymph node metastases, mostly 2–5 cm in size and located along the lesser curvature. Grossly, early GC can be divided into type 1 (protruded growth), type II (superficial growth), type III (excavating growth), and type IV (infiltrating growth with lateral spreading). Histologically, most of the early gastric cancers have tubular or papillary architecture and are well-differentiated. The 5-year survival of early gastric cancer is around 90%, depicting an excellent prognosis. The advanced-stage GC invades into muscularis preppie and beyond. Grossly, it could be ulcerating, fungating, infiltrative, or combined. Pathologically, several histological patterns coexist, and there is marked architectural and cytological heterogeneity in advanced-stage gastric cancer. It carries a poor prognosis with a 5-year survival of 60% or less [13].

Histopathological classifications sometimes guide therapy but are insufficient to guide personalized treatment, which is seriously needed given the wide heterogeneity of gastric cancer.

The hereditary diffuse gastric cancer (HDGC) accounts for approximately 3% of the GC cases and harbor germline mutations in CDH1 that encodes for E-cadherin in a significant proportion of cases. It has a penetrance rate of 80%, making it a high-risk for developing into a diffuse gastric cancer. Additional familial syndromes associated with GC include hereditary nonpolyposis colorectal cancer II (HNPCC/Lynch syndrome II) with MSH2 (MutS protein homolog 2) and MLH1 (MutL homolog 1) mutations, mostly Li-Fraumeni syndrome with TP53 (tumor protein 53) mutations, adenomatous *polyposis coli* (APC) mutations, and Peutz-Jeghers syndrome (STK11 or serine/threonine kinase 11). Majority of the GC occurs sporadically. Some of the important risk factors include blood group A (20% increased risk compared to other blood groups), *H. pylori*, and EBV (Epstein-Barr virus) infection. Clustering of *H. pylori* can explain the increased incidence of gastric cancer in certain families [13].

3.2.1 Genomics of Gastric Cancer

Recent advances in genomic technology have greatly facilitated the GC biology to be studied at molecular levels thus identifying candidate driver genomic alterations such as gene expression, epigenetic disturbances, chromosomal alterations, and transcriptional changes (as depicted in Fig. 3.2) [10].

3.2.2 Gene Mutations in Gastric Cancer

The systemic analysis and high throughput of genetic alterations in the genome can be facilitated by next-generation sequencing (NGS), which has identified novel gene mutations in the GC. Mutations in the genes involved in chromatin remodeling, genome integrity, cell adhesion/cytoskeleton/motility as well as *Wnt* and RTK signaling pathway have been identified in GC. The genomic instability plays important role(s) in tumorigenesis and can be caused by the mutations in "caretaker/tumor suppressor genes" such as *TP53* and *BRCA2* (breast cancer susceptibility gene 2), which are primarily involved in the DNA damage detection and repair and are frequently mutated in GC. Chromatin alteration is another emerging cellular mechanism of carcinogenesis which impairs DNA accessibility to transcriptional factors and thus greatly affects gene expression. SW1-SNF (switch/sucrose nonfermentable) is a chromatin-remodeling complex, and *ARID1A* (AT-rich interactive domain 1A gene) encodes a subunit of this complex. *ARID1A* is a commonly mutated chromatin-remodeling gene in GC. The genes involved in the cell adhesion/motility/cytoskeleton regulate the cell-extracellular matrix and intercellular interactions. The mutations in these genes [including *CDH1*, *CTNNA1* (rabbit

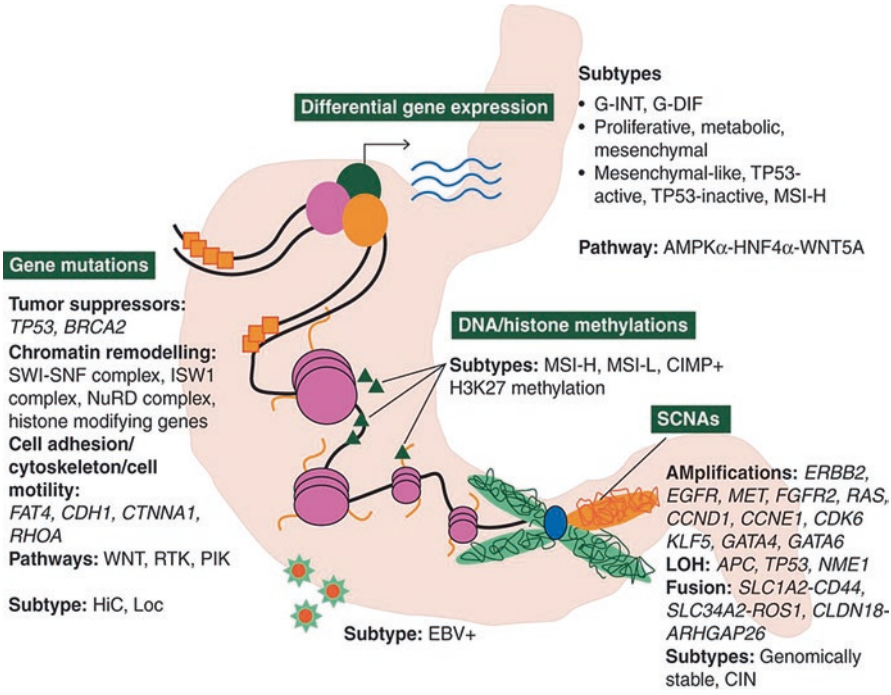

Fig. 3.2 Genetic and epigenetic modifications of gastric cancer (GC). The genetic alteration that contributes to GC involves gene mutations, differential gene expression as well as somatic copy number alterations (SCNAs). The epigenetic modifications involve DNA as well as histone methylation. The subtypes highlighted in red are reported in The Cancer Genome Atlas (TCGA) study [10]

catenin alpha-1), and *RhoA* (ras homolog gene family member A)] are frequently seen in the diffuse type of gastric cancer. While *CDH1* belongs to the E-cadherin family and regulates cell-cell adhesion, *CTNNA1* encodes a protein involved in the cell adhesion to the cytoskeleton.

The NGS has also identified key aberrant cellular pathways in GC. Two major pathways involved are the Wnt signaling pathway and the RTK-associated pathways. Activating mutations in CTNNB1, inactivating mutations in APC, and ring finger protein 43 (RNF43) are involved in the generation of dysregulated Wnt signaling pathway. Similarly, for RTK-associated pathways, mutations in ErbB3 RTK and Neuregulin 1 (NRG1)/EebB4 ligand/RTK pair have been reported in >10% of the GC cases. The phosphatidylinositol 3-kinase (PIK3) is another pathway downstream of the RTK signaling and is responsible for cell growth, survival, and proliferation. The PIK3 catalytic subunit alpha (PIK3CA), main catalytic component of the PIK protein, is found to be frequently mutated in microsatellite instability (MSI) and EBV-positive GC subtypes. The molecules targeting the Wnt signaling and TRK signaling pathways are some of the promising targets for GC treatment [10].

3.2.3 Chromosomal Instability

Somatic copy number alterations (SCNAs) result from the alterations in the DNA copy number that leads to the structural variation in DNA. Specific SCNAs are associated with histological type in GC; for example, intestinal-type GC is associated with gain in the gene copy at 8q, 17q, and 20q, whereas the diffuse-type GC is associated with gains at 12q and 13q. Deng et al. [24] reported that GCs exhibit frequent focal SCNAs such as amplifications in genes involved in the RTK/RAS/MAPK signaling pathway. Many of the genes involved in this pathway such as *ERBB2*, *EGFR*, *MET*, and *FGFR2* can be targeted by novel medications. Medications targeting the RTK/RAS/MAPK signaling pathway can potentially treat 37% of the GC population [10]. Other SCNAs and amplifications involved in GC include Janus kinase 2 (JAK2), programmed death-ligand (PDL)1/2 (immune checkpoint inhibitors), frequently mutated in EBV-positive subtype of The Cancer Genome Atlas (TCGA) network, and transcription factors including Kruppel-like factor 5 (KLF5), GATA4, and GATA6. The KLF5/GATA4/GATA6 transcription factor mutations have been found in 30% of the GC cases. Another marker of chromosomal instability is the loss of heterozygosity (LOH), which can result in the loss of tumor suppressor genes such as *APC* and *TP53* genes. The high-level LOH has been associated with the intestinal or mixed-type GC and low-level LOH with diffuse-type GC [10]. Genomic instability varies in patients from the different geographical locations indicating possible heterogeneous biological mechanisms at different locations.

3.2.4 Transcriptional Changes in Gastric Cancer

Gene expression profiling using the NGS and microarrays can be used to define the transcriptional changes in GC. It has identified pathways involved in the cell migration, metastases, cell cycle, and the cytoskeletal organization to be upregulated in GC that has both diagnostic and prognostic significance. Several studies have identified expression signatures based on the gene expression profiling to predict survival independent of the tumor, node, and metastasis (TNM) staging, the gold standard for prognosis in GC. Lei et al. [14] classified GCs based on the expression signatures into proliferative, metabolic, and mesenchymal subtypes showing the genetic and molecular differences as well as response to the therapy offered. The metabolic subtype has shown increased sensitivity to 5-fluorouracil and phosphatidylinositol 3-kinase-Akt-mTOR inhibitors, respectively, whereas the proliferative subtype has a higher rate of TP53 mutation and genomic instability [14].

3.2.5 Epigenetic Modifications in Gastric Cancer

Epigenetic dysregulation of gene expression can lead to the development of malignant cell transformation. Hypermethylation of promoter regions results in transcriptional silencing of the mismatch repair (MMR) and tumor suppressor genes – for

example, hypermethylation of hMLH promoter region can cause MSI phenotype in GC. CpG island methylation phenotype (CIMP) characterized by the genome-wide methylation of CpG islands rather than any single gene is also demonstrated in GC. Kim et al. [15] demonstrated that CIMP is seen in about 35% of the GC cases; occurs in relatively younger patients; is associated with oncogene mutations including *KRAS*, *ERBB2*, *PIK3CA*; and has a worse prognosis. The DNA-demethylating drugs such as azacitidine and decitabine, which are clinically used for myelodysplastic syndrome (MDS), could also be promising for the epigenetic aberrations in GC [15]. Histone modification is another mechanism responsible for the epigenetic aberration in GC. Understanding of the epigenetic profiling of the cells can help stratify gastric cancer and also serve as potential targets for treatment [10].

3.2.6 Molecular and Genomic Stratification of Gastric Cancer

Recently, TCGA network carried out a landmark study for the genetic and molecular characterization of GC, including 295 gastric cancers samples [based on 6 molecular platforms including array-based somatic copy number analysis, whole-exome sequencing, messenger RNA sequencing, microRNA (miRNA) sequencing, array-based DNA methylation profiling, and reverse-phase protein array (RPPA)] [16]. Based on the integrative analysis, TCGA classifies GC in four subtypes: EBV-positive tumors, MSI tumors, genomically stable tumors, and chromosomal instability tumors [16].

The EBV subtype is suggestive of viral etiology of gastric cancer and is detected in about 9% of the malignant cells in gastric cancer. DNA hypermethylation was the most prevalent in the EBV-positive tumors in TCGA. The EBV-positive tumors have a very strong predilection for PIK3 mutation, and 80% cases have non-silenced PIK3CA mutation in this subset [16]. The rate of PIK3CA mutation ranges from 3% to 42% in other subtypes. It also exhibits a mutation in ARID1A and Bcl6 corepressor (BCOR) and has a high frequency of amplification of PD-L1/2 and JAK2 genes [17, 18]. The MSI tumors exhibit a high prevalence of MLH1 promoter hypermethylation and occasional mutations in PIK3CA, ERBB2, ERBB3, and EGFR. Tumors lacking higher rates of mutation or hypermethylation and aneuploidy were regarded as genomically stable. They were predominantly present in the diffuse histological subtype. Molecular alterations in TCGA GS subtype include abnormalities of CDH1 and RHOA signaling pathways [11, 17]. Tumors with chromosome instability (CIN) show marked aneuploidy and frequently exhibit amplifications in RTK-RAS pathway resulting in its activation [11, 18].

The Asian Cancer Research Group (ACRG) conducted another similar landmark study on 300 gastric cancer samples using targeted gene sequencing, genome-wide copy number microarrays, and gene expression profiling and classified GC into four subtypes: MSI, MS stable/epithelial to mesenchymal transition (MSS/EMT), MSS/TP53 +ve (intact TP53 activity), and and MSS/TP53 –ve (functional loss of TP53). The MSI tumors have the best prognosis of all the ACRG subtypes, comprise intestinal-type tumors frequently, contain hypermutation at a molecular level, and

are diagnosed at an early stage of the disease [17, 18]. The MSS/EMT subtypes have the worst prognosis and highest recurrence rate of all the subtypes. It frequently comprises diffuse-type cancers and harbor CDH1 and/or RHOA mutations corresponding to the GC tumors in TCGA classification [11, 17]. The MSS/TP53 active subtype has a better prognosis than the MSS/TP53 inactive tumors. The MSS/P53+ subtype, corresponding to TCGA EBV-positive subgroup, exhibits a higher prevalence of the mutations in *ARID1A*, *APC*, *PIK3CA*, *KRAS*, and *SMAD4* mutations. Amplification of ERBB2, CDNE1, CCND1, and MDM2 is enriched in MSS/P53 inactive subtype, corresponding to TCGA CIN subtype and could be the targets of novel therapies currently available or are under trials such as trastuzumab, CDK2 inhibitors, CDK4/6 inhibitors, and MDM2 inhibitors, respectively [17–19]. Although there are similarities between the two classification systems, some differences exist as well in terms of the demographics and molecular mechanisms which indicate that these are overlapping but distinct classification systems. The ACRG classification complements the TCGA classification and uses additional incorporation of the two key molecular mechanisms (TP53 activity and EMT) to stratify gastric cancer patients further [11, 19].

Multiple genetic and epigenetic aberrations characterize gastric cancer and are likely responsible for the heterogeneous and complex nature of this disease. Advancements in the genomic and molecular analyses of gastric cancer have created a preliminary road map to stratify the heterogeneous disease and to develop targeted therapies for the distinct group of patients with the eventual goal of improving survival in gastric cancer.

3.3 Role of Tyrosine Kinases, Receptors, and Inhibitors in Gastric Cancer

Protein kinases are enzymes catalyzing transfer of phosphoryl group from adenosine triphosphate (ATP) to proteins specifically either serine/threonine or tyrosine side chains of the protein, playing important roles in signal transduction and other cellular cross-talk processes [20]. The RTKs are a family of 56 membrane-bound proteins, which fall into 21 subfamilies. They are characterized by an extracellular ligand-binding domain, a transmembrane portion, and a cytoplasmic tyrosine kinase motif. All known RTKs, with the exception of insulin receptors, form monomers in the cell membrane. The RTKs play key roles in the cell cycle regulation, cell proliferation and differentiation, survival and metabolism, cell adhesion, and migration [21, 22]. Mutations in the RTKs and alterations in the downstream signaling pathways have been associated with almost all cancers, inflammation, angiogenesis, and arteriosclerosis [22]. Overexpression and/or activation of the RTKs transforms cells and plays significant role(s) in the development and progression of cancers. These causal associations led to the development of TKIs and render them the targets for many immunological treatments such as trastuzumab (ERBB2 receptor inhibitor) and cetuximab (EGFR2 blocker) and other TKIs such as imatinib and gefitinib [21].

The ligand-induced dimerization results in autophosphorylation of cytoplasmic tyrosine kinase moieties, and the downstream signaling proteins lead to activation of RTKs [21, 23]. Activation mainly involves two processes with autophosphorylation being critical for both activities, amplification of intrinsic catalytic activity of RTKs and creation of the recruitment sites for the downstream signaling proteins [23]. Previously, ligand-induced dimerization was thought to be a simple mechanism of a bivalent ligand simultaneously binding to two receptors and cross-linking them into dimeric complex. Recent studies have provided more insights into the four most plausible mechanisms of receptor dimerization [22, 23]: either entirely receptor-mediated without much contribution from ligand to the dimer interface or entirely ligand-mediated without direct contact between the two receptor molecules. Alternatively, dimerization could involve both the ligand-mediated and receptor-mediated components. All tyrosine kinase domains (TKDs) have a C-lobe and an N-lobe with the crystal structure of activated TKDs being similar for most of the TKs. In all activated TKs, "activation loop" and alpha-C helix in N-lobe exhibit a specific configuration necessary for the phosphoryl transfer. Each TKD is cis-autoinhibited uniquely through several molecular interactions, and the release of cis-autoinhibition following the ligand-mediated receptor dimerization is the major event triggering the RTK activation [22].

3.3.1 Receptor Tyrosine Kinases in Gastric Cancer

Of the 56 known RTKs falling into the 21 families, several RTKs including the EGFR family (ErbB1 to B4), VEGFR subtypes, the FGF receptor family, and the PDGF receptor family have been found in gastric cancer growth and progression and thus serve as potential targets for novel therapies [21, 23]. Deng et al. [24] reported alterations in 37% of gastric cancers with FGFR2 being the most frequently amplified RTK (9.3%), followed by KRAS (8.8%), EGFR (7.7%), and ERBB2 (7.2%). Amplification of the RTKs has been regarded as an independent poor prognostic marker in gastric cancers [24].

3.3.2 Role of EGF Receptor/HER Tyrosine Kinase Family in Gastric Cancer

The EGF family of receptors consists of four subtypes including HER1/Erb B1, also called EGFR, HER-2/Erb B2, HER 3/Erb B3, and HER 4/Erb B4 which are encoded by *Erb* oncogenes [23]. The EGF family of receptors like other tyrosine kinase receptors has an extracellular ligand-binding domain (I–IV), a transmembrane component, and intracellular tyrosine kinase domain except Erb B3. The binding of oncogenes such as *EGF*, transforming growth factor alpha (*TGF-α*) and heparin binding EGF activates EGF receptors by either homodimerization (binding to a similar receptor class) or heterodimerization (binding to the receptor of a different class). This, in turn, results in autophosphorylation of the TK residues and initiation

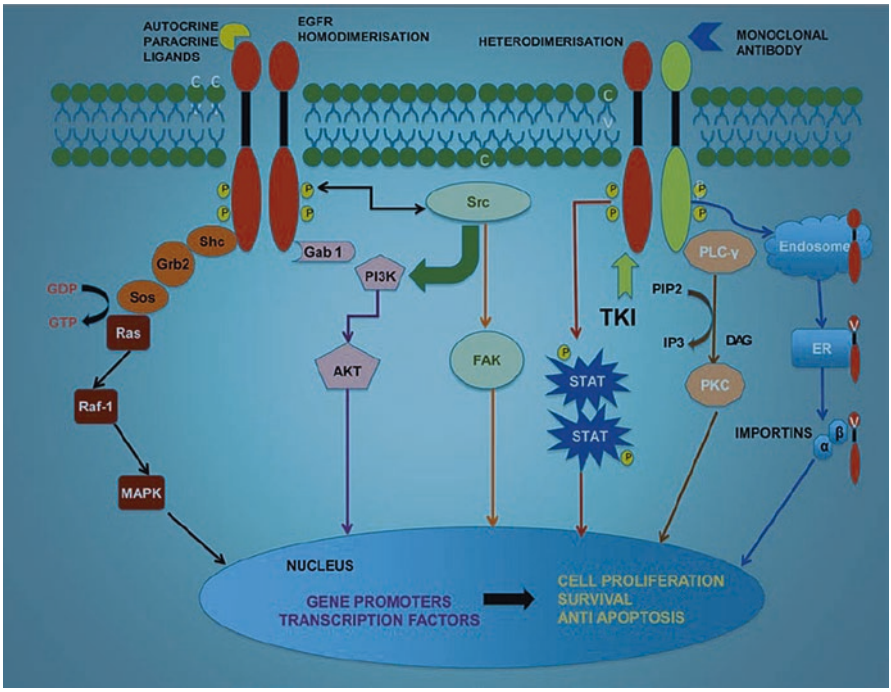

Fig. 3.3 The EGFR signaling: EGFR is activated by ligand binding and subsequent receptor heterodimerization or homodimerization, which results in autophosphorylation of tyrosine residues and binding of adaptor molecules like Shc and Gab-1 to the cytoplasmic domain. Intracellular signaling pathways include RAS/Raf-1/MAPK, P13K/AKT, and PLC-y/PKC pathways which require the adaptor molecules for signaling. The Src and STAT pathways are directly activated by the phosphorylated receptors. Alternately, the activated receptors can undergo endocytosis followed by the importin-mediated translocation to the nucleus and co-transcriptional of the key genes like Cox-2, iNOS, aurora kinase-A, and cyclin-D1. All these pathways lead to changes in the gene expression and stimulation of cell proliferation, survival, invasion, and metastasis. The EGFR manipulation can be approached extracellularly by monoclonal antibodies through inhibition of the ligand binding and intracellularly by the TKIs, which compete with the ATO binding to receptor kinase for activation [25]. *Abbreviations: EGFR* endothelial growth factor receptor, *Shc* Src homology-2 domain containing transforming protein-1, *Grb2* growth factor receptor bound protein-2, *Sos* son of sevenless, *Ras* rat sarcoma, *Raf-1* rapidly accelerated fibrosarcoma, *MAPK* mitogen-activated protein kinase, *Gab-1* Grb2-associated binding protein-1, *P13K* phosphoinositide 3 kinase, *AKT* protein kinase B, *Src* sarcoma gene, *FAK* focal adhesion kinase, *STAT* signal transducer and activator of transcription, *TKI* tyrosine kinase inhibitor, *PLC-y* phospholipase C-y, *PIP2* phosphatidylinositol 4,2 biphosphate, *IP3* 1,3,5-triphosphate, *DAG* 1,2-diacylglycerol, *PKC* protein kinase C, and *ER* endoplasmic reticulum

of the downstream signaling cascade. The signaling cascade is a complex pathway with multiple passages and includes Ras/Raf/mitogen-activated protein kinase (MAPK)/cyclin-D1 pathway, PI3K/Akt pathway, and signal transducers and activators of transcription (STAT) signaling pathways involved in the cell differentiation and proliferation [25] (Fig. 3.3).

The EGF receptors also promote tumor growth independent of the abovementioned pathways. The EGF receptor is internalized after the activation and is transported to the nucleus by cytoplasmic importin-ß where it activates many gene promoters [25]. Of all the EGF family of receptor tyrosine kinases, the HER-2 targeted therapy has been most successful and well-studied treatment for advanced gastric cancer. HER-2/Erb B2 is overexpressed in 10–38% of gastric cancers [23]. The EGFR overexpression has been detected in 27–64% of gastric cancers. Elevated levels have been detected in the advanced-stage gastric cancer with poor prognostic factors (T3/T4, G3, lymph node +ve, diffuse subtype) [21, 23]. Treatments targeting EGFR/Erb B involve small molecule kinase inhibitors and monoclonal antibodies. In contrast to the kinase inhibitors, monoclonal antibodies against EGFR/ErbB2 have inherent ability to recruit inflammatory cells via binding of antibody Fc domain to the tumor cell-specific receptors [21].

Trastuzumab (Herceptin®) is a humanized IgG1 monoclonal antibody against the extracellular domain IV of HER-2/neu receptor. It prevents activation of its intracellular tyrosine kinase domain and downstream signaling pathways, thus interrupting cell cycle progression and growth of tumor cells. Postulated mechanisms include activation of natural killer cells, antibody-dependent cellular cytotoxicity (ADCC) and destruction of the tumor cells bound to the Fc domain of trastuzumab [25, 26]. The ToGA (trastuzumab for gastric cancer) trial was a phase III, international, randomized controlled, open-label trial which evaluated the role of trastuzumab in the treatment of advanced gastric cancer. Results from ToGA trial demonstrated that trastuzumab in combination with standard chemotherapy improves the overall survival by more than 1 year in advanced gastric cancer and has proven to be the landmark study for advanced gastric cancer [26, 27]. Ado-trastuzumab emtansine (T-DM1) is an antibody-drug conjugate of trastuzumab with microtubule polymerization inhibitor, derivative of maytansine. It undergoes receptor-mediated internalization after the binding to HER-2 and releases DM1 into cytoplasm, which can induce apoptosis and ADCC. An ongoing phase II study of T-DM 1, the trastuzumab-emtansine conjugate, in advanced gastric cancer is being conducted as this conjugate drug has shown to inhibit the gastric cancer cells in vitro and in vivo [25].

Cetuximab is a chimeric monoclonal antibody against EGFR, which blocks EGF and TGF-α ligand binding to EGFR and activation of TKR. In gastric cancer cell lines, it inhibits EGF-induced EGFR and HER-2 phosphorylation, EGFR homodimerization, EGFR and HER-2 heterodimerization, and signal transduction via the MAPK and Akt pathways. In contrast to cetuximab, which is a chimeric antibody, panitumumab is a full humanized monoclonal antibody against EGFR and has minimal immunogenicity and is unable to stimulate ADCC.

Pertuzumab is another fully humanized monoclonal antibody binding to HER-2 domain II instead of domain IV by trastuzumab. It inhibits HER-2 heterodimerization with other EGFR receptors and activates ADCC. Combination of trastuzumab and pertuzumab has been studied to be more effective than therapy alone in the xenograft models, and currently clinical trials are being done to evaluate this combination in advanced gastroesophageal cancers [25].

Gefitinib and *erlotinib* are the EGFR receptor tyrosine kinase inhibitors inhibiting phosphorylation step that follows the EGFR receptor dimerization and thus hinders the downstream signaling proteins [25]. These drugs have already been approved for the treatment of non-small cell lung cancer (NSCLC) when the first-line therapy failed but have not shown promising results yet in a phase II trial for gastric cancer [4].

Lapatinib is a dual reversible tyrosine kinase inhibitor blocking both HER-2 and EGFR. In contrast to erlotinib and gefitinib, it has slower receptor dissociation rates and binds to the inactive EGFR conformation. Lapatinib produces its effect through survivin, an apoptosis inhibitor protein. It causes growth inhibition in HER-2 amplified gastric cell lines in both in vitro and in vivo studies. Currently, two phase III trials are evaluating its role as a first-line and second-line therapy for advanced gastric cancers [23, 25].

3.3.3 Vascular Endothelial Growth Factor Receptor

Abnormal angiogenesis is ubiquitous in all the malignant tumors with VEGF secreted by the tumor cells being the key mediator of angiogenesis. VEGFR promotes cell growth and metastases and is frequently overexpressed in the gastric cancer cell lines [21, 26]. Kinase insert domain receptor (KDR) is one of the main VEGF receptors involved in physiological and pathological angiogenesis, and VEGF-KDR signaling pathway is a potential therapeutic target for cancer treatment since VEGF expression is associated with high recurrence of gastric cancer. Bevacizumab is a monoclonal antibody against VEGF-A and interrupts the signaling pathways by neutralizing the VEGF ligand instead of directly binding to the tyrosine kinase receptor [21]. Ramucirumab is a humanized monoclonal antibody against VEGFR-2 and interrupts downstream pathways for angiogenesis. Apatinib is a small molecule TKI that targets VEGF-2, which has been shown to improve the overall survival in heavily pre-treated metastatic gastric cancer which has failed two or more chemotherapy agents in phase II and III trials. It is the first VEGFR tyrosine kinase inhibitor to have a small but clinically significant effect on advanced gastric cancer among the Asian patients [26].

3.3.4 Fibroblast Growth Factor Receptor

Growth factors of FGF family, which are secreted by fibroblasts, result in tumor proliferation in scirrhous gastric cancer. Gastric cancers expressing high levels of FGF2 mRNA (basic FGF) have demonstrated higher microvascular density, tumor progression, and worse outcomes. Orally active inhibitor of FGF autophosphorylation has shown efficacy in animal models, but additional clinical/translational studies in humans are pending [28].

3.3.5 Platelet-Derived Growth Factor Receptor

PDGF family members are often expressed at high levels in malignant tumors. They promote tumor growth by either directly stimulating certain cell lines, stimulating angiogenesis, recruiting pericytes, or controlling stromal interstitial fluid pressure influencing trans-vascular transport and lymph node metastases. Kodama et al. [29] showed that high expression of tumor cell-secreted PDGF-B and stromal cell-secreted PDGF-B receptor is associated with lymphatic metastases in gastric carcinoma [29]. Imatinib mesylate, an inhibitor of PDGFR tyrosine kinase, has not shown efficacy by itself in gastric cancer cells but may serve as an important chemosensitizer with antitumor drugs targeting PDGF/PDGFR pathway in tumorigenesis and angiogenesis [30].

3.3.6 Combined VEGFR and PDGFR Tyrosine Kinase Inhibitors

Sorafenib is a multi-target inhibitor of BRAF, VEGF, PDGFR, and the Ras/Raf/MERK/ERK pathways. A phase II trial determined safety of sorafenib with oxaliplatin in advanced gastric cancer but did not support phase III study. Sunitinib suppresses PDGFR, Kit, rearranged during transfection (RET), Flt-3, and VEGFR. Sunitinib has not shown efficacy as a second-line agent in a phase II trial for gastric cancer [23].

3.3.7 Hepatocyte Growth Factor Receptor Tyrosine Kinase

HGF receptor tyrosine kinase, also known as c-Met receptor tyrosine kinase, is a receptor for *HGF/c-Met* oncogene, which has an extracellular alpha subunit and a transmembrane beta subunit. The binding of *Met* oncogene to extracellular domain results in phosphorylation of intracellular tyrosine kinase domains. The phosphorylated MET (p-MET) recruits various downstream proteins and activates signaling pathways such as PI3K/AKT and extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathways and plays significant role(s) in the tumor growth, survival, angiogenesis, and metastasis [31]. Amplification of c-Met and co-amplification of c-Met and c-Myc have been implicated in gastric cancers. Co-expression of c-Met and Erb-B2 has been associated with poorer survival in gastric cancer as compared to the overexpression of either one [32].

Thus, various RTKs are implicated in the pathogenesis of gastric cancer and are potential targets for ongoing therapy against gastric cancer in addition to the currently available chemotherapy agents. Figure 3.4 summaries the targets for various RTK therapies in gastric cancer.

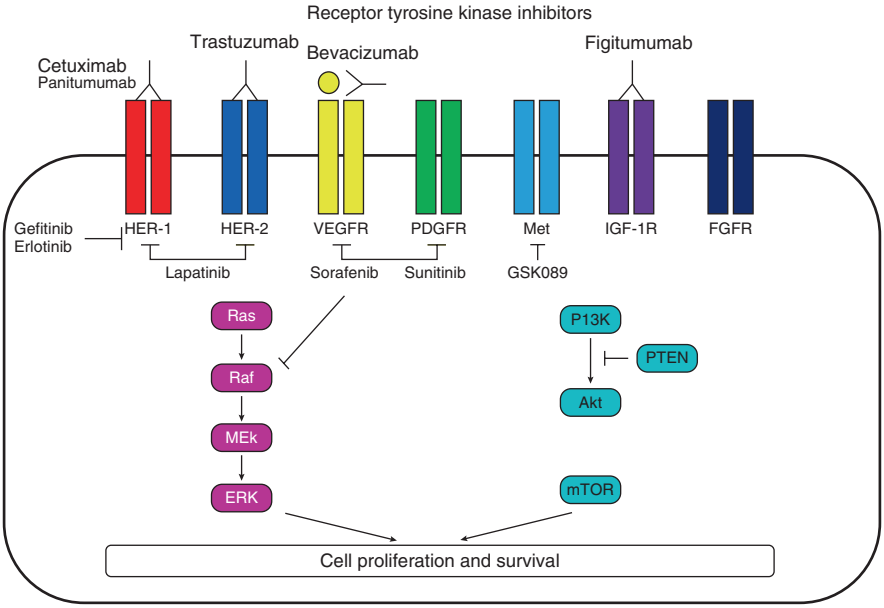

Fig. 3.4 Schematic representation of the targets for various receptor tyrosine kinase inhibitors. *Abbreviations: VEGFR* vascular endothelial growth factor receptor, *PDGFR* platelet-derived growth factor receptor, *IGF-1R* insulin-like growth factor 1 receptor, *FGFR* fibroblast growth factor receptor, *HER* human epidermal growth factor receptor, *PTEN* phosphatase and tensin homolog, *mTOR* mammalian target of rapamycin, *PI3K* phosphoinositide 3-kinase, and *Akt* a serine/threonine-specific protein kinase B, also known as Akt

3.4 Targeted Therapies and Ongoing Clinical Trials

Gastric cancer is a not uncommon cancer; however, there are limited treatment options, and therefore the prognosis is often poor. Patients diagnosed with stages III and IV gastric cancer have 5-year overall survival rates of only 9.2–19.8% and 4.0%, respectively [33]. GC is one of the most common causes of cancer-related mortality, and its deadly nature can be accounted for various reasons. First, like many other neoplasms, GC typically presents with advanced-stage disease, and in most counties, there is no population-based screening. Second, there are no gold-standard treatment regimens available; therefore, the treatment varies from center to center. Third, GCs are notorious for a high degree of heterogeneity at the histologic and molecular level, which plays a vital role in terms of tumor behavior and response to therapy. Finally, the lack of unanimous classification schemes can lead to significant inter-observer variability between the pathologists, thereby leading to difficulties in reproducibility. Several classification systems have been proposed for GC, and the most widely used GC classification system is the Lauren classification which classifies GCs into “intestinal,” “diffuse,” and “mixed” subtypes. The other GC classification schemes include the WHO classification, which subdivides DC into papillary, tubular, mucinous, and poorly cohesive subtypes [34].

In recent times, increased availability of molecular sequencing has paved the way for newer classification based on the molecular characteristics of the tumors and with the identifications of the molecular targets, creation of new therapeutic targets will potentially provide personalized (precision) prognosis and treatment. The TCGA study performed sequencing of 295 gastric cancer samples and clustered GC into 4 groups: EBV positive (9%), tumors with MSI (22%), genomically stable tumors (20%), and tumors with chromosomal instability (50%) [16].

As noted earlier, the RTKs play pivotal roles for the targeted therapies in gastric cancer. Deng et al. [24], with the use of high-resolution single-nucleotide polymorphism (SNP) arrays, profiled the copy number alterations in a panel of 233 gastric cancers and found enormic alterations in RTKSs in at least 37% of cases that are potentially treatable by the RTK/RAS directed therapies. Those genomic alterations included FGFR2 (9% of tumors), KRAS (9%), EGFR (8%), ERBB2 (7%), and MET (4%) [24].

3.4.1 Targeted Therapies for HER-2 Overexpressed Gastric Cancers

HER-2 is a transmembrane tyrosine kinase and a member of the EGFR family, which promotes cell proliferation, adhesion, migration, and differentiation. It begins with heterodimerization with other members of the HER family causing activation of the RAS-MAPK and PI3K/AKT pathways. The HER-2 gene is located on chromosome 17q21 [35]. HER-2 overexpression is seen in 15–30% of gastric cancers, and its prevalence is based on the histology and location of the tumor. It is more common in the intestinal-type and in gastroesophageal junction tumors [27].

HER-2-positive gastric cancers have been targeted successfully by the anti-HER-2 monoclonal antibody trastuzumab. It causes inhibition of the MAPK and PI3K/Akt pathways causing suppression of cell growth and proliferation. Other anti-HER-2 agents that have been studied in the treatment of HER-2-positive advanced gastric cancer include lapatinib, a dual anti-EGFR and anti-HER-2 tyrosine kinase inhibitor, and pertuzumab, a monoclonal antibody that binds the extracellular dimerization domain of HER-2 preventing its dimerization. Currently, a double-blind, placebo-controlled, randomized, multicenter, international, parallel-arm study is underway to evaluate the efficacy of pertuzumab combined with trastuzumab and chemotherapy (cisplatin plus a fluoropyrimidine) (NCT01774786).

3.4.2 Ongoing Trials for HER-2 Targeted Therapies

As noted earlier, the phase III ToGA studied addition of trastuzumab in the first line of chemotherapy treatment in patients with HER-2-positive advanced gastric cancer. Patients were randomized to fluorouracil-based chemotherapy and cisplatin with or without trastuzumab. Patients in the trastuzumab arm had an overall survival benefit [median overall survival (OS) 13.8 vs. 11.1 months (HR 0.74, CI 0.60–0.91;

$p = 0.0046$)] without any increase in grade 3 or 4 adverse events [27]. The objective response rate was also higher with trastuzumab, at 47% vs. 35% in the standard arm. In the ToGA population, there was a proportion of patients (22%) who were fluorescence in situ hybridization (FISH) positive but immunohistochemistry (IHC) 0-1-; this subset of patients did not benefit from the addition of trastuzumab [27].

In the TRIO-013/LOGIC trial, patients with advanced or metastatic HER-2-positive gastric cancer were randomized to capecitabine and oxaliplatin with lapatinib or placebo [36]. The primary endpoint of the overall survival benefit was not reached. Median overall survival was 12.2 months in the lapatinib group and 10.5 months in the placebo group (HR 0.91, 95% CI 0.73–1.12, $p = 0.35$). Progression-free survival (PFS) was also not significantly improved [median PFS 6.0 vs. 5.4 months (HR 0.86, 95% CI 0.71–1.04, $p = 0.10$) [36]. TyTAN trial compared paclitaxel alone with lapatinib plus paclitaxel and again did not show any significant benefits in such outcomes such as the median overall survival, PFS, or time to progression (TTP) [37].

3.4.3 Targeted Therapies for the VEGF-Positive Gastric Cancers

VEGF stimulates the formation of blood vessels and promotes carcinogenesis by angiogenesis and neovascularization. Therefore, the VEFGR signaling pathway is deemed a strategic therapeutic target. The VEGF overexpression is a common feature in gastric cancers, seen in up to 58% of the cases with this disease [38].

Ramucirumab is a human IgG1 monoclonal antibody against VEGFR2 and has been recognized as a second-line treatment of metastatic gastric cancer. Another VEGF-directed monoclonal antibody for GC is bevacizumab (Avastin®), but less promising results have been observed. In the AVAGAST study, cisplatin and capecitabine were given with bevacizumab or placebo, and there was no overall survival benefit from the addition of bevacizumab [median OS 12.1 months vs. 10.1 months (HR 0.87, 95% CI 0.73–1.03, $p = 0.1002$)], although PFS and objective response rates (ORR) were clinically improved [39].

3.4.4 Trials for VEGF Targeted Therapies

The REGARD study was a phase III trial, which compared ramucirumab with the best supportive care in the second-line advanced gastric cancer. The results revealed an overall survival benefit of 1.4 months (5.2 vs. 3.8 months, HR 0.776, 95% CI 0.603–0.998, $p = 0.047$) [40].

The phase III RAINBOW study compared the use of ramucirumab vs. placebo, in combination with paclitaxel, in patients with advanced gastric cancer that had progressed on the first-line chemotherapy of fluoropyrimidine/platinum with or without an anthracycline [41]. The results revealed significant overall survival benefit with ramucirumab of 9.6 months vs. 7.4 months (HR 0.807, 95% CI

0.678–0.962, $p = 0.017$), with an increase of 1-year overall survival from 30% to 40%. The PFS, ORR, and the disease control rates (DCR) were also improved [41].

3.4.5 Anti-EGFR-Targeted Therapies and Trials

Epidermal growth factor receptor (EGFR) is a protein that spans across the cell membrane and is activated when epidermal growth factor binds on it. After the EGFR is activated, it then phosphorylates and triggers the intracellular protein-tyrosine kinase activity, which in turn, causes activation and signaling of further intracellular proteins. Subsequently, several signal transduction cascades, predominantly the MAPK, AKT, and JNK pathways, are initiated, resulting mainly in DNA synthesis and cell multiplication but also partake in the cell migration, adhesion, and proliferation [42].

Waddell et al. [44] in the REAL3 study, a randomized open-label phase III trial, evaluated the addition of panitumumab (an anti-EGFR antibody) to the regimen of epirubicin, oxaliplatin, and capecitabine (EOC). The REAL3 study found that the addition of panitumumab to EOC does not increase survival [43]. Currently, a phase III study (NCT01813253) is evaluating the addition of monoclonal antibody nimotuzumab in combination with irinotecan and then comparing the effects of irinotecan alone in patients with advanced gastric and gastroesophageal cancers [44].

3.4.6 Targeted Therapies for Gastric Cancers with FGFR2 Amplification

FGFR2 (fibroblast growth factor receptor type 2) with its ligand fibroblast growth factor, promotes mitogenesis, cell proliferation, and angiogenesis. Various studies have shown FGFR2 amplification in 3–16% of gastric neoplasms. FGFR2 is found to be associated with the diffuse-type gastric cancers and advanced stages and carries poor prognosis [45].

Drugs that target the FGF receptors have shown promising results in some preclinical studies. For instance, dovitinib (TKI258), which is a multi-targeted RTK inhibitor of FLT-/c-Kit, FGFR, VEGFR, and colony-stimulating factor, is being actively studied. Niantao Deng et al. [24] in their study found that dovitinib has potent inhibitory activity against gastric cancers that overexpress FGFR2. They also observed that dovitinib reduced the mean tumor size in an FGFR2-amplified human by inducing apoptosis by inhibiting several intracellular proto-oncogenes [24].

3.4.7 Clinical Trials for FGFR2-Targeted Therapies

Dovitinib alone or in combination is being tested currently by few clinical trials of gastric neoplasms overexpressing FGFR2. In one phase II study (NCT01719549), the efficacy and safety of dovitinib monotherapy is being evaluated when the

first-line chemotherapy failed to control the growth of gastric cancers with FGFR2 amplification. Another phase I/II study is investigating the combined effects of docetaxel with dovitinib in patients with gastric neoplasms (NCT01921673).

The SHINE study assessed the efficacy and safety of AZD4547, a selective FGFR1-3 inhibitor, and compared the results with paclitaxel treatment in patients with advanced gastric cancers [47]. A total of 960 patients were enrolled in the SHINE study, among which the prevalence of the FGFR2 amplification was 9%. The results of this study showed 1.8 months of overall median PFS on the AZD4547 arm and 3.5 months for paclitaxel. On the other hand, the PFS was 1.5 months in the 9% of patients with FGFR amplification on AZD4547 arm and 2.3 months for paclitaxel (NCT01457846) [46].

3.4.8 Targeted Therapies for the PI3K Pathway and Clinical Trials

The PI3K/Akt/mTOR pathways are crucial for the cell growth and survival pertaining to both the physiological as well as in pathological conditions. During the time of cellular stress, for instance, the tumors have stressful intrinsic environment owing to the limited supply of nutrients and oxygen. The PI3K/Akt pathway plays key regulatory role(s) in the survival of cancer cells during the cellular stress [47]. In the TCGA study, the rate of the PIK3CA mutations has been reported from 0.8% to 20% overall.

Although currently various trials are studying the PI3K inhibitors in gastric cancer, but definitive clinical results are still lacking. LY294002 is a commonly used inhibitor of PI3K/Akt pathway when used with vincristine showed synergistic inhibition of the tumor cell growth by inducing apoptosis. BEZ235 and BKM120 in another study by Mueller et al. have also shown potential pro-apoptotic effects in the cells with PI3KCA mutation [48, 49]. Other currently ongoing clinical trials of the PI3K pathway inhibition include PI3K inhibitor BYL719 in combination with HSP90 inhibitor AUY 922 in advanced or metastatic gastric cancer, (NCT01613950) and LDE225 in combination with BKM120 in cases with advanced solid tumors (NCT01576666).

Everolimus, an mTOR inhibitor, in one multicenter phase II trial has shown promising results such as the disease control rate (DCR) of 56% and median PFS of 2.7 months [50]. The GRANITE-1, a randomized, double-blind, phase III study, subsequently investigated the effects of everolimus vs. placebo in patients with advanced gastric neoplasms. A total of 646 patients were enrolled in this study, and the median OS with everolimus was 5.4 months vs. 4.3 months with placebo. With everolimus, the median PFS was 1.7 months and 1.4 months with placebo. Looking at the above results, the GRANITE-1 study showed that everolimus do not significantly improve overall survival in cases of advanced gastric cancers [51].

3.4.9 Targeted Therapies and Clinical Trials for MET-Amplified Gastric Tumors

The c-MET is a RTK and HGF is its ligand, which after binding with its ligand activates a wide range of intracellular signaling pathways, including those involved in the cell proliferation, motility, migration, and invasion. The overexpression of MET is found in 0–23% of gastric neoplasms; therefore, it can be used as a target by the MET inhibitors [52]. AMG 337, rilotumumab, and onartuzumab are a few prominent MET inhibitors that are currently being tested in various clinical trials (as summarized below).

NCT02016534 is a multicenter phase II trial evaluating the role of AMG 337, a c-MET inhibitor, in patients with gastric and gastroesophageal cancers with MET overexpression. Rilotumumab, a human monoclonal antibody to HGF (hepatocyte growth factor), was tested in the RILOMET-1 study, but the study was halted prematurely as it did not meet its primary endpoints, and risk of death was higher with the test drug [53].

Onartuzumab is an antibody against the MET-amplified gastric neoplasms and was assessed in a phase III study (METGastric trial) in combination with mFOLFOX6, but this study was also terminated after the study revealed that the test drug is associated with serious toxicities such as neutropenia [54].

3.4.10 Immunotherapy for Gastric Cancer

Due to the limited availability and success of the targeted chemotherapy for gastric cancer, immunotherapy is being developed as one of the newer approaches for cancer treatment. Tumor infiltrated cells usually escape from the immune detections and destruction by involving the pathway of PD-1/PD-L1 (programmed cell death protein-1/programmed death-ligand-1). Some tumors cells also interact with cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and its ligands. The binding of the PD-L1 to its receptor transmits an inhibitory signal, which in turn, suppresses T-lymphocyte proliferation causing apoptosis of the tumor-specific T-cells. As per the TCGA study, PD-L1 and PD-L2 are specially overamplified in the EBV-positive gastric cancers; hence, anti-PD-1 immunotherapy would prove to be crucial in treating the EBV-positive gastric cancers [24].

During the past several decades, the use of monoclonal antibodies (mAbs) to target cancer cells has been well tested. The mechanisms by which mAb works include blocking the growth factor/receptor interactions, downregulating proteins required for tumor growth, and activating effector mechanisms of the immune system including complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity [55].

3.4.11 Clinical Trials on Immunotherapy for Gastric Cancer

Pembrolizumab (Keytruda®) was evaluated in the KEYNOTE-012 phase 1b trial. The results of the KEYNOTE-012 study have shown the overall response rate (ORR) of 22% by the central review and 33% by the investigator review. Noticeable antitumor activity and manageable toxicities also made pembrolizumab a promising immunotherapeutic agent (NCT01848834) [56].

Nivolumab (Opdivo®), a human anti-PD-1 monoclonal antibody, is another exciting immunotherapeutic agent that works as a checkpoint inhibitor, blocking a negative regulatory signal of the T-cell activation, thus allowing the immune system to identify and destroy the tumor cells (NCT02267343). Another trial is currently testing the effects of nivolumab alone and in combination with anti-CTLA-4 antibody ipilimumab (Yervoy®) for the patients with advanced solid tumors (NCT01928394). There is one other ongoing multicenter study by Jose Lutzky et al. evaluating a human IgG1 monoclonal antibody against PD-L1 durvalumab (MEDI4736) in patients with advanced solid tumors. Preliminary results have shown acceptable safety profile and measurable tumor shrinkage [57, 58].

3.5 Toxicities Associated with Various Commonly Used Agents/Regimens in Gastric Cancer

Below we provide a summary of the toxicities associated with some of the key agents/regimens that have been investigated in patients with gastric cancers in regard to tyrosine kinases.

3.5.1 Trastuzumab

As noted earlier, trastuzumab is a monoclonal antibody used for the treatment of HER-2 receptor-positive breast cancer and gastric cancer. Common toxicities of trastuzumab mainly include the involvement of cardiorespiratory systems. Respiratory toxicities include dyspnea, bronchospasm, and reduced oxygen saturation, whereas a decrease in the left ventricular ejection fraction or congestive heart failure is an important cardiac toxicity associated with trastuzumab treatment. A meta-analysis of the randomized clinical trials in patients treated with anthracycline-based chemotherapy and trastuzumab in the adjuvant setting showed higher risks of congestive heart failure (CHF) and asymptomatic cardiotoxicity [59]. Limited data are available on the effect of trastuzumab during pregnancy and lactation. Few cases of trastuzumab treatment during pregnancy and development of oligohydramnios and reversible fatal renal failure have also been reported [60].

3.5.2 Lapatinib

Lapatinib is a dual TKI targeting the EGFR and HER-2. Gastrointestinal disorders such as nausea, vomiting, and diarrhea and skin conditions such as rash, hand-foot syndrome, and dry skin are the most frequently reported adverse events for lapatinib. The adverse events largely are not lethal; however, failure to recognize and prompt the treatment can potentially lead to decrease treatment adherence, treatment discontinuation, and thus poor quality of life [61].

3.5.3 Ramucirumab

Ramucirumab is an FDA-approved monoclonal antibody that binds to the VEGFR-2 and thus blocks its anti-angiogenic activity in such cancers as gastric or GE junction adenocarcinoma. Ramucirumab in combination regimens is associated with higher toxicity. For example, in the RAINBOW trial, ramucirumab plus paclitaxel was reported to be associated with an increased chances of developing neutropenia than paclitaxel with placebo [62]. Specific toxicities related to ramucirumab include hypertension, hemorrhage, and thromboembolic disease. In the REVEL trial, hypertension was found in about 6% of the cases treated with ramucirumab [63].

3.5.4 Bevacizumab

Bevacizumab, like ramucirumab, is a monoclonal antibody targeting the VEGF. Preliminary results of some phase III clinical trials have noted somewhat greater degrees of bleeding, arterial thromboembolic events, gastrointestinal perforation, altered wound healing, proteinuria, and hypertension with bevacizumab as compared with placebo [64].

3.5.5 Cetuximab

Cetuximab is a monoclonal antibody against the EGFR. The more common adverse reactions associated with cetuximab are skin disorders such as dermatitis acneiform/rash, dry skin, and paronychia. Other drug-related toxicities include electrolyte abnormalities (e.g., hypomagnesemia, hypocalcemia, and hyperkalemia), cardiotoxicity (e.g., myocardial infarction, cardiac failure, right cardiac failure, and coronary spastic angina), diarrhea, and leukopenia [65].

3.5.6 Dovitinib

Dovitinib is a multi-targeted RTK inhibitor that includes potent inhibitory effects to the FGF receptors as well. The most common adverse reactions of dovitinib are gastrointestinal such as nausea, diarrhea, vomiting, decreased appetite, and fatigue. Skin and subcutaneous tissue toxicities were also commonly described in the patients taking dovitinib, and other less common events such as hypertension, hypertriglyceridemia, non-cardiac chest pain, pulmonary embolism, hemiparesis, neutropenia, and cerebrovascular accident are also reported [66].

3.5.7 Rilotumumab

Rilotumumab is a human monoclonal antibody against the human HGF, and it functions by blocking the signaling via the MET receptors. Early phase II clinical studies reported fatigue, nausea, peripheral edema, and constipation as some of the main treatment-related side effects. Other rare serious adverse reactions reported include edema, deep vein thrombosis, pulmonary embolism, and diarrhea [67].

3.5.8 Pembrolizumab

Pembrolizumab is a humanized antibody used in cancer immunotherapy, which targets the PD-1 receptor of lymphocytes and thereby allows the immune system to destroy cancer infiltrated cells. The most common adverse events were fatigue, pruritus, and decreased appetite. The inflammatory or immune-mediated toxicities noted were infusion-related reactions, hypothyroidism, and pneumonitis [68].

3.6 Conclusions and Future Perspectives

Gastric cancer ranks fifth worldwide as the most frequent malignancy, and the majority of patients are diagnosed at the advanced stages, which along with the relatively limited treatment options makes this a poor prognostic cancer. Recent advances in the gastric cancer genomics and sequencing have discovered many potentially targetable mutations, which are being used for the creation of promising therapies – and with the development of these therapies, our hopes in improving the patients' survival have also increased.

Amid advancement in the field of molecular therapies, immunotherapies have also gained wider recognition in the treatment of gastric cancer due to the presence of high levels of somatic mutations in gastric cancer. Though molecular therapies and immunotherapies are being developed, we still should succeed various challenges before any gold-standard treatment become available for the treatment of advanced gastric cancer. Some of those challenges include genomically complex

nature of gastric tumors, tumor heterogeneity, and the development of molecular classification system for gastric cancer.

Molecular therapeutic agents targeting RTKs are constantly being developed and have been effective in various clinical trials in patients with advanced gastric carcinoma. For example, trastuzumab, a TKI targeting EebB2, for the treatment of human ERBB2-positive advanced gastric cancer patients has already been approved. Activation of other RTKs has been associated with gastric carcinoma, and these include EGFR, VEGF, PDGFR, c-Met, IGF-1R, and FGFR2.

Various trials of TKIs are currently underway, which can have a greater impact (positive outcomes) on the treatment of gastric carcinoma soon. A trial examining bevacizumab, a TKI inhibiting VEGF, showed longer PFS in gastric carcinoma patients, although it did not seem to meet its primary goal of increasing the OS duration. Other clinical studies, especially phase III trials that have tested the drugs targeting RTKs such as EGFR, combined targeting of HER-2 and EGFR, VEGFR, and combined targeting of VEGFR and PDGFR, have shown modest effects against gastric 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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PIK3-AKT and Its Role in Pancreatic Cancer

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Abstract

The pancreas plays an important role in our body to help with maintaining and regulating homeostatic conditions. One of the cellular pathways that play a major role in pancreatic cell growth and division is the phosphatidylinositol-3-kinase-AKT pathway. According to current research studies, even the smallest changes to the phosphatidylinositol-3-kinase-AKT can cause abnormal cellular growth leading to the development of cancerous cells. Currently, there are many treatment options which are targeting the PI3K-AKT signaling pathway. This chapter examines the function of phosphatidylinositol-3-kinase-AKT pathway in pancreatic cancer and discusses current research studies that focus on potential treatment options.

Keywords

Pancreatic cancer · Growth · PI-3K · AKT

4.1 Introduction

Pancreatic cancer is described when cells divide and grow abnormally resulting in cancerous cells polluting the pancreatic tissue. The pancreas is an abdominal organ located behind the stomach that produces pancreatic juices and enzymes through

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exocrine means and that releases hormones through endocrine means [1]. Some hormones that are released by the pancreas include glucagon secreted by the alpha cells and insulin secreted by the beta cells. Both hormones are crucial to the regulation of blood glucose levels. Glucagon stimulates the liver to metabolize glycogen into glucose in order to increase glucose serum levels. On the other hand, insulin is generally stimulated during food consumption; insulin binds to cells, and through secondary messenger systems within the cells, GLUT transporters ultimately bind to the plasma membrane to allow for cellular glucose uptake and utilization. Another type of cell the pancreas generates is the delta cells which secrete somatostatin, a hormone used to inhibit hormonal release (e.g., growth hormone). All of the pancreatic juices, enzymes, and hormones are important for maintaining homeostasis in the human body. Even mild adjustments in the pancreas and to overall homeostatic conditions in blood glucose levels can cause catastrophic events in the human body.

Additionally, there are other delicate mechanisms and pathways within the pancreas that need to also be modulated in order to prevent disruptions and diseases, such as cancer. One such pathway that plays an important role in pancreatic cell growth and division is the phosphatidylinositol-3-kinase-AKT pathway.

4.2 PI3K-AKT Cellular Mechanism and Pancreatic Cancer

Phosphatidylinositol-3-kinase-AKT pathway plays an important role in the maintenance of cells, and disruptions in this pathway can cause cancer. PI3K is a lipid kinase and an important secondary messenger that is used for the displacement or translocation of AKT to the plasma membrane [2]. At the membrane, AKT becomes phosphorylated and hence stimulated by phosphoinositide-dependent kinases, PDK1 and PDK2 [2]. AKT plays a key role by phosphorylating substrates in order to maintain cellular proliferation and cell survival.

More specifically, the AKT gene is activated by growth factors binding to the tyrosine kinase receptor or to G-protein-coupled receptors which leads to immediate stimulation of the phosphorylation of PI3K. PIK3 then converts phosphatidylinositol-4,5 bisphosphonate to phosphatidylinositol-4,5 trisphosphate [PI(4,5)P2 to PI(4,5)P3] [2, 3]. AKT then moves toward the plasma membrane and interacts with the PI(4,5)P3 through the Ph domain [2, 3]. AKT is then phosphorylated at the Thr308 and Ser473 domains by the phosphoinositide-dependent kinases, PDK1 and PDK2, and integrin-linked kinase, ILK [3]. This entire process of phosphorylation of AKT leads to the overall development and survival of the cell.

Overall, PI3K-AKT phosphorylates the Plk1-Ser99 (polo-like kinase 1), which is a serine-threonine kinase that also monitors mitotic cell division and apoptosis. This phosphorylation is crucial for the metaphase to anaphase transition in cells. Furthermore, the Plk1-dependent phosphorylation of IRS2-S556 decreases AKT activity in order to prevent mitotic division [4, 5]. Any changes to the phosphatidylinositol-3-kinase-AKT or PI3K-AKT signaling pathway can cause irregular cell growth leading to cancerous cells in the pancreas. In current studies, through the processes of immunohistochemistry and tissue microarray, there is a

strong a correlation between the overexpression of the AKT gene and cancerous pancreatic cells [2, 3]. One potential theory is that there is an amplification of the PIK3C gene which codes for PI3K and AKT gene [3]. Another potential theory is that pancreas cancer results from mutations within the DNA, primarily focusing on the mutations of chromosome 10 on phosphatase and tensin homologue [3]. Deleting PTEN results in the inhibition of the AKT gene because it has the ability to reverse the conversion of phosphatidylinositol-4,5 bisphosphonate to phosphatidylinositol-4,5 trisphosphate [PI(4,5)P2 to PI(4,5)P3] [2, 3, 6]. In order to ultimately reverse the effects of pancreatic cancerous cells, the PI3K-AKT signaling pathway must be manipulated to inhibit cellular growth and to induce apoptosis.

Apoptosis or programmed cellular death has a crucial role in the development of most organisms; however, at the same time, if it is not closely modulated, it can result in tumors. Apoptosis is generally induced by an intrinsic pathway and sometimes an extrinsic pathway. It is reported that AKT phosphorylates XIAP at residue serine 87 that leads to resistance of cisplatin-induced XIAP degradation, activation of caspase-3, and most importantly apoptosis [6]. Furthermore, other investigations have shown that activation of the Bcl-2 family within the pancreas can result in apoptosis [2, 3]. Lastly, there are studies that also indicate that the deregulation of Plk1 could result in increased regulation of apoptosis.

4.3 RLIP76 Modulates PI3K-AKT Signaling Pathway

Unlike colorectal cancer, the results and patient outcomes of pancreatic cancer are not as promising. Therapy is often both disrupted and counteracted by overexpression of growth factors, including epidermal growth factors, transforming growth factors, and also insulin-like growth factors [7, 10]. Furthermore, the PI3K-AKT signaling pathway plays a crucial role in signal transduction involving growth factor receptors and oncogenic K-ras and also in determining basal survival and resistance to cell death in chemoradiotherapy [11, 12]. Due to its significant role in pancreatic cancers and other types of cancers, the PI3K-AKT signaling pathway has become an attractive study for treatments for cancer [8, 9]. Understanding both the mechanism and regulation of this pathway will allow for potential therapeutic agents to aid in bringing the pathway to homeostatic levels and treat cancerous cells in the body.

In recent studies, there is a strong correlation between RLIP76 (from the mercapturic acid pathway) and the PI3K-AKT signaling pathway. Mercapturic acid pathway functions to modulate the effects of antioxidants on cells and resistance to drugs involved with chemotherapy [13]. More specifically, glutathione, which is a sulfur-containing molecule that is present in all cells, aids in preventing spontaneous apoptosis [13]. In recent studies, it has been shown that RLIP76 plays a significant role as a transporter in the primary mercapturic acid pathway; the pathway plays a major role in apoptosis. RLIP76 is a major glutathione-conjugate transporter that is strongly correlated to counteracting with chemotherapeutic agents by allowing cells to resist cellular death [13]. Studies show that a decrease in RLIP76 levels led to the activation of the PI3K-AKT signaling pathway. Additionally, a decrease

in RLIP76 levels effectively killed cancerous cells and left normal body cells unharmed.

Since there is a strong, inverse correlation between RLIP76 and PI3K-AKT signaling pathway, studying how to effectively monitor and balance the levels of RLIP76 and the activation of the PI3K-AKT signaling pathway can be beneficial to the search for a permanent solution to pancreatic cancer.

4.4 Conclusion and Further Research

Along with chemotherapy and radiation, currently, there are many treatment options being developed that focus on the PI3K-AKT signaling pathway. Targeting the PI3K-AKT signaling pathway can be a potential therapeutic approach in pancreatic cancer, specifically by designing substances that can induce the inhibition of the mitotic cellular division of these cancerous cells and/or increase apoptosis of pancreatic cancerous cells. However, if there is a way to use molecular substances to inhibit the PI3K-AKT signaling pathway either in vivo or in vitro, then in the future, such substances can be used as drugs to treat pancreatic cancer. Nonetheless, further research on the inhibition of the PI3K-AKT signaling pathway needs to be completed.

This includes researching which molecular substances function optimally in terms of inhibition, studying which mode of transmission would be beneficial when incorporating these substances into the human body, and whether there is a way to convert this treatment plan into drugs or medications for effective patient use. Furthermore, studying how to balance the levels of RLIP76 and activation of the PI3K-AKT signaling pathway needs to be further analyzed. As we have seen, RLIP76 and the PI3K-AKT signaling pathway share an inverse relationship; the PI3K-AKT signaling pathway can be activated by decreasing levels of RLIP76. Reduced levels of RLIP76 can lead to the death of cancerous cells; however, it may also lead to the overexpression of PI3K-AKT, which is harmful to the body. Further research needs to be conducted on how to balance these two factors.

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Tyrosine Kinase Inhibitors and Their Clinical Prospective in Pancreatic Cancer

5

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Abstract

Cancer is the leading cause of death in the world. Pancreatic cancer is reported to be the third leading cause of cancer-related death in the United States. This is due to unreliable early detection system and also lifestyle changes. Most of the patients hospitalized with pancreatic cancer are already in the advanced stages of cancer. Pancreatic cancer is caused by cigarette smoking, alcohol abuse, late onset of diabetes, obesity, and hereditary pancreatitis. Surgical removal alone is effective in the patients where cancer is exclusively located in the pancreas and of which 25–40% of the patients shown an overall 5-year survival. In majority of the clinical cases, pancreatic cancer is already in metastatic state, wherein cancer is spread to the surrounding blood vessels and liver that severely impedes the surgical intervention. Hence, the clinician relies on therapeutic intervention for pancreatic cancer.

Tyrosine kinase is an intramembranous moiety of EGFR and VEGFR complex and plays an active role in activation and induction of EGFR and VEGFR pathway. Tyrosine kinases are kinases that phosphorylate on amino acid "tyrosine." Phosphorylation is part of posttranslation modification important to protein activity. Several chemical/monoclonal antibodies such as erlotinib, cetuximab, and panitumumab tested on EGFR pathway were proven to be effective treatments for pancreatic cancer. Compared to other drugs developed on EGFR pathway, erlotinib, an EGFR tyrosine kinase inhibitor, is reported to be effective when given along with gemcitabine for treating patients with metastatic cancer and increased median survival.

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There are also some other drugs/monoclonal antibodies such as sorafenib, axitinib, and lapatinib developed by different companies on VEGFR pathway that are currently under phase I and phase II trials.

Keywords
Pancreatic cancer · Tyrosine kinase inhibitors · Epidermal growth factor receptor (EGFR) · Vascular endothelial growth factor (VEGFR) · Erlotinib · Gefitinib · Cetuximab · Sorafenib · Axitinib · Lapatinib

5.1 Pancreatic Cancer

World Health Organization (WHO) reports suggest that cancer is one of the leading causes of death happening worldwide. According to WHO report published in 2012, nearly 14 million people were diagnosed of cancer, of which 8.2 million were reported death incidents [1]. Trend over the years is shown to be increasing with each passing year and expected to affect more than 23 million people worldwide by 2030.

Although lung and breast cancer are more common, pancreatic cancer poses more challenges for the clinicians. Currently, it causes the third leading cancer-related death in the United States, and situation is not promising either in other parts of the world; in the European Union (EU), pancreatic cancer is expected to surpass breast cancer-inflicted death and thus to become the third leading death caused by cancer after lung and colorectal by 2017 [2]. According to GLOBOCAN, 310,000 deaths per year are reported to be caused by pancreatic cancer which is projected to become the second leading cancer-related death in the United States by 2030 [3, 4].

Etiology of pancreatic cancer includes several life choices such as excessive alcohol consumption and cigarette smoking and obesity. Pancreatic cancer is also caused by other long-term ailments such as chronic and hereditary pancreatitis and late onset of diabetes mellitus [5]. Due to poor prognosis, pancreatic cancer initial symptoms are very general such as jaundice, poor appetite, weight loss, nausea, and vomiting which are also associated with other organ ailments. The pancreas is located deep inside the intraperitoneal cavity where its head is attached to the duodenum and its tail is loosely attached to the spleen; thus its location poses a challenge in collecting biopsies, and hence it causes high risk of sampling errors. Pancreatic cancer survival outcome has shown to increase in patients who have undergone surgery; even then 5-year survival is between 25% and 40% [6]. Surgically pancreatic cancer can be divided into resectable (surgically removable), unresectable, and metastatic. Pancreatic cancer (PC) is considered resectable if it is solely localized to the pancreas without any invasion. Unresectable PC is where tumor is invaded in pancreatic surroundings especially blood vessels. Metastatic pancreatic cancer is when the tumor metastasizes to the liver, intestine, and peritoneal surfaces. Patients with metastatic pancreatic adenocarcinoma often live less than 1 year. Only an experienced pancreatic surgeon can distinguish the difference using CT scan. High hospital costs and need of expertise at the clinics severely

restrict pancreatic cancer surgery. Due to stringent criteria for surgery, only 15% of overall patients can be considered for surgery. Hence, intervention by chemotherapy is considered as a more viable and cost-effective option for pancreatic cancer treatment, but so far none of the standardized chemotherapies such as gemcitabine is totally effective; hence, it is very important to understand the pathways and mechanism involved in pancreatic cancer.

An elaborate and detailed genomic analysis of patients with pancreatic cancer found that a majority of genetic alterations affected core set of total twelve important signaling pathways and processes. These pathways are as follows: RAS pathway, epidermal growth factor receptor (EGFR) pathway, vascular endothelial growth factor (VEGF) pathway, gastrin and cholecystokinin receptor pathway, PI3K/AKT pathway, cyclooxygenase pathway, TGF beta and SMAD4 pathway, hepatocyte growth factor receptor (HGF) pathway, insulin-like growth factor (IGF-1) pathway, focal adhesion kinase [7] pathway, Src pathway, hedgehog pathway, notch pathway, and Wnt pathway [8]. Out of all the abovementioned, tyrosine kinases play an active role in activation and induction of EGFR and VEGF pathways.

5.2 Protein Phosphorylation and Tyrosine Kinase

Posttranslation modification (PTM) is an enzymatic modification that leads to a mature and active functional protein, and it is a key process in protein synthesis. There are several different kinds of modification such as phosphorylation, methylation, acetylation, amidation, glycosylation, hydroxylation, ubiquitylation, and sulfation and also some other lesser-known kinds of modification. Of all the posttranslation modifications (PTM), phosphorylation showed more frequent than the rest of the modification using proteome-wide modification information from the Swiss-Prot database [9]. The amino acids such as tyrosine, threonine, and serine are most frequently phosphorylated, whereas amino acids such as histidine, arginine, lysine, cysteine, aspartic acid, and glutamic acid are less frequently phosphorylated [10]. Phosphorylation is a reversible process where phosphorylation is carried by kinase and dephosphorylation is carried by phosphatase. This reversible phosphorylation process is one of the most significant and best-studied mechanisms that regulates protein activity involved in many functional aspect of cell. In general, proteins are active when phosphorylated and inactive when dephosphorylated. If the phosphorylation is carried on tyrosine moiety, then such kinases are known as protein tyrosine kinase (PTK) or also known as tyrosine kinase; on the other hand, dephosphorylation is carried out by protein tyrosine phosphatase (PTP).

5.3 EGFR Signaling Pathway

Epidermal growth factor receptor (EGFR) belongs to HER family of receptor tyrosine kinases. EGFR is a transmembrane receptor glycoprotein that is activated by selective binding of the ligands on its extracellular domain resulting in

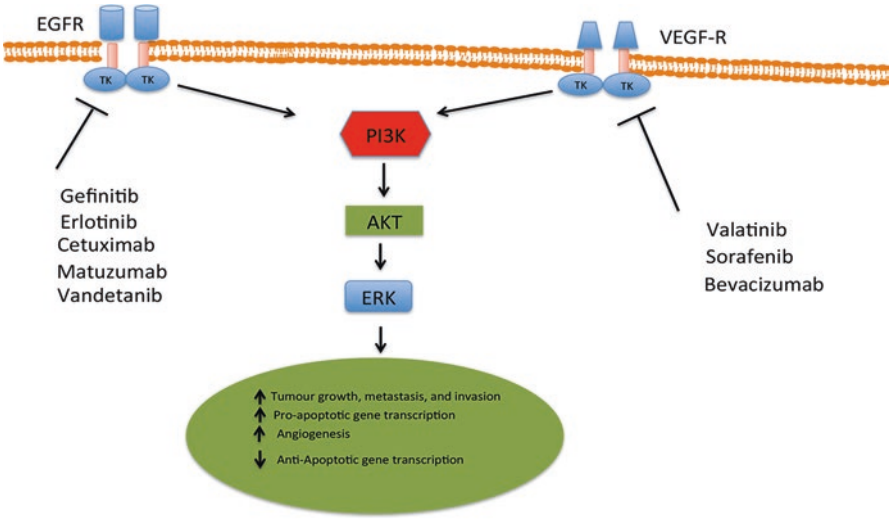

Fig. 5.1 EGFR and VEGFR pathway activation in pancreatic adenocarcinoma. Ligand binding leads to conformation changes that activates intracellular tyrosine kinase domain, which is phosphorylated and activated. This activation transduces the signal that activates PI3 kinase and Akt pathways that finally induce tumor growth and metastasis. *EGFR* epidermal growth factor receptor, *VEGFR* vascular endothelial growth factor receptor, *TK* tyrosine kinase domain

homodimerization. This further induces conformational change that allows phosphorylation of tyrosine residues in the intracellular domain. Phosphorylation at tyrosine turns the EGFR complex to an active state, which further triggers the downstream pathways such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase/Akt pathways that are involved in angiogenesis. Pancreatic cancer patient screening routinely showed overexpression of EGFR as well as its corresponding ligands [11, 12]. Hence, it is the ideal and most targeted pathway for therapeutic intervention (Fig. 5.1).

5.4 Drugs Targeted on EGFR Signaling Pathway

A total of over five different EGFR pathway-targeted therapies are available ranging from chemicals to monoclonal antibodies. Chemical compounds such as erlotinib and gefitinib were developed against EGFR, and whereas monoclonal antibodies such as cetuximab and panitumumab raised against EGFR. Lapatinib is reported to be inhibit EGFR/HER2.

Erlotinib, developed by Genentech Inc., marketed as Tarceva®, is a small molecule EGFR tyrosine kinase inhibitor. It acts by competing with ATP binding site in the catalytic domain of the EGFR tyrosine kinase and thus prevents phosphorylation and activation of EGFR, which results in inhibition of the downstream pathway [13]. Compared to gemcitabine, erlotinib (100 mg/day) along with gemcitabine

proved to be more effective in treating metastatic pancreatic cancer. This combination treatment increased the median survival of the patients to 6.24 months from 5.91 months [14]. The Food and Drug Administration (FDA) approved erlotinib usage for treating pancreatic cancer. Erlotinib + gemcitabine treatment combined with paclitaxel and radiotherapy improved median survival to 14 months in patients with advanced pancreatic cancer [15]. The combination therapy has its own side effects such as cutaneous rash. These early results from erlotinib were promising and prompted to production of monoclonal antibodies against EGFR.

Cetuximab is one such a monoclonal antibody raised against HER-1/EGFR [16]. Cetuximab acts by blocking extracellular domain to prevent receptor activation and further downstream signaling [17]. Cetuximab along with gemcitabine treatment in pancreatic xenograft mice models showed inhibition of tumor growth and metastasis [18]. Cetuximab alone and in combination with gemcitabine was found to be tolerable in phase I studies. Patients experienced rash as side effect as in case of erlotinib [19]. Phase II trial with an initial dose of 400 mg/day for a week and then followed by 250 mg/day along with gemcitabine regiment followed by week rest from chemotherapy and then weekly administration of cetuximab increased overall survival to 1 year [20]. Whereas in phase III randomized trial, the results of cetuximab or in combination with gemcitabine for advanced unrespectable pancreatic cancer were not as promising, whereas the results from phase II trial shown an overall survival of 6.5 months in combination therapy compared to 6 months in gemcitabine alone [21]. Later on, cetuximab regimen was also tested with radiotherapy and or cisplatin treatment but has not shown any conclusive significant improvement of the patients' overall survival [22].

There are some other tyrosine kinase inhibitors such as gefitinib that are under testing in combination with gemcitabine in phase II trials [23], and it is being used in another multicentered clinical trial in combination with docetaxel [24]. Gefitinib is developed and marketed by AstraZeneca. Lapatinib along with gemcitabine and oxaliplatin is currently being tested in phase I clinical trial for treating advanced pancreaticobiliary cancer [25]. It is also tested in another multidrug trial in combination with 5-fluorouracil, irinotecan, and leucovorin that are currently in phase I trial [26].

In summary, erlotinib, an EGFR tyrosine kinase inhibitor, is the only agent shown to be significantly beneficial for treating pancreatic cancer patients. FOLFIRINOX protocol is a combination of drugs that include 5-fluorouracil (5-FU), oxaliplatin, irinotecan, leucovorin, and gemcitabine and was initially used to treat colorectal cancer as a first-line treatment in phase II trial [27]. In the last 5 years, FOLFIRINOX protocol treatment was shown effective for treating pancreatic cancer patients vs gemcitabine alone treatment [28]. Modified FOLFIRINOX protocol was used to treat resectable as well as unresectable stage III pancreatic cancer.

5.5 Vascular Endothelial Growth Factor (VEGF) Pathway

Vascular endothelial growth factor and its receptors were reported to be as overexpressed in pancreatic cancer promoting tumor growth [29]. VEGF is reported to be the most important factor that induces angiogenesis. The VEGF protein includes

family of a total of six proteins, i.e., VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor, out of them VEGF-A is the predominant member. VEGF-A has two different receptors, VEGF-receptor 1 and VEGF-receptor 2. The structural analysis of VEGF receptor reported that it has three distinct domains: the first is an extracellular domain that has seven immunoglobulin-like regions, the second is a transmembrane domain, and the third one is an intracellular tyrosine kinase domain. Ligand binding activates VEGF receptor resulting in dimerization and the signal is transduced to intracellular tyrosine kinase domain which results in phosphorylation of which is active form of tyrosine kinase. The tyrosine kinase domain activation further amplifies intracellular pathways such as MAPK and PI3 kinase [30]. VEGF and VEGF receptors are reported to be overexpressed in 90% of patients with pancreatic adenocarcinoma [31].

5.6 Drugs Targeted on VEGF Signaling Pathway

Sorafenib developed by Bayer Pharmaceuticals, marketed as Nexavar®, is a multikinase inhibitor that was initially tested in advanced renal carcinoma and later used in phase II trial in pancreatic cancer. It was tested in combination with gemcitabine (1000 mg/m^2) and sorafenib, with dosage of 400 mg twice a week. The trial found no significant improvement in the overall survival and concluded that regimen is inactive in advanced metastasis pancreatic cancer [32]. Despite negative results from the above study, it is still being used as multidrug regimen in other multicentered trials in early pancreatic cancer.

Axitinib is another VEGFR-targeted small molecule developed by Pfizer. It is currently tested for treatment of advance pancreatic cancer. Randomized phase II trial in combination with gemcitabine has proven to be effective in increasing overall survival compared to the gemcitabine alone, and patients show good tolerance for the regiment [33]. Promising phase II results prompted to conduct controlled phase III trial of axitinib + gefitinib which is planned in advanced pancreatic cancer patients.

Bevacizumab is an anti-VEGF antibody used in clinical trials. Bevacizumab was previously used for treatment of colorectal [34], breast, and renal carcinoma [35, 36]. In phase II trial, bevacizumab in combination with gemcitabine increased mean survival for treatment of patients with metastatic pancreatic cancer. Phase III trial is currently evaluating effect of bevacizumab in combination with gemcitabine and erlotinib in Europe and Canada [36]. Bevacizumab in combination with radiotherapy was proved to be safe in acceptable dose in phase I and phase II trials [37].

Valatinib is another inhibitor developed against VEGF-R tyrosine kinase. Valatinib in combination with gemcitabine has shown to decrease the tumor growth and metastasis in pancreatic cancer models. Other small molecules targeted against VEGFR such as sunitinib, developed by Pfizer and marketed as Sutent, and lapatinib developed by GlaxoSmithKline and marketed as Tykerb are underway in phase II trial.

5.7 Conclusion

Several tyrosine kinase inhibitors are generated and tested for treatment of pancreatic cancer that are currently in different stages of clinical trials. These are mainly targeted against two pathways, i.e., EGFR and VEGFR pathways. Of all the drugs so far tested, erlotinib has proven to be effective when given along with gemcitabine for treating patients with metastatic pancreatic cancer.

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RON Receptor Tyrosine Kinase in Pancreatic Cancer Progression

6

Tapan K. Barik and Surya N. Swain

Abstract

The receptor d'origine nantais (RON) is a tyrosine kinase (TK) receptor, an oncogene expressed on several tissue occupant macrophage populations. Overexpression as well as constitutive actuation of RON receptor TK has been identified in a variety of tumors including pancreatic cancer, leading to tumor progression. RON is among the two individuals that belongs to MET receptor tyrosine kinase family, along with parent receptor MET. In pancreatic cells, RON is an essential K-Ras effector, and its biological response is intervened by authoritative of its ligand, macrophage-stimulating protein/hepatocyte growth factor-like protein. Under physiological conditions, ligand-mediated receptor activation and its stimulation through its receptor-binding sites are the significant reasons for RON activation. Various oncogenic signaling pathways involved in cell growth, migration, apoptosis, and survival were instigated by activated RON. However, in pancreatic cancer, overexpression and mutations, generations of splicing variants, and, seldom amplified gene copy numbers are responsible for RON activation. The pathobiological noteworthiness of RON overexpression in pancreatic cancer presently cannot seem to be fully elucidated. This chapter explains the contemporary state of information about RON biology in relation to pancreatic cancer and also reviews its probable role as a therapeutic target.

Keywords

Pancreatic cancer · Tyrosine kinases · Receptor d'origine nantais · Progression

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6.1 Introduction

Cell surface receptors play a vital role in pleiotropic cellular functions by way of external signals like peptide hormones as well as growth factors [35, 38]. Many of these external signals are regulated by receptor tyrosine kinases (RTKs). All RTKs possess three essential domains: an N-terminal extracellular ligand-binding domain, an intracellular cytoplasmic C-terminal domain that possesses TK catalytic activity, and a single membrane-spanning α-helix domain [15, 47]. The ligand binding to RTK is responsible for the development of receptor dimers as well as in stimulating the receptor kinase domain with phosphorylation of the receptor and downstream targets.

Pancreatic cancer is an aggressive form of cancer with pancreatic ductal adenocarcinoma (PDAC) as a major category and one of the most lethal malignancies in humans, which is thought to arise from pancreatic ductal epithelial cells. Pancreatic cancer is mainly subdivided into exocrine and endocrine pancreatic tumor. PDAC develops through a sequence of precursor lesions, called the pancreatic intraepithelial neoplasia (PanINs) and are categorized into three major grades: PanIN-1A/1B, PanIN-2, and PanIN-3. The advancement from normal pancreas to PanINs and PDAC is marked by a series of genetic alterations. One such type of alteration is the activation of mutation in the KRAS2 oncogene, and since this activation process is both an early and prevalent event, it is thought to have a pivotal role in the creation of pancreatic tumors. It has been revealed that pancreatic tumor cells also overexpress various RTKs such as EGFR, VEGFR, PDGFR, FGFR, c-KIT, Src, and RON [24]. Upon overexpression of RTKS, different biological processes are modified in pancreatic tumor cells, which include cell growth, activation of downstream signal transduction events, motility, and alterations of reactive oxygen species (ROS). With the novel therapeutics accessible against these RTKs, excitement has been generated for testing these compounds against pancreatic cancer. In view of these new findings, we will portray the role of RON RTK in pancreatic cancer.

The RKT *receptor d'origine nantaise* (RON), also called human macrophage-stimulating 1 receptor (MST1R) or stem cell-derived tyrosine kinase (STK), is a cell surface receptor protein, expressed by *MST1R* gene, which has a place in the c-MET proto-oncogene family [25]. The RON receptor was first identified in 1993 from primary human foreskin keratinocyte (HFK) cDNA library [32]. A few orthologs of human RON receptor have been identified and affirmed in murine and rodent [22], zebrafish [4], and feline [14] recommending its conservation all through evolution.

The ligand for RON is a member of the plasminogen-linked growth factor family [41], which was at first recognized as a serum chemotactic protein having the ability of instigating macrophage contour alteration and phagocytosis and subsequently separated from human serum and named as macrophage-stimulating protein [MSP] [26]. Interaction of MSP with RON results into the activation and phosphorylation of receptor tyrosine, which is an important event in signal transduction from cell surface to inside cell [11].

6.2 RON Receptor: Structure and Expression

The RON gene in humans is situated on chromosome 3p21.3 and contains 20 exons and 19 introns [2]. The protein is deciphered as a 190 kD glycosylated single-chain polypeptide antecedent [2] that is proteolytically managed by furin-like proprotein convertase before being conveyed to the cell surface [19]. On the outer cell surface, RON is expressed as a disulfide-related heterodimer comprising of a 40 kD α-chain as well as a 150 kD β-chain. RON coordinated as a single-chain precursor, pro-RON, intracellular proteolytic cleavage at a fundamental amino acid site KRRR, converts pro-Ron into mature, two chain heterodimeric receptor [19]. The α-chain is completely extracellular and is linked by disulfide bonds with the β-chain. The β-chain spans the membrane and includes an intracellular region, an extracellular domain, and a transmembrane domain consisting of an efficient tyrosine kinase-signaling region. The SEMA domain located in extracellular region of the RON receptor regulates phosphorylation, ligand binding, and receptor dimerization [3, 20]. The intracellular TK domain of the RON receptor shares about 63% organizational homology with the c-MET TK domain, representing similarities between these receptors [22] along with overlapping functions, signaling, and engage in cross talk. RON and c-MET have practically identical C-terminal multifunctional docking sites with two tyrosine kinase-signaling residues, a highlight characteristic of the c-MET family of TKs (Fig. 6.1).

The RON-specific transcript expression has been found during early stages of developing embryo and along within the epithelium of normal esophagus, stomach, duodenum, small intestine, colon, rectum, gallbladder, pancreas, spleen, testes, skin, brain, and bone marrow tissue [18, 28]. RON is also expressed during macrophage terminal differentiation [23].

6.3 RON Ligand (HGFL): Structure and Expression

The macrophage-stimulating protein (MSP) is the ligand for RON, also called HGF-like protein (HGFL), located on chromosome 3p21.31. It is secreted and released into blood stream in an inactive form as pro-HGFL, an 80 kD single-chain inactive precursor. Pro-HGFL works in an endocrine fashion, when locally cleaved by proteases to form an active heterodimer comprising of a disulfide-associated α- (53 kD) as well as β-chains (25 kD). The activated α-chain consists of the kringle domain and influences the RON signaling activities such as proliferation and macrophage cell scattering, while the β-chain encodes serine proteases which regulate ligand receptor-binding activity [9, 40]. Membrane-bound proteases produced by macrophages were moreover seemed to have specific and nonspecific pro-HGFL proteolytic activity, with the true objective that both activation and degradation of pro-HGFL occurred at the cell surface [42]. Recently, specific membrane-bound protease that is accountable for the activation of pro-GHFL at the cell surface has been identified on normal tissues, malignant cell lines, and multiple type of cancer tissues.

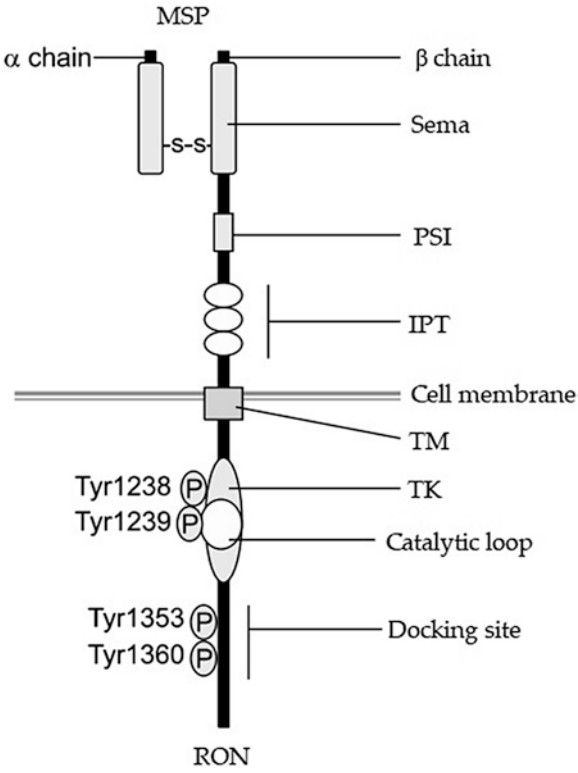

Fig. 6.1 Structure of human RON. *MSP* macrophage-stimulating protein, *Sema* semaphoring, *PSI* plexin-semaphorin-integrin, *IPT* immunoglobulin-plexin-transcription factor, *TM* transmembrane, *TK* tyrosine kinase catalytic site

Overexpression of HGFL has recently been found to promote breast tumor progression and metastasis [46]. The additional HGFL expression significantly increased the initial growth rate of mammary tumors, but the most striking effect of ligand overexpression was the increased range of metastasis.

6.4 RON Signaling: Role in Inflammation and Oncogenic Signal Transduction

RON expression levels in normal tissues and cells has been characterized to define the normal cells and signaling pathways that are activated during the conversion from normal to tumor cell. Inflammation plays a vital role in normal cellular functions as well as pathogenesis. The role of macrophage-specific RON expression in mediating inflammation was first characterized in mice deficient in RON signaling which subordinately shows defects in inflammation. Earlier reports suggest that the peritoneal macrophages isolated from RON-deficient mice were shown to synthesize excess nitric oxide (NO) upon interferon-γ (IFNγ) stimulation and lipopolysaccharide (LPS) injection [8]. Furthermore, macrophage RON activation was shown to limit LPS-induced NF-κB activation which subsequently diminishes the cytokine

expression [31]. Altogether, the above outcomes recommend that macrophage-specific RON expression is a negative controller of inflammation and in this way serves to enhance the potential tissue-damaging effects of macrophage-produced inflammatory responses.

The role of inflammation in interceding oncogenesis is widely accepted. For instance, chronic inflammation has been shown to be a precursor for prostate cancer progression [21]. Recent investigations have revealed that macrophage-specific expression of RON is closely involved in controlling inflammation that promotes tumorigenesis. In RON signaling-deficient mice, the tumor growth was arrested due to loss of expression level of tumor-associated macrophage (TAM)-specific Arg-1 [36]. Further, it was studied that inhibition of prostate tumor growth might be regulated by loss of myeloid-specific RON. Altogether, these outcomes demonstrate the unique anti-inflammatory and pro-tumorigenic effects of RON signaling in macrophages.

Transduction of a range of signaling pathways is mediated by activated RON [43]. The signaling proteins which are triggered by RON are SOS, Grb2, Ras, PI-3K, JNK, β-catenin, FAK, integrins, and NF-κB [7, 10, 12, 13, 27, 30, 50]. Signaling proteins, activated by RON, are the effector molecules accountable for cell replication, matrix invasiveness, transformation, and migration.

Activation of RON receptor involves binding of its ligand HGFL to the extracellular binding domain, which results in receptor dimerization and trans-autophosphorylation of tyrosine residues at position 1238 and 1239 within the TK domain. Phosphorylation of these tyrosine residues leads to the formation of bidentate motif, a multifunctional docking site, composed of a conserved sequence of two tyrosines at position 1353 and 1360 (Y^{1353}VQL-XXX-Y^{1360}MNL-) [33]. Mutational studies showed that this docking position is required for RON signaling, as both the tyrosine residues failed to engage SH2 domain consisting of signaling proteins that leads to a loss of transforming activity [30]. Some recent investigations reveals that some RON mutants like RON^{M1254T} undergo phosphorylation at position Y^{1198} in the kinase domain and able to deliver cell transformation and metastatic activities without the docking site [33, 45].

The RON receptor can also be activated through heterodimerization with other receptors. In the same manner, epidermal growth factor receptor (EGFR) has been shown to cross talk with the RON receptor. The interactions with other receptors can occur through both the TK domain and their multifunctional docking site. Cell signaling through multiple downstream targets such as c-SRC, mitogen-activated protein kinase, β-catenin/TCF, phosphatidylinositol 3-kinase/AKT, and some other known/unknown signaling molecules is induced by activated RON through its ligand (Fig. 6.2) [6]. These cell signals favor many cellular processes like proliferation, adhesion, motility, as well as apoptotic resistance. Earlier report suggests that the invasive activity of tumors is correlated with elevated RON kinase expression [51]. Suppression of both TGF-β-induced apoptosis and RON pathways is mediated by RON signaling which may promote the transition from epithelial to mesenchymal cell [44].

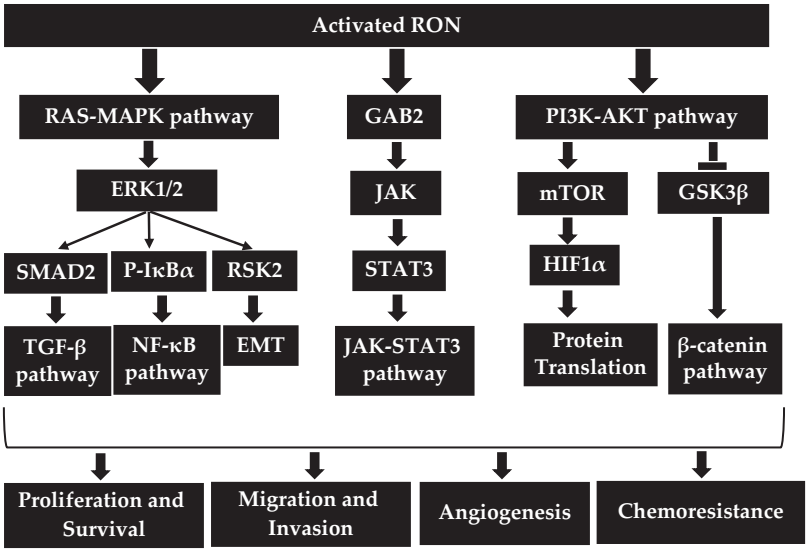

Fig. 6.2 Signaling (downstream) of RON activation. *MAPK* mitogen-activated protein kinases; *GAB2* GRB2-associated-binding protein 2; *PI3K* phosphoinositide 3-kinase; *ERK1/2* extracellular signal-regulated kinase; *JAK* janus kinase; *mTOR* mammalian target of rapamycin; *GSK3β* glycogen synthase kinase 3 beta; *IκBα* nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, alpha; *STAT3* signal transducer and activator of transcription 3; *HIF1α* hypoxia-inducible factor 1-alpha; *TGF-β* transforming growth factor beta; *NF-κB* nuclear factor kappa light chain enhancer of activated B cells; *EMT* epithelial mesenchymal transition

6.5 RON Signaling in Pancreatic Cancer Progression

Approximately 90% of pancreatic malignancies are pancreatic ductal adenocarcinoma (PDAC). Several investigations have revealed the functional significance of RON signaling in normal and cancer cells in controlling epithelial cell growth, survival, migration, and epithelial to mesenchymal transition. Overexpression of RON is reported in a variety of cancers along with macrophages and epithelial cells [29]. For instance, overexpression of RON and matriptase-1 in human breast cancer patients has linked with higher rate of metastasis and death [46]. Likewise, in other cancers, overexpression of RON in pancreatic cancer can be associated with cancer progression. A molecular mechanism encompassing RON signaling in macrophages concerning pancreatic cancer progression has yet to be deciphered. RON expression has been studied in pancreatic cancer cell lines and found that various cell lines such as BXPC-3, ASPC-1, Capan-2, Hs766.T, HPAC, and L3.6pI express RON receptor.

RON has also been shown to be an important KRAS effector and mediator of KRAS oncogene addiction. Downregulation of RON can trigger pancreatic cancer cells to first line of drug therapy for pancreatic cancer treatment. Furthermore, RON has been shown to inhibit hemidesmosome formation, a complex cell used to attach

to the extracellular matrix, by disrupting the interaction between plectin and ITGβ4 [48]. This data strengthens the correlation between RON expression and pancreatic cancer. Additionally, RON expression has been linked to enhanced β-catenin expression.

In β-catenin signaling, phosphorylation at serine 33 and 37 causes stabilization of β-catenin, which further leads to nuclear translocation. The phosphorylated β-catenin can act as a transcription factor in the nucleus. The phosphorylated β-catenin also stimulates the development of a degradation complex, which subsequently triggers proteasomal degradation. Alternatively, RON has been shown to phosphorylate β-catenin on tyrosine 654 and 670. This type of phosphorylation also stabilizes β-catenin and can lead to nuclear translocation [49]. In the nucleus, β-catenin acts as transcriptional activator of cyclin-D1, c-myc, and MMP7, all of which are upregulated in pancreatic cancer [39].

Elevated expression of matrix metalloproteinases such as MMP7 also linked with increased metastasis in pancreatic cancer [5]. MMP7 is capable of degrading extracellular matrix proteins like gelatins, fibronectin, laminin, elastin, as well as E-cadherin. Degradation of these compounds is thought to facilitate the motility of cancer cells and promote metastasis. As MMP7 is a transcriptional target of β-catenin, this hypothesizes that the RON expression may be greatly associated with elevated incidence of metastasis. Additionally, it could be hypothesized that elevated MMP7 expression downstream of RON signaling could activate Notch1, thereby leading to pancreatic cancer.

More evidence suggests that Notch signaling exhibits a vital role in pancreatic cancer growth [16] and exerts oncogenic as well as tumor-suppressive effects, depending on the cellular context. Reactivation of Notch pathway is detected in early pancreatic cancer and continues through the disease progression, suggesting that Notch could be used as a prognostic biomarker [37]. Metalloproteinases, specifically MMP7, have been shown to cleave Notch family proteins by γ-secretase [34]. This activity is sufficient to induce pancreatic cancer. On contrary, cell-expressing pancreatic duodenal homeobox protein 1 (PDX-1) is sensitive to Notch expression. Notch signaling maintains the PDX-1 expressing cells in an undifferentiated state and controls endocrine differentiation [1]. During pancreatic cancer progression, the cells expressing PDX-1 differentiate rapidly and increase in number and target RON overexpression forming Pdx-1-RON complex.

Recently, heat-shock protein 90 (HSP 90) was identified as an imperative target for the treatment of cancer because of its vital role in oncogenic signaling. Remarkably, earlier report indicated that RON could be a unique HSP 90 client, as mutated RON is highly sensitive to HSP 90 inhibitor facilitating degradation [17].

6.6 RON Signaling Pathway as a Therapeutic Target

Targeted therapies are designed to focus on oncogenic signaling molecules required for tumor progression and survival, a process described as oncogenic addiction. Most tumors acquire drug resistance with the assistance of stromal cells present in

the tumor microenvironment. Better therapeutic efficacy could be achieved through the inhibition of factors supporting tumorigenesis by targeting tumor as well as stromal cells at the same time. This suggests the potential use of multiple kinase inhibitors that have the ability for targeting tumor as well as stromal cells. As reviewed above, the RON signaling pathway is overexpressed in many tumors including pancreatic cancer and regulates various oncogenic functions such as proliferation, migration, survival, and invasion. Drugs that target the RON receptor will be valuable for promoting tumor regression through targeting various tumor cell functions of RON.

6.7 Future Prospectives

Our understanding on the role of RON has expanded significantly in the field of physiology and pathogenesis during last few decades. The disclosure of unusual expression and activation of RON in cancer cells indicates its involvement in the oncogenesis of epithelial tumors. Moreover, this unusual expression of RON is a very critical signal in regulating malignant phenotypic events of tumor. In this direction, it is highly essential to determine the tumorigenic role of RON in pancreatic cancer progression. Furthermore, it is also worth pursuing to clarify the relationship between the unusual RON expression and phenotypic events of pancreatic cancer. In this context, basic information should be provided about the methods involving monoclonal antibodies, small molecule inhibition to inactivate RON or any of its variants, and their tumorigenic roles in pancreatic cancer. Therefore, highlighting the role of RON in pancreatic cancer progression offers a chance to reveal the molecular mechanisms of its pathogenesis.

6.8 Conclusion

In summary, there is abundant and growing evidence to elucidate the vital role of RON receptor in human cancer especially in pancreatic cancer. The hypotheses explained in this chapter outline the functions of RON receptor signaling in epithelial cell and describe its vital role as a mediator of inflammation and oncogenesis. Further, this chapter explains that the tumor immunity can be regulated by RON expression in malignant cells and silencing of RON expression in these cells enhances antitumor immune responses and renders as a probable immunotherapy target.

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VEGFR and PDGFR Targeting in Pancreatic Cancer

7

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Abstract

Numerous studies have confirmed that angiogenesis acts as a momentous process in pancreatic cancer (PC) developmental stages in tumor growth, proliferation, differentiation, and metastasis. Proangiogenic factor overexpression such as fibroblast growth factor (FGF), tumor necrosis factor (TNF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) initiates progression of angiogenesis in the tumor cells. Among these VEGF and PDGFR have been confirmed as strong angiogenic factors. Overexpression of these factors has an imperative function in each step of angiogenesis development during tumor progression, recurrence, and fewer prognoses in pancreatic carcinomas. This chapter covers elementary biology of VEGF and PDGFR and their expression as prognostic biomarkers in pancreatic cancer. Overexpression of VEGF–PDGFR-mediated signaling pathways associated with pancreatic cancer metastasis and accumulating diverse therapeutic targets of VEGF and PDGFR complex are discussed.

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7.1 Introduction

Pancreatic cancer (PC) is considered to be the most aggressive cancer around the world. It is the fourth most common cancer in both developed and developing countries [1]. By 2020, it is estimated that this devastating cancer would occupy the second leading position in the USA and developed countries [2]. Common risk factors for PC are chronic pancreatic family history, excess amount of alcohol consumption, smoking, diabetes mellitus, infections with hepatitis B virus, and intake of low vegetables and processed meat with high fat [3]. Nonspecific symptoms include weight loss or painless disruptive jaundice, abdominal epigastric pain roaring at the back, and dark urine (Cancer Research UK). The screening and diagnosis of PC is illustrated in Fig. 7.1. The high mortality rate of PC patients is mainly due to the poor diagnosis in the early stages, and the reported survival rate is 1–5% [1, 2]. Various experimental studies on PC have provided data supporting that a minimum of 5 years are needed for a benign tumor to turn into metastatic stage [2, 4]. This information clearly indicates that early diagnosis makes PC curable, improving survival and decreasing mortality. One of the most important reasons for this is that some tumors are not detected, either because of small tumor size or complexity in description, and are diagnosed at a later malignant stage when compared to other types of cancer. Another reason is tumor size underestimation or overestimation.

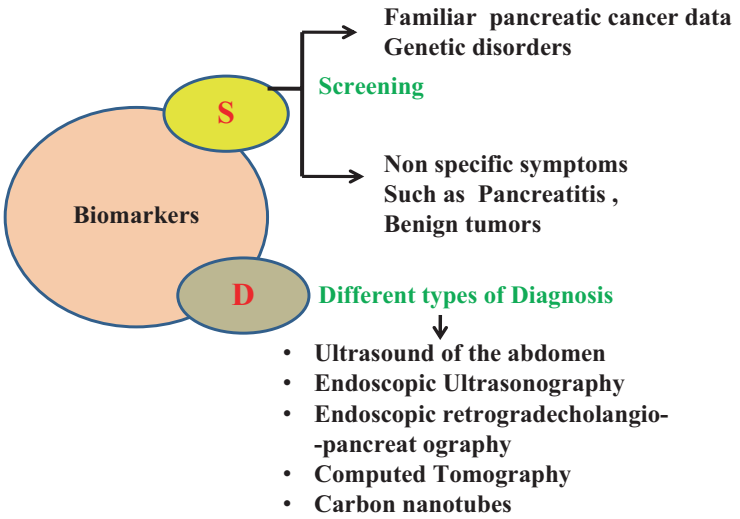

Fig. 7.1 Different approaches and methods to screen and diagnose for pancreatic cancer

Histopathological studies of PC tissue specimens showed that there are mainly three precursors lesions which lead to the development of PC. They are IPMN (intraductal papillary mucinous neoplasms), PanIN (pancreatic intraepithelial neoplasia lesions), and MCN (mucinous cystic neoplasms) [5, 6]. IPMN are borderline cystadenomas and intraductal papillary mucinous invasive carcinomas. In this type, modifications of cellular and histological pan epithelial cells take place leading to development of invasive cancer by accretion of increased atypia, methylations, and genetic modifications in tumor suppressor genes [6, 7]. EUS (endoscopic ultrasound), MDCT (technological advances in multidetector computed tomography), and magnetic resonance with cholangiopancreatography are used to diagnose cystic premalignant lesions like IPMN and MCN. Image techniques are not used for diagnosis of PanIN lesions as they are microscopic. Though all cystic lesions do not lead to development of cancer, their identification itself can result in incorrect recommendation for surgery due to difficulty in clinical supervision [2].

As part of the treatment, surgery is one of most sought out methods in early stage of the PC, as it responds very slowly to radiation and chemotherapy. However, the chances of recurrence are high, and survival rate is very low. Many researchers study the cellular and molecular mechanisms involved in PC carcinomas and different elements associated with PC tumor growth, proliferation, metastasis, and survival [8, 9]. To predict or identify PC, different molecular biotech techniques and immunohistochemical reviews and their impact on clinical pathology management are in progress. Several prognostic biomarkers are identified based upon the stage of the tumor, type of the tumor, grading, complete localized resection, and negative resection of stage I and II disease patient to increase the survival prognosis of PC [6, 8]. The biomarkers include cell cycle signaling regulatory molecules (cyclins, CD44, SMAD4, P^{21}, P^{53}, P^{16}, and P^{27}), growth factors (EGFR, EGF, FGF, TGFβ and HB-EGF, TMSF), transcriptional factors (HIF-1α, PIPk, Bax, Bcl-2, STAT-3, MMp, uPA, and c-erbB2) [8], miRNAs, methylation biomarkers (CA119-9, CD1D, KRAS, CLEC11A, PKRCB, KCNK12), carbohydrate antigen 19-9 [2], cell adhering molecules (heparanase, lamininns, cathepsin, E-cadherin, catenin, and integrins), and angiogenic proteins and their receptors (VEGF, PDGF, and IL -8) [9]. Still a large number of biomarkers are evaluated to make PC preventable and curable and increase the survival proficiency.

Tumor angiogenesis depends on the growth, proliferation, and metastasis of the tumor cell. This requires a number of intermediated connecter malignant cells, resident of adjacent tissue cells, and migration cells. Numerous molecules are required for initiation of angiogenesis in tumor and host cells. Among the various dynamic molecules, VEGF (vascular endothelial growth factor) is also a glycoprotein linked with heparin; it acts as a mitogen angiogen and increases the vascular permeability functions of endothelial cells; however it is a main player in angiogenesis [10]. Normal cells and overexpression of tumor cells both secrete VEGF in cellular process of angiogenesis and lymphangiogenesis. New vessel formation in normal cells as well as tumor cells by binding of tyrosine kinase receptor in signal transduction pathway shows endothelial cell development and migration [11]. Regulating the angiogenesis by decreasing the expression levels of VEGF by inhibiting the transcriptional factors HIF-1α, PI3k/Akt kinase pathway can lead to the apoptosis of PC

cells [12]. Tyrosine kinase receptors related to VEGFR and PDGFR are regulating the cell abundance, migration, and differentiation of pancreatic cells. Therefore, in vasculature of PC tumor cells, VEGF/PDGFR is one of the rational targets to treat and diagnose PC and increase the survival rate.

7.1.1 Biology of Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is part of the platelet-derived growth factor supergene families, and it is known as a vascular permeability factor which is initially explained as an endothelial cell-specific mitogen [13]. In angiogenic architecture, the involvement of VEGF has an important function in normal cells and in tumor cell growth [13]. It is secreted from several types of cells like platelets [14], macrophages [15], and tumor cells. In vivo studies explain that the VEGF induces the cell growth, division, and circulation in blood vessels and stimulates cell death in tumor cells [16].

At present, VEGF family entails eight members with same homology domain and core region consisting with cystine knot motif: VEGF-A, PlGF-1 and PlGF-2 (placental growth factor), VEGF-B, VEGF-C, VEGF-D, VEGF-E, and VEGF-F [17]. These all differ in molecular mass and cell surface receptor biological properties.

7.1.2 VEGF-A

In 1983 Ferrara and Henzel found evidence of VEGF-A as a VPF (vascular permeability factor), but later it was defined an endothelial-specific cell mitogen [17]. VEGF-A has been identified in mouse, rat, zebra fish, birds, and mammals [18]. VEGF-A is a master regulator for vascular homeostasis of islets and islet vascular development, and it is secreted by the endocrine islet cells [19]. VEGF-A is a precursor angioprotein for endothelial cell proliferation and migration by activating through two tyrosine kinase receptors VEGFR1 and VEGFR2 binding [20]. VEGF-A, a angioprotein has four isoforms by splicing contains number of amino acid sequence vary in their isoforms such as $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ in that $VEGF\text{-}A_{165}$ is qualitatively as well as quantitatively significantly main initiative angioprotein for angiogenesis [21].

7.1.3 VEGF-B

VEGF-B is structurally analogous to VEGF-A and placental growth factor and exits in two isoforms $VEGF\text{-}B^{167}$ and $VEGF\text{-}B^{186}$, but their COOH terminal amino acid sequence is different. It is identified in the heart, adult myocardium, skeletal muscle, nervous system, and pancreas and expressed with VEGFR-1 [22, 23]. VEGF-B regulates the fatty acid cellular metabolism and transport of lipids to adipose tissues, heart, and skeletal muscle [24, 25].

In vivo studies explained under pathological conditions that VEGF-B does not promote the blood vessel growth but acts as a survival factor not as angiogenic factor [26]. Deficiency or inhibition of VEGF-B in in vitro and in vivo experiments showed very poor vessel survival and greater cell apoptosis [26]. Therefore, VEGF-B is a critical marker for anticancer therapies and neovascular diseases.

7.1.4 VEGF-C

In humans VEGF-C is encoded by the *VEGFC gene*, and it is positioned at chromosome locus 4q34 [27]. Previously it was mapped as a 4q34-35 because of its proximity to the aspartylglucosaminidase gene of humans [27]. Mostly found in the heart, placenta, lung, skeletal muscle, small intestine, and the thyroid gland, it consists of seven exons. It acts as paracrine signaling in lymph angiogenesis process on lymphatic endothelial cells. It incorporates three domains – the central VEGF homology domain (VHD), the N-terminal domain (propeptide), and a C-terminal domain (propeptide) – which are required for lymphangiogenic process [28]. VEGF-C mainly works as a ligand for both FLT4 receptor tyrosine kinase, VEGFR-3, and KDL receptor tyrosine kinase, VEGFR-2. Structurally and functionally there are many close similarities between VEGF-C and VEGF–D [21].

7.1.5 VEGF-D

Another name of VEGF-D is C-fos-induced growth factor (FIGF); it is known and encoded as the *FIGF gene* in humans [29]. VEGF-D binds and activates the tyrosine kinase receptors VEGFR2 and VEGFR3 and through this receptor signaling actively participates in lymphangiogenesis and angiogenesis [30]. It is a concerned protein formed by macrophages and fibroblast which stimulates lymphangiogenesis through VEGF-3 receptor signaling [30]. VEGF-D is rich in adult tissues, particularly the lung, colon, skeletal muscle, heart, and small intestine [31].

7.1.6 VEGF-E

VEGF-E induces angiogenesis in lesions on the skin when sheep and goats are affected with infection and rarely in humans [21]. [32] identified a novel VEGF-E in Orf virus which is part of the zoonotic species parapox virus family [32]. Meyer et al. [33] explained in their in vitro studies that in *E.coli* culture VEGF-E_{D1701} (OV strain) expression was observed and identified as a heat-stable, secreted dimer having with 34 kDa molecular weight. VEGF-E shows similar bioactivities like VEGF-A such as inducing tissue factor, cell growth, explosion, chemotaxis, and developing vascular endothelial cells in cultured *E.coli*, as well as angiogenesis in vivo studies. In another study, VEGF-E controls occupation of keratinocytes by increasing the epidermal thickening and epidermal regeneration. Their study found that like VEGF-A, VEGF-E also induces the reepithelialization in non-healing

wounds [34]. Like VEGF-A, VEGF-E binds to VEGFR-2 (KDR) receptor with high affinity and promotes mitogenic activity without heparin [21, 35]. Purified VEGF-E protein injection stimulates epidermal thickening, increasing keratinocytes, and the area of neo-epidermis; this therefore increases rate of reepithelialization of skin wounds. As VEGF-E exposure to wounded skin increases, the rete ridges projecting from neo-epidermis increased in number and length. Above all viral VEGF itself can promote epidermal changes like in Orf virus infection. VEGF-E may support viral growth by viral replication and the regenerative response within the epidermis [36].

7.1.7 VEGF-F

VEGF-F is called snake venom VEGF protein, called as Vammin and VR1. These are isolated from *Daboia r. russelii* and *Vipera a. ammodytes* venoms [37]. Biologically and physiologically, VEGF-F is similar to VEGF-$A_{165.}$ Like VEGF-A, VEGF-F binds with high affinity to VEGFr1/KDR. Two VEGF-F isoforms of *Tfsv*VEGF include *Pm*VEGF identified in *Trimeresurus flavoviridis* (lethal poisonous Habu snake venom) and *Protobothrops mucrosquamatus* snake venoms [37, 38]. *Tfsv*VEGF increases the vascular permeability and shows a very low act activity to cell proliferation, and it is a strong stimulating molecule for vascular permeability. Further [38] hypothesized that it is clinically useful to increase the capacity of anticancer drug penetration in t-tumors and suppress the tumor angiogenesis.

7.1.8 Placental Growth Factor

The placental growth factor is coded by the *PGF gene* on chromosome 14 [39] which is homologous to VEGF and is found in placenta and in very low levels expressed in the heart, lungs, and kidney. The splicing of PIGF mRNA produces four different isoforms which are PIGF-1, PIGF-2, PIGF-3, and PIGF-4 [40]. PIGF binds to VEGFR-1 (Flt) and co-receptors of Npn-1 and Npn-2 (neuropilin 1 and 2) at high affinity [40]. Tumor malignant stage PIGF employs multiple jobs in stimulating tumor cell division, growth, proliferation, migration, angiogenesis, and resistance to antiangiogenic therapy [40].

7.2 VEGFR and Their Ligands

VEGFs interact with the tyrosine kinase receptors (RTKs) that activate the signal transduction through autophosphorylation. VEGFRs belong to class III cell membrane protein tyrosine kinases. There are three subtypes of VEGFRs, i.e., VEGFR1, VEGFR2, and VEGFR3. VEGFRs play a crucial role in drug discovery and have different target sites to treat the cancer and other pathological diseases (Table 7.1). The structure of VGFRs (Fig. 7.1) involves seven immunoglobulin (Ig) domains

Table 7.1 Functions of VEGF and their receptors

VEGF proteins	Location on chromosomes	Isoforms of VEGFs	Binding with receptors	Main functions
VEGF-A	6p21.3 (8 exons and 7 introns)	$VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$	VEGFR1, VEGFR2 and neuropilin1	Angiogenesis, chemotactic factor, vasodilatation
VEGF-B	11q13 (7 exons and 6 introns)	$VEGF_{167}$, $VEGF_{186}$	VEGFR1	Embryonic angiogenesis
VEGF-C	4q34 (7 exons)		VEGFR2, VEGFR3	Lymphangiogenesis
VEGF-D	Xp22.31 (7 exons)		VEGFR2, VEGFR3	Lymphangiogenesis
VEGF-E	Nonhuman Orf parapoxvirus		VEGFR2	Angiogenesis
VEGF-F	Nonhuman snack venom vegf	*Tfsv*VEGF and *Pm*VEGF	VEGFR1, KDR	Vascular penetrating factor
PIGF	14q24 (7 exons)	PIGF-1, PIGF-2, PIGF-3, and PIGF-4	VEGFR1, neuropilin- 1	Inflammation, embryo angiogenesis, vasculogenesis

present in extracellular region to bind the VEGFRs and intracellular portion containing split tyrosine kinase domain and cytoplasmic domain [21, 41]. Homologues of VEGFRs have been identified in zebra fish, *Drosophila melanogaster*, chicken, quail, and frogs [42].

VEGF started to binding to extracellular VEGFR domain which is located space for binding to receptor domains, this process forms VEGF-VEGFRs complex and ensuing the activation of tyrosine molecules to autophosphorylation by receptor dimerization either by homo- or heterodimers placed in the intracellular membrane [42]. Later, a number of signaling proteins that bind to VEGFRs generate large protein-ligand complexes, and then these signal transduction pathways initiate different molecular and cellular activities.

7.3 VEGF and PC

Strengthening this investigation, elevated levels of VEGF-C expression promote intratumoral lymphangiogenesis, and upregulation of intratumoral lymph vessel density (iLVD) renders malignant development of pancreatic endocrine tumors (PET). These results significantly showed expression of VEGF-C mediating VEGFR-2 and VEGFR-3 involved in maintaining PET growth and metastasis [43, 44]. In another study [45] explained by experimental studies both in vivo and in vitro showing results of VEGFR1 and VEGFR-2, upregulation of VEGFR2 leads to tumor growth, proliferation, angiogenesis, and survival of tumor spread in PC, whereas VEGFR1 can involve tumor migration.

Many studies elucidate overexpression of VEGF protein family components triggers increased angiogenesis in PC. In this VEGF protein family, mainly VEGFR2 is a vital marker to evaluate the angiogenesis in PC [46]. Normal cells as well as tumor cells which show expression of VEGF are maintained by several external and extracellular molecules such as growth factor receptors, cytokines, gonadotropins, cell cycle regulatory molecules, and transcriptional regulating molecules, e.g., hypoxia, acidosis, and hypoglycemia [10]. In JAK-STAT signaling pathway, STAT-3 is a transcriptional membrane protein acting as an oncogene and which participated grave significance in tumor development, proliferation, and angiogenesis. [47] observed in vitro studies on PC cell lines and Pan tissues specimens and by using Western blot analysis confirmed overexpression of VEGF was irreversible to STAT expression by activation of STAT3 which binds to the VEGF gene promoter region [47]. Shi et al. [10] elaborated that low tumor pH that upregulated the elevated levels of VEGF expression can lead to tumor angiogenesis in PC by their in vitro assay on cultured FG human PC cells incubated for 24 h, and after harvesting, cells were treated with fresh media at different pH levels, i.e., 7.4, 7.1, 6.9, and 6.7, and incubated 6–12 h. After completion of harvesting, cells under longer incubation periods showed increased levels of VEGF expression at pH 6.9 and 7.1 and considerably reduced levels at pH 6.7. This data suggests that the (low pH) acidic microenvironment upregulated the VEGF expression in PC [10].

In another in vitro study explained, the RON (recepteur d'origine nantais) signaling pathway stimulates the overexpression of VEGF in PC. RON receptor is a heterodimer unit with 150 and 35 kDa molecular weight transmembrane glycoprotein connected with disulfide bonds. RON endures autophosphorylation by activation of HGFL (hepatocyte growth factor-like protein) which induces the overexpression of VEGF, which results from cell growth, proliferation, recruiting cells for circulation, apoptotic resistance, invasiveness, and angiogenesis in PC [48].

7.4 Biology of PDGF

PDGF (platelet-derived growth factor) is a significant regulatory molecule for blood vessel formation in angiogenesis and tissue regeneration for normal cells as well as tumor cells. PDGF was identified in the year 1970 and is a substantial serum growth factor for glial cells, fibroblast, and smooth muscle which are formed from the platelets [49]. PDGF has homo and hetero cationic dimeric forms which are linked with disulfide-bonded polypeptide chains of A and B. Genes of A and B polypeptide chains of PDGF are situated on human chromosome numbers 7 and 22 [50].

PDGFs are made up of PDGF-A, PDGF-B, PDGF-C, and PDGF-D; these are connective tissue cells used in skeletal muscles, mitogens, and fibroblast. Structurally PDGFs have a dimeric nature with disulfide-bonded A and B polypeptide chains conserved with cystine knot protein domain [51]. There are five dimeric compositions of PDGFs, and all are homodimeric (PDGF-AA, PDGF-BB, PDGF-CC, and PDGF-DD) except one which is a heterodimer (PDGF-AB) [49]. A major form of PDGF-AB identified is in platelets; when blood clotting occurs, it is released into the serum [52].

Receptors corresponding to PDGF are α and β receptors which are class III tyrosine kinase receptors and have 170 and 180-kDa molecular weight [49]. These receptors are organized into five Ig-domain segments extracellularly and are labeled D1–D5 and tyrosine kinase domains intracellularly enclosed with distinctive sequences [50, 51]. The α-receptor binds to domains 2 and 3 of PDGF-A, PDGF-B, and PDGF-C at a high affinity. Consequently PDGF-AA generates the αα-homodimer, PDGF-BB and PDGF-DD generate ββ-homodimer, and PDGF-AB generates αβ-heterodimer. PDGFR-β receptor binds with PDGF-B and PDGF-D [53]. PDGFR-α receptor has the gene situated on chromosome locus 4Q12, and VEGFR2 and SCF receptors are very close with this; PDGFR-β gene is located on chromosome number 5 [50, 54].

PEGFR dimerization occurred with activation of tyrosine kinase residues undergoing autophosphorylation in the intracellular parts of juxtaposes membrane. PDGFR undergoes two major activities after dimerization; one side is a tyrosine kinase a-receptor Tyr-849, and b-receptor Tyr-857 undergoes phosphorylation inside of kinase domains and provides catalytic efficiencies for PDGF-b receptor, fibroblast growth factor, insulin, and hepatocyte growth factor. The other side, autophosphorylated tyrosine kinase molecules, binds with outside kinase domains which are opened signal transduction protein with SH2 domains landing sites. PDGF α and β receptors bind SH2 domains consisted of signal transduction proteins which activate signal transduction pathways. These all involve several cellular process such as cell growth, division, migration, angiogenesis, anti-apoptotic, and chemotaxis [53].

7.4.1 PDGF and PC

Mutant tumor suppressor protein P^{53} obstructs the actual p^{53} functions and gains oncogenic functions contributed to upregulate the elevated expression of PDGFR-b inducing cell development, migration, existence, and metastasis in pancreatic cancer [55]. In PC activation of PEGFR-β binds to PDGF-D, stimulates elevated levels of increased PDGF-D expression and stimulates tumor development, proliferation, migration and angiogenesis by up regulation of Notch–1 pathway and binding to NF-κB, which activated the target gene VEGF and MMP-9 [56, 57].

7.5 Current Therapeutic Drugs for PC Treatment

In the architecture of the angiogenesis, VEGF is an essential inducer for tumor proliferation, division, invasion, transportation, and metastasis by activation of the tyrosine kinase pathways. Therefore inhibition of the VEGF is sticking a therapeutic target for PC.

A multi-kinase inhibitor of foretinib acts as an antiangiogenic drug which inhibits the VEGFR2-meditated angiogenic pathway, HGF (hepatocyte activity), c-MET receptor, and receptors of VEGFR3 and TIE 2 which are key players for lymphangiogenesis. Chen et al. [58] demonstrated in their in vivo studies on xenograft animal models containing Panc-1 cells that animals treated orally with 30 mg/kg of

foretinib were observed considerably to have reduced tumor size than in the control group, signifying its utilization for treating PC. Still clinical trials are undergoing for PC treatment [58]. Another drug developed for PC treatment use is gemcitabine, which suppresses the signaling transduction molecules which are VEGF, PDGFR, and EGFR associating tumor growth, proliferation, migration, and angiogenesis; it enhances the survival rate of PC patients [59].

New inventions came for treatment to PC, such as the combination of drugs like gemcitabine with bevacizumab, gemcitabine with capecitabine, and bevacizumab and gemcitabine with cisplatin and infusional fluorouracil which completed phase II trials. The occupation of combinational drugs inhibiting the angiogenic proteins and inducing apoptosis via various cellular process increases the survival rate and resistance to PC [60–61].

Weissmueller et al. [55] observed inhibition of PDGFRb by using imatinib, which is approved by the FDA and significantly moderates anti-metastasis in PC [55]. Bevacizumab is an antiVEGF monoclonal antibody that inhibits VEGF-A-mediated signaling cellular process involved in tumor proliferation, transportation, angiogenesis, and increased cell death and dissolves all the VEGF isoforms [17].

Numerous research investigations are going on plant-derived phytochemicals which are beneficial to treat PC without any aberrations such as curcumin, resveratrol, and genistein. [64] demonstrated that the polyphenolic compound CDF (curcumin-derived analogue-diferuloylmethane) is a potential agent to treat PC by deregulating VEGF, IL -6, and HIF-1-α expressions under hypoxic conditions and significantly inhibiting tumor growth, proliferation, and angiogenesis [64]. To support this hypothesis is another in vitro investigation in which clinical trials and in vivo studies were conducted by using a combination of curcumin with gemcitabine on PC cell lines. Significant improvement of tumor growth and angiogenesis was seen by suppressing angiogenic growth factor VEGF-PDGF-mediated signaling pathways NF-κB, JAK/STAT, PI3k/Akt, and Notch 1 and their signaling genes [65].

In another study, a multitargeted agent kinase inhibitor resveratrol suppressed the self-regeneration activity of PC cells through the deactivation of Bcl-2, cyclinD1, and XIAP, which resulted in resveratrol stimulating apoptosis and cell cycle arrest (G0/G1, S, and G2/M) in PC [66].

In the experimental era of oncology, molecular targeted therapies have been successful in treating cancer and have improved survival rates. Clinically these molecular target drugs induce the interruption of normal cellular activation, and some results shown in patients have included adverse side effects and graceful sensitizing to the drugs [67].

7.6 Conclusion

Still researchers investigate the involvement of growth factors VEGF and PDGF contributing to new perception in cellular and molecular levels of pathophysiological processes of pancreatic cancer. Theoretically pancreatic cancer improvement and development are controlled by several signaling paths affecting cell

development, abundance, differentiation, movement, and angiogenesis. These studies provide better understanding and extra insight to develop photochemical and bioactive drugs with capabilities of penetration (drugs with anti-VEGF-F) in tumors and drug modulations without any aberrations and with improved survival rates.

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EGFR and Cytoplasmic Kinase Src Targeting in Pancreatic Cancer

8

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Abstract

Pancreatic cancer (PC) is one of the most devastating malignancies in the world and the fourth leading cause of fatalities associated with cancer in the United States. PC has an extremely poor prognosis and a 5-year survival rate ranging between 1% and 5%. In emerging PC treatment, tyrosine kinases and its inhibitors signify a new generation of therapeutic drugs that specifically target tumor pathways that are associated with tumorigenesis such as cell cycle mechanism, signal transduction, apoptosis, and angiogenesis. Tyrosine kinases such as EGF and Src kinases were specifically used to target PC progression and metastasis. In this chapter, we discuss the EGFR and Src role in progression PC.

Keywords

Pancreatic cancer · Progression · Metastasis · EGFR · Src

8.1 Introduction

Pancreatic cancer (PC), attributable to its late presentation and early metastases and its resistance to radiation and chemotherapy, has become one in all deadly cancers prevalent in humans. With a survival percentage of less than 5%, it remains the

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fourth common reason behind human cancer deaths [1]. Presently surgical medical care is the sole form of treatment related to future survival in PC. Substantial studies to recognize the molecular genetics of PC have predicted common alterations and genetic mutations [2]. An average PC genome has a single mutation per mega base and is a heterogenous tumor [3]. Even before one will understand the communications of multiple signaling pathways concerned within the initiation and maintenance of the cancer, one ought to perceive the genetic complexity of this cancer.

The lack of effective therapeutic procedures and dismal prognosis of PC has been associated with various factors. Mostly, PC displays an aggressive physiological phenotype categorized by initial invasion of encompassing structures as well as fast metastasis. PC also exhibits a strong immunity toward chemo- and radiotherapy [4]. To boost the prognosis of patients suffering from PC, early diagnosis must be made, and curable stages that suggest a lot of biological markers are needed for early detection of PC [5].

The EGFR family is a member of the RTKs that include the epidermal growth factor receptor (EGFR) or HER1 (or ErbB1), HER2 (or ErbB2), HER3 (or ErbB3), as well as HER4 (or ErbB4) receptors. Further, activation of transmembrane glycoproteins (EGFRs) by ligands is highly expressed and mutated in various cancer cells including PC. Nevertheless, before discussing the association between PC and EGFR, it is extremely vital to recognize the advancement, genetics, and prognosis of PC and the function of EGFR in various tumors.

8.2 Advancement and Genetics of Pancreatic Cancer

The most collective form of PC is the pancreatic ductal adenocarcinoma (PDA) that arises within the exocrine region (or the acinar and duct tissue) of the pancreas. Some initial lesions of PC include the pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasia, as well as the intraductal papillary mucinous neoplasia [6]. A most well-known precursor of PDA is PanIn. Although genetic engeneering and molecular studies recommend that acinar to ductal metaplasia (ADM) may be a sign to the growth of the initial stage of PDA [7, 8].

A spectrum of mutations that appears within the cancer has been processed by the intensive characterization of the molecular genetics in spite of the very fact that only few therapeutic choices are available for treatment of PC. A far-reaching genetic examination of 24 humanoid PDAs uncovered that, typically, a mature PC cell consists of around 63 genetic variations per tumor exome. The primary variation in PDA is the activation of mutations within the KRAS proto-oncogene [2]. Virtually 95% of humans have these kinds of mutations and induce the impairing capability of KRAS which hydrolyzes guanosine triphosphate (GTP) into guanosine diphosphate (GDP) by fastening KRAS in an activated conformation [9].

8.3 EGFR

Overexpression of EGFR in chronic pancreatitis could be a risk factor for PC. Since EGFR plays a diverse role in the growth, advancement, and survival of PC cells, it is widely associated with antitumor therapies [7]. ERBB receptors undergo numerous sorts of alteration in human tumors. Gene amplification resulting in EGFR overexpression is found in human cancers. Furthermore, in several tumors, EGF-associated growth factors either are released by the cancer cells themselves or are accessible from adjacent stromal cells, resulting in constitutive EGFR stimulation.

The receptor HER4 is structurally like the EGFR and has the capacity of ligand-dependent homodimerization as well as heterodimerization. Whereas, HER2 has no familiar direct ligand and is known to initiate dimerization that is both ligand-dependent and ligand-independent [10]. Functional kinase domain lack in the HER3 receptors and thereby need heterodimerization for active signal transduction however will undergo ligand-dependent dimerization.

8.4 EGFR in Cancer

EGFR initiation in PC can occur via several mechanisms like ligand-dependent as well as ligand-independent activation, partial deletions, overexpression, and point mutations [11]. EGFR along with its group of receptors is located in the nucleus of the cancerous cell of different tumor cancer types including ovarian, breast, lung, and oropharyngeal. Stimulation of EGFR signaling occurs at the cell surface and is later moved to the nucleus through the cytoplasmic intermediates.

Apart from the vital role played by EGFR signaling in PC growth, it additionally plays another significant role in tumor microenvironment (TME) and can stimulate a number of angiogenesis-associated factors like VEGF, IL-8, and FGF [12]. EGFR thereby shows a central function in PC cell proliferation, angiogenesis, as well as metastasis [13]. Erlotinib and gefitinib are the two highly documented EGFR-targeting TKIs that are used in clinical treatment. EGF and HER2 receptors are the monoclonal antibodies developed due to frequent activation of EGFR.

The research pertaining to the study of effect of EGFR on PC has been carried out either in preclinical trials involving mouse models or in cell culture due to the level of difficulty in PDA diagnosis. An analysis of immune histochemical (IHC) of PanIN2 shows an overexpression of HER2 in 80% of PanIn1a lesions [14]. The first ever direct evidence of EGFR signaling in PDA was evident from the transgenic mice that overexpressed the EGF ligand TGF in the pancreas [15].

EGFR role in tumor maintenance and movement is uncertain; nonetheless, different examinations have been distributed using persistent specimens. The two receptors and ligands are perceived to be overexpressed in straightforward

carcinomas, be that because the importance of these perceptions stays disputable. Trials analyzing the impact of EGFR in cells have demonstrated that cells can experience cell cycle capture and apoptosis or stay uninterested, contingent upon the cell line and examine utilized. Despite various endeavors to distinguish biomarkers discerning of reaction, there are incompatible outcomes with respect to the prescient estimation of receptor overexpression, quality enhancement, and receptor phosphorylation. The foremost-organized method researched the affectability of around 639 cancer cell lines, containing 17 PC cell lines, for affectability to erlotinib, afatinib, lapatinib, and gefitinib. In spite of promising outcomes, for example, the relationship of lapatinib affectability with cells with either HER2 overexpression or transformation, there was no cover of qualities foreseeing affectability or protection for each one of the four inhibitors tried. The affiliation that most nearly approaches importance is that all-around recorded part for KRAS/NRAS changes in protection from EGFR-focused therapies. While this information could mirror the characteristics of each inhibitor, they in any case underscore the overly complicated nature of biomarker revelation.

8.5 The Role of EGFR Group of Receptors in PC Progression

Overexpression of EGFR in PDA is anywhere between 30% and 95% [16, 17]. Even though there is proof of EGFR overexpression in PDA and signs that it can assume a part in metastatic advancement as well as in TME, the therapeutic importance of these discoveries stays indeterminate owing to incompatible outcomes in most patients.

Overexpression of HER2 is less common in PDA as compared to EGFR expression though when overexpressed it is shown to possess a shortened life [18, 19]. A post hoc analysis of patients for HER2 showed very low response rate. Thus, due to the conflict between HER2 enhancement and IHC articulation of HER2, there can be alternative dynamic dysregulated malignancy pathways adding to the overexpression of HER2. The experiments so far reveal that anti-HER2 treatment may not be compelling unless joined with alternative molecularly targeted agents, with solid logical methodology of reasoning behind the underlying combination.

The function of HER3 and HER4 in PDA though under examined as compared to HER2 and EGFR reveals that overexpression of HER3 is related with very low survival rate in pancreatic cancer patients [20].

Studies show that HER4 is expressed in normal ductal cells; however its expression is reduced in the advanced stages of PC suggesting that it may be imperative in the initial stages of PC but insignificant during the later stages [21, 22]. Treatments that focus on HER3 and HER4 are being developed, because it is important to better comprehend their part in the movement of pancreatic growth to settle whether this may be potentially useful alternative for patients. Regardless of whether this could be achieved in composed translational clinical trials or by utilizing cell lines and mouse models of PC stays to be resolved.

8.6 Targeting EGFR and Src in PC

The failure of standard chemotherapeutic administrations to deliver any significant effect on survival in patients with pancreatic disease due to advanced metastatic disease features an urgent demand for novel treatment systems. Over the previous decade, studies have established that procedures specializing in molecular irregularities involved in pancreatic oncogenesis could invoke inhibition of PC development in preclinical investigations. But these outcomes have neglected to convert into clinical advantage in various Phase III trials of molecularly focused treatments in patients with cutting-edge PC when combined with gemcitabine.

KRAS mutations have been found to be the most effective therapeutic target as they are found commonly in PDA. But the biochemical properties of KRAS protein have made it extremely difficult to target this as there are no effective KRAS inhibitors [23]. The two best documented EGFR-targeted TKIs that are utilized as part of clinical treatment of patients are erlotinib and gefitinib. Numerous EGFR TKIs have the ability to block many receptors of the EGFR family, such as lapatinib that has the ability to reversibly inhibit HER2 as well as EGFR. Receptor's extracellular ligand-binding region completely binds the monoclonal antibodies against in its inactive state. Upon binding, ligand binding and receptor dimerization are prevented, thereby blocking the activation of the endogenous ligand of EGFR in an extremely specific manner.

The explanation to target Src and EGFR signaling relies on the fact that EGFR- and Src kinase-specific activity both are increased in the majority of PC cases and are also involved in PC progression and metastasis [24]. One of nine constitutes of the Src group of non-receptor protein tyrosine kinases is the Src. Under typical circumstances, Src is a cytoplasmic protein, which is kept up in a dormant frame. It isn't stimulated by mutation; however Src plays an important role in facilitating various signal transduction pathways along with numerous proteins including G-protein-associated receptors and RTKs, for example, EGFR and integrins, which make it an ideal focus for therapeutic interventions [25].

Src squarely regulates EGFR work via phosphorylation of tyrosine residues on EGFR, which permits coupling to downstream flagging occasions [26]. In addition, it is used to be known that mechanisms of resistance to Src inhibition appear to be linked with a lack of inhibition of initiated STAT3 signaling. The effects of dasatinib, erlotinib, and gemcitabine on cell motility, migration, and invasion show that it has optimal wound closure when used in combination as compared to individual use [27]. Interestingly, PC cell lines, which are sensitive to dasatinib, are also sensitive to erlotinib and gemcitabine treatment, whereas cell lines that were more resistant to dasatinib also showed greater resistance to erlotinib and gemcitabine therapy, suggesting an inherent resistance to individual cytotoxic or targeted therapies. In addition, the interplay between tumor cells and surrounding cells such as vascular endothelial cells as well as pericytes, fibroblasts, and immune cells adds to the complexity of reformed cellular signaling to trigger tumor growth, which clearly suggests that targeting a single constituent will not affect the sustained tumor growth inhibition. In this way, focusing on various flagging pathways engaged with tumor

development can possibly conquer either essential or gained protection from focused monotherapy and improve the probability of managed reaction by influencing distinctive systems of activity related with growth advancement and upgrade the impacts of traditional cytotoxic chemotherapy.

8.7 Clinical Studies of EGFR in Pancreatic Ductal Adenocarcinoma

As mentioned earlier, EGFR is RTK of the EGFR family, which is abnormally triggered in the epithelial tumors. The conventional EGFR receptor is called HER1 or ERBB-1 [28]. Additional constituents of the EGF family are ERBB2 (or HER2 or HER2/neu), ERBB3 (or HER3), and ERBB4 (or HER4), all of them share an equivalent molecular structure [29, 30].

Studies show that TGF-α and EGF are recognized as the foremost vital ligands of EGFR (Table 8.1). TGF-α ligand binds with the EGFR to bring receptor homo- or heterodimerization at the cell surface followed by acquisition of the dimerized receptor. Once dimerized, phosphorylation of the intracytoplasmic EGFR tyrosine kinase domain is induced. Phosphorylated tyrosine kinase residue is the binding sites for enrollment of signaling molecules, for instance, RAS (rat sarcoma viral oncogene). These signaling fragments can phosphorylate alternative "downstream" particles [31, 32].

In PC, EGFR is overexpressed or its mutant forms could manipulate downstream signaling. A method to control the EGFR system is by setting the extent of activity slightly below the threshold that is essential for the enlistment of control machineries. EGFR in PC can be related to either structural or numerical modifications of chromosome. A typical characteristic of PC is that by the time of clinical trials, patients accumulate various genetic variations. KRAS mutations and EGFR gene

Table 8.1 The EGFR group of receptors as well as ligands

EGFR group	Ligands
ErbB1	EGF, TGF-α, AREG, Epigen
ErbB2	None
ErbB3	NRG1, NRG2
ErbB4	NRG3, NRG4
ErbB1/ErB4	HB-EGF, BTC
ErbB3/ErB4	NRG1, NRG2
EbrB1/ErB3/ ErbB4	EPR

Abbreviations: *AREG* amphiregulin, *BTC* betacellulin, *EGF* epidermal growth factor, *EGFR* epidermal growth factor receptor, *EPR* epiregulin, *ErbB2* v-erb-b2 avian erythroblastic leukemia viral oncogene homolog, *HB-EGF* heparin-binding EGF-like growth factor, *NRG* neuregulin, *TGF* transforming growth factor

amplification most likely occur early, followed by p16 inactivation. In PC, Tzeng and partners studied 30 micro-dissected pancreatic samples, corresponding to peripheral blood samples and 9 PC cell lines that were treated with erlotinib. This examination concluded that short EGFR intron 1 CA repeat length is related with worse PC clinical diagnosis and in vitro response to erlotinib. The investigation established that the utilization of EGFR mutation standing for predicting prognosis and response to anti-EGFR therapy appears to be less useful in PC [33].

Erlotinib is a first-generation EGFR TKI and presents the sole Food and Drug Administration (FDA)-approved targeted agent for advanced PDA. The National Cancer Institute of Canada conducted clinical trials to check the consequences of combination of erlotinib and gemcitabine. It showed 18% relative decrease in the risk of death and 23% decrease in the risk of progression or death with erlotinib therapy [34].

8.8 Conclusion

Regardless of promising preclinical information, EGFR restraint with small molecule EGFR TKI inhibitors or mAbs has been to a great extent unsuccessful treatment system in advanced PDA, and this being the reason that most of the studies have been done on molecularly unselected patients, and hence analysis of many trials for discovery of predictive biomarkers did not allow firm conclusions. A few examinations have investigated modifications in the EGFR pathway in PC, which are prescient components for EGFR mutations, for example, EGFR mutations and amplifications. These reports have neglected to record an important pervasiveness of such changes. These discoveries feature the need to investigate elective clarifications for unusual EGFR pathway initiation in pancreatic growth.

Pancreatic tumor has demonstrated exceedingly impervious to EGFR-focused treatments through a few proposed instruments with EGFR mutational status, quality duplicate number and EGFR overexpression, and cross talk with other flag transduction pathways embroiled in directing reaction to treatment. Moreover, PC is habitually hypovascular and may confine drug conveyance. The future lies in all-around planned trials that consolidate numerous natural endpoints to evaluate the novel focuses under scrutiny. It is likely that, as opposed to giving incremental advantages in all patients, EGFR-focused treatments will give huge advantages to a minimal subset of patients. In vitro and in vivo studies have shown that the multiple targeting of Stat3 and EGFR or Stat3 and Src has the potential to induce strong antitumor responses in PC [35].

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VEGFR and PDGFR: Their Targeting in Liver Cancer

9

A. J. Bastien

Abstract

Pro-angiogenic factors such as bFGF, VEGF, and PDGF play a significant role in the invasion, metastasis, and neovascularization of hepatocellular carcinoma (HCC) cells. The expression levels of VEGFR, FGFR, and PDGFR along with the expression of their respective ligands are elevated in HCC. Increased expression levels of bFGF, not acidic FGF, are observed in HCC patients showing capsular infiltration of tumorous cells. Overexpressed VEGF and VEGFR are correlated to progression, angiogenesis, metastasis, tumor recurrence, and poor prognosis in HCC patients. Overexpressed levels of PDGF are associated with an increase in the metastatic potential of HCC. In this chapter, I will discuss VEGF and PDGF roles in metastatic properties of HCC.

Keywords

Liver cancer · Angiogenic factors · VEGFR · PDGFR

9.1 Introduction

Today, primary liver cancer, emanating in the liver, is the sixth most diagnosed cancer [1]. Furthermore, hepatocellular carcinoma (HCC), a primary liver malignancy, was reported to be the third cause of cancer-related deaths in 2012. However, HCC incidence and mortality rates vary vastly around the world [2]. The dominant risk factor for HCC is cirrhosis due to chronic hepatitis B or hepatitis C. Other risk factors include, but are not limited to, age, having a body mass index higher than 30,

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diabetes mellitus, and related nonalcoholic fatty liver disease [3]. Like many cancers, the best approach to treating HCC is its prevention.

However, if cancer does develop, HCC is known to be a highly vascularized cancer. The process of developing new blood vessels from pre-existing vessels known as angiogenesis is thought to contribute to HCC's development and progression. VEGF (vascular endothelial growth factor), characterized in angiogenesis, levels have been shown to be helpful in diagnosing and monitoring patients with HCC ([4].

9.2 VEGF Overview

There are a total of nine proteins in the immediate VEGF family [5]. VEGFs are highly conserved in all vertebrate species; for example, VEGF-A has been identified in zebrafish, frogs, birds, and mammals [6]. This high level of conservation strongly suggests that VEGF, especially its isoform VEGF-A, may be involved with an important role in biological processes.

In fact, VEGF and its receptors are known for their vital role as regulators of angiogenesis and their involvement in vascular permeability. Currently, nine family proteins have been identified, but due to alternative splicing, many isoforms exist; for example, VEGF-A undergoes alternative splicing leading to nine different subtypes. Interestingly, it is thought that each different VEGF isoform plays a distinct role in vascular and arterial development. For example, VEGF-A has been shown to interact with VEGFR-1 and VEGFR-2; VEGF-1 is characterized more so in pathological conditions such as cancer, ischemia, and inflammation, while VEGFR-2 is involved in endothelial growth and survival signals, but both acting as tyrosine kinase receptors (Fig. 9.1) [7].

VEGF family members transduce their signal intracellularly via a membrane-bound tyrosine kinase receptor. VEGF-A and VEGF-B share a stronger affinity to receptors VEGFR-1; in addition, VEGF-A, VEGF-C, VEGF-D, and VEGF-E are capable of binding and activating VEGFR-2, while VEGF-C and VEGF-D bind preferentially to VEGFR-3 [8]. The activation of VEGF receptors is essential for angiogenesis.

Many of the VEGF family members are regulated by hypoxia-inducible factor (HIF) [9]; hypoxia initiates expression of many growth factors including VEGF and other angiogenetic factors. In liver cancer, HIF-1α is highly expressed at levels significantly higher than levels in normal liver tissues [6]. Other metabolic regulators and transcription factors include E-twenty-six growth factor and reactive oxygen species which regulate the expression of VEGF family of ligands and its receptors [10, 11].

9.3 PDGF Overview

Platelet-derived growth factor (PDGF) is a key area of research in cancer development and progression. An abundance of PDGFR activity may increase tumor growth. It has been shown that throughout the progression of HCC in combination

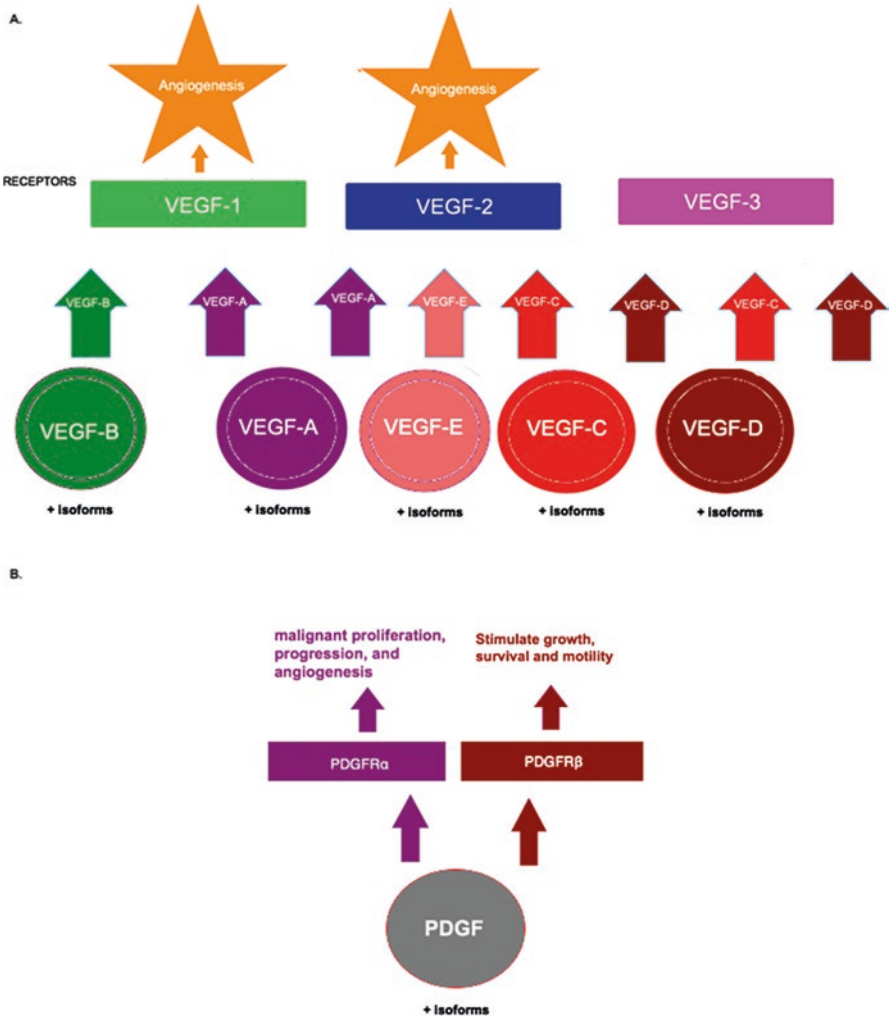

Fig. 9.1 Overview of VEGF and PDGF and their respective receptors. Drugs such as lenvatinib and sorafenib are used as inhibitors of downstream proteins of these receptors. Research continues to study angiogenesis and the associated proteins in hopes to develop better treatments for patients with HCC

with epithelial-mesenchymal transition, levels of PDGF-A, PDGFRα, and PDGFRβ were both increased (Fig. 9.1) [12].

First, it is important to understand PDGF normal structure and function. PDGF is a dimeric molecule that has disulfide-bonded A and B polypeptide chains. The chains can homo- and heterodimerize. Their cellular effects are mediated by binding to their tyrosine kinase receptors known as the alpha-receptor (PDGFRα) and the beta-receptor (PDGFR β) [13]. The family isoforms are known to stimulate

growth, survival, and motility in many cell types and play an important role in adult tissue homeostasis [13].

PDGF signaling is evident in epithelial cancers; the signaling leads to stromal recruitment which possibly helps initiate epithelial-mesenchymal transition and, as a result, increases tumor growth, angiogenesis, invasion, and metastasis [14].

9.4 VEGF and HCC

Meta-analysis, conducted by Zhan et al., studied VEGF levels and its possible effect on the prognostic significance in patients with HCC. Their data suggests that above normal levels of VEGF was associated with poor overall survival in HCC patients [15]. Using an enzyme immunoassay, plasma VEGF levels in varying stages were analyzed and measured. The later stages (Stage IVB) had levels of VEGF measured as high as 103.1 ± 123.2 pg/ml [16]. Strengthening this finding, Jinno et al. findings show similar data that advanced metastasis in patients with HCC has increasingly higher levels of VEGF compared to patients at earlier stages.

In another study, VEGF-A and its receptor VEGFR-1 had significant higher levels in HCC patients compared to controls ($p < 0.001$). However, in serum there was no significant difference in measured levels of VEGF-C and its receptor VEGFR-2 [17]. These findings support that different VEGF members have different biological roles and may help in targeting therapies to specific ligands and their receptors mediating their effects. This targeting of VEGF-A and VEGFR-1 may prove to be beneficial.

9.5 PDGF and HCC

In a study using HCC whole-cell lysates, the majority of HCC tissues measured PDGFRα levels contained a large (sevenfold) increase compared to their controls. Higher levels in PDGFRβ were only characterized in 6 of 22 tumors but were higher in samples associated with cirrhosis [18]. PDGFRα is essential in the development in several tissues, proliferation, morphogenesis, angiogenesis, and epithelial-mesenchymal interactions [19]. It has been previously established that PDGFRα is associated with malignant proliferation, progression, and angiogenesis.

Using an in vivo assay using hepatoma cells, overexpression of PDGFRα led to high tumorigenic potential; these samples also included increased microvessel density compared to the controls [20].

When injury occurs or vascular damage presents, thrombosis occurs. Platelets become activated, adhered to the injured area, aggregate together, and secrete platelet granules. These granules can contain several factors including both VEGF and PDGF (and others). Since both these molecules are elevated in HCC patients, it suggests that platelets may play a significant role in tumor development and metastasis [21]. The role of platelets and their granules has been investigated and characterized in many types of cancer. Furthermore, targeting VEGF and PDGF and their respective receptors may be useful in treating patients with HCC and better patient prognosis.

9.6 Current Treatments for HCC

The process of angiogenesis is essential for cancer development and its metastasis. Since VEGF is critical for angiogenesis to occur, VEGF-targeted agents have already been developed as potential treatments for patients with HCC.

One agent developed for cancer treatment use was sorafenib. Sorafenib is an orally active multikinase inhibitor. The function of the drug is to block many important cellular factors involved in tumor cell proliferation and angiogenesis and has been found to increase the rate of apoptosis [22].

Sorafenib, a multikinase inhibitor, prevents the serine-threonine kinase activity of Raf-1 and B-Raf and the receptor tyrosine kinase activities of VEGFR-1, VEGFR-2, VEGFR-3, and PDGFR-β (Fig. 9.1) [22, 23]. Utilizing mouse xenografts, it has been shown that administering sorafenib reduces angiogenesis and increases cancerous cell apoptosis [24]. Furthermore, in a phase III trial called Sorafenib Hepatocarcinoma Assessment Randomized Protocol (SHARP; ClinicalTrials.gov number, NCT00105443), administering sorafenib increased median survival and the time to progression by 3 months in patients with HCC [25]. In fact, as of 2017, sorafenib is currently the sole systemic agent approved in the United States for HCC treatment. As with all the treatments, there are downsides. Not only is the drug costly; it has been shown to have considerable drug-related symptoms for little benefit [26]. Also some patients exhibit resistance or intolerance to sorafenib.

Another drug, lenvatinib, is a different but very similar inhibitor to sorafenib. Lenvatinib specifically targets VEGFR, FGFR, PDGFR-β, RET, and KIT [27]. A recent 2017 study investigated sorafenib versus lenvatinib as the first recommended therapy for unresectable HCC. The study concluded that lenvatinib showed noninferiority in overall patient survival and had improvements in secondary end points, for example, in time to progression [27]. Sorafenib being the only approved drug treatment for HCC patients needs to change. As trials continue with lenvatinib and other potential treatments, hopefully the prognosis improves for patients with HCC.

Relatively new imaging equipment and radiofrequency ablation (RFA) instruments have changed treatments available. For example, the use of RFA may be an alternative to surgical resection. Its benefits are numerous including it is minimally invasive, it is easy to operate, and the procedure is repeatable. It also has been seen to increase immunity and reduce the levels of VEGF in serum [28].

9.7 Conclusion

For cancer to thrive, it needs to create its own blood supply. This process of angiogenesis and the expression of VEGF are critical for tumor development. There has been a plethora of evidence showing that VEGF and PDGF serum levels, especially the ligands and receptors, are overexpressed in HCC and are highly characterized and present. We conclude that these still remain important targets for future treatments in patients with HCC. As lenvatinib trials continue, drugs continue to be

developed and other treatment methods utilized; hopefully HCC will no longer have such a high prevalence of cancer-related deaths.

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Functional Consequences and Clinical Significance of Tyrosine Kinase Inhibitors in Advanced Colorectal Cancer

10

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Abstract

Colorectal cancer (CRC) is an important public health issue as the 5-year prognosis is <20% for newly diagnosed metastatic CRC (mCRC). In recent years, screening modalities have led to early detection of the disease, which has shown some promise for improved survival. The advancements in adjunctive treatments and aggressive surgical treatment are also partly responsible for this success, but the deeper understanding of carcinogenesis and targeted molecular therapy has made a stronger impact with the emergence of newer targets in the recent past. Particularly, the development and FDA approval of newer drugs, including capecitabine, irinotecan, oxaliplatin, monoclonal antibodies that block either VEGF (bevacizumab, aflibercept, and ramucirumab) or the EGFR (cetuximab and panitumumab), and most recently, trifluridine/tipiracil and regorafenib (TAS-102), have been remarkable in this area of research. The clinical benefits of these drugs are now generally acceptable/established for mCRC patients, with the median overall survival of >30 months. Currently, limitation in the effectiveness of tyrosine kinase inhibitors (TKIs) is due to (i) combination chemotherapy use that necessitates lowering of the dose density for toxicity profile management, and (ii) these drugs have mainly been developed in molecularly unselected population. The main challenge now is the identification of more reliable and

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116 specific predictive biomarkers for selecting the most suitable therapy for mCRC. So far, the only well-established/reliable biomarker for mCRC treatment is RAS mutational status, which predicts negative response to anti-EGFR therapy. Current recommendation for the BRAF mutational status has also been given by the NCCN and the ESMO. Unlike VEGF inhibitor therapy, the resistance mechanisms in the EGFR inhibitor therapy are well understood, as are the drugs blocking the downstream RAS-MAPK pathway. Notably, a number of clinical trials on targeting the RAS signaling pathway have revealed promising efficacy in chemo-refractory mCRC. This chapter discusses the role of TKIs in advanced CRC from both translational and clinical research points of view.

Keywords
Colorectal cancer · Tyrosine kinase inhibitors · EGFR · VEGF

10.1 Introduction

Colorectal cancer (CRC) is an important public health issue, and the cases reported per year are approximately 1.2 million. Among men, it is the third most common cancer, while it stands second among women. Regarding the mortality rate, it stands third with nearly 600,000 deaths per year. The 5-year prognosis is less than 20% for newly diagnosed metastatic colon cancer [1]. Screening modalities like colonoscopy and DNA stool test have led to the early detection of the disease, and thus, treatments at an early stage of the disease are resulting in improved survival. Over the past decades, there has also been improvement in 5-year survival rate due to the improved management of metastatic colorectal carcinoma (mCRC).

The advancements in adjunctive treatments and aggressive surgical treatment are also partly responsible for this success, but a stronger impact has been made by the deeper understanding of carcinogenesis and targeted molecular therapy with the emergence of newer targets in the recent past. Systemic treatment of mCRC is rather important as >25% of patients with CRC present with metastasis.

In view of the above noted facts, in this chapter, we study and recapitulate the role of tyrosine kinases in the pathogenesis of CRC and the use of their inhibitors as anticancer therapy. We also discuss the challenges such as resistance to these agents, success achieved so far, ongoing clinical trials, and future expectations/perspectives.

10.2 Genetic and Epigenetic Alterations in Colorectal Carcinoma

The genetic as well as epigenetic changes in colorectal malignancy are responsible for the perceived carcinogenesis. Proposed in the 1980s, adenoma-carcinoma sequence was the description of normal colonic epithelium transformation to a non-malignant tumor and finally to a progressive and metastatic carcinoma.

Three discrete pathways of the genomic instability that has been identified so far in CRC are *CpG* island methylator phenotype pathways, chromosomal instability, and microsatellite instability [2]. Recently, mutations of transforming growth

factor-β (TGF-β) receptor and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) genes have also been proposed.

10.2.1 Role of Chromosomal Instability

The pathway of chromosomal instability, otherwise termed as adenoma-carcinoma sequence, shows predictable growth of genetic along with other corresponding histologic modifications. The genetic alterations involve proto-oncogenes stimulation as well as inactivation of some tumor suppression genes, i.e., loss of APC, p53 loss, and heterozygosity loss for the long arm of chromosome 18 [3]. In the familial/inherited and sporadic colon, the most common initial gene mutated is APC. It is still controversial whether the genomic instability is responsible for the commencement of the adenoma-carcinoma sequence or it originates during the process and catalyzes the progression to carcinoma [4]. Chromosomal instability as well as microsatellite instability (MSI) can be detected in nonmalignant tumors [5]. Thus, it seems that the genomic instability exists during the commencement of adenoma prior to the APC gene mutation and evolution to frank malignancy [6].

10.2.2 Role of APC/Wnt/B-Catenin Pathway

The major role is played by the APC/Wnt/B-catenin pathway in the pathogenesis of sporadic and hereditary forms of CRC. Either frameshift or nonsense mutations form 98% of the APC mutations resulting in the production of truncated protein. Approximately 30–70% of the sporadic adenomas as well as sporadic colorectal malignancies have this mutation [7–9].

Normally, the G1 to S phase transition is blocked by the APC tumor suppressor gene. The native stem cells in the base of colonic crypts are maintained in their undifferentiated state by the Wnt signaling pathway. B-catenin plays a key role in the Wnt signaling. Normal APC is responsible for the degradation of B-catenin, and hence, it negatively regulates this pathway [10, 11]. The prolonged activated state of the Wnt pathway results from the sustained levels of B-catenin intracellularly in CRC cells with APC mutation. B-catenin plays a role in the migration of stem cells from the crypts to the surface. B-catenin accumulation in enterocytes precursors (as a result of APC inactivation) results in the accumulation of a stem cell makeup that prevents their migration toward the surface for shedding off. The homogeneous cells abnormally accumulate inside colonic crypts resulting in polyp formation. Additional mutations subsequently accumulate involving Kirsten rat sarcoma viral oncogene homolog (*KRAS*) along with tumor protein-53 (*TP53*) genes as suggested by the adenoma-carcinoma sequence model, giving rise eventually to carcinoma.

10.2.3 TP53 Mutation and Colorectal Cancer

Among the most frequently mutated genes in CRC is *TP53* gene, which is involved in the regulation of apoptosis and cell cycle [12, 13]. The p53 protein arrests G1 cell

cycle as well as promotes DNA repair before a cell enters the DNA replication. Apoptosis is induced, if the DNA repair fails. It is believed that TP53 mutation occurs during the adenoma to carcinoma transition.

Conflicting results have been achieved from various investigations that attempt to explain the prognostic importance of TP53 alteration in mCRC. Adrover et al. concluded that p53 overexpression in advanced stages of CRC gives an improved overall survival to patients [14]. According to Popat et al., the prognostic importance of p53 as well as the status of thymidine synthase as a biomarker toward the overall survival (OS) in the additional treatment of mCRC in around 1000 patients. Ninety percent of the cases received adjuvant chemotherapy, and 60% had rectal cancer in this study. Notably, 60% of the tumors had overexpression of TP53 with no substantial prognostic value during the adjuvant examination [15].

10.2.4 18q Loss of Heterozygosity and Colorectal Cancer

The loss of heterozygosity (LOH) in 18q21 site is often found in progressive colorectal cancer. LOH means the loss of either of the two alleles of a gene. A mutation every so often affects the residual allele. The long arm of chromosome 18 is the location for DCC (deleted in colorectal carcinoma) gene. The DCC encodes the transmembrane protein. During ligand absence, the DCC inhibits cell progression, i.e., netrin-1, contrary to the other common transmembrane receptor types. The LOH is present in nearly 70% of colorectal malignancies in the region of DCC gene. Netrin-1 concentration falls as the differentiated epithelial cells move toward the surface. This concentration difference is thought to be the contributor of the normal apoptosis process and epithelial cells shedding. The DCC apoptotic effects can be overcome by netrin-1 overexpression, which has been reported in advanced CRC patients [16]. An inverse relationship has been found between the 18qLOH and survival in some studies [17, 18].

10.2.5 Microsatellite Instability/Mismatch Repair Pathways/ Colon Cancer

The DNA replication is semiconservative, i.e., the DNA polymerase reads one DNA strand and uses it as a template to synthesize an identical copy. The DNA polymerase, while synthesizing the identical strand, scans continuously for errors in the synthesized part and corrects them through its endogenous exonuclease activity. Despite this proofreading mechanism, the mismatch repair (MMR) system scans and corrects the overlooked mistakes by the DNA polymerase. The microsatellite instability (MSI) is most common in Lynch syndrome or the hereditary nonpolyposis colorectal cancer (HNPCC) with >95% of HNPCC containing this genetic abnormality [19]. Contrasting are the sporadic CRC where mechanism of chromosomal instability remains unclear, and only 15–20% of the cases are due to MSI [19].

Throughout the genome are scattered hundreds of nucleotides in the form of short stretches with repetition called short tandem repeats (microsatellites). These

are made up of multiple nucleotide repeats – mono-, di-, tri-, and tetra-. The strand slippage leads to the DNA stutter during cell replication, and this phenomenon is more common in regions with microsatellite.

Malfunctioning the MMR system results in the formation of microsatellites, which are either long or short as compared to their parent cell, an occurrence called MSI. The mismatch occurring within the coding part of the gene leads to induction of point mutation, which could affect the gene function [20]. The MMR enzymes' inactivation can occur in two ways: (i) either through the promoter CpG islands' aberrant methylation of MLH1 gene or (ii) through the mutation in any member of the MMR group. In sporadic colorectal malignancies, the epigenetic silencing of MLH1 genes by the promoter hypermethylation is responsible for most of the MMR defects.

Generally, CRC develops by the age of 40 years in patients with germline mutations in mismatch repair capability in 80% of the cases [21]. Site for the MSI tumors is proximal colon with mucinous histology and lymphocytic infiltration like Crohn's disease. The National Comprehensive Cancer Network (NCCN) recommends the MMR testing for all newly diagnosed patients younger than 50 years old with stage II disease due to the increased probability of Lynch syndrome in this population [22].

10.2.6 Epigenetic Instability and CpG Methylation in Colon Cancer

The DNA expression is regulated by the multiple epigenetic mechanisms without changing the sequence of nucleotides. Inappropriate methylation of the promoter regions of genes leading to aberrant epigenetic regulation is common in CRC and is as important in inactivating tumor suppressor genes as the DNA mutations. Abnormal hypermethylation includes the covalent bonding of a methyl group with cytosine at the 5′ position and occurs in the repetitive dinucleotides CG or CpG-rich stretches inside the promoter region of DNA.

Mostly, the normal cells have unmethylated CpG. Genes are expressed normally in the absence of methylation. The promoter methylation results in the downregulation of gene transcription. The tumor suppressor gene silencing results when hypermethylation of the promoter region involves both the tumor suppressor gene alleles or a combination of one allele loss through deletion or combination with the allele silencing through the promoter hypermethylation. The MLH1 abnormal methylation occurs in 80% of the MSI sporadic CRC.

10.2.7 Tyrosine Kinase Enzymes/EGFR/KRAS/Cellular Proliferation and Survival

The phosphorylation of tyrosine is controlled by the tyrosine kinase enzymes (TKs, or tyrosine kinases), which are important mediators of the cellular signal transduction and its functions, i.e., cell proliferation, survival, migration, apoptosis, etc.

Tyrosine kinases are grouped in a family of signaling molecules with implication in almost every cancer type, which forms the basis for the development of modern targeted therapies. The mutant tyrosine kinases are selectively targeted with targeted therapies, which resulted in many significant clinical benefits, including the improved survival for patients with the disease.

Remarkably, at least 90 TKs are expressed by the humans, in which 58 are receptor tyrosine kinases (RTKs) [23]. The activation of RTKs is via the extracellular domain binding of the ligand such as growth factors and cytokines. The RTK dimerizes/oligomerizes as a result of this ligand binding, and phosphorylation of tyrosine occurs subsequently [24].

Epidermal growth factor receptor (EGFR) is one of the receptor tyrosine kinases and major catalyst for the growth and progression of colorectal malignancies [25, 26]. It transduces signals intracellularly via the two parallel pathways (i.e., RAS/RAF/MAPK and PI3K/AKT pathways), thereby activating the cellular survival and development. Epidermal growth factor (EGF), the ligand for EGF receptor (EGFR), binds to the EGFR extracellular domain resulting in the receptor dimerization. This dimerization results in the intracellular domain autophosphorylation activating several downstream effectors of the RAS/RAF/MAPK and PI3K/AKT pathways. These signals reach the nuclear DNA and leads to the induction of metastasis, cell proliferation, motility of the cell, and angiogenesis [27, 28].

The mitogen-activated protein kinase (MAPK) signaling is a prominent signal transduction pathway, which induces the cellular proliferation. A number of intermediate effectors proteins such as the RAS, RAF, and MEK are involved. RAS functions as a fundamental distributor of signals by the stimulation of the cascade through PI3K (phosphoinositol kinases) as well as RAF. The PI3K activation inhibits apoptosis, while the RAF activation results in the cellular growth. This cascade is responsible for the regulation of growth signals, cancer invasion, and cell survival. Thus, the EGF pathway inhibitors become useful for the treatment of carcinoma. However, the mutations in KRAS can independently activate these effectors despite the inhibition of EGFR receptor resulting in the cell survival and proliferation. Hence, the EGFR inhibition therapeutically becomes ineffective as KRAS is located downstream. Codon 61 on exon 3 and codons 12 and 13 on exon 2 are mostly mutated in the case of RAS. Most commonly affected is codon 12 with the missense mutation. Livere et al. reported that as a self-regulating prognostic factor, KRAS mutations were prevalent in 27% of the advanced CRC patients treated with cetuximab [29].

10.2.8 PI3K/AKT Pathway, PTEN, and TGF-β

An alternative EGFR signaling pathway is PI3K/AKT/mTOR (mammalian target of rapamycin) [30]. The PIK3CA mutation is present in over 25% of CRC [31]. PI3K activates AKT via phosphorylation. During the activation, up to 100 other proteins are phosphorylated by phospho-AKT, including mTOR. PIK3CA activating mutations (gene encoded with the catalytic subunit of PI3K) are recognized as newer mechanisms for the induction of oncogenic PI3K signaling. The presence of

PIK3CA mutations were correlated with significantly elevated mortality rates specific to colon cancer in patients with wild-type KRAS.

In contrast, the PI3K/AKT signaling pathway activation plays no significant role in tumor aggressiveness with no KRAS gene activation [32]. The phosphatase and tensin homolog (PTEN) is a protein, which regulates negatively the PI3K/AKT pathway by de-phosphorylation of the PIP3 [phosphatidylinositol (3,4,5)-trisphosphate] to block the activation of AKT through PI3K signaling hyperactivation [33]. The genome is protected from instability by the PTEN, and therefore the PTEN loss was linked with either tumor response absence or the overall survival worsening. Subsequently, the current evaluations of the molecular status of the KRAS and PIK3CA/PTEN signaling pathways are capable of identifying up to 70% of the metastatic colon cancer patients not likely to respond to mAbs against EGFR.

10.2.9 Role of Transforming Growth Factor-β

TGF-β is a protein, which is multifunctional that controls many cellular developments via binding to TGF-β receptors. There are three main types of TGF-β receptors that are recognized in most cells [34]. TGF-β RII (transforming growth factor-β receptor type II) exhibits mutation in approximately 90% of the MSI CRC tumors [34]. TGF-β RII is known to function in distinct ways during tumorigenesis. During initial stages of carcinogenesis, TGF-β RII mediates tumor suppression, but in the later stages, it promotes tumor progression via blocking the cell death cancer cells and immune repression. TGF-β RII also promotes EMT (epithelial-to-mesenchymal transformation), which is well-known to stimulate invasion of tumor, progression, and metastasis [35]. The TGF-β RII mutation hinders with EMT as well as decreases the invasiveness, growth, and metastatic ability of tumors. The MSI CRC has been shown to have impaired EMT. The MSI tumors without the TGF-β RII mutations have the ability to undergo EMT in response to TGF-β RII that shows that TGF-β RII and not the MSI status could serve as a key indicator of invasion, metastasis, as well as prognosis [36, 37].

Watanabe et al. [18] found that TGF-β RII mutation marginally improves 5-year OS ($p < 0.06$) in stage III CRC. The 5-year disease-free survival (DFS) was 79% in stage III CRC patients with both the TGF-β RII mutations and MSI in comparison to 40% in those with MSI but no TGF-β RII gene mutation. The TGF-β RII measurement has no established clinical applications currently in the CRC management, and data obtained from the investigations of PI3K- and PTEN-targeting drugs are currently premature.

10.3 Introduction to the Targeted Therapies for Metastatic Colorectal Cancers

Management of mCRC has considerably improved over the period of past years, which has resulted in a significant improvement in the 5-year survival of patients. Aggressive surgical management and advancements in the adjunctive treatments are

partly responsible for this success, but a powerful impact is created by the more understanding of carcinogenesis and discoveries of new molecular therapeutic targets.

The mainstays of mCRC treatment are cytotoxic agents along with irinotecan or oxaliplatin combined with 5-FU (fluorouracil) and leucovorin or capecitabine (FOLFIRI/FOLFOX or CAPIRI/CAPOX regimens). This combination of the treatment results in the patients' average survival of 18 months. Targeted therapy has increased the overall survival from 22 to 29 months in patients with mCRC [38]. Below we discuss targeted molecular therapies with focus on tyrosine kinase inhibitors and a few ongoing trials for potential targets.

Monoclonal antibodies targeted against the EGFR and VEGF have become the essential component of first-line treatment for mCRC. Targeted therapies such as EGFR mAbs (cetuximab and panitumumab) have been approved by the US Food and Drug Administration (FDA) as well as the European Medicines Agency (EMA) for the treatment of mCRC patients with wild-type RAS tumors. For the RAS-mutant mCRC, anti-VEGF such as ramucirumab mAb (anti-VEGFR2), bevacizumab mAb (anti-VEGF), and ziv-aflibercept, along with regorafenib, has received approval. Ramucirumab (anti-VEGFR-2 mAb) and aflibercept in combination with chemotherapy are being used as monotherapy in patients who have been previously treated as second-line therapy. In refractory setting, regorafenib (a multi-kinase inhibitor) is used [39, 40]. These agents are further discussed below in details.

10.4 Anti-EGFR Drugs/Cetuximab and Panitumumab

Mechanism of Action Cetuximab and panitumumab are anti-EGFR antibodies. Figure 10.1 schematically demonstrates the family of EDGFs on the cell surface. Table 10.1 also summarizes a few key anti-EGFR drugs used in advanced CRCs.

Trials Showing Effectiveness Several phase III trials were conducted to investigate these anti-EGFR antibodies, which showed efficiency in respect to progression-free survival, OS, response rate (RR), as well as quality of life among various treatment options [41, 42]. Survival prolongation was shown by these antibodies in mCRC patients when used as monotherapy or in combination with irinotecan among the refractory patients [43]. The RR of only 10% was achieved when unselected patients were treated with cetuximab and panitumumab, although they demonstrated a strong benefit as noted above [28].

Primary or Intrinsic Resistance to Anti-EGFR The presence of genetic changes in EGFR or downstream effectors of EGFR pathway or in receptor tyrosine kinases other than these is the cause of resistance to these antibodies, known as primary or intrinsic resistance [44].

Acquired or Secondary Resistance to Anti-EGFR The EGFR blockage induces genetic changes, which result in the positive selection of independent clones or treatment-induced mutations leading to intrinsic tumor genomic instability that is

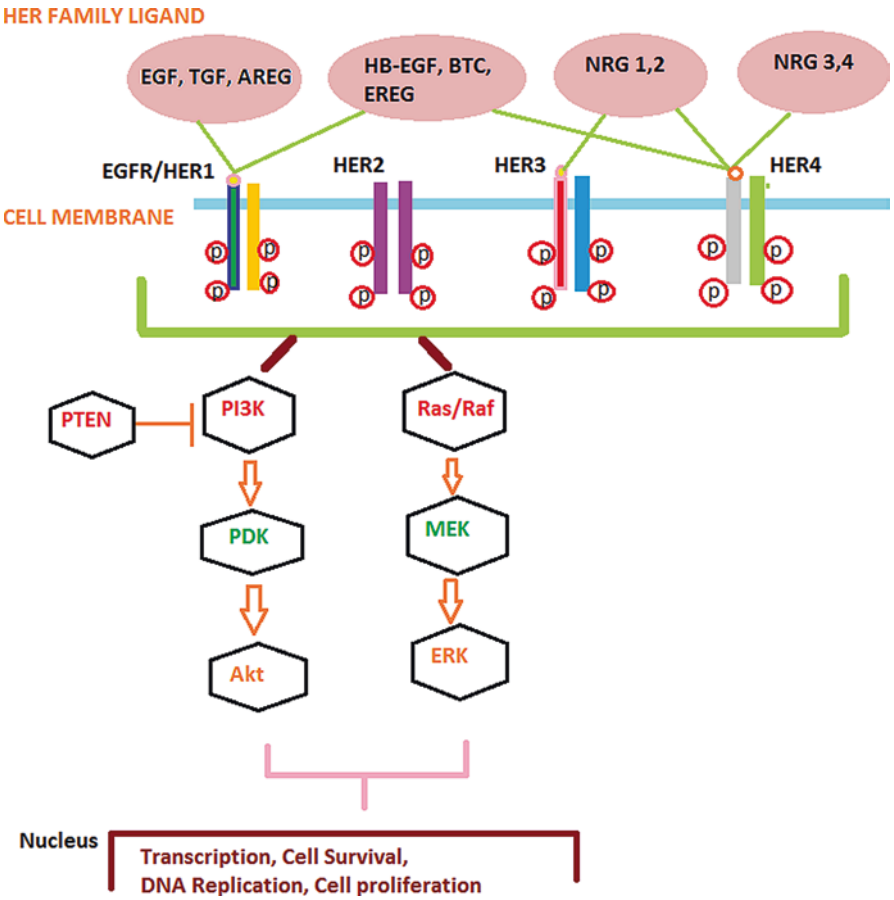

Fig. 10.1 Schematic representation of the family of epidermal growth factor on cell surface. (Adapted and modified from the Reference Material to Improve Cancer Measurements: https://www.nist.gov/%3Cfront%3E/reference-materials-improve-cancer-measurements)

associated with secondary resistance development to anti-EGFR therapeutics at treatment failure [45].

Adverse Effects of Cetuximab and Panitumumab Cetuximab may cause dry/cracked/swollen skin, changes in one's fingernails or toenails, mild itching or rash, diarrhea, headache, mild nausea, vomiting, upset stomach, weight loss, and/or sore throat. The most common side effects of panitumumab include skin reactions (redness, acne, itching, or rash), nausea, vomiting, constipation, growth of eyelashes, abdominal pain, tiredness, etc.

Table 10.1 A summary of the anti-EGFRs used in advanced colorectal cancer

Currently used anti-EGFR for colorectal carcinoma						
Drug name	**Mechanism of action**	**Type**	**Origin**	**Study author(s)**		**References**
Cetuximab	Anti-EGFR	IgG1	Chimeric mAb	Piessevaux et al.		[46, 47]
				Bokemeyer et al.		
Panitumumab	Anti-EGFR	IgG2κ	Human mAb	Douillar et al.		[48, 49]
				Schwartzberg et al.		
Anti-EGFR under clinical trials for colorectal carcinoma						
Drug name	**Mechanism of action**	**Type**	**Origin**	**Primary measures**	**Secondary measures**	**Identifier at (clinicaltrials.gov)**
CMAB009	Anti-EGFR	IgG1	Chimeric mAb	PFS	Best ORR, OS, duration of response	NCT03206151
KL-140	Anti-EGFR	IgG1	Chimeric anti-EGFR	PFS	TTF, Best ORR, OS	NCT03426371

Abbreviations: *EGFR* epidermal growth factor receptor (human), *PFS* progression-free survival, *ORR* overall response rate, *TTF* time-to-treatment failure, *OS* overall survival, *IgG* immunoglobulin G

10.5 Anti-angiogenesis Agents

Over four decades ago, anti-angiogenesis was suggested as an anticancer therapy [40]. It is known that invasive tumor progression and metastasis require angiogenesis and are also an essential part of the carcinogenesis process [50]. Vascular endothelial growth factor (VEGF) is primarily responsible for the angiogenesis, and its abnormal regulation can lead to several disorders, including cancer. VEGF, a heparin-binding growth factor, specifically acts on the vascular endothelial cells that are capable of angiogenesis induction in vivo [51]. So far, three anti-VEGF therapeutic agents have been approved by the FDA for the treatment of mCRC, which are discussed here. Several other medications have also been used, which inhibit angiogenesis and are currently under clinical trials. Table 10.2 summarizes a few important anti-angiogenic agents that are commonly used in advanced CRCs.

10.5.1 Bevacizumab

Mechanism of Action It is one of the most common anti-VEGF used currently for mCRC. It is a recombinant humanized IgG-1 antibody, acting counter to the soluble VEGF-A. Bevacizumab blocks the VGEF-A and, in turn, prevents it from interacting with the vascular endothelial cells resulting in the stoppage of the abnormal downstream signaling.

Table 10.2 Summary of the key anti-angiogenesis agents used in advanced colorectal cancer

Drug	Mechanism of action	Type	Origin	Study authors	References
Bevacizumab	Anti-VEGF	IgG-1	Humanized mAb	Cremolini et al.	[52, 53]
				Giantonio et al.	
Ziv-aflibercept	Anti-VEGF	VEGFR domains are fused with IgG-1		Tabernero et al.	[56]
Ramucirumab	Anti-VEGF	IgG-1	Humanized mAb	Tabernero et al.	[57]
Regorafenib	VEGF/TIE2 receptor TKI	Oral receptor TKI		Grothey et al.	[58, 59]
				Li et al.	

Abbreviations: *VEGF* vascular endothelial growth factor, *TIE2* tyrosine kinase with immunoglobulin-like and EGF-like domain 2, *EGF* epidermal growth factor, *TKI* tyrosine kinase inhibitor, *IgG* immunoglobulin, *mAb* monoclonal antibody, *VEGFR* VEGF receptor

Trials Showing Effectiveness It has been used in combination with other agents. One of the longest OS periods so far was shown by a clinical trial conducted recently, which examined the usage of FOLFOXIRI along with bevacizumab as a first-line therapy – and the results showed a better PFS and RR than FOLFIRI in combination with bevacizumab [52].

FOLFOX4 plus bevacizumab combination gives better median survival (12.9 months) as compared to FOLFOX4 or bevacizumab monotherapy in the second-line setting, an Eastern Cooperative Oncology Group study (E3200) showed [53]. In CAIRO3 study, capecitabine and oxaliplatin plus bevacizumab (CAPOX-B) were used for the initial treatment, and then the maintenance therapy was continued with capecitabine and bevacizumab. The PFS benefit was shown without affecting the quality of life in patients as compared to observation by this study [54].

Adverse Effects Hypertension, epistaxis, proteinuria, and thrombosis are the common antagonistic effects linked with the use of bevacizumab [55]. Hypertension is usually managed with the standard use of antihypertensives.

10.5.2 Ziv-Aflibercept

Mechanism of Action Human extracellular VEGFR domains are fused with IgG 1 Fc portion to form a fusion protein named as ziv-aflibercept. This fusion protein is known to trap VEGF-B, VEGF-A, and PIGF (phosphatidylinositol glycan anchor biosynthesis class F).

Trial Showing Effectiveness A significant increase in the OS was found in a phase III trial which investigated the effectiveness of aflibercept plus FOLFIRI combination as compared to FOLFIRI plus placebo in mCRC patients treated previously with an oxaliplatin-based regimen [56]; median survival was 13.50 and 12.06 months, respectively. Efficiency was preserved with a similar safety profile. Hence, the use of aflibercept in combination with FOLFIRI was approved by EMA after oxaliplatin-based therapy.

Adverse Effects Diarrhea, headache, sores on the mouth and lips, nosebleeds, fatigue, hoarse voice, high blood pressure (hypertension), low white blood cell count (neutropenia), weight loss, increased liver enzymes, loss of appetite, stomach or abdominal pain, etc.

10.5.3 Ramucirumab

Mechanism of Action Ramucirumab is a completely humanized immunoglobulin 1 monoclonal antibody, which has a high affinity for the extracellular domain of VEGFR-2, which binds VEGF. This avid bonding of ramucirumab prevents VEGF form binding and activating the receptors. This blockage of VEGF-mediated activation of VEGFR-2 gives ramucirumab an antitumor activity in several malignancies as the only agent under in vivo models or along other drugs in combination.

Trials Showing Effectiveness The EMA and the FDA agencies gave approval to ramucirumab as a second-line therapy for patients with the disease progression on first-line regimens containing bevacizumab, oxaliplatin, and fluoropyrimidine. This approval was based on the results of RAISE trial with the enrollment of 1072 participants randomized into 2 groups of 536 (each receiving either ramucirumab or placebo) [57]. It was found that the median OS was 13.3 months (95% CI 12.4–14.5) for patients on ramucirumab treatment vs 11.7 months (95% CI 10.8–12.7) for placebo-treated group (HR = 0.844; 95% CI 0.730–0.976; log-rank p = 0.0219). Significant improvement in the PFS was observed in the treatment group than the placebo with the median PFS of 5.7 vs 4.5 months.

Adverse Effects Decreased neutrophils, high blood pressure, elevation of proteins in the urine, tingling, pain, redness and edema of hands and feet, high blood pressure, diarrhea, low energy, head pain, nosebleed, etc.

10.5.4 Regorafenib

Mechanism of Action *Regorafenib* is an oral multi-kinase inhibitor, developed by Bayer, Inc. It is known to affect the stromal, angiogenic, and oncogenic receptor tyrosine kinases (RTK). *Regorafenib* has anti-angiogenic activity because of its dual targeted VEGFR2-TIE2 TK inhibition properties.

Trials Showing Effectiveness Numerous clinical trials are under way to show the effectiveness of regorafenib. One of the trials conducted by Grothey et al. showed that the median OS was 6·4 months in the regorafenib-treated group as compared to 5·0 months in the placebo group (HR 0·77; 95% CI 0·64–0·94; p = 0·0052) [58]. Similarly, another trial conducted by Li et al. demonstrated that after a median follow-up of 7·4 months [interquartile range (IQR) 4·3–12·2], the OS was significantly increased with regorafenib treatment than it was observed with placebo (HR 0·55; 95% CI 0·40–0·77; one-sided p = 0·00016; median OS was 8·8 months [95% CI 7·3–9·8] in the regorafenib-treated group vs 6·3 months [95% CI 4·8–7·6] in the placebo group) [59].

Adverse Effects Weakness/fatigue, diarrhea, weight loss, stomach pain, hand-foot skin reaction, infection, hypertension, etc.

10.6 Resistance to Anti-EGFR Therapy

10.6.1 Role of KRAS Mutations

The renin-angiotensin system (RAS) is a downstream effector in tyrosine kinase pathway. Therefore, anti-EGFR therapy can become ineffective if mutation affects the *RAS* gene. The *RAS* gene mutation is often present in mCRC with KRAS (Kirsten rat sarcoma viral oncogene) mutation most frequent. Nearly 40% of the CRCs have *KRAS* gene mutation with single-nucleotide point somatic mutations in

codons 13 and 12 of exon 2 of *KRAS* gene specifically and codon 61 in a trivial percentage resulting in the constitutive activation of MAPK pathway [60]. These mutations have established the predictive value in the resistance detection to anti-EGFR mAb treatment in mCRC [as shown by the retrospective analysis from the randomized controlled trials (RCTs)]. Therefore, initially only the patients with KRAS exon 2 wild-type CRC were approved for the treatment with cetuximab and panitumumab by the EMA and the FDA [61].

The significance of the patient selection based on RAS has been underlined by retrospective and prospective clinical studies. Notably, the PRIME trial was retrospectively analyzed for the assessment of expanded RAS status (NRAS and KRAS) and found that panitumumab plus FOLFOX4 regimen had efficacy in relation to the OS, PFS, and objective RR in comparison with the chemotherapy alone for RAS wild-type (non-mutated) mCRC as first-line treatment [62].When the range of patients with mutation was transformed from approximately 15% to 53% in other phase II and III trials' analyses showing the refractoriness of this population to anti-EGFR therapy [38]. Identification of several other biomarkers, which are responsible for the resistance to anti-EGFR therapy, in addition to the mutations of KRAS exon 2, has helped to regulate a more suitable patient's choice. Precisely, KRAS exon 3, codon 59/61, and exon 4, codon 117/146, and NRAS exon 2, 3, and 4 presence corresponded to the loss of anti-EGFR mAbs efficacy.

A systematic review and meta-analysis was performed for nine RCTs to assess anti-EGFR mAbs therapy in all lines of mCRC treatment [60]. The analysis showed that the RAS mutation-negative tumors had a suggestively superior treatment consequence with mAb EGFR treatment than the RAS mutation-positive tumors.

Entrectinib, a selective tyrosine kinase inhibitor, targets cancers which harbor activating mutations in ALK, NTRK1/2/3, or ROS1. Entrectinib is an extremely potent TKI clinically and does not have any off-target activity. This TRK (tropomyosin receptor kinase) inhibitor is currently in a phase II clinical trial studying CRC mutations which are responsive in targeting receptor kinases. This clinical trial is an open-label, multicenter, global, registration-enabling phase II clinical trial of entrectinib. It uses a basket design with patient tumor samples screening for the relevant targets. The full advantage of entrectinib is taken by such basket design whose clinical activity has been established across a variety of tumor forms as well as molecular targets.

These results show that RAS status is important as a prognostic marker in managing mCRC. Indications of cetuximab and panitumumab were restricted to the "RAS wild-type" CRC tumors by the EMA and the FDA in 2013 [38].

10.6.2 New Medications Used in Colorectal Cancer with RAS Mutations

A common way to inhibit RAS is by the identification of downstream effectors and MEK and PIK3CA. A number of upstream signaling pathways can be blocked at the MAPK-ERK pathway, which is a convergence point. Trametinib (an anti-MEK) and

palbociclib (an anti-CDK4/6) combination was studied in animal model obtained from the KRAS-mutant CRC patients, and the results revealed that the treatment is well tolerated with high efficacy. Nonetheless, confirmation of this preclinical data needs further clinical evaluations and validation [63, 64].

A naturally occurring, non-enveloped, ubiquitous human reovirus (Reovirus Serotype 3 – Dearing Strain: Reolysin®) is capable of replication in the RAS-transformed cells to induce cell lysis and is being investigated for a possible role in targeting KRAS in mCRC. In a multicenter phase I clinical trial, Reolysin is used with FOLFIRI plus bevacizumab testing in FOLFIRI-naïve patients with KRAS-positive KRAS.

10.7 Other Biomarkers of Resistance

Several mechanisms of primary resistance have been identified, which are briefly summarized below.

BRAF Mutation There has been a significant percentage of wild-type (WT) RAS mCRC patients that are unresponsive to the anti-EGFR treatments in spite of the appropriate selection criteria used. This could be explained as a result of the mutations, which have occurred in the downstream effectors of the KRAS/NRAS signaling pathway. BRAF, a serine/threonine protein kinase, is one of such effectors, which is mutated in about 12–15% of the mCRC patients [65]. The most common BRAF mutation is a point mutation, which is considered to be mutually exclusive with exon 2 KRAS mutations. Constitutively active protein, encoded by the $BRAF^{V600E}$, would be responsible for resistance to mAbs cetuximab or panitumumab. The BRAF mutation, as a poor prognostic indicator, has been highlighted by several clinical trials. For example, the median OS for BRAF-mutant mCRC patients reported was 10.4 months in comparison with 34.7 months for BRAF wild-type mCRC [66]. Two-thirds of the BRAF-mutant mCRC are found in the right lateral of the colon and commonly linked with the peritoneal and distant nodal involvements, as shown by a retrospective analysis. Moreover, a fairly good proportion of the literature established the association of $BRAF^{V600E}$ mutation with a relatively poorer prognosis [47, 67].

10.7.1 Treatment Strategies for the BRAF Mutations

Present studies are working on the simultaneous or dual blockage of BRAF as well as EGFR or downstream effector pathway [68]. Preliminary results have shown that vemurafenib (a BRAF inhibitor) in combination with panitumumab (a EGFR inhibitor) is safe but has a modest response. It is believed that the extracellular signal-regulated kinase (ERK) inhibitors can suppress the MAPK activity and can also override the resistance to the RAF inhibitors. It may be used as a treatment strategy. Anti-EGFR mAbs combined with the BRAF and MEK inhibitors have also been

investigated recently with very interesting findings [69]. Van Cutsem et al. [70] reported the results which revealed that the $BRAF^{V600E}$-mutant mCRC patients treated with triple regimens dabrafenib, trametinib, and panitumumab had an improvement in the best overall response/prolonged PFS as compared to panitumumab plus dabrafenib or trametinib group.

10.7.2 PI3KCA

Predictive biomarkers other than the NRAS/KRAS and BRAF mutations also predict resistance to mAbs [71]. For example, several RTKs, including EGFR, are associated with *PIK3CA/AKT/mTOR* signaling pathway. The PIK3CA activating mutations are present in approximately 10–20% of mCRCs, primarily in exons 9 and 20, and are the cause of unresponsiveness to anti-EGFR mAb therapy [72, 73]. Correlation between the mutations of PI3KCA and resistance to cetuximab or panitumumab in KRAS wild-type mCRC was demonstrated in a retrospective analysis of 110 mCRC patients [74].

Because of the concurrent presence of BRAF or KRAS mutations, the role of PI3KCA mutations is still not fully clear. However, two main results were yielded by a retrospective analysis of 1022 tumor samples of patients treated with cetuximab: mutations of exon 20 PI3KCA have predictive resistance to cetuximab in subgroup with wild-type KRAS mutations and PIK3CA exon 9 mutations have a secondary role in cetuximab resistance, which is suggested by the association of PIK3CA exon 9 and KRAS mutations [61]. In patients who relapse after the EGFR-targeting mAbs, the *PIK3CA* mutations have a role in the secondary resistance [75]. Mortality due to colon cancer is increased in PIK3CA-mutant mCRC as compared to the wild-type mCRC showing a prognostic value of PIK3CA mutations [76].

The loss of phosphatase and tensin homolog (PTEN) can lead to the activation of PIK3CA pathway in 30% of mCRCs, and the lack of tumor response is associated with these, as well as the OS is worse in patients with KRAS wild-type mCRC treated with cetuximab-containing regimen [77].

Combination of everolimus (an mTOR inhibitor) with panitumumab and irinotecan is the new potential treatment option that was investigated recently (available from ClinicalTrials.gov, Identifier: NCT01139138). Aspirin through its COX-2 inhibition showed a benefit in the survival of PIK3CA-mutant patients in some preliminary results [78].

10.7.3 HER2/HER3

The human epidermal growth factor receptor 2 (HER2) is a driver of oncogenesis and belongs to the EebB family for which the targeting therapy is trastuzumab mAb in breast tumor and gastric carcinoma treatments [79]. The heterodimer of HER2/HER3 is a strong stimulator of intracellular signaling [78]. HER2 has also been studied in the RAS/BRAF wild-type and xenograft models with cetuximab

resistance and, as a result, is also proposed as a target in CRC. In quadruple wild-type population, i.e., KRAS, NRAS, BRAF, and PIK3CA wild type, it has been found that HER2 gene amplification has been recognized to be a potential mechanism for primary resistance to cetuximab mAbs [80]. The HER2 amplification was found in 2–3% of the mCRC patients without genetic selection. HER2 is known to be the first druggable target to date in mCRC with good predictability of response to the targeted treatments [81].

The HER 2 amplifications are not the only molecular change responsible for the hyperactivation of HER2. The HER3 ligand (heregulin) overproduction may give resistance to anti-EGFR therapy. HER3 may also be a biomarker for the resistance predictability with 11% of the CRC patients harboring this mutation resulting in the decreased responsiveness to anti-EGFR therapy (even in the absence of HER2 amplification) [82]. MEHD7945A (duligotuzumab) with dual anti-HER3/anti-EGFR action has been shown to be superior to anti-EGFR mAbs in multiple xenograft models. Thus, more than a single molecular driver is implicated in the secondary resistance and RAS mutations, which are the most common with 50–80% of the CRC patients.

10.7.4 Role of S492R and Additional EGFR Mutations

Mutations which affect the EGFR extracellular domain are responsible for the secondary resistance to cetuximab. A missense mutation was identified by Montagut et al. in codon 492 (S492R), which seems to delay the cetuximab binding [83]. Identification of this allele has never been made in previously treated cancer samples. There has been no identification of this allele in earlier treated CRC samples, suggesting that its mutation is an exclusive biomarker of the secondary resistance. Tumors with S492R mutations still respond to panitumumab as it binds an epitope, which is different – and this result may be rather important clinically. A patient with EGFR S492R mutation was reported by the researchers and had disease progression after the initial response to cetuximab. Patient then achieved a response of 5 months when panitumumab was used. However, further analysis was not conducted. Two patients with acquired cetuximab resistance were identified with new mutations in the EGFR extracellular domain: R451C and K467T [75].

10.8 Immunotherapy

Cancer immunology is currently thought to be the most attention-grabbing area with considerable outcomes in the management of various tumors. For instance, blockage of PD-1 signaling pathway with antibodies targeted against the PD-1 has shown extraordinary clinical benefits in patients with several cancer types such as melanomas, Hodgkin's lymphoma, renal cell carcinoma, non-small cell lung carcinoma, and ovarian and bladder cancers. PD-1 ligand expression on the tumor cell surface or immune cells is a vital biomarker for the prediction of response toward the PD-1

inhibition. Unfortunately, colorectal malignancies appear to have unique molecular characteristics, and the response rate to PD-1 inhibition is relatively lower [84]. However, further studies are desirable to substantiate these preliminary observations.

Pembrolizumab is a humanized anti-PD-1 antibody which was investigated by Le et al. [85] in a phase II trial in a stage IV CRC population refractory to treatment. The immune-based rate of objective response and immune-based PFS rates were 40% and 78% for MSI-H CRCs and 0% and 11% for MSS CRCs, respectively. Disease progression occurred in only 1 out of 10 MSI-H CRC patients in comparison to 11 of 18 patients with MSS CRC. Efficacy of nivolumab (an anti-PD-1) as monotherapy or in addition with ipilimumab (an anti-cytotoxic T-lymphocyte antigen 4) was investigated in CheckMate-142 trial, and the results were presented at the 2016 European Society of Medical Oncology (ESMO) annual congress, which showed a significant advantage and tolerable toxicity profile [86].

For the enhancement of susceptibility of MSS colon cancer to inhibitors of immune checkpoint, more research is required. Identification of the treatments for this group of patients with MSS CRC was attempted by Bendell et al. in a phase IB clinical trial and presented at the 2016 annual meetings of the American Society of Clinical Oncology (ASCO). Taking into consideration the low activity of atezolizumab monotherapy, which is an engineered mAb that avoids the binding of PD-L1 with its receptors PD-1 and B7.1 in mCRC, the MEK-inhibiting agents have been found to have an association with immune checkpoint blockers, because intratumoral T-cell infiltration can be induced by them and the PD-L1 activity enhancement (which has been confirmed in a preclinical setting). Cobimetinib combination with atezolizumab at the maximum dose administration was tolerated well in chemo-refractory KRAS-mutant mCRC patients. A higher clinical response rate was achieved with the above combination MSS disease patients as compared to that expected from either atezolizumab or cobimetinib alone. It was observed that the combination also gave a 17% ORR and an OS of 6 months in 72%, resulting in the extension of the phase IB trial. A phase III trial investigating cobimetinib and atezolizumab combination against atezolizumab or regorafenib alone in advanced unresectable patients or CRC with metastasis is currently under investigation [87].

Additionally, recent study conducted by Ahn et al. [87] revealed a subgroup of stage II/III CRC patients with a DNA polymerase epsilon (*POLE)* gene mutation have a better prognosis. Possible explanation for this observation can be the higher immunologic activity in tumors having the *POLE* mutations with $CD8^+$ lymphocyte infiltration increased, effector cytokines, and cytotoxic T-cell marker expression as observed in cancers with MSI. Although it is not common (and is seen in few), in 66/6448 (1.0%) CRC samples, *POLE* mutations were found to be significantly related to several patients and tumor factors, including gender, age, tumor location, disease stage, and without the mismatch repair deficiency [87]. A multivariable study has showed an association that is statistically significant between the mutation in *POLE* gene and a greater risk reduction in the disease recurrence. This risk reduction was specifically greater in stage II disease and the MSI-H linked tumors, a biomarker accepted as indicator of good prognosis in this setting [87]. A comprehensive list of the several key immunotherapeutic options for patients with CRC that are in various phases of clinical trials is summarized in Table 10.3.

Table 10.3 Summary of the various key treatment options for colorectal cancer that are in different phases of clinical trials

Drug	Target/mechanism of action	Primary measures of the study	Secondary measures of the study	Identifier at Clinicaltrials.gov
Tivozanib	VEGF receptor TKI	Investigator-assessed PFS	TTF, best ORR, OS time	NCT01478594
Erlotinib	VEGF receptor TKI	Determine the tumor response rate	Determine the time to disease progression and TTF	NCT00940316
Binimetinib	Small molecule MEK inhibitor	ORR	PFS, OS, evaluation of the safety and tolerability of pembrolizumab, binimetinib, and bevacizumab	NCT03475004
Pembrolizumab	Humanized antibody, IgG4, anti-PD-1	ORR	PFS, OS, evaluation of the safety and tolerability of pembrolizumab, binimetinib, and bevacizumab	NCT03475004
Trifluridine/tipiracil hydrochloride	Trifluridine, a nucleoside analog, and tipiracil, a thymidine phosphorylase inhibitor	PFS	ORR, DR, DCR, OS	NCT02743221
Vismodegib	(GDC-0449, hedgehog pathway inhibitor)	PFS	PFS	NCT00636610
Apatinib	TKI that selectively inhibits the VEGFR-2	PFS	OS, ORR	NCT03271255
Reolysin®	Reovirus Serotype 3 – Dearing Strain affecting Ras-transformed cells causing cell lysis	Phase 1, dose-limiting toxicity Pharmacokinetic parameters	CEA and ORR, clinical benefit rate (PR, CR, SD), PFS, and OS	NCT01274624
Atezolizumab	PD-L1	PFS	ORR, OS	NCT02873195
Raltitrexed	Inhibitor of thymidylate synthase	Evolution of raltitrexed plasma levels	Treatment-related adverse events, ORR	NCT02821559
Enzastaurin	Inhibits protein kinase C-beta, an enzyme involved in the induction of VEGF-stimulated neo-angiogenesis	PFS	Safety and adverse events, OS	NCT00612586
Simvastatin	Competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase	PFS	OS, response rate	NCT02026583

(continued)

Table 10.3 (continued)

Drug	Target/mechanism of action	Primary measures of the study	Secondary measures of the study	Identifier at Clinicaltrials.gov
Vandetanib	Kinase inhibitor of the VEGFR, the EGFR, and the RET tyrosine	Maximum tolerated dose of vandetanib	Effect of vandetanib on the pharmacokinetics, dose-limiting toxicities associated with the combination	NCT00532909
Nivolumab	Human IgG4 anti-PD-1 monoclonal antibody	PFS	ORR, disease control rate, duration of response	NCT03414983
NANT colorectal cancer (CRC) vaccine	Combination immunotherapy, including various vaccines	Incidence of treatment-emergent AEs and serious SAEs, ORR	PFS, OS	NCT03169777
Avelumab	Human IgG1 monoclonal antibody directed against the human immunosuppressive ligand PD-L1	PFS	Safety, immunologic analysis of samples from peripheral blood, ORR	NCT03050814
Regorafenib	Dual targeted VEGFR2-TIE2 tyrosine kinase inhibition	The rate of evaluable patients alive and not progressed at 6 months	Worst grade toxicity per patient, OS	NCT02619435
Ramucirumab	Human monoclonal antibody against VEGFR2	OS	PFS	NCT01183780
Vismodegib (GDC-0449)	Hedgehog pathway inhibitor antineoplastic agent	Percentage of participants who experienced at least one AE	Incidence and severity of all AEs and SAEs	NCT00959647
Sorafenib	Blocks the enzyme RAF kinase	PFS rate	Response rate, OS	NCT00826540
Dasatinib	Oral dual Bcr/Abl and Src family TKI	To determine the maximum tolerated dos	Dose-limiting and non-dose-limiting toxicities	NCT00920868
Linifanib (ABT-869)	Potent inhibitor of RTKs, EVEGF, and platelet-derived growth factor	PFS	OS, (12-month OS rate)	NCT00707889

Abbreviations: *VEGF* vascular endothelial growth factor, *TKI* tyrosine kinase inhibitor, *PFS* progression-free survival, *OS* overall survival, *TTF* time-to-treatment failure, *ORR* overall response rate, *MEK* mitogen-activated extracellular signal-regulated kinase, *DR* duration of response, *DCR* disease control rate, *PD-L1*programmed death ligand 1, *RTK* receptor tyrosine kinase, *AE* adverse event, *SAE* serious adverse event, *EGFR* epidermal growth factor, *IGG1* immunoglobulin G1, *CEA* carcinoembryonic antigen

10.9 Conclusions and Future Perspectives

Significant advances have been made for improving the survival of mCRC patients. This has been possible and achieved mainly by the development and approval of newer drugs, including capecitabine, irinotecan, and oxaliplatin, and several monoclonal antibodies (humanized) that block either VEGF (bevacizumab, aflibercept, and ramucirumab) or the EGFR (cetuximab and panitumumab), and, most lately, trifluridine/tipiracil and regorafenib (TAS-102). The clinical benefits of these drugs are now generally acceptable/established for the mCRC patients, with the median OS of greater than 30 months.

Currently, the limitation in the effectiveness of TKIs is due to the two principal reasons: firstly, combination chemotherapy use that necessitates lowering of the dose density for toxicity profile management and, secondly, these drugs that have mainly been developed in molecularly unselected population. The main challenge now is the identification of more reliable and specific predictive biomarkers for selecting the most suitable therapy for a patient. So far, the only well-established and reliable biomarker for the mCRC treatment is RAS mutational status, which predicts negative response to anti-EGFR therapy. Current recommendation for the BRAF mutational status has also been given by the National Comprehensive Cancer Network (NCCN) [88] and the ESMO. Unlike VEGF inhibitor therapy, the resistance mechanisms in the EGFR inhibitor therapy are well-understood, as are the drugs blocking the downstream RAS-MAPK pathway. Of course, a number of clinical trials conducted recently (Tables 10.2 and 10.3) on targeting the RAS signaling pathway have revealed promising efficacy in chemo-refractory mCRC. Further research is the utmost important need of the time to discover newer biomarkers coupled with genomic/immunotherapeutic/bioinformatic approaches for identifying appropriate therapy (precision/personalized) for a selected patient population.

Conflict of Interest None of the authors has any potential financial or commercial conflict of interest associated with this research manuscript (chapter).

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EGFR and FGFR in Growth and Metastasis of Colorectal Cancer

11

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Abstract

Colorectal cancer (CRC) is the most important cause of tumor-related fatalities around the world, and its distant metastasis is responsible for 40% of mortalities in the USA as well as around the world. CRC is not a single disease; it is rather an assortment of multiple cancers. Metastatic CRC develops from the relapse period after the therapy, where the cancer cells develop resistance. Due to the heterogeneous biology, clear descriptive study at molecular level about the mechanisms, which takes place during CRC invasion and proliferation, is necessary. These studies can help understand the factors affecting the increased risk of CRC progression and help deduce novel therapeutic strategies. This chapter includes the mechanism of EGFR and FGFR in CRC, which are common targets for therapy since they induce cell proliferation and cell division and inhibit apoptosis. Their overexpression in CRC is associated with metastasis including invasion and angiogenesis.

Keywords

CRC · EGFR · FGFR · FGF · EGF · Tyrosine kinase

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11.1 Introduction

Colorectal cancer (CRC) is among the three most common malignancies and is the second deadliest type of malignancy in both genders. According to the annual incidence rate in the USA, an estimated 151,000 cases of CRC has resulted in 51,000 cases of fatalities annually. In developed countries like Australia or New Zealand, 55% of the patients are diagnosed with CRC. Patients treated are in a constant fear for the sequel and recurrence. The high mortality and morbidity rate is because of the metastatic conditions; however, it controls if there is an early detection of the disease followed by an effective treatment. The genetic alterations, molecular abnormalities, and overexpression of growth factors and their receptors like EGFR, VEGFR, PDGFR, and FGFR are the unfavorable conditions leading to colorectal tumorigenesis and metastasis.

CRC may be benign or malignant, which originates from the epidermis of the colon or rectum as a polyp (adenomatous or hyperplastic) and mainly develops into a tumor. Possible risks for CRC include patient having ulcerative colitis or Crohn's disease and the patient's social history. Modern lifestyle, consuming alcohol, smoking, and intake of high protein and fats are also risk factors contributing toward CRC. CRC stage or extent of spread is easily diagnosed and detected using colonoscopy. CRC patients are subject to surgery followed by adjuvant chemotherapy. Nowadays, targeted therapy has proved to be an effective method. Overexpressed EGFR and FGFR in CRC, leading to cancer cell survival, proliferation, metastasis, invasion, and angiogenesis, are mainly targeted using monoclonal antibodies (MAbs). Thus, EGFR and FGFR have become attractive targets for therapy. Even biological agents such as tyrosine kinase inhibitors are used as a therapeutic target. This chapter discusses the biology and expression of EGFR and FGFR and their function in CRC growth as well as its malignant properties.

11.2 Biology of Growth Factors Receptors

11.2.1 Epidermal Growth Factor Receptor Family

Epidermal growth factor receptor (EGFR) is a part of the ErbB family of RTKs (receptor tyrosine kinases), which comprises of four member, namely, ErbB (EGFR or HER1), ErbB2/HER2/Neu, ErbB3/HER3, and ErbB4/HER4 (Table 11.1) [1, 2]. The EGFR family of transmembrane glycoproteins molecular weight ranges from 170 to 185 KDa [3]. Proteins from the EGFR family are characterized by the EGF corresponding domain that is composed of intramolecular group with three disulfide bonds. They confer specificity in binding additional structural resembling immunoglobulin-like domains, heparin-binding sites, and glycosylation sites. EGFR and ErB4 are the receptors with enhanced functional ability to bind with the ligand as well as autophosphorylate C-terminal tails via intracellular tyrosine kinase domain, but the ErbB3 has no intrinsic tyrosine kinase activity; it holds substitutions of critical amino acids and transduces signals by interacting with kinase active receptors like EGFR, ErbB2, and ErbB4 [4].

Table 11.1 EGFR location and their expression

Name of the receptor	Amino acids	Gene symbol/other names	Gene location	Count of exon	Expression of EGFR
ERBB1	1210	EGFR/ERBB/ HER1/mENA/ ERBB1/PIG61/ NISBD2	7p11.2/7p12	30	Broad expression in the placenta, skin, and 22 other tissues
ERBB2	1225	ERBB2/NEU/NGL/ HER2/TKR1/ CD340	17q11.2–q12	32	Ubiquitous expression in the kidney, skin, and 24 other tissues
ERBB3	183	ERBB3/HER3/ LCCS2/ MDA-BF-1/ p180-ErbB3	12q13.2	28	Broad expression in the small intestine, duodenum, and 17 other tissues
ERBB4	1292	ERBB4 HER4/ ALS19/p180-erbB4	2q33.3–q34	31	Biased expression in the kidney, brain, and 8 other tissues

Among the ErbB family of RTKs, HER2 is exclusive and incapable of binding to any of the ligands, but it is still preferred as the dimerization partner for EGFRs [4–6].

11.2.2 Structure of EGFR/ErbB-1/HER1

The EGFR gene is present on chromosome 7p11.2 and has 30 exons coding for 464 amino acids translating transmembrane proteins. In the 30 exons, exon 5–7 and 13–16 exon code for ligand binding domain and exons 18–24 for TK domain. The regions exons 25–28 are encoded for autophosphorylation [7]. The developed EGF receptor consists of a sole polypeptide chain, composed of 1186 amino acid residues and structurally consisting of an extracellular binding domain with N-terminal and cysteine-rich arm involved in dimerization, a hydrophobic transmembrane domain, and a cytoplasmic C-terminal tyrosine kinase domain that are highly conserved with numerous phosphorylation sites intracellularly [8–10]. The extracellular region of EGFR can be again divided into four main domains L1, CR1, L2, and CR2 (Fig. 11.1) [4, 11]. CR1 comprises a β-hairpin loop, which is necessary for the receptor's functionality.

EGFR conveys growth-inducing signals to its cognate ligand (TGFα and EGF), binds with it, and gets activated, endocytosed or degraded in lysosome, or recycled into the plasma membrane [11, 12]. This consecutively regulates cell growth, differentiation, and proliferation and contributes a multifarious signaling cascade in healthy cells and also adapts tumor survival, signaling, differentiation, adhesion, and metastasis in malignant cells [4]. The overexpression and upregulation of EGFR genes lead to poor prognosis and high risk of metastatic colorectal cancer in 80% of CRC cases.

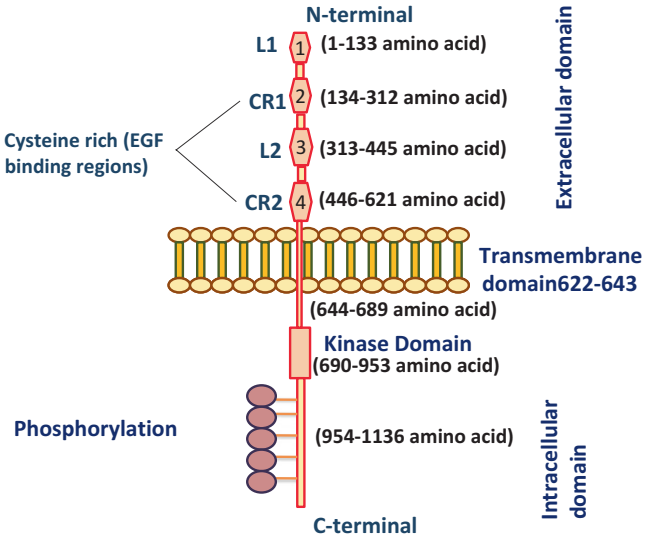

Fig. 11.1 The structural details of EGFR

11.2.3 Expression of EGFR

Overexpression of EGFR is related to the high involvement of mesenteric lymph node, which is more advanced in tumor stage, and its scarcity in signaling pathways causes Alzheimer and many other aggressive diseases [13–15]. From the measurement of immunohistochemistry, EGFR reactivity is not homogenous throughout the tumor and is in the deepest region of primary CRC and peripheries of metastasis. Its expression also specifies that the primary CRC is consistent with metastases irrespective of the site and occurrence. Spano et al. [16] compared the tumor-node-metastasis (TNM) tumor stage (T3) study with expression of EGFR and confirmed that EGFR expression and tumor invasion are related. The overexpression of HER1 and HER2 results in solid tumor cells in small-cell lung cancer (SCLC) and colon cancers. ADAM17, a disintegrin metalloproteases 17, controls the activity of EGFR via shedding of the EGFR ligands. Its overexpression along with the co-expression of EGFR suggests tumor growth and angiogenesis (Table 11.1) [17].

11.2.4 Fibroblast Growth Factor Receptor and Expression

The signaling pathway of FGF is a central element in the development of embryo, regulating cellular progression, differentiation, proliferation, migration, and survival, but its aberrant activity causes in the pathology of human including metabolic disorder and skeletal and cranial diseases as well as cancer. They control the pathological process like angiogenesis, organogenesis, and tumorigenesis.

11.2.5 FGFR Structure

Like EGFR, fibroblast growth factor receptor (FGFR) is also a part of the family of receptor tyrosine kinase. FGFR structurally has a N-terminal extracellular domain containing three Ig-like subdomains (D1, D2, and D3), a transmembrane α helix domain, and an intracellular kinase domain. They play a significant role in transmitting signals, but FGFR5 lacks tyrosine kinase domain and inhibits the activity of FGF in the mechanism of ERK1/2 (extracellular signal regulated kinase 1/2) by binding with other proteins [18–20]. There is an acid box present in between the domain of Ig-II and Ig-I which plays a key role in auto-inhibition of receptor. The Ig-II and Ig-III form the site for ligand to bind. The family of FGFR consists of members FGFR 1–4 and exists as seven isoforms FGFR (1b, 1c, 2b, 2c, 3b, 3c and 4) [21, 22]. FGFR4 is expressed as FGFR4-3c isoform and contains cofactor β-Klotho and interacts only with FGF19 ligand [23–25].

11.2.6 FGFR Expression

The expression levels of FGFR1 and FGFR2 are seen in CRC and colorectal adenoma, where the transformation of colon adenomas to carcinoma occurs (Table 11.2) [26–28]. FGFR2IIIb isoform expression is reported widely in most of the cancers like breast, gastric, pancreatic, and CRC. The interaction of ligands FGF7 and 10 with FGFR2IIIb through autocrine and paracrine causes CRC proliferation and tumor angiogenesis. They even play essential role in metastasis [29]. From the analysis of immunohistochemical test, it was demonstrated that expression of FGFR2 in the epithelium of colorectal leads to the growth and variation of cells [29]. The activity of FGFR3 in CRC is observed in patients through their aberrant splicing and cryptic splice sequence [30, 31]. The splice sequence of FGFR4 is also reported, however not determined [32]. The overexpression of FGFR4 is observed at the RNA and protein level of CRC. The overexpression of FGFR4 is observed at the RNA and protein level of CRC. It is even determined that its interaction with ligand FGF19 followed the ERK pathway with substrate FRS2 causing tumorigenesis in CRC [33]. The FGFR1 is overexpressed, and FGFR3 is downregulated in CRC, but commencing of FGFR1 siRNA will disrupt the overexpression of FGFR1 and provoke the expression of FGFR3 [34].

Table 11.2 FGFR their location and expression

FGFR	Chromosomal location	Count of exon	Expression
FGFR1	8p11.23	25	Ubiquitous expression in the colon, ovary, and other tissues
FGFR2	10q26.13	26	Broad expression in the skin and thyroid. Less in the colon
FGFR3	4p16.3	19	Biased expression in the skin and very less in the colon
FGFR4	5q35.2	19	Broad expression in the lung and kidney, also expressed in the colon

11.3 Ligands

11.3.1 EGF

The ligands belong to the polypeptide family. The essential step of a ligand is binding with the ectodomain, i.e., domain 3 of the receptor for dimerization. Eventually, activating the receptor thereby provokes signaling pathways in both healthy and tumor cells. Activation of the receptor family performed by 13 ligands are distinguished into three subgroups. The first group consists of the EGF, TGF-α (transforming growth factor alpha), and AR (amphiregulin), which bind distinctively to the EGFR. The second group comprises of BTC (betacellulin), HB-EGF (heparin-binding EGF-like growth factor), and EPR (epiregulin) that show dual specificity by binding to EGFR as well as to ErbB-4. Third group involved the EPG (epigen) and 1–6 NRG (neuregulins) [35, 36].

In general, the motif of a ligand has six cysteines and is conserved, which creates three peptide bonds through the disulfide bonds common in conformation of its receptor. An extended N-terminus is also present for AR, HB-EGF, and BTC. Stanley Cohen, A Nobel Prize Laureate in Physiology 25 years ago, revealed the first growth factor, the epidermal growth factor (EGF), which is a cytokine consisting of 53 amino acids weighing 6.2 kD. EGF is secreted by ectodermic cells, monocytes, kidneys, and duodenal glands. EGF stimulates the growth of epidermal and epithelial cells. Ligands EGF, TGF-α, AR, BTC, and EPR bind distinctively to HER1, but HER2 is incapable of binding to any of the ligands (Fig. 11.2). From the studies, it was determined that the protein expression of AR (ligand), HER1, and HER2 exhibited the prevalence of CRC and thus can be a promising prognostic marker for liver metastasis of CRC. BTC, HB-EGF, and protein of the HRG group also bind explicitly to HER4 [37]. In addition to the three groups of ligands, heregulin is a growth factor ligand, which phosphorylates the EGFR and localizes (ErbB2 and ErbB3) to the cytoplasm and nucleus, but ErbB4 can be detected only in the cytoplasm. This process does not follow the traditional signaling pathway for translocation. Thus, the autocrine loop of heregulin-ErbB is considered as a target treatment of CRC [37].

11.3.2 FGF

The fibroblast growth factor (FGF) is the largest family of ligands comprising of 22 polypeptide FGFs. These are heparin-binding growth factors subdivided into canonical (cFGFs, FGF7 to FGF10, FGF16 to FGF20, FGF22), intracellular (iFGFs, FGF11 to FGF14), and hormone-like (hFGFs, FGF9, FGF21) [38]. FGF is activated by endothelial cell surface receptors and leads to stimulate the activity of tyrosine kinase of FGFR (Table 11.3). Binding with other proteins like integrins and heparin-sulfated proteoglycans or heparin sulfate glycosaminoglycans (HSGAGs), FGF promotes angiogenesis and fibroblast proliferation and responds to repair of tissues and wound healing.

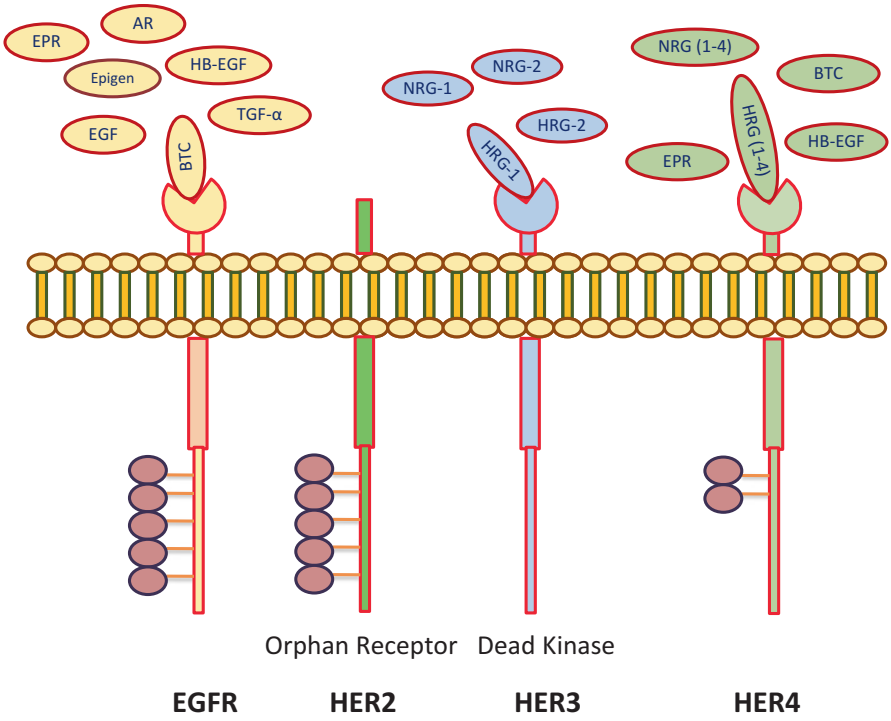

Fig. 11.2 EGFR and their ligands. EGFR binds with ligands EGF, TGF-α, AR, BTC, and EPR. HER2 receptor has no ligand. HER3 lacks the fundamental tyrosine kinase domain and binds to heregulin-1, 2 (HRG-1 and 2). HER4 binds to HRG1-4, NRG1-4, HB-EGF, BTC, and EP

Table 11.3 FGFR and their ligands

Receptor variant	FGFR1		FGFR2		FGFR3		FGFR4	FGFR5
Isoforms	IIIb	IIIc	IIIb	IIIc	IIIb	IIIc		
Ligands	FGF1, 2, 3, 8, 10	FGF1, 2, 4, 5, 6, 8	FGF1, 3, 7, 10	FGF1, 2, 4, 5, 6, 8	FGF1, 9	FGF1, 2, 4, 8, 9	FGF1, 2, 4, 6, 8, 9	FGF1, 2

Among 23 of FGFs in humans, 18 (FGF1–10 and 16–23) serve as mitogenic signaling molecules that bind with high affinity to FGFRs (Table 11.3) [21]. Canonical FGF reduces the diffusion through extracellular membrane by tightly binding with heparin or heparin sulfate proteoglycans (HSPGs) and serves as a cofactor to regulate the affinity for signaling FGFR. The hormone- or endocrine-like FGFs require protein cofactors for their binding with receptor since they show less affinity for heparin. While the iFGFs are the first to show high affinity toward heparin, it limits the diffusion and regulates the binding with receptor. Proteins like MAPK protein kinase and NEMO (NF-κB modulator) bind directly to iFGFs.

The expression of FGF2 is observed in most of the tumors like prostate cancer, CRC, head and neck cancer, etc. [39–47]. It induces angiogenesis by expressing in submucosal tissues [26–28]. FGF1 is expressed mostly in epithelial-mesenchymal transition (EMT), invasion, and metastasis as well as in tumorigenesis [48, 49]. FGF1 and FGF2 being acidic and basic, respectively, with their receptors involving autocrine and paracrine loops are found in cancers like CRC, prostate cancer, breast cancer, etc.; these disrupted loops promote high proliferation and survival of cancer cells [50, 51]. The expression of FGF7 and FGF10 is intricate in the pathway of MAPK for proliferation of ameloblastoma cells [52]. Angiogenesis results from the interaction of ligand FGF7 with the isoform FGFR2IIIb, and it also enhances the adhesion to collagen type IV in CRC [53]. FGF8 expresses through Yes-associated protein1 (YAP1) and shows its activity in proliferation and metastasis of CRC [54]. FGF9 plays its essential role in anti-apoptosis and is secreted by cancer-associated fibroblast (CAFs) [55]. FGF13 regulates resistance to drugs [56]. FGF16 and FGF22 play a role in cancer phenotype of ovarian cancer cell and pro-oncogenic role in the skin [57, 58]. FGF20 shows activity in high proliferation rate in epithelial progenitor cells [26–28]. However, the expression of FGF6 to FGF9 and FGF11 to FGF16 in malignancy is unknown.

11.3.3 How Dimerization Happens?

The most essential step for a signaling pathway is the homo- or heterodimerization where a ligand binds with the ectodomain of the receptor (EGFR), followed by autophosphorylation of the tyrosine kinase domain [59].

11.3.4 Dimerization in EGFR

The EGF ligand/receptor system or the process of dimerization is involved in the initial embryonic growth and renewal of the stem cells in normal tissues like the gut, skin, and liver. The activation of ErbB receptors can be done by autocrine secretion or through paracrine secretions with growth factors [60, 61]. Two different confirmations have shown by the crystal structure of the Her-4, Her-3, and the EGFR ectodomains during the process of dimerization: an active/open confirmation and closed/inactive conformation. Interaction amid domains II and IV is prevalent inside the later (closed) confirmation at the intermolecular levels. This prevents the interaction between domains I and III with their respective ligands [12, 62]. Both confirmations maintain equilibrium with one another [35, 63] irrespective of the ligand binding. However, HER2 is unique and incapable of binding to any of the ligands. Ultimately, signaling the downstream in promoting cell proliferation and increasing cell survival. Structurally the TK domain is bilobed, and a dimeric complex is formed with ATP binding between the two lobes. Activation of RK occurs when the N-lobe of one TK domain interacts with the C-lobe of another Fig. 11.3 [64].

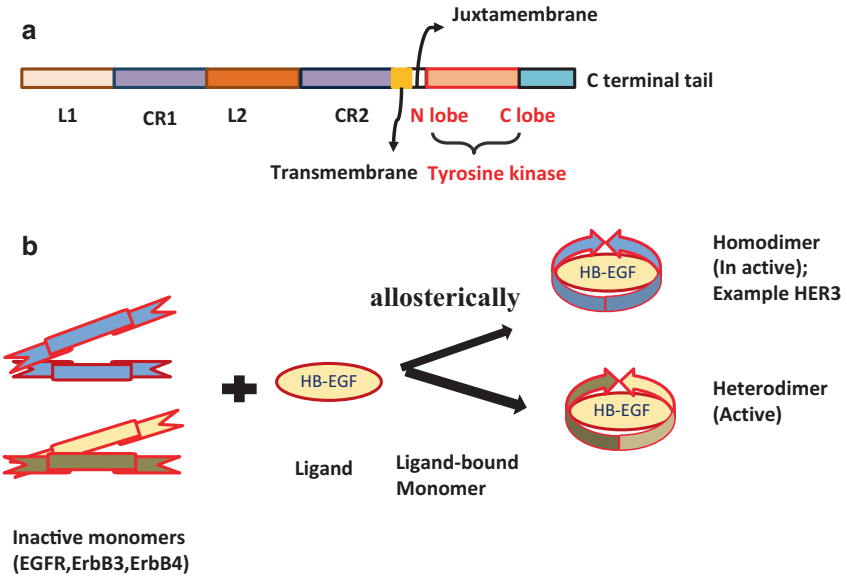

Fig. 11.3 (**a**) Linear representation of ErbB receptor domains. (**b**) Schematic overview of the structural basis for ErbB receptor dimerization and activation

11.3.5 Dimerization in FGFR

Dimerization of the receptor is a critical step for kinase activation. During this process the tyrosine kinase domain of the two receptors comes in close proximity and gets cross phosphorylated [65]. The phosphorylated kinase binds with the adaptor proteins and controls the downstream cascade signaling for cell growth and proliferation by activating various cytoplasmic substrates, but the mutation and gene amplification in the receptor drive abnormal morphogenesis resulting in progression of cancer like CRC.

11.3.6 Signaling Pathways

EGFR, a multifunctional receptor, plays a key role in stimulation of downstream signaling cascade activated by ligand. Tyrosine phosphorylation is an essential step here. The initiation of EGFR primes at the stimulation of downstream signaling cascade like the Ras/MAPK (involved in cell proliferation), PI(3)K/Akt (for apoptosis), PLCγ1/PKC, and STAT.

The signaling pathways in **FGFR** include many biological processes like cell apoptosis, cell proliferation, and cell migration [65]. The binding complex of FGF-FGFR-HS (cofactor) phosphorylates intracellular tyrosine kinase and initiates the cascade of downstream signaling cascades like RAS-MAPK, PLCγ, PI3K-AKT, and STAT controlling mitogenesis and differentiation [21, 66]. There are several

intracellular proteins and regulators acting all through the pathways. The intracellular proteins like signal transducer and activators of transcription (STAT), PLCγ (phospholipase Cγ), FRS2, Src kinase, RSK (ribosomal S6 protein), adaptor GRB2 (growth factor receptor-bound protein), etc. show their activity. GRB2 exhibits its activity in RAS/MAPK pathway and PI3K/AKT pathway, by binding with the phosphorylated FRS2.

The regulators, which monitor the output of the signaling pathways, include positive and negative regulators. The positive regulators are FLRT1, FLRT2, and FLRT3 (fibronectin leucine-rich transmembrane protein), and the negative regulators are MKP3 (mitogen-activated protein kinase phosphatase 3), sprout proteins, and SEF (similar expression to the FGF). The sprout protein attenuates signaling by directing the FGF to lysosome.

11.4 The RAS/ERK Pathway

11.4.1 EGFR

The dimerized receptor with ligand results in EGFR phosphorylation, thus creating sites for Grb2 and Src homology2(Shc2) binding, which activates the Ras/Raf/MAPK signaling cascade via Son of Sevenless (Sos) initiating DNA synthesis and cellular growth [67, 68]. SOS displaces guanosine diphosphate (GDP) fragments from the RAS to guanosine triphosphate (GTP) fragments. Active GTP-RAS can gather the RAF proteins (A-RAF, BRAF, and C-RAF) toward cell surface [69]. RAF protein is inhibited in the cytosol by 14-3-3 proteins. Nevertheless, after GTP-RAS binding, the RAF protein activates the pairing of RAF among them, forming heterodimers. Hence, they are able to bind to KSR1 enzyme [70]. The heterodimer RAF proteins can thus phosphorylate MEK by binding to them, which is connected through KSR1 enzyme; MEK in turn activates ERK1 and ERK2.

The ERK/MAPK enters into the nucleus and activates a series of transcription factors, namely, JUN, NF-kappaB, and FOS [70], also as an inhibitor of apoptosis protein (IAPs) thus binding to the AP-1 DNA domain of the nucleus and transcribing genes that promote cell proliferation and survival [71]. This progression is normal for a healthy non-cancerous cell and terminated through the RAS-GTPase-activating (GAP) proteins. These GTPase enzymes are found within RAS and thus hydrolyze GTP to GDP and hence switch the RAS off [70].

11.4.2 FGFR

The FGFR substrate FRS2α bound with FGFR intracellularly initiates the pathway by binding with the CRKL and phosphorylates via FGFR tyrosine kinase. The activated FRS2α binds with the adaptor GRB2 followed by the binding with SOS. SOS activates the MAPK pathway by activating RAS GTPase. MAPK pathway phosphorylates the transcription factors like ETS (E26 transformation specific), which

interacts with the DNA and controls gene expression of target and negative regulators in the nucleus. The activity of GRB2, which is inhibited by SPRY, followed by inhibition of the RAS-MAPK pathway and SEF (similar expression of FGF) is an antagonist in FGF signaling and aids in blocking the nuclear translocation of MAPK. The GRB2 decreases the receptor kinase activity by inhibiting SHP2 and get docked in the FGFR2 at its C-terminal domain.

11.5 PI3 Kinase/AKT Pathway

11.5.1 EGFR

The other important pathway for cell survival is PI(3)K/AKT pathway. Just like MAPK pathway, PI3K activates and signals downstream. There are four different classes of PI3Ks; the most important among them is IA in human cancer. The class IA PI3k is a heterodimer of P110 catalytic subunit as well as a p85 regulatory subunit [72]. The subunit of PI(3)K, p85, is stimulated mainly with the receptors of ErbB3 and ErbB4 since they encompass the p85-binding sites [73]. The regulatory subunit, p85, confers to the phosphotyrosine residues and/or to various other adaptors that are instituted on the RTKs. The catalytic subunit of the PI3Ks, p110, phosphorylates PIP2 into PIP3 [74]. This phosphorylated PIP3 phosphorylates PDK1 and AKT by PDK1 and AKT kinases and generates downstream signals; these signals are involved in cellular growth and apoptosis [74]. EGFR might also stimulate the PI(3)K signaling pathway via adaptor protein, Gab-1 [75]. RAS can trigger the cascade physiologically via directly binding to p110 [76]. Back gain, the tumor-suppressor protein PTEN is dephosphorylated to PIP2 from PIP3, hence terminating the process of signaling [74].

11.5.2 FGFR

The phosphorylated FRS2α binds with the adaptor protein GRB2, which in turn binds with GAB1, and activates the enzyme PI3K. PI3K phosphorylates AKT enzyme and stimulates the PI3K/AKT pathway. AKT, a protein kinase, activates the mTOR complex and inhibits cytoplasmic TSC2 (tuberous sclerosis complex2) resulting in proliferation, cell growth, and inhibition of FOXO1 (forkhead box class transcription factor) causing it to exit the nucleus and promote cell survival. GRB2 regulates the PI3K-AKT pathways by binding with SPRY.

11.6 PLCγ1/PKC and Stat Pathway

EGFR has the capability to initiate **PLCγ**, leading to the activation of protein kinase C (PKC) followed by the activation of c-Jun and MAPK that controls cell proliferation [77]. The phosphorylated **FGFR** produces IP3 as well as DAG

(diacylglycerol) through the hydrolysis of PIP2 by activating the enzyme PLCγ. IP3 and DAG play a key role in downstream signaling; the IP3 meant for the release of calcium ions from the intracellular region, and DAG activates PKC enzyme. GRB14 inhibits the activity of PLCγ.

STAT3 is an important element in maintaining the polarity as well as adhesion of the epithelial cell. STAT3 binding with stimulated EGFR leads to its dimerization and translocation to the nucleus in order to control gene transcription [78]. Likewise, STAT5 also binds to EGFR and ErbB4 [73]. STAT pathway is stimulated by the activation of STAT1, 3, and 5, which is in turn activated by phosphorylated tyrosine kinase of FGFR. STAT3 is regulated by FGFR4, which acts as a transducer and regulates apoptosis through the anti-apoptic inhibitory protein cells FLICE (c-FLIP) and B-cell lymphoma-2(Bcl-2) [23]. The activated pathway in both EGFR and FGFR regulates the expression of gene in the nucleus.

11.6.1 Receptor Cross Talk

The mechanism, which assists various human tumors to escape the inhibitory properties of tyrosine kinase inhibitors, is known as the ErbB cross talk. G-protein-coupled receptors (Frizzled family) that are initiated by ligands like the lysophosphatidic acid, endothelin-1, bombesin, angiotensin-II, and thrombin transactivate the EGFR via activation of the ADAM family cell surface MMPs (metalloproteinases) that leads to cleaving of various membrane-bound EGF precursors such as HB-EGF [79–83].

Alternatively, the tyrosine kinase Src is activated via the Frizzled family of G-protein-coupled receptor, which in turn phosphorylates EGFR. Activated growth hormone also activates the Jak2, which is involved in phosphorylation of EGFR (Fig. 11.3). Cellular Src kinase (C-src) also transactivates ErbB2 by integrins. C-src with integrins holds the ability to stimulate several intracellular signaling cascades. These integrins are known to play a vital role in indigenous tumor invasion, progression, and metastasis.

11.6.2 Nuclear Localization of ErbB Members

The internalized receptor makes its way to the nucleus. The internalization of the EGFR could be translocated either completely or partly. It can even form into an endosome. Translocated ErbB regulates the transcription and thereby upregulates many genes involved in cancer biology like cyclins and transcription growth factor.

11.6.3 Endocytosis

In the process of signal termination where the tyrosine is dephosphorylated, the EGFR is internalized through various proteins like adaptin (Grb2), clathrin, and dynamin forming vesicles. EGFR fuses with the early endosome (EE). The fate of the EGFR is decided in the endosome in either tending to recycle back to the cell surface, degraded by the lysosome, or translocate to the nucleus.

In the EE, EGFR binds together with the adaptor molecules like Grb2, casitas B-lineage lymphoma proto-oncogene (Cbl), and an E3 ubiquitin ligase (parkin). Eps 15 and cbl are the downstream substrates of EGFR, involved in endocytosis of EGFR [84]. The lysosomal degradation of EGFR is promoted by E3 ubiquitin ligase by ubiquitination. The homodimers are highly stable in the EE and remains as a complex with cbl under mild acidic environment, but the heterodimers like EGFR-HER2 are less stable and disassociate from the ligand complex. This makes its way back to the cell surface [85]. The proteins have nuclear localization sequence (NLS) complexes with importin alpha/beta, interact with nucleoporins in the nuclear pore complex of the nucleus, and translocate into the nucleus [11]. Sec61beta, an endoplasmic reticulum membrane protein translocator, present in the inner nuclear membrane is involved in EGFR translocation into the nucleus.

ErB4 follows a unique mechanism for translocating to the nucleus. Once dimerized with the ligand, ErB4 at the extracellular surface is proteolyzed by the ADAM17 metalloproteinase TACE [35]. Presenilin/γ-secretase separates ErbB4 at the transmembrane domain and also releases the active dimeric tyrosine kinase domain s80/E4ICD into the cytoplasm [63]. S80 acts as a chaperon for WW domain of Yes-associated protein (YAP-1), which is a transcriptional regulator [35, 36]. In the S80 complex, YAP-1 is seldom translocated into the nucleus and can stimulate transcription. WW domain protein called the WWOX will now balance s80 inside the cytosol [3]. The early endocytic system in the FGFR signaling, which is regulated by the src target Eps8, is a novel mechanism in regulating the trafficking of FGFR but remains elusive. The dimerization can also attenuate the signal. HSP90 binds at the N-lobe of the catalytic tyrosine kinase domain of ErbB2. It maintains a condition of homeostasis by degrading excess dimer at the extracellular surface by ubiquitinylation and proteasomal degradation.

11.6.4 Mutation

CRC is a heterogeneous disease caused due to mutation, effecting various tumor-suppressor genes, DNA repair genes, and oncogenes involved in the signaling pathways of EGFR, VEGF, and FGFR and resulting in genetic and epigenetic alterations [86]. Marmol et al. [87] classified CRC depending on the origin of mutation as sporadic (70%), inherited (5%), and familial (25%).

Sporadic mutations are carried out by point mutation aiming at different genes, initiating in the formation of adenomas and ending with the formation of carcinoma. APC (adenomatous polyposis coli), a tumor-suppressing gene, is the first

gene-targeted mutation leading to benign adenoma called polyps followed by mutations in KRAS, TP53, and DCC [86].

In inherited mutation, if one of the alleles of mutated gene is affected and the other is targeted with point mutation, this causes tumor. Sporadic mutation is further classified as polyposis or familial adenomatous polyposis, which is described as the malignant polyps formed in the colon. Hereditary nonpolyposis CRC (HNPCC) is characterized as the mutations in DNA repair mechanism.

The molecular genetic pathways explained in this chapter are regulating CRC due to these major molecular characteristics: CpG island methylator phenotype (CIMP), chromosomal instability (CIN), and microsatellite instability (MSI). These interact with the oncogenes involved in the signaling pathways of RAS/RAF/MAPK, BRAF, and PI3K/PTEN/AKT of EGFR occurring independently in these genetic pathways [88]. On the basis of clinicopathological survival, Domingo et al. [89] added mutations in NRAS, PIK3CA, TP53, and FBXW7/CDC4, but no classifications had clinical impact.

The mutation in the DNA mismatch repair genes causes DNA microsatellite instability **(MSI)** phenotype. Nearly 15–20% of cases of CRC are due to MSI and 90% of CRC cases in patients suffering with Lynch syndrome or hereditary nonpolyposis colorectal carcinoma (HNPCC). MSI plays a key role in repairing the errors in DNA replication and thus promotes genetic stability. The inactivation of this gene is because of hypermethylation in the mismatched repair genes. MSI are of two forms, namely, sporadic MSI tumor, which is caused due to promoter hypermethylation of the mismatch repair genes, MLH1 [90], and familial MSI form that is hereditary called HNPCC or Lynch syndrome, which is caused by germline mutation. The mismatch repair genes are MLHI, PMS2, MSH6, and MSH2.

The genomic array revealed that the abnormalities of somatic copies that are increased with structural aberrations are the markers of **CIN** (chromosomal instability) acquired in cancer cells [91]. This frequently affects the cancer-associated genes like APC, TP53, KRAS, CTNNB1, LOH, and PIK3CA which are present on chromosome 18q and tumor-suppressor genes such as SMAD2, DCC, and SMAD4 [92–97]. Seventy percent to 85% of CRCs is due to CIN and is described as MSS CRCs (microsatellite stable) [98]. They are illustrated as the imbalance in the number of chromosomes leading to aneuploid tumors and loss of heterozygosity (LOH). It has been suggested that genomic aberrations are due to abnormalities in DNA damage repair genes, centrosome function, telomere function, or mitotic checkpoint or loss of heterozygosity (LOH). LOH is found within chromosomes 1, 5, 8, 17, and 18 [92–94].

The **CpG** island methylator phenotype (CIMP) is characterized as epigenetic instable due to hypermethylation of promoter CpG island sites leading to silencing of tumor-suppressing genes and tumor-related genes and thus loss of protein expression [87]. The genome CpG (cytosine preceding guanine) islands present in the promoter sites rich in CpG dinucleotide shows DNA methylation [99]. DNA methylation is an enzymatic process in the presence of DNA methyltransferase, a process in which methyl group is added to the 5th position of cytosine and produces 5-methylcytosine [100]. Methylation of CpG islands within and outside the promoter

region leads to transcriptional silencing and transcriptional activation, respectively [101–103]. Genes like CDKN24 (gene coding for p16, tumor suppressor), CXLC12 (gene responsible for metastasis), and MLH1 (mismatch repair gene) are affected by methylation [104, 105].

The combination effects of genetics and epigenetics in CRC development and the presence of BRAF mutations and MSI in many CIMP tumors have been described [104]. From the classification of CRC done by Weisenberg et al. [104], CRC is divided into CIMP-positive and CIMP-negative using MethyLight technology and correlated BRAF mutation with CIMP cancer. The work from Shen et al. [103] basing on MSI, BRAF divided CIMP CRC into three groups – CIMP1 tumors are MSI tumors (80%) and also have BRAF mutation (53%), CIMP2 tumors have KRAS mutations (92%) but seldom have MSI and BRAF and CIMP-negative.

11.6.5 Mutation in EGFR

EGFR is a transmembrane growth factor receptor and a glycoprotein. The activation of EGFR after dimerization with the ligand enables ATP binding with the tyrosine kinase (TK) domain at its ATP cleft and phosphorylation of tyrosine residue in the intracellular domain of EGFR. Thus, phosphorylation makes residues the best docking sites for various proteins. This results in the activation of various signaling pathways intracellularly and causes malignant tumors in the lung, colon, breast, bladder, kidney, and pancreas [6]. This affects cell growth, proliferation, angiogenesis, metastasis, and death [106, 107]. Mutation in EGFR occurs as a deletion in mRNA that codes for both extracellular and intracellular. No mutation is observed in the transmembrane. In the extracellular domain, there are three mutations observed; they are EGFR variants I, II, and III (EGFRvI, II, III). But most frequently observed is the EGFRvIII [108]. Mutation in EGFR kinase domain exons 18–21 response to TKIs causes lung cancer [109–111], and the somatic mutations in the TK domain of EGFR gene causes 12.1% of CRC at exon 19 and 20. A prediction that mutation induces the conformational alteration and stabilizes the dimer results in the activation of EGFR without binding of ligand. However from the work of Bo young, Oh et al. [112] compared the EGFR mutations on exons 18 (codon 719), 19 (codon 747–750), and 21 (codon 858) and predicted that 22.41% of patients showed mutations in exon 20 in 13 (G → A transitions). These mutations also build oncogene resistance to therapy [6].

Genes affected by the mutations involved in EGFR pathways are EGFR, KRAS, BRAF, MAP2KI, PIK3CA, PIK3R1, PTEN, etc. and proteins EGFR, PI3K subunits p110α and p85α, PTEN, phosphor AKT, and phosphoMEK1.

11.6.6 Mutation in KRAS

KRas, a proto-oncogene, was first identified in Kristen rat sarcoma virus [113]. It encodes to GTP (guanosine5′-triphosphate) binding protein. Its molecular weight is

21 kDa. About 30–40% of colorectal cancers are due to somatic mutations in KRas [97, 114–117]. The most often codon 12 and 13 in exon 2 are the affected codons evading the GTPase activity, resulting in approximately 85% of KRAS mutations in CRC. Poor prognosis and low survival rate with mutation in codon 13 exon 2 in advanced tumors and metastasis with mutated codon 12 exon 2 are reported [118, 119]. The mutations identified were glycine to aspartate on p.G12D, glycine to valine on p.G12V of codon 12, and glycine to aspartate on p.G13D of codon 13 [120]. Codons 61, 117, and 146 in exon 3 and 4 result in the remaining 15% of KRAS mutations. The mutations in KRAS lead to resistance therapy agents.

11.6.7 Mutation in BRAF

BRAF is a 766-amino acid proto-oncogene referred to as B-Raf and v-Raf murine sarcoma viral oncogene homologue B [121, 122]. It is a gene encoding B-Raf protein, a threonine/serine-protein kinase B-Raf. BRAF mutation occurs in exon 15, codon 600. These proteins are involved in cell growth by down streaming on MAPK signaling pathways of KRas. CRC due to BRAF mutation is linked with MSI (microsatellite instability). Five to 22% of CRC is due to mutation in BRAF, but from MSI (microsatellite instability) pathway, it was identified that the colorectal cancer ranges of about 40–52% by BRAF mutation with good prognosis [123–126] and microsatellite stability are 5% of CRC [123] with less survival rate of patient or poor prognosis. The most often reported mutation is valine-to-glutamic acid amino acid substitution [127]. The KRAS mutations wharf to 5–15% of BRAF mutations at 12/13 codon [128, 129]. A new BRAF gene in codon 600 of exon is identified as BRAF V600E which is mutated by missense mutation. BRAF V600E is a poor analytical factor in metastatic cancer. KRAS mutation with less common BRAF mutation develops therapy resistance, and thus BRAF V600E and KRAS mutations are always mutually excluded [130]. However, the combination with inhibitors of BRAF V600E and MAPK/PI3K pathways shows effective treatment in CRC metastasis. CE et al. [131] explained that DNA hypermethylation in cancer is caused due to continuous signaling of oncogenic BRAF in serrated polyps.

11.6.8 Mutation in PI3K Pathway

The PI3K/AKT pathway is a pathway regulating the cell cycle. This pathway is regulated by PIK3CA (p110 subunit), PTEN, and AKT genes. Mutation in these genes causes overactivation of PIK3CA and AKT and inactivation of PTEN, thereby deregulating the pathway. The PIK3CA gene encodes for PI3K. The genes of PI3K family code for three classes of phosphatidylinositol 3-kinases (PI3Ks). PI3K is the key signal transducer for PI3K-AKT pathway. It consists of heterodimeric subunits p85 and p110 and is found to be mutated in CRC. Fifteen percent to 18% of CRC patients are reported due to mutation in PI3K. It is detected that exons 9 and 20 of α isoform of p110 subunit are mutated and exon 20 showed lower response to the

therapy. The PI3K activation even confers drug resistance and promotes cell survival.

PTEN (phosphatase and tensin homologue) is a lipid phosphatase, which showed mutation in exons 1, 3, 5, 7, and 8. It is a tumor suppressor and an antagonist for PI3K and thus downregulates the PI3K-dependent signaling [132]. Loss of PTEN builds resistance to therapeutic agents like cetuximab in CRC [133]. Mutation in PTEN can be due to various mechanisms like promoter hypermethylation, allelic loss, point mutation, and deletion [134, 135]. Approximately 13–19% of CRC is due to PTEN mutation [132, 136]. Work on combined profile of KRAS, PTEN, and PI3K showed metastasis and developed resistance against anti-EGFR antibodies [137].

11.6.9 Mutation in FGFR

Deregulation can occur at the level of gene/protein expression of receptors and ligands of FGF due to mutation, which in turn results from aberrant signaling pathways. The mutation, gene amplification, and aberrant expression of FGFs result in indulgence of signaling and finally lead to cancer. In human colon and endometrial cancer, FGF9 ligand shows lack of β-catenin activation and homozygosity due to somatic mutations [138]. FGF9 also undergoes frameshift/nonsense mutation, whose overexpression leads to non-small-cell lung cancer. The overexpression of FGF8 causes CRC [139].

Gene amplification of the receptors causes overexpression by mutation or by gene fusion [140]. The missense mutation of FGFR3 causes CRC and many other cancers [141]. Its overexpression also leads to colon cancer (FGFR3c) [142]. The missense mutations in FGFR2 are observed in gastric and endometrial cancer [143–145]. The expression of at least one allele in G388R variant of FGFR4 is associated with poor prognosis [146]. It is identified from the tumor DNA of CRC that it shows two somatic mutations occurring at the region of third Ig-like loop present in all the receptors. One in exon 7 and the other in exon 9 showed a transition of G →A, resulting in the substitution of Lys for Glu [147].

11.6.10 Metastasis

Regardless of various diagnoses, surgical techniques, and advancements, deaths associated with cancer are the major hitch due to reoccurrence of tumor and spreading of cancer. The spreading of cancer to other parts of the body is metastasis, and it is becoming crucial to understand the cellular as well as molecular factors that are responsible for metastasis and tumor survival. The course of tumor metastasis is dependent on several interactions between the tumor cells and the host cells (EGFR, FGFR, VEGFR, etc.).

The metastasis is a heterogeneous disease where the tumor cells migrate from their primary site to distant site and form a secondary tumor with poor prognosis. It

is common in patients with advanced colon cancer and can be diagnosed at stage IV CRC. The cancer cells make its way by proliferating and invading into other healthy cells, spreading through the walls of lymph nodes. The survival and proliferation of tumor cells depend on the body supplying adequate amount of nutrients and oxygen [148]. This process is facilitated by angiogenesis. Angiogenesis is the process that leads to the development of new blood vessels and plays an important role in the development of solid tumors ultimately leading to metastasis. The EMT is another way for invasion and promoting metastasis. The EMT is a biological process, which shows transition of the polar epithelial cells into phenotype mesenchymal cell, thus enhancing the ability of invasiveness and resistance to apoptosis.

11.6.11 EGFR

The progression of cancer metastasis and resistance to chemotherapy are correlated with the overexpression of TGFα and EGFR [149–151]. Under hypoxic conditions, TGFα is secreted by CRC cells. Binding of TGFα (ligand) to EGFR promotes the generation of angiogenic proteins like VEGF (vascular endothelial growth factor) and IL-8 (interleukin-8). These play a crucial role in provoking angiogenesis and thus leading to metastasis. MMP-2 and MMP-9 (matrix metalloproteinases) are proteolytic enzymes that play a key function during angiogenesis. Macrophages also provoke the growth of VEGF. Asporin, an oncogene, enhances TNM stage and lymph node metastasis by encouraging phosphorylation of EGFR/src/cortactin signaling pathways [152]. Migration of CRC cells also occurs through β-catenin pathway. β-catenin is an E-cadherin-related protein involved in inhibiting cell-cell adhesion, hence a substrate for metastatic potential [153]. From the work of Loupakis et al. [154], mutation in KRAS and primary tumor leads to 95% of metastasis. Bazan et al. [155] also found that mutation in codon 13 KRAS is aggressive and includes advanced stage, metastasis in lymph nodes. From the analysis of bioinformatics, Huang et al. identified mutation in genes KRAS, BRAF, SMAD4 and P53 showed their role in CRC metastasis including lymphatic and distant metastases; this is even supported by other articles [156]. Colon cancer usually spreads to the liver and lungs. Thus, an understanding of the cellular and molecular aspects that promote tumor metastasis is extremely significant. PEAK1, a pseudopodium-enriched atypical kinase 1 and a non-receptor tyrosine kinase, is induced by the activation of EGFR and associated with tumor invasion and metastasis. PEAK1 is vital for CRC cell progression and metastasis induced by KRas. However, the overexpression of PEAK1 can be regulated by the ectopic expression of miR-181d [157].

11.6.12 FGFR

The microenvironment developed by tumor cells consists of immune cell, derived bone marrow cells, and carcinoma-associated fibroblast (CAF) [158]. The CAFs are characterized as heterogeneous and fibroblast-activated proteins (FAP), whose

contact with tumor causes tumor cell proliferation, survival, invasion, and metastasis [159]. Activated fibroblast increases the expression of FGF1. Thus, it gains importance as a target for novel therapy and as a marker for early tumor invasive CRC. The association of FGF2 and FGFR-integrinαβ-SRC signaling causes migration as well as invasion in cancer cells in association with fibroblast. The variant form G388R of FGFR4 significantly expresses advanced tumor stage and lymph node metastasis [33, 160–162]. About 27% of CRC was expressed due to immunoreactivity of isoform FGFR2IIIc and correlates with distant metastasis, poor prognosis, and decreased adhesion to extracellular matrices with EMT transition [163]. The work of Tsutomu Sato correlated the overexpression of FGFR1 gene with liver metastasis in CRC patients; thus it can be considered as a predictor for liver metastasis [31].

11.7 Therapeutic Potentiality

11.7.1 EGFR

Due to the multidimensional role of EGFR, it has become an attractive target for therapy. The EGFR inhibitor showed their effect by preventing the neoplasm cells from growing and targeted in treating CRC either as a single or in combination with chemo agent. The biological agents evaluated for the therapy are EGFR monoclonal antibodies as well as tyrosine kinase inhibitors. These agents differ in their efficacy as well as in the target molecular site of EGFR in the process of their treatment in CRC.

However, the effect of EGFR MAb drugs against KRAS exon 2 wild type is benefited as compared to the KRAS mutation, which showed resistance to EGFR-targeted antibodies. Thus, the combination of cetuximab with FOLFOX-4 in the treatment of first-line patients suffering from KRAS wild-type CRC confirmed that KRAS mutation was effective and can be used as a biomarker [164]. EGFRvIII can be taken as a good indication in various radiation therapies to raise radioisotopes against it along with other antibodies [165]. Combination therapy is another process to control CRC, for instance, Ryan et al. combined dabrafenib, panitumumab, and trametinib (for MEK inhibition) against BRAFv600E, a mutant expressed due to the reactivation of MAPK signaling. BRAFv600E signaling is mediated by EGFR. The combination therapy resulted in increased MAPK suppression; however MAPK is a resistance mechanism [166]. In another case of therapy for a CRC patient treated with 5-FU, irinotecan, bevacizumab, and oxaliplatin resulted in resistant disease. Two new mutants KRAS c.1633G>C and c.1645G>C were detected after the therapy (patient initially detected with mutation in KRAS, NRAS, BRAF, PI3KCA). This shows that mutation is worsening the disease, and preventive measurements should be taken [167]. Recently identified monoclonal antibody tomuzotuximab was detected safe and showed antitumor activity in patients having advanced metastasis [168]. The use of natural chemopreventive agents like ginseng, green tea, and curcumin also showed good result in suppressing EGFR signaling and inducing apoptosis in vivo.

11.7.2 FGFR

The use of anti-FGFR2 therapy and their specific isoforms is considered as an innovative treatment option for CRC patients. CRC growth, invasion, and migration can be inhibited by targeting shRNA to FGFR2 [169]. Inhibitors PD-161570 and PD-173074 prevent the elongation of fibroblast-induced cancer cells. FGFR2IIIc expression results in poor prognosis; the human anti-FGFR2IIIc monoclonal antibody (HuCAL GOLD) is used to inhibit CRC growth. Thus, it can be used as therapeutic target for CRC [170]. Therapy involving combination of recombinant form FGFR1 protein vaccine and gemcitabine in lower dose suppresses the growth of tumor and promotes anti-angiogenesis [171]. Chen et al. [172] attached the self-designed truncated form of FGF peptide with cationic liposomal doxorubicin and paclitaxel, which resulted in inhibition of tumor growth and good survival rate of tumor mice. Doxorubicin, a DNA-damaging agent, has been used in order to upregulate the activity of FGFR4 [161]. The interaction of FGF19-FGFR4 which signals for CRC can be inhibited by using a blocking antibody similar to FGF19 (IA6) and inhibits the CRC cell lines (HCT116) and xenograft tumor (Colo201) by binding to FGFR4 [33]. However, the chemoresistance of the FGFR4 against chemo agents (5-FU) led the researchers to bring novel therapy plans. Silencing of FGFR reduced the activity of STAT3 that leads to downregulating c-FLIP protein expression. Thus, combination therapy of 5-FU and oxaliplatin with silenced FGFR4 can be taken as a potential therapeutic route to control the disease [173].

Angiogenesis is a biological process, which leads to metastatic CRC; therefore inhibiting angiogenesis is an effective treatment for CRC. Ramucirumab, aflibercept, and bevacizumab are some effective antiangiogenic agents available [174, 175]. The use of nintedanib as an oral inhibitor of angiokinase, which targets FGFR, showed marginal increase in progression for CRC. Clinical development is still ongoing in the context of CRC [176]. FGF1 and FGF2 are the key aspects in angiogenesis, but in this case, a mutant form R50E is identified from the integrin-binding FGF1 complex. R50E suppresses the formation of complex, thereby preventing the angiogenesis and tumor proliferation in vivo by FGF1 (from CAM assays). Thus R50E, a mutant form, can be used as an anticancer and anti-angiogenesis agent [177].

11.8 Conclusion

Designing drug delivery system and targeting various overexpressed proteins such as FGFR, EGFR, COX-2, and CD44 are used as therapeutic targets to overcome CRC. However, the heterogeneity nature of cancer cells is still a limitation and complicates the process. Although there is significant development in chemotherapy and types of agents that are being used, the overall survival rate of metastatic CRC remains poor [178].

The resistance mechanism developed by cancer cells affects the outcome of clinical treatment performed by the blockade of receptors (EGFR, FGFR) with

anti-receptor antibodies/monoclonal antibodies along with chemotherapy agents. In their relapse period, patient eventually develops mutations from resistant cancer cells like RAS, RAF, and BRAF or at the domains of a receptor. However, clonal evaluation of mutant or tumor cells in such a process is being developed to detect the type of mutation and clinical and molecular outcome of CRC patients [179]. Thus, developing a profile with updated changes in mutations at molecular level of cancer cells is essential for the researchers and an appropriate novel ideology focused for the benefit of the patient.

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EGFR and Its Role in Colorectal Cancer

12

Saimila Momin and Ganji Purnachandra Nagaraju

Abstract

Colorectal cancer is one of the most common and fatal types of cancers which congregates in the colon or rectum regions of the body. One of the ligand-receptor complexes that plays a major role in the progress of colorectal cancer includes the epidermal growth factor receptor (EGFR). EGFR is expressed on the cellular surface of a variety of cells such as epithelial and muscle cells. Upon binding to a ligand, the overall EGF-EGFR complex leads to a cascade of signaling within the cell to ultimately allow for multiple cellular functions including proliferation, differentiation, and dedifferentiation. When the EGFR activation is overexpressed, it can lead to colorectal cancer. Currently, there are many investigations aiming to study how the complex can be manipulated especially through inhibitors and antibodies to reverse the advancement of colorectal cancer. This chapter aims to understand the EGF-EGFR complex and explore the current research studies investigating EGFR and its role on colorectal cancer.

Keywords

EGF · EGFR · Colorectal cancer

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12.1 Introduction

Colorectal cancer is described as a cancer that is aggregated in the colon or rectum of the large intestine; depending on its location, colorectal cancer is also known as colon cancer and rectal cancer, respectively. Under normal conditions, the colon aids in absorbing nutrients and water back into the system and in transporting wastes to the rectum. In most cases either an adenomatous or hyperplastic polyp emerges on the inner lining of the colon which can lead to colorectal cancer [1]. Cancerous polyps can eventually extend from the walls of the colon to the outermost layer and ultimately make its way toward the blood vessels [1]. Colorectal cancer is the third most common and threatening types of cancers for both males and females in the United States [2, 3]. However, there are ways to prevent this cancer either through lifestyle changes and treatment options.

There are many risk factors associated with colorectal cancer that can be studied and identified in order to prevent colorectal cancer. Risk factors for colorectal cancer include genetic mutations in oncogenes or tumor suppressor genes; environmental contact, such as alcohol consumption and radiation; genetic lineage of the disease, such as Lynch syndrome or familial adenomatous polyposis syndrome; and any other associated disease states linked to colorectal cancer, such as Crohn's disease or even diabetes [4]. However, colorectal cancer can be both easily prevented and detected with the epidermal growth factor receptor (EGFR) as it is overexpressed during the cancer's development. By designing a form of treatment that includes targeting this receptor, colorectal cancer mortality rates can be greatly reduced.

12.2 Epidermal Growth Factor Receptor

Currently, colorectal cancer is first screened by examining stool samples and performing colonoscopies to determine the location of the polyps. Depending on the anatomical location of the growth, the cancer is identified as either colon or rectal cancer. The two cancers share many striking features; however, due to their location, they have different molecular characteristics. Upon detection of colorectal cancer, a pathological assessment of the cancer is completed in order to see if resection of the tumor or chemotherapy is the best treatment option. Treatment depends on how early the tumor is detected and if the tumor will respond to treatment options such as FOLFOX chemotherapy or anti-EGFR monoclonal antibodies [4]. Another possible form of treatment may become available in the future by studying the epidermal growth factor receptor and its role in colorectal cancer.

The epidermal growth factor receptor (EGFR) plays a major role in the development and advancement of many types of cancers. EGFR is a 170-kDa transmembrane protein that closely resembles the tyrosine kinase receptors and is one of the four distinct members of this family of receptors [3, 4]. The receptor is expressed on the cellular surface of epithelial, stromal, glial, and muscle cells. Within the ligand-receptor complex, there are two cysteine domains and an alpha-helix-shaped

transmembrane domain which bind the ligand-binding region to the intracellular receptor which consists of three areas or domains [4]. EGFR plays a crucial role in cellular proliferation and programmed cellular death and cellular migration, differentiation, and dedifferentiation and during fetal development in organogenesis [5]. EGFR not only serves for cellular growth and development, but it also is a strong prognostic biomarker for multiple cancer types, including colorectal cancer (Fig. 12.1).

12.3 Cellular Mechanism of EGFR

When a ligand binds to EGFR, it leads to a cascade of signaling within the cell to ultimately allow for cellular growth, development, and division. The different functions of EGFR are controlled through ligands such as EGF, TGF, amphiregulin, heparin-binding EGF, betacellulin, or epiregulin, which in turn modulate various signaling pathways including PLC-gamma-1, RAS-RAF-MEK-MAPKs, phosphatidylinositol-3 kinase and AKT, Src, the stress-activated protein kinases, PAK-JNKK-JNK, and the signal transducers and activators of transcription [4]. The release of EGFR ligands is strictly monitored by the metalloprotease enzymes of the ADAM family; ADAM17 plays a major role in the release of these EGFR ligands through enzyme modulation [6]. In order to activate the EGFR signaling pathway, the ligand-induced receptor dimerization is initiated and followed by phosphorylation of tyrosine in the C-terminal of one of the EGFR by the other tyrosine, which is within the kinase domain of the other associating EGFR [7]. This phosphorylation allows for a platform where effector proteins can congregate, primarily occurring through the Src homology 2 and phosphotyrosine binding (PTB) [7]. Effector proteins and adaptor proteins can now trigger other signaling pathways within the cell, such as KRAS-BRAF-MEK-ERK and STAT signaling pathway (anti-apoptotic), AKT kinase pathway, and phospholipase C gamma protein pathway, and stimulate other kinases, such as phosphoinositide 3-kinase [7]. Under normal conditions, the EGFR pathway causes cellular proliferation, angiogenesis, migration, survival, and adhesion [4, 7]. However, when this pathway is not properly modulated, often as a result of mutations, its actions play a prominent role in the advancement of cancerous cells.

Overall, when ligands bind to EGFR, the receptor autophosphorylates the kinase domain in the cytoplasm of the cell, which in turn amplifies the cellular signal through secondary messenger systems. The now phosphorylated tyrosine complex serves as a site for adapter and/or effector proteins which have Src homology 2 domains or binding zones for protein tyrosines [4, 8, 9]. Ultimately, signals are sent to the nucleus, and transcription results in cellular activities such as proliferation and mitotic division.

EGFR activation is necessary for normal body function; however, in the case of colorectal cancer, it can be overexpressed. The cellular mechanisms that result with overactivation of this receptor, which lead to colorectal tumors, can be linked to disruptions in cellular division and the promotion of tumor-like factors. Depending

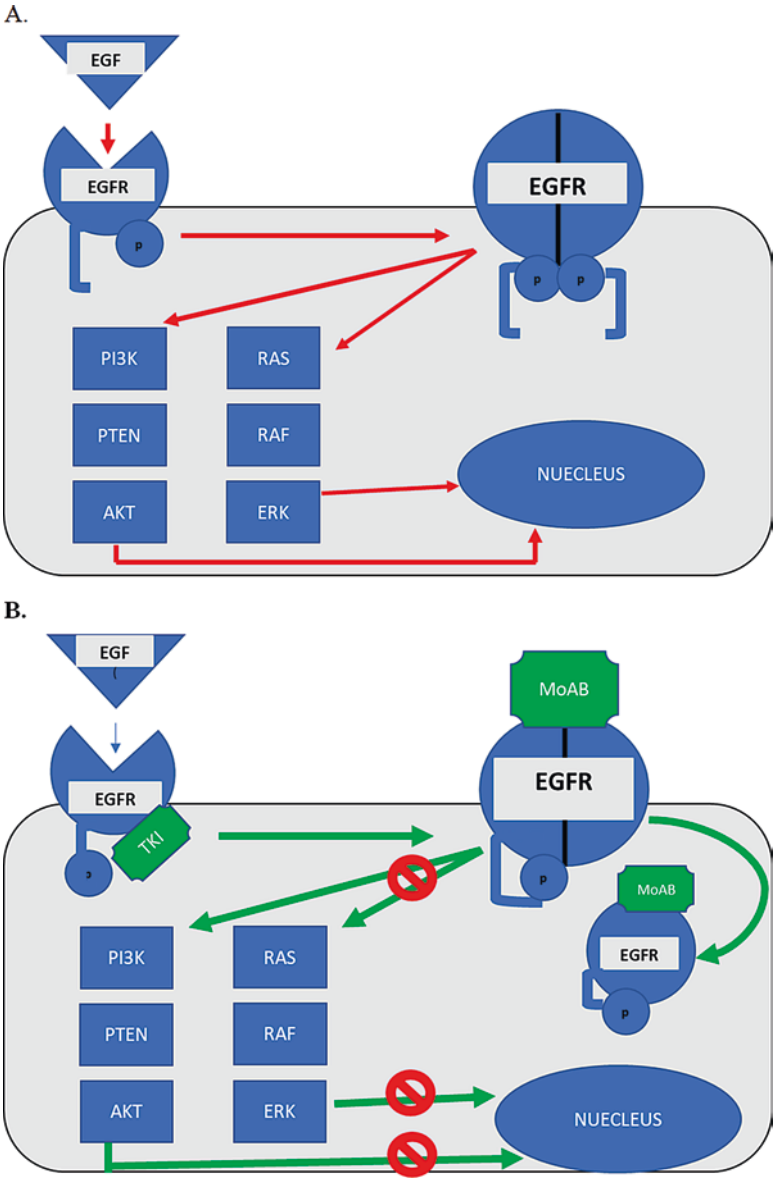

Fig. 12.1 EGF-EGFR receptor-ligand complex (**a**). Diagram outlining the intracellular mechanism of the epidermal growth factor receptor or EGFR activity. In the nucleus, the signaling pathway is able to enable cellular mitotic division and cellular proliferation under normal conditions (**b**). Diagram outlining the intracellular mechanism of the epidermal growth factor receptor or EGFR activity when inhibited by monoclonal antibodies and/or inhibitors. In the nucleus, the signaling pathway is able to enable cellular mitotic division and cellular proliferation. *EGF* epidermal growth factor, *EGFR* epidermal growth factor receptor, *p* phosphor, *RAS* rat sarcoma, *PTEN* phosphate and tensin homologue deleted at chromosome 10, *ERK* extracellular signal-regulated kinase, *RAF* rapidly accelerated fibrosarcoma, *PI3K* phosphatidylinositol 3-kinase, *TKI* tyrosine kinase inhibitor, *MoAB* monoclonal antibodies

on the cancer type, the mechanisms resulting in EGFR expression are different. For example, in breast cancer, overexpression of EGFR activity is strongly correlated to increased blood vessel formation (angiogenesis) and cellular proliferation. However, in the case of colorectal cancer, significant decreases in microRNA-143 and microRNA-145, which are due to environmental exposures, diet, and upregulation of RAS and MYC genes strongly contribute to the negative regulation of G1 factors and consequently lead to the disruptions in cellular mitotic activity [4, 10]. The relationship between EGFR activity and colorectal cancer can be used to generate treatment and preventative options.

12.4 Deactivation of the EGF-EGFR Receptor-Ligand Complex

The EGFR complex is not a continuous process that occurs in the cell; there are many processes involved to diffuse the signaling pathway. Overall, the pathway and the receptor can be deactivated by undergoing the following processes: internalization, ubiquitination (in which a protein is deactivated by ubiquitin), and degradation of the receptor-ligand complex [7, 11]. During the end of the signaling pathway, clathrin-coated pits from the cell's membrane come into play and internalize the EGF-EGFR receptor-ligand complex by allowing the generation of endocytic vesicles, which will fuse with early endosomes to ultimately discharge the receptor-ligand complex into the early endosomes [7]. In the early endosome, EGFR interacts with clathrin-binding protein AP-2 along with other interacting proteins that results in a modified EGFR [7, 11, 12]. Adapter molecules, especially Casitas B-lineage lymphoma proto-oncogene, will bind to the receptor to promote EGFR ubiquitination [7, 11]. At this point, the complex will either be recycled to be reused by the cell surface, or it will be engulfed by intraluminal vesicles to be degraded.

12.5 Correlation Between Western Diets and Overexpression of EGFR Activity

According to research studies that are investigating the relationship between colorectal cancer and EGFR activity, there is a strong correlation between colorectal cancer and western diet style, suggesting that this diet style contributes to the overexpression of EGFR activity. Western diets typically contain high contents of fat and increased consumption of red meat. Azoxymethane or AOM results in the O6 methylation of DNA guanine bases which then causes the activation of mutations in K-ras and CTNNB1 [4, 13]. The aforementioned results were exhibited when tested in mice by feeding them a western-style diet. Furthermore, in their investigation, they reported increased levels of proto-oncogenes CTNNB1, MYC, CNND1, and PTGS2 and the EGFR ligand TGFα [8]. Through research, it has been concluded that western diets have the ability to significantly increase the expression of ADAM17 and overregulate TGF-α and amphiregulin ligands, resulting in an

overexpression of EGFR activity [14]. Therefore, studies suggest that diet control may be a strong preventable method in inhibiting EGFR overexpression before it leads to a cancerous body state.

12.6 Tyrosine Kinase Inhibitors and Other Therapeutic Agents

Currently, EGFR pathway signaling inhibition appears to be an appealing therapeutic agent in treatments for a variety of cancers, including colorectal cancer [16, 17]. Since EGFR has been strongly linked to colorectal cancer, many treatment strategies are focused on targeting EGFR with tyrosine kinase inhibitors, such as gefitinib and erlotinib, and monoclonal antibodies such as cetuximab [2, 4, 9]. These inhibitors and antibodies show promising results in clinical trials as they are both biologically available and can be easily introduced to the human body.

Kinase inhibitors bind to EGFR and prevent the ligand-receptor complex from cascading a series of reactions through secondary messenger systems leading to the inhibition of all cellular activity that is modulated by the nucleus. Monoclonal antibodies undergo a similar mechanism as kinase inhibitors target the tyrosine kinase domains of the intracellular region. Overall, there are a strong correlation and link to EGFR overactivity and colorectal cancer. The disruptions in the signaling pathways of EGFR can play a crucial role in the progression of colorectal cancer along with other cancer types, such as breast cancer.

Both tyrosine kinase inhibitors and monoclonal antibodies have been used in clinical trials to investigate the effect of these therapeutic agents on cancers from epithelial origin, including colorectal cancer and lung cancer. Phase I and II clinical trials have been completed, and the results suggest that treating patients with both a tyrosine kinase inhibitor, such as gefitinib, and a chemotherapy drug, from the beginning of diagnosis, yielded the most promising results, with a response rate of 70% [12, 15]. Many studies have been conducted to see how these agents affect the cancer at both molecular and cellular levels and also how these agents can be used for treatment; however, the studies are quite limited for colorectal cancer. Therefore, further research needs to be conducted in order to better understand which set of drugs and agents are optimal in treatment.

12.7 Conclusion and Further Research

Even though we understand the underlying mechanisms that strongly link EGFR activity and various cancers, additional research needs to be completed on how EGFR can be manipulated to prevent various cancer types in the body but, specifically, in colorectal cancer. Even though EGFR is an excellent prognostic biomarker and antibodies such as cetuximab have shown positive results as treatment options with colorectal cancer, additional research to holistically understand the role of EGFR needs to be conducted.

Further studies include but are not limited to defining the specific molecular mechanisms that lead to the abnormal function and overexpression of EGFR; how kinase inhibitors and monoclonal antibodies can be converted into therapeutic agents for fighting cancer; ways in which we can manipulate or alter the cellular mechanisms associated with the EGFR, for example, limiting the production of growth factors; and antagonists that bind to EGFR to either decrease or completely cease cellular proliferation. Additionally, we can also investigate ways to promote degradation of the EGF-EGFR receptor-ligand complex. In other words, we clearly identify molecules and signals that enhance or lead toward degradation. Research of these questions could ultimately lead to the design of drugs, medications, or treatment plans that can fight against colorectal cancer and reverse its adverse effects.

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TGF-ß and Tyrosine Kinases: Context in Colorectal Cancer

13

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Abstract

Tyrosine kinases and transforming growth factor-ß (TGF-ß) are known to be the hallmark molecules that drive many metastatic cancers, including colorectal cancer (CRC). There is an urgent need to understand the role of these molecules (and their underlying molecular mechanisms) that regulate CRC disease progression. This chapter highlights recent progress made in our knowledge of the molecular mechanisms that underlie the TGF-ß signaling pathway in CRC growth. The role of TGF-ß in promoting pro-angiogenic events such as epithelial to mesenchymal transition, invasion, and migration is revealed. We also discuss the importance of different tyrosine kinases as metastatic drivers of TGF-ß-regulated CRC pathogenesis, as well as noting different therapeutic products and genes that can inhibit the TGF-ß signaling pathway, which itself contributes to CRC progression. In short, this essential chapter discusses the overall role of tyrosine kinases in TGF-ß-implicated CRC progression.

Keywords

Colorectal cancer · Tyrosine kinases · Transforming growth factor-ß · Metastasis

Abbreviations

CD24	Cluster of differentiation 24
CRC	Colorectal cancer

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EC	Endothelial cells
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
LEF	Lymphoid enhancer factor
PDGFRB	Platelet-derived growth factor receptor B
STYK1	Serine threonine tyrosine kinase 1
TGF-ß	Transforming growth factor-ß
TSP-4	Thrombospondin-4
VEGF	Vascular endothelial growth factor
WNT	Wingless-type MMTV integration site family member

Colorectal cancer (CRC) is one of the most important cancers, in terms of its rising incidence and rising mortality. Indeed, CRC mortality rates are increasing every year in the western world. One of the main causes of CRC is the alteration of tumor-promoting and tumor-suppressing genes [1]. The incidence of CRC is higher in developed countries, especially in regard to CRC associated with such factors as obesity, physical inactivity, and smoking [2]. Although CRC has many causative factors, the gut microbiota play a key role in its evolution, particularly through their metabolization of protein and fat residues into pro-inflammatory and tumorigenic metabolites [3]. Regardless of its causes, many treatments are now available for the treatment of both primary and metastatic CRC [4].

Transforming growth factor-ß (TGF-ß) is one of the most important cytokines that drive many critical cellular processes, such as proliferation, migration, adhesion, and epithelial-mesenchymal transition (EMT) [5]. Regulation of the TGF-ß pathway is initiated through the affinity of TGF-ß ligands for TGF-ßI and TGF-ßII receptors, which then phosphorylate the downstream regulators Smad 2/3, and their association, together with Smad4, directs them to the nucleus, thereby targeting TGF-ß-responsive proteins [6, 7]. TGF-ß is context-dependent in terms of its role as either a tumor repressor or a tumor supporter. In the beginning phases of tumor growth, TGF-ß functions as a suppressor, but during the final course it promotes cancer spread. Aberrant changes in the TGF-ß pathway lead to many pro-inflammatory functions that are critical in processes such as angiogenesis, metastasis, and tumor progression [8]. TGF-ß has a profound effect in driving cancer progression, by enhancing EMT and metastasis via both the canonical SMAD signaling pathway and non-canonical signaling pathways [9–14]. One important note is that patients with advanced stage rectal tumors that express low levels of TGF-ß have a poor prognosis [15]. The existing literature on TGF-ß as a tumor inhibitor indicates that it affects apoptosis; also, the migration of interstitial epithelial cells revealed that suppression of the TGF-ß pathway led to controlled migration and apoptosis resistance [16]. There is an increased risk of CRC growth, owing to genetic variations, along with deregulation of the TGF-ß pathway [17].

The growing proof of the effects of TGF-ß during the course of CRC mainly concerns its contribution to changing the fate of a normal cell, allowing the cell to

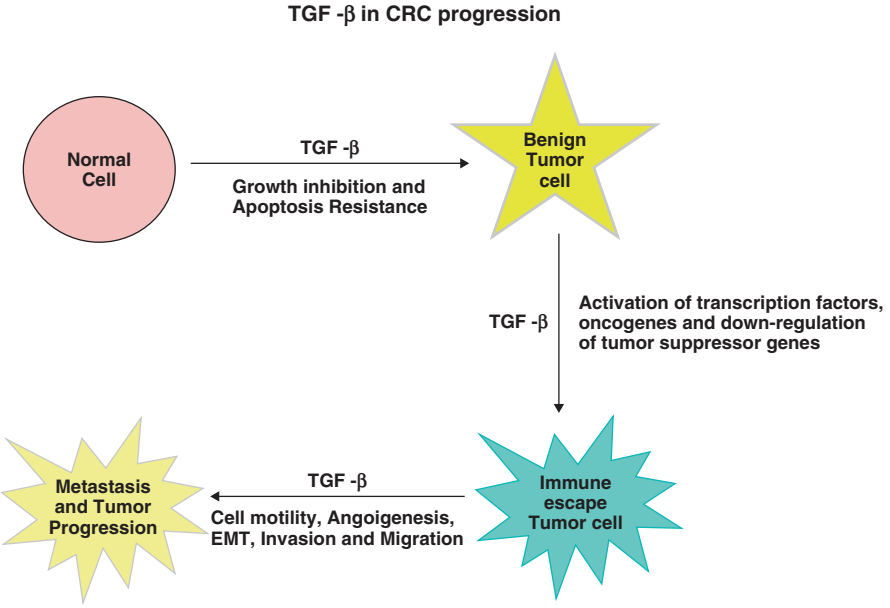

Fig. 13.1 The impact of transforming growth factor-ß (TGF-ß) in transforming a normal cell to a benign-stage cancer cell and permitting it to escape from immune surveillance, allowing the cell to become invasive, migrate, undergo epithelial-mesenchymal transition (EMT), and finally metastasize into the lungs and liver, leading to tumor progression. All these events are, in part, facilitated by TGF-ß

escape its normal growth and apoptosis stages and to undergo pro-inflammatory processes in which the cell loses its epithelial polarity and undergoes EMT, becoming more migratory and invasive, and able to transmigrate through the bloodstream to metastasize to the lungs and liver, thereby enhancing tumor progression (Fig. 13.1).

All these abovementioned important findings associated with TGF-ß in CRC progression enhance its impact as a potential therapeutic target; thus, targeting this molecule would definitely be helpful in the treatment of both early-stage and metastatic-stage CRC.

13.1 TGF-ß as an EMT Regulator

EMT occurs naturally at the time of cell differentiation and also during growth. However, benign cancer cells express low levels of epithelial markers and high levels of mesenchymal markers. TGF-ß acts as a regulator of EMT by promoting the regulation of EMT-relevant proteins, including Snail1, Slug, and ZEB1. Downstream partners of the TGF-ß signaling pathway, such as SMADs, are also known to interact with EMT-associated genes to promote EMT. This interaction induces EMT,

which contributes to the increased invasion and migration of tumor cells associated with metastasis [18–20]. A recent study notes that TGF-ß promotes EMT in the presence of WNT protein [11]. The downstream effectors of TGF-ß, i.e., SMAD proteins and the WNT effector LEF-1, cooperatively regulate EMT, which shows cross-talk between the two pathways [21, 22]. The neural growth factor Neuropilin-2 induces EMT of CRC cells in a TGF-ß-dependent manner; accordingly, therapeutic targeting of this interaction may be crucial for elucidating multiple tumorigenic processes like EMT [23]. A recently identified protein named damaged DNA-binding protein (DDB)-2 is downregulated in CRC and is a potential player in suppressing EMT and the metastasis of CRC cells; specifically, it inhibits EMT promoted by TGF-ß [24]. Studies indicate that TGF-ß-induced EMT is also able to induce a cancer stem cell phenotype, by forming tumor spheres that promote transendothelial migration and invasion, whereas the blockage of TGF-ß signaling by the anti-metastatic peptides P17 and P144 reversed these effects. These investigations highlight the importance of P17 and P144 in TGF-ß-supported metastatic CRC [25]. Another important study on TGF-ß showed that the TGF-ß-associated promotion of the adhesion and migration of colon adenocarcinoma cells was inhibited by N-hydroxycinnamide derivatives of osthole, mainly via the suppression of Smad2, which is a TGF-ß family member [26].

In addition, genetic and epigenetic modifications and alterations in the TGF-ß signaling pathway and its associated components are strongly implicated in the spread of several malignant tumors, including CRC. Almost 40–50% of CRCs are diagnosed with mutations in TGF-ß receptors I and II and downstream mediators Smad2, Smad3, and Smad4 [27–29]. Specifically, Smad4 mutations account for 16–25% and Smad2 mutations account for about 6% of CRC incidence [30].

Accordingly, critical understanding of the relevance of each component of the TGF-ß signaling pathway, which has a substantial effect in inducing EMT, will provide insights for the development of novel therapies to treat metastatic CRC.

13.2 TGF-ß as a Regulator of Migration and Invasion

Recent studies report that the pro-inflammatory effects of TGF-ß on CRC are suppressed via the inhibition of EMT [31]. A tripartite motif containing 25 (TRIM25) plays an important task as an E3 ubiquitin ligase, an oncogene that promotes pro-inflammatory processes like invasion and proliferation through TGF-ß signaling [32]. It has been stated that TGF-ß either acts independently to promote migration and invasion, or it depends on cross-talk with other signaling pathways or through microRNAs; for example, TGF-ß promotes the migration of CRC cells through miR-130b [33]. The tumor repressor N-myc-downstream-regulated-gene2 (NDRG2) inhibits TGF-ß-induced pro-metastatic processes by inhibiting EMT, thereby attenuating the invasion and migration of CRC cells [34]. Recent literature notes that TGF-ß-associated pro-metastatic effects have been inhibited by a novel signaling regulator protein (km23-1) in CRC, suggesting that these inhibitors could have a potential role in curing CRC via inhibiting the tumor promoter TGF-ß [35].

Plasminogen activator inhibitor 1 (PAI-1), a target of TGF-ß superfamily members, regulates the migration and adhesion of CRC; an alkaloid named oxymatrine inhibits PAI-1 as well TGF-ß/Smad family proteins, suggesting that some drugs could indirectly target TGF-ß-responsive genes to inhibit TGF-ß pro-metastatic responses [36].

TGF-ß has tumor invasion-promoting activity, as it regulates E-cadherin by decreasing its expression, along with with that of invasion-related integrins such as αIIIß1 integrin, during carcinogenesis [37, 38]. The paradoxical nature of TGF-ß in relation to cancer cells is shown by the cells undergoing alterations and gaining resistance to the tumor-suppressive behaviors of TGF-ß such as its anti-apoptotic effect, and also undergoing tumor-promoting effects such as adhesion, migration, and invasion [39, 40]. Many studies of CRC indicate that TGF-ß has a primary role in inducing invasiveness and metastasis, thus increasing pathogenicity [39].

Cancer cell migration and invasion are the hallmarks of CRC progression and they will have a considerable effect on the final patient outcome. Understanding the molecular mechanisms that lead to these pro-angiogenic properties of the cancer cells will definitely be helpful in developing anti-metastatic drugs to attenuate the fatal stage of metastasis.

13.3 TGF-ß as a Regulator of Metastasis

It has been reported that TGF-ß can foster a microenvironment around tumor cells that can enhance metastasis and tumor progression [41]. Inhibition of the TGF-ß signaling pathway will certainly be useful to treat metastasis-driven cancers. It has been shown that SMAD4 mutations are associated with the progression of colon cancer [42]. It has also been shown that glucose-regulated protein 78 (GRP78) activates the TGF-ß pathway, which strengthens cell-matrix adhesion and EMT in colon cancer cells [43]. Recent studies demonstrate that microRNAs also promote carcinogenesis by targeting TGF-ß signaling family proteins such as SMADs [44]. Some important regulators that drive colon cancer cells to metastasize to the liver are induced by TGF-ß, e.g., platelet-derived growth factor receptor C (PDGF-C), interleukin (IL)-11, proteoglycan-4, and periostin [45]. Research on TGF-ß downstream proteins shows that activin, a member of the TGF-ß family, has a critical role in TGF-ß-induced pro-metastatic actions in CRC [46].

Another important factor contributing to metastasis is angiogenesis. It has been stated that thrombospondin-4, an extracellular matrix protein that is induced by TGF-ß in endothelial cells [47], contributes to tumor growth stimulation through TGF-ß [48]. The consequences of multiple malfunctions in metastasis that occur during CRC progression are studied to combat CRC. One such malfunction is mediated by a central molecular network that includes entities such as microRNA-21, CD24, and Src, which are essential in the regulation of CRC progression [49]. An important anti-metastatic agent named curcumin is able to inhibit inflammatory processes and the metastasis of CRC [50]. Ample evidence reveals that curcumin can be an effective treatment that inhibits the TGF-ß-induced tumor invasiveness of CRC [51]. TGF-ß enhances EMT in stromal cells and the cross-talk between

stromal fibroblasts and cancer stem cells can be suppressed by curcumin, thereby inhibiting the EMT and metastasis that is partly mediated by TGF-ß [52].

Metastasis is a consequence of events like invasion, migration, and EMT, which are controlled by factors such as cytokines, deregulated signaling pathways, genetic and epigenetic mutations, the tumor microenvironment, immune surveillance cells, pro-angiogenic factors, and apoptosis regulators. The existing evidence shows that many of the metastatic events in CRC are partially mediated by TGF-ß. Although much research has been devoted to delineate the actions of TGF-ß in CRC promotion, there is a knowledge gap as to how TGF-ß is involved as a complicated carcinogenesis regulator. To fill this knowledge gap, the paradoxical role of TGF-ß has to be clearly investigated.

13.4 Role of Tyrosine Kinases in TGF-ß-Induced CRC Progression

13.4.1 Platelet-Derived Growth Factor Receptor B (PDGFRB)

Protein tyrosine kinases are important signaling regulators that have diverse roles in many cellular processes [53]. Platelet-derived growth factor receptor B (PDGFRB) is a kinase family member that is a key player in metastasis. Studies have reported that epithelial cells, after undergoing EMT, require PDGFRB to further acquire the pro-metastatic behavior that leads to liver metastasis. It is interesting to note that the inhibition of TGF-ß signaling reverses the EMT process and inhibits the expression of PDGFRB [54]. Signaling pathway alterations give rise to cancer progression, and one such metastatic disease promoter is the epidermal growth factor receptor (EGFR) signaling pathway [55]. In addition, serine/threonine pathway kinase receptors transduce signals through TGF-ß family members [56], particularly TGF-ß and activins [57]. Somatic mutations associated with the receptor tyrosine kinases are associated with CRC progression; it has been shown that serine/threonine kinase 11 (STK11) is a tumor inhibitor, and its genetic changes and loss of heterozygosity highlight its role in driving CRC progression [58].

13.4.2 Serine Threonine Tyrosine Kinase 1 (STYK1)

Increased expression of serine threonine tyrosine kinase 1 (STYK1) is reported in many cancers, e.g., leukemia, prostate cancer, and CRC, which are partly regulated by TGF-ß. High expression of STYK1 leads to metastasis and is a marker of poor longevity in CRC [59–61]. STYK1 has 30% similarity with fibroblast growth factor and PDGF [62], which indicates their common multiple roles in cancer progression. It has been observed that STYK1 messenger levels were upregulated in CRC [63]. More importantly, SYTK1 leads to CRC progression and metastasis and can be an important prognosticator in CRC [59].

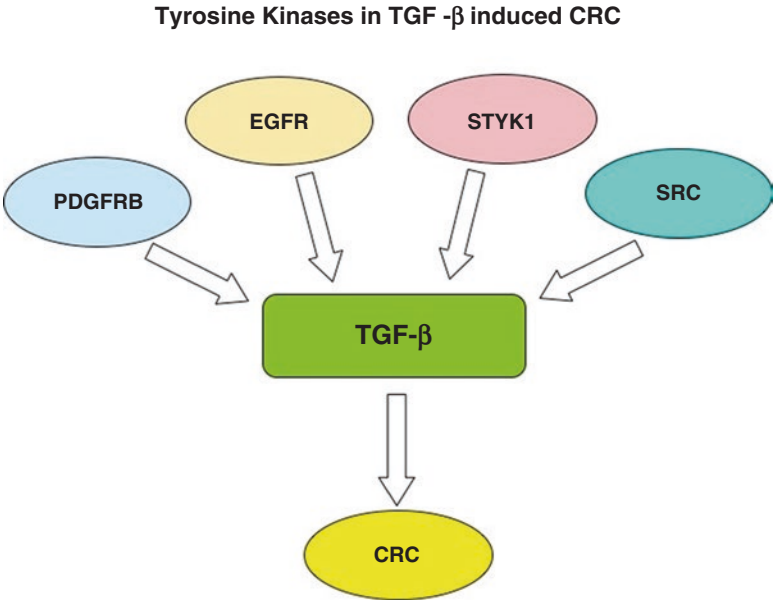

Fig. 13.2 The mediation of tyrosine kinases in TGF-ß-induced colorectal cancer (CRC). Many important tyrosine kinases are shown: e.g., platelet-derived growth factor receptor B (PDGFRB), epidermal growth factor receptor (EGFR), serine threonine tyrosine kinase 1 (STYK1), and non-receptor tyrosine kinase (SRC)

13.4.3 Src and Epidermal Growth Factor Receptor (EGFR)

Src has a potential role in regulating cellular differentiation and cancer-related processes like migration, invasion, proliferation, and angiogenesis. It has been shown that Src is activated more in metastatic tumors than in primary tumors. There are studies connecting the combined role of Src and EGFR together in the signal transduction process. Although many factors affect the transactivation of EGFR, Src is the most common transactivator [64]. Epidermal growth factor receptors correspond to ErbB family kinases. The EGFR pathway is the main pathway that is upregulated in colon cancers. In CRC cell lines Src activation is correlated with high expression of EGFR [65]. Studies indicate that vascular endothelial growth factor can stimulate Src, which can regulate cellular migration [66, 67].

To summarize, studies show that Src and its close relationship with EGFR are involved in advancing CRC progression. In accordance with this finding, current studies have also emphasized that combined drugs acting on EGFR and Src might be more beneficial than single-target drugs for the chemotherapy of CRC patients (Fig. 13.2).

13.5 TGF-ß as a Therapeutic Target in CRC

In order to develop therapeutic strategies against the TGF-ß signaling pathway, its interaction with other genes and signaling pathways has to be thoroughly understood, as do the indirect effects of tumor suppressors or promoters on TGF-β. It has been shown that galunisertib (LY2157299 monohydrate), a TGF-β receptor I inhibitor, abrogates the specific inhibition of a canonical signaling pathway, thereby proving its anti-tumorigenic properties in many cancers [68]. Another TGF-ß receptor I inhibitor, SB-431542, elicited anti-tumor responses in immune cells associated with the increased activity of TGF-ß [69]. TGF-ß provides many therapeutic approaches owing to its interaction with several signaling pathways, such as Hippo, wnt, and akt [70]. A recently identified protein-bound polysaccharide can inhibit the TGF-ß pathway through suppressing Smad2 protein, thus attenuating TGF-ß-induced EMT and metastasis [71]. A recent study has shown that a drosophila dachshund homolog (DACH1) is an important therapeutic target for treating CRC, as its loss induces cell invasion and cell growth through TGF-ß-mediated EMT [72]. Taken together, the findings of TGF-beta mediated cellular processes indicate that TGF-β is a tumor promoter and an autonomous prognosticator of CRC [73]. The depletion of Lim and SH3 protein 1 (LASP1) significantly inhibited the pro-inflammatory effects induced by TGF-ß, thereby indicating that the role of these genes could be taken into consideration for clinical intervention in patients [74]. It is interesting to note that even when CRC patients have an inactivated TGF-ß signaling pathway, they have high TGF-ß production; this is because TGF-ß influences the microenvironment, which can further develop into a high-risk CRC with relapses. Further understanding of the fundamental process that underlies the pro-metastatic pathway regulated by TGF-ß will definitely be helpful for the treatment of relapsed CRC [75]. Another important finding has demonstrated that resveratrol, a grape extract, inhibits the EMT of CRC cells by suppressing the TGF-ß/Smad signaling pathway; this finding clearly denotes the function of TGF-ß in promoting the EMT of CRC cells [76]. To draw our attention to the paradoxical role of TGF-ß, dual kinase inhibitors are available. One of these is LY2019761, which targets both Smad and non-Smad signaling pathways, thereby inhibiting CRC liver metastasis [77].

In conclusion, to develop better treatments for CRC patients, the molecular mechanisms underlying the TGF-ß signaling pathway—a pathway that drives pro-inflammatory processes such as EMT, migration, and invasion—have to be further delineated.

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Drug Resistance Against Tyrosine Kinase Inhibitor in Gastrointestinal Malignancies

14

L. V. K. S. Bhaskar and L. Saikrishna

Abstract

Gastrointestinal cancers are heterogeneous and complex among the most common human cancers. In spite of this complexity, certain types of genetic alterations are linked to specific pathological lesions. Genomic and transcriptomic analyses have disclosed molecular subtypes that are characterized by specific genetic aberrations and expression signatures. Identification of better molecular markers to assist detection and prognostic evaluation of the cancer is therefore required. Tyrosine kinases are enzymes responsible for the activation of many proteins by signal transduction cascades. Inhibitors of tyrosine kinases (TKIs) have been effectively used for clinical treatment of certain types of cancer. Chronic exposure to gradually increasing concentrations of the TKI over a period of time, cells by activating modified signaling pathway can replace the lack of signal in target therapy, leading to the development of drug resistance. In recent years, researchers have specified different subsets of tyrosine kinase inhibitors' potential resistance mechanisms in various gastric cancers. This chapter intends to provide an overview of the most recently identified molecular mechanisms of acquired resistance to tyrosine kinase-targeted therapy in various gastrointestinal malignancies.

Keywords

Gastrointestinal cancer · Tyrosine kinase inhibitors · TKI resistance · EGFR · VEGF · HER2

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Abbreviations

ABC	ATP binding cassette
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
bFGF	Basic fibroblast growth factor
CML	Chronic myeloid leukemia
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ErbB2	Epidermal growth factor receptor II (Her 2)
ERK	Extracellular signal-regulated kinase
FGFR	Fibroblast growth factor receptor
FISH	Fluorescent in situ hybridization
FLT3	FMS-like tyrosine kinase 3
FOLFOX	Folinic acid (FA)-fluorouracil (5FU)-oxaliplatin (OX)
GC	Gastric cancer
GI	Gastrointestinal
GOJ	Gastroesophageal junction
HCC	Hepatocellular carcinoma
HGF	Hepatocyte growth factor
hOCT1	Human organic cation transporter type 1
IR	Insulin receptor
JAK	Janus kinase
mAbs	Monoclonal antibodies
mCRC	Metastatic colorectal cancer
MET	Mesenchymal-epithelial transition
mPC	Metastatic pancreatic cancer
nRTKs	Non-receptor tyrosine kinases
OS	Overall survival
PDGFR	Platelet-derived growth factor receptors
PFS	Progression-free survival
PIK3CA	Phosphatidylinositol 3-kinase catalytic subunit
PPARdelta	Peroxisome proliferator-activated receptor delta
PTEN	Phosphatase and TENsin homolog deleted on chromosome 10
RTKs	Receptor tyrosine kinases
TGF-α	Transforming growth factor α
TKI	Tyrosine kinase inhibitor
TKs	Tyrosine kinases
T_m	Melting temperature
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptors

14.1 Introduction

Gastrointestinal (GI) cancers are a group of highly aggressive malignancies [1]. Gastrointestinal cancers represent a heterogeneous, complex array of disorders and diseases that include tumors of the esophagus, stomach, pancreas, gallbladder, liver, bile duct, anus, colon, and rectum. Although the underlying causes for different types of gastrointestinal cancers vary, there is a critical interplay of genetic and environmental factors that play a role in mediating the conversion of normal tissue to malignant tissue [2]. Majority of gastric cancer patients are diagnosed with advanced disease because GI cancers rarely show symptoms in the early stage. Different types of treatment are available for patients with gastrointestinal cancers. Surgical resection is one of the most effective therapies for GI cancers, in which tumor along with nearby lymph nodes is removed. Further, introduction of multiple cytotoxic drugs and combination regimens showed a significant improvement in the prognosis and progression-free survival of cancer patients. However, several adverse effects and narrow therapeutic index have blunted their potential therapeutic utility. This represents a need for developing more specific drugs.

14.2 Tyrosine Kinases and Their Signaling

Tyrosine kinases (TKs) are a subclass of protein kinase enzymes involved in the phosphorylation of select tyrosine residues in target proteins, by transferring a phosphate group from ATP. Tyrosine phosphorylation is an important covalent posttranslational modification that involved in normal cellular communication and facilitated maintenance of cellular homeostasis [3]. Tyrosine kinases are primarily classified as transmembrane receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (nRTKs). In humans, over 100 genes encode protein TKs, among them 58 genes encode RTKs which fall into 20 subfamilies, including epidermal growth factor receptor (EGFR), platelet-derived growth factor receptors (PDGFRs), fibroblast growth factor receptor (FGFR), FMS-like tyrosine kinase 3 (FLT3), and the insulin receptor (IR). About 32 genes encode soluble cytoplasmic nRTKs that fall into 10 subfamilies, including well-known Src, C-abl, FAK, and JAK. Tyrosine kinases are known to modulate the key signaling pathways that orchestrate cancer cell proliferation, apoptosis, and angiogenesis [4, 5]. Abnormal kinase activity plays a critical role in neoplastic development and progression. Abnormalities in kinase activity may arise due to either mutations or changes in their expression level of TKs [6]. As many cancers are caused by mutations in TK genes, a new class of drugs that block or attenuate TKs activity has been developed.

14.3 Tyrosine Kinase Inhibitors

Several small molecules, which can compete with the ATP binding site of the tyrosine kinases, have been identified [7]. These small molecules affect different sites of cancer cells and shutdown the subsequent signaling of attached tyrosine kinases. Since then, tyrosine kinase inhibitors (TKIs) play increasingly important role in the treatment of cancer and giving promising results. Most of TKIs are hydrophobic compounds; thus they can rapidly reach their specific intracellular targets to arrest aberrant signaling pathways in malignant cells [8]. Tyrosine kinase inhibitors are typically very well-tolerated medicines, and due to their more specific mechanism of action, the side effects on normal tissues are minimal or clinically insignificant. Due to their safety profile, TKIs showed better response in combination with chemotherapy and radiation [9–11]. Based on the binding site, TKIs can be largely classified into four categories: (1) type 1 ATP competitive inhibitors that bind to the active conformation of the kinase [12], (2) type 2 ATP competitive inhibitors that bind to the non-active conformation of the kinase [13], (3) allosteric inhibitors that bind outside ATP binding site which disrupt the interaction between ATP and the kinase pocket [14], and (4) covalent inhibitors that bind covalently to ATP binding site of kinase [15]. Further, monoclonal antibodies (mAbs) kill tumor cells by blocking cell surface receptor function and by recruiting immune cells and complement to the antigen-antibody complex. The monoclonal antibodies and small-molecule receptor tyrosine kinases with therapeutic use are given in Fig. 14.1.

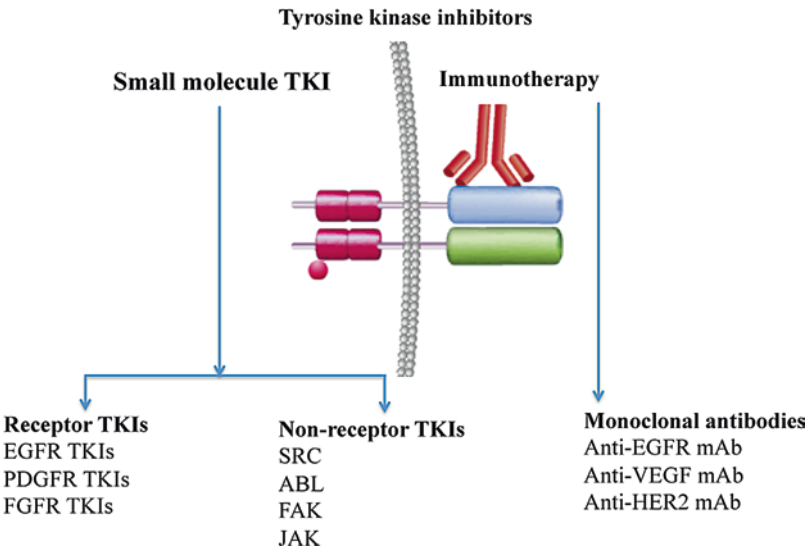

Fig. 14.1 Classification of tyrosine kinase inhibitors

14.4 Kinase Inhibitor Selectivity

Investigating intracellular targets, target effectiveness and validation of a small-molecule kinase inhibitor are essential for understanding the potential mechanistic basis of particular inhibitor for its anticancer activity in various tumors [16]. The initial evaluation of a new kinase inhibitor includes tests for their potential to inhibit kinase-catalyzed phosphotransfer from ATP to a substrate protein. Kinase specificity profiles of many kinase inhibitors have been evaluated using this approach [17]. The ability of a test kinase inhibitor to alter the melting temperature (T_m) of a kinase also reflects the binding affinity of inhibitor to kinase [18]. A larger shift in T_m can occur when there is higher affinity of the inhibitor to the kinase. Several engineered cell lines are in use to assess the inhibitory effect and cellular selectivity of kinase inhibitors [19]. Assessment of on- and off-target effects of the TKI is only possible through evaluation of its selectivity on an organismal level by determining organ or compartment distribution of the drug and monitoring the pharmacodynamic end points [20].

14.5 Mechanisms of TKI Resistance

The cytotoxic effects of the kinase inhibitors exert a selective pressure that drives cells to acquire resistance through forced mutations in the kinase gene [21]. In addition, non-mutational TKI resistance mechanisms such as alteration in gene copy number, modification of signaling pathway, and overexpression of ATP-binding cassette (ABC) transporter protein have been documented [22, 23]. Basic mechanisms of therapeutic resistance to tyrosine kinase inhibitors are given in Fig. 14.2.

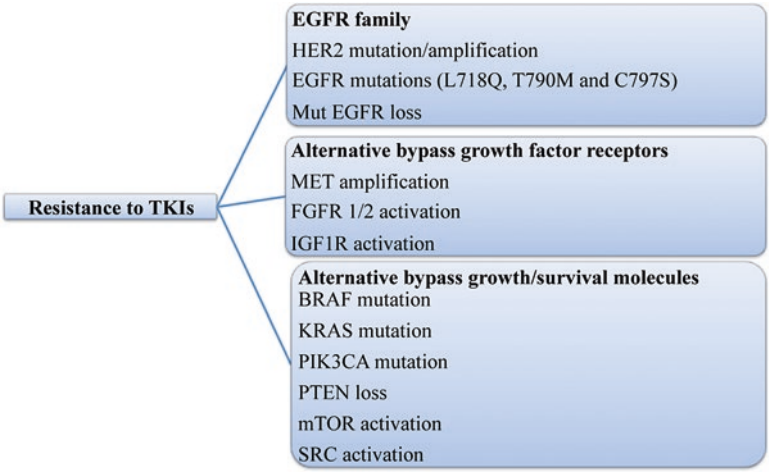

Fig. 14.2 Mechanisms of therapeutic resistance to tyrosine kinase inhibitors

14.5.1 Mutations

Point mutations in the kinase domain are the most common mechanism of acquired resistance to TKIs. The mutations in the kinase domain decrease the affinity of the TKIs to binding domain without changing its catalytic activity. The mutations that occur around binding site may cause conformational changes and prevent TKI approach through steric hindrance. Further, few mutations enhance the binding affinity of kinase for ATP [24]. There are two main schools of thought regarding the emergence of mutation in tumors after TKI therapy. The first school of thought believes that the development of mutation is due to the selection of preexisting cell population [25]. The second school of thought greatly believes that the addiction of a cell on a specific oncogenic survival pathway forces genomic instability and allows the induction of mutations [26].

14.6 Alteration in Gene Copy Number

Proper balance between active and repressed states of genes is essential for cell viability. Malignant cells treated with TKIs tend to acquire persistent genetic alterations to tolerate inhibition. An important example for this alteration is the activation of mesenchymal-epithelial transition (MET) factor gene, encoding receptor kinase for hepatocyte growth factor. Gaining MET extra copies have a selective advantage under the selective pressure of the drug to protect cell from DNA damage. Fluorescent in situ hybridization (FISH) analysis revealed that the acquired copies of MET are localized on marker chromosome [27]. MET amplification is often complemented with EGFR or KRAS amplification leading to TKI therapy failure [28, 29]. In this context, modest restoration of sensitivity to EGFR inhibitors was achieved when MET signaling was inhibited [30]. Overexpression of hepatocyte growth factor (HGF) in the resistant specimen is indicating that the HGF alone modulates the induction of drug resistance [31]. Blockade of EGFR and the downstream pathways can overcome HGF-mediated resistance.

14.7 Activation of the Bypass Pathways

Several line of evidences demonstrated that synchronous activation of redundant kinases could induce resistance via activation of alternative pathways. The activation of an alternative RTKs is known to bypass the inhibitory effects of TKI. Imatinib-resistant gastrointestinal stromal tumor cells overexpressed AXL receptor tyrosine kinase, which in turn activates the AKT pathway to overcome c-KIT inhibition [32, 33].

14.8 Differences in Cellular Drug Influx and Efflux

Among the mechanisms of resistance to TKI, the modulation of the influx and efflux of drug is particularly important due to their role in maintaining the effective intracellular concentration of a drug. The human organic cation transporter type 1 (hOCT1) is a critical factor that regulates availability of imatinib in the cell [34]. Other genetic aberrations that promote drug efflux could also be linked to imatinib resistance. Consistent with this hypothesis, increased expression of P-glycoprotein efflux pumps has been observed in patients with chronic myeloid leukemia (CML) resistant to imatinib [35, 36].

14.9 Tyrosine Kinases Inhibitors and Gastrointestinal Cancers

Several inhibitors of TKI were in use for the treatment of gastrointestinal cancers. The Food and Drug Administration (FDA) of the United States has approved imatinib for gastrointestinal stromal tumors [37, 38]. Following these several other tyrosine kinase inhibitors such as sunitinib [39], regorafenib [40, 41], sorafenib [42], and erlotinib [43] have been approved to treat specific types of gastrointestinal cancers (Table 14.1). Monoclonal antibody (mAb)-induced downregulation of tyrosine kinases is an attractive strategy for various gastrointestinal cancers (Table 14.2).

14.10 Colorectal Cancers and TKIs

Overexpression of EGFR is frequently detected in colorectal cancers [44, 45]. Hence, small-molecule EGFR-TKIs such as erlotinib and gefitinib have been tested in colorectal cancer patients. Erlotinib is a quinazoline derivation that acts by selectively and reversibly blocking the ATP-binding site of the EGFR-TK domain. Gefitinib shows similar efficacies of erlotinib in inhibiting EGFR and also targets ERK 1/2 phosphorylation [46]. Further, anti-EGFR monoclonal antibodies (mAbs) including cetuximab and panitumumab bind to extracellular domain of *EGFR* and block ligand binding leading to inhibition of its downstream signaling events [47]. Both cetuximab and panitumumab are important treatment options in patients with metastatic colorectal cancer [48]. Furthermore, antiangiogenic TKIs [anti-vascular endothelial growth factor receptor (VEGF)] potently inhibit c-KIT and flt-3 kinase activation and exhibit myelosuppressive effects [49]. Bevacizumab, a humanized mAb that binds to all isoforms of VEGF-A, has been approved for use in the treatment of metastatic colorectal cancer (mCRC) [50, 51]. Aflibercept, an antiangiogenic fusion protein that targets the other ligands in the VEGF pathway, has been approved for the treatment of mCRC [52–54].

Table 14.1 Therapeutic small-molecule tyrosine kinase inhibitors studied for treatment of various gastrointestinal cancers

Agent	Treatment benefit			
	CRC	HCC	PC	GC
EGFR inhibitors				
Erlotinib	Demonstrated 39% of stable disease [159]. Increased OS and PFS in combination with bevacizumab [160]	Manifested disease control in 59% of the patients [161]. Erlotinib is an efficacious and well-tolerated treatment [162]	Showed significant survival benefit in combination with gemcitabine [43]. Erlotinib monotherapy failed [163]	Not predictive of the patient therapeutic response [139]. Increased FAK inhibition in combination with RNA interference [164]
Gefitinib	Enhanced the antitumor efficacy of FOLFOX-4 chemotherapy [165]. Gefitinib inhibited viability and promoted apoptosis only in human CRC cells [166]	Prevents HCC development in rats [167]. Induced apoptosis and cell cycle arrest in human HCC cells [168]. Gefitinib plus irinotecan showed potential benefit in HCC [169]	Inhibited cell growth, invasion, and colony formation in PC cell lines [170]. Gefitinib and gemcitabine combination showed antitumor activity in APC patients [171]. Gefitinib in combination with docetaxel showed limited efficacy in PC patients who have failed gemcitabine treatment [172]. Reversed MDR protein gene expression, which sensitized resistant cells to gemcitabine [173]	Increases the sensitivity of tumor cells to radiation [174]. Gefitinib treatment is limited by the rare EGFR mutations in gastric cancers [175]
HER2 inhibitors				
Lapatinib	As second-line monotherapy, lapatinib showed limited activity in patients with mCRC [176]. The combination of trastuzumab and lapatinib is active and well-tolerated in patients with HER2-positive mCRC [177]. Lapatinib in combination with regorafenib showed beneficial effect in CRC patients [178]	Lapatinib is well-tolerated but seems to benefit only a subgroup of patients [179] and did not meet the predefined efficacy rate [180]. Lapatinib induced autophagic cell death and the growth of human hepatoma cells [181]. The combination of celastrol and lapatinib provides strong anticancer synergy in the treatment of HCC [182]	Lapatinib is not effective in PC [183, 184]. Lapatinib may not enhance the antitumor effects of gemcitabine for pancreatic cancer [185]. The combination of lapatinib and capecitabine is a tolerable regimen for PC patients progressed on first-line gemcitabine-based therapy [186]	Lapatinib has activity in GC patients with HER2-amplified disease, and addition of trastuzumab showed increased antitumor efficacy [187]. As a single agent, lapatinib is well-tolerated and showed modest activity in advanced or metastatic GC patients [188]. Lapatinib showed improvement in PFS rates in Asian GC patients under age 60 years [189]

VEGF and/or VEGF receptor inhibitors				
Axitinib	Axitinib is well-tolerated in combination with FOLFOX and FOLFIRI in mCRC [190]. Addition of neither continuous axitinib nor the axitinib/bevacizumab combination to FOLFOX-6 failed to improved PFS or OS [191, 192]	Compared with the placebo plus best supportive care, axitinib combined with best supportive care resulted in significantly longer PFS and higher clinical benefit in HCC [193]. In treatment with axitinib, the patient experienced improved quality of life, with better physical conditions, and alleviated cancer pain [194]	Addition of gemcitabine did not increase safety profile or OS in advanced PC [113, 114]. Axitinib and gemcitabine combination showed antitumor activity in advanced PC [195]. Compared with the APC patients receiving gemcitabine alone, this combination did not provide survival benefit [196]	Axitinib given on a week-on/week-off schedule combined with FOLFIRI or FOLFOX was well-tolerated in patients with gastrointestinal tumors [197]. Axitinib alone or in combination with 5-fluorouracil or cisplatin has potent antitumor activity against human GC in vitro and in vivo [198]. In patients with untreated advanced GC, addition of axitinib to capecitabine-cisplatin chemotherapy resulted in stable disease [199]
Apatinib	Apatinib is active for the treatment of refractory mCRC with a manageable tolerability profile [200]. Apatinib regulates colon cancer cells autophagy and thus inhibits the malignant phenotype [201]	Apatinib has potential survival benefit in patients with advanced HCC [202]. Apatinib in combination with transcatheter arterial chemoembolization (TACE) prolongs the PFS of HCC patients [203, 204]	A case report and literature review suggested that the apatinib might be a potential treatment option for PC [205]. Apatinib administration, improved PFS in PC patients [206]. Further, pancreatic liposarcoma was successfully treated with apatinib [207]	Apatinib showed improved PFS and OS in mGC patients experienced treatment failure [146, 147, 208]. The combination of apatinib and aspirin may be a potential therapeutic strategy for GC treatment [209]. Further, no treatment-related death was documented during the apatinib administration [210]

Table 14.2 Monoclonal antibodies for targeting receptor tyrosine kinases in various gastrointestinal cancers

	Treatment benefit			
Agent	CRC	HCC	PC	GC
EGFR inhibitors				
Cetuximab	As a single agent, cetuximab showed modest activity and is well-tolerated [211]. Improved OS and PFS in whom other treatments have failed [212]. Cetuximab monotherapy is associated with grade 3/4 adverse events [213]. Cetuximab monotherapy is beneficial in patients with RAS/BRAF wild-type tumors [214]	Demonstrated no antitumor activity [215]. Gemcitabine plus oxaliplatin (GEMOX) combined with cetuximab appears to be active [216]. Combination of capecitabine/oxaliplatin/cetuximab was associated with a modest response rate in HCC [217]. Combination of cetuximab and rapamycin inhibited the progression of HCC both in vitro and in vivo [218]	Combination of cetuximab and gemcitabine showed clinical benefit in APC patients [108]. Cetuximab addition to a combination of gemcitabine-cisplatin chemotherapy did not increase survival [219]. The efficacy of cetuximab treatment for APC is not consistently reported in phase II and phase III clinical trials [220]. Addition of cetuximab to docetaxel-irinotecan therapy did not show clinical beneficial [221]	Cetuximab in combination with IL-2 shows significant antitumor activity [222]. Cetuximab and FOLFOX4 combination therapy is active and well-tolerated in GC [223]. A meta-analysis of clinical trials over regimens with or without cetuximab indicated that the cetuximab addition to chemotherapy does not improve OS or disease control rate [224]
Panitumumab	Panitumumab monotherapy improved PFS in wild-type KRAS patients [225]. Panitumumab addition to bevacizumab and chemotherapy for the first-line treatment of mCRC showed harmful when compared with bevacizumab and chemotherapy alone [226]. A recent review denoted that the panitumumab is a beneficial treatment option for RAS WT mCRC [227]	The panitumumab in combination with mFOLFOX6 is effective in those patients with multiple liver metastases from colorectal cancer [228, 229]	Panitumumab and trastuzumab therapy significantly induced growth-inhibitory effects of trametinib in PDX tumors [230]. Analysis if in vivo sensitivity in PDX of KRAS wild-type and mutant PC tumors revealed panitumumab sensitivity in KRAS wild-type tumors [231]	Addition of panitumumab to pirubicin, oxaliplatin, and capecitabine chemotherapy does not increase OS [135]. Panitumumab addition to docetaxel-based chemotherapy did not increase antitumor activity and resulted increased toxicities in esophagogastric cancer [232]

Nimotuzumab	Nimotuzumab in combination with radiotherapy and concurrent capecitabine showed moderate or good tumor regression in locally advanced rectal cancer [233]	Nimotuzumab resulted in a complete remission in an 87-year-old patient [234]	Nimotuzumab has been shown to be safe and well-tolerated in PC patients [235]. Nimotuzumab along with gemcitabine showed 55.6% disease control rate and is safe [236, 237]. Pancreatic cancer cells with EGFR high expression were more sensitive to nimotuzumab in vivo [238]	Nimotuzumab combined with DCF plan is effective in treating late-stage gastric cancer [136]. Combination of nimotuzumab and S-1-cisplatin provided no additional benefit in metastatic gastric cancer patients [239]. Addition of nimotuzumab to irinotecan is not beneficial [240]. Nimotuzumab combined with chemotherapy showed promising clinical benefit in metastatic esophageal cancer [241]
HER2 inhibitors				
Trastuzumab	Trastuzumab is not effective in patients with low overexpression rate of HER-2 [242]. Trastuzumab and lapatinib combination has shown to be well-tolerated in HER2-positive treatment-refractory mCRC patients [243]	No benefit of trastuzumab treatment in patients with HCC [244, 245]. The combination of trastuzumab and 9-cis-retinoic acid is effective for the treatment of HCC [246]	Trastuzumab exert its antitumor effects in high HER-2-expressing PC [247]. Combination therapy with trastuzumab and S-1 is effective for HER2-overexpressing PC patients [248]. Combination therapy with trastuzumab and capecitabine did not improve PFS and OS [249]. No objective response was observed in a combination therapy with trastuzumab and cetuximab given to advanced PC patients after first-line gemcitabine-based chemotherapy failure [250]	Addition of trastuzumab to chemotherapy improved OS in patients with HER2-positive advanced GC and GOJ cancer [128]. The addition of trastuzumab to chemotherapy was effective and safe for advanced gastric cancer [251]

(continued)

Table 14.2 (continued)

Agent	Treatment benefit			
	CRC	HCC	PC	GC
Pertuzumab	Both in vitro and in vivo studies suggest antitumor activity of the pertuzumab on human colon cancer cells [252]. Combination of pertuzumab and cetuximab in refractory CRC was associated with potential antitumor activity and toxicities [253]	Pertuzumab in combination with trastuzumab showed antitumor activity in metastatic biliary tumors [254]	The pertuzumab/9F7-F11 combination enhanced tumor inhibition and the median survival time in mice xenografted with HER3-expressing pancreatic cancer cells [255]	Pertuzumab and trastuzumab combination showed clinical benefit in patients with HER2-positive GC [256]. Combination therapy with pertuzumab and T-DM1 showed significant antitumor activity in HER2-positive gastric cancer patients [257]. A dose-finding study revealed 840 mg q3 was a safer dose for a phase III study of pertuzumab, trastuzumab, and chemotherapy in HER2-positive GC patients [130]
VEGF and/or VEGF receptor inhibitors				
Bevacizumab	Axitinib with FOLFOX plus 5 mg/kg bevacizumab experienced dose-limiting toxicity [190]. Patients of bevacizumab arm had the longest treatment exposures and the highest rates of peripheral neuropathy [191]. However, bevacizumab-based regimens are well-tolerated than axitinib [192]. Treatment with FOLFOX/bevacizumab followed by maintenance axitinib as first-line treatment for mCRC produced a median PFS [258]	Bevacizumab treatment seemed to be fairly well-tolerated [259]. Bevacizumab showed significant clinical and biologic activity in nonmetastatic HCC [260]. Bevacizumab combined with erlotinib had minimal activity in patients with advanced HCC [261]. Bevacizumab plus erlotinib was tolerable and showed a signal of survival benefit for patients with advanced HCC [262]. Bevacizumab combined with chemotherapy was an effective and safe option for the treatment of children affected by HCC [263]	Bevacizumab chemotherapy developed gastric perforation in PC patients [264]. The bevacizumab and erlotinib combination seems to be safe but relatively ineffective in gemcitabine-refractory mPC patients [265]. Bevacizumab treatment benefit is more in PC patients with normal baseline serum albumin [266]. Addition of bevacizumab and cetuximab to gemcitabine, cisplatin, and fluorouracil may represent an effective treatment option for pancreatic cancer [267]	Intraperitoneal administration of bevacizumab inhibits peritoneal metastasis of MNK-45P gastric cancer in mice [268]. Bevacizumab may suppress peritoneal dissemination from gastric cancer [269, 270]. Bevacizumab administration followed by chemotherapy was a more effective therapeutic method for GC [271]

Ramucirumab	Ramucirumab may enhance the efficacy of modified FOLFOX-6 chemotherapy with an acceptable safety profile in metastatic mCRC [272]. Ramucirumab plus FOLFIRI significantly improved OS in patients with mCRC [273]. Ramucirumab in combination with chemotherapy represents a valid option in second-line treatment of advanced CRC patients [274]. Metastatic CRC patients with low carcinoembryonic antigen obtain greater benefit from ramucirumab treatment [275]	Ramucirumab monotherapy may confer anticancer activity with an acceptable safety profile in advanced HCC [276]. Second-line treatment with ramucirumab did not show significant improvement in survival of HCC patients [277]. No difference in treatment safety between East Asians and non-East Asians [278]. Ramucirumab treatment improved OS in Japanese patients with a baseline alpha-fetoprotein level of 400 ng/mL or greater [279]	Results of a phase II randomized trial of mFOLFIRINOX +/− ramucirumab in advanced PC patients are awaited [280]	Ramucirumab is the first FDA-approved therapy for advanced GC after prior chemotherapy [281]. Ramucirumab plus paclitaxel significantly improves OS and PFS in AGC patients [282, 283]. Ramucirumab conferred improvements in efficacy across age groups with a tolerable safety profile in GC patients [284]

14.11 Colorectal Cancers and TKI Resistance

14.11.1 KRAS Mutations and Resistance to TKIs

About 35–40% of patients with colorectal cancers have KRAS mutation [54]. Codon 12 and codon 13 mutations of KRAS gene were identified and correlated with the resistance to EGFR-targeted monoclonal antibodies [55–57]. Since then, these mutations were used to exclude EGFR-targeted therapy for patients with colorectal cancer [58]. Further, several oncogenic mutations of KRAS gene (codons 59, 61, 117, and 146) have been found in 80% of colorectal cancer samples [59, 60]. In addition to this, 3–5% of colorectal cancer samples showed mutations in codons 12, 13, and 61 of the NRAS isoform. All these activating mutations of KRAS and NRAS are known to activate the ERK signaling, even if the EGFR is blocked [61]. These data suggest that EGFR-targeted therapy currently in use will be ineffective against CRC that has a mutation in either KRAS or NRAS [62]. As KRAS mutations are a major cause of TKI resistance, simultaneous inhibition of multiple KRAS effects may overcome resistance to anti-EGFR therapy in CRC patients with KRAS mutations.

14.11.2 BRAF Mutations and Resistance to TKIs

The *BRAF* gene is a proto-oncogene located on chromosome arm 7q34, composed of 18 exons. About 10% of CRC patients are characterized by mutations in BRAF gene [55]. It is interesting to note that the KRAS and BRAF gene mutations occur in a mutually exclusive manner and tumors with these mutations show poor prognosis and survival in colorectal cancers [56]. There are more than 30 different *BRAF* mutations [57]. The most common activating mutation is found in exon 15 (V600E/p.Val600Glu/c.1799T>A) that accounts for 90% of all activating *BRAF* mutations [58]. Multiple line of evidences revealed that the presence of BRAFV600E mutation is correlated with resistance to cetuximab or panitumumab in CRC patients [59–63]. Further studies revealed that the CRC patients harboring BRAF mutations at codon 594 or 596 had markedly longer overall survival when compared with BRAF wild-type and BRAF V600E-mutated CRC patients [64].

14.11.3 PIK3CA Mutations and Resistance to TKIs

The phosphoinositide 3-kinases (PI3Ks) constitute a lipid kinase family and are part of an important signaling pathway downstream from EGFR. Although the acquired *PIK3CA* mutation related to EGFR-TKI treatment is rare, many point mutations that elevate the enzymatic activity were found in about 10–30% of CRC tumors [65]. The *PIK3CA* mutation clusters in the helical domain (c.1624G>A/p.E542K and c.1633G>A/p.E545K) and the kinase domain (c.3140A>G /p.H1047R) [66]. Analysis of primary tumor specimens collected from patients with CRC indicates that the *PIK3CA* mutations are not associated with clinical or pathological factors in CRC patients [67].

14.11.4 Gene Amplification and Resistance to TKIs

Analysis of DNA copy number alterations in primary gastric tumors and gastric cancer cell lines revealed *KRAS* gene amplification in gastric cancer [68]. Analysis of *KRAS* copy number variation in mCRC patients treated with first-line cetuximab revealed that the copy number gains and losses were, respectively, found in poor progression-free survival and good responders [69]. Further, screening of large number of CRC samples revealed that the KRAS amplification is an infrequent event and mutually exclusive with KRAS mutations in CRC [70]. The Cancer Genome Atlas Research Network uncovered very low frequency of copy number alterations in BRAF, ARAF, CRAF, and NRAS genes, but their association with response to anti-EGFR therapy in colorectal tumors has yet to be elucidated [71]. Several other molecular changes in PTEN/PI3K/AKT genes also act as the predictor of response to EGFR-targeted therapy. Phosphatase and TENsin homolog deleted on chromosome 10 (PTEN) is involved in the homeostatic maintenance of PI3K/Akt signaling originating from EGFR activation. PTEN loss of function was noted in 20–30% of CRC cases through various genetic alterations including mutations, deletions, and hypermethylation of the *PTEN* promoter region [72, 73]. One of the important nongenetic mechanisms that involved in driving acquired resistance to EGFR blockade is the paracrine network of TGFα and amphiregulin [74]. This paracrine protective mechanism might be therapeutically exploitable for attaining the effective anti-EGFR therapies.

14.12 Resistance to Anti-VEGF Therapy

Antiangiogenic therapy with antibodies against VEGF (bevacizumab) or VEGFR2 (ramucirumab) has been proven efficacious in colorectal cancer (CRC) patients. It is now recognized that the VEGF axis-dependent alteration is one of the important mechanisms of resistance to antiangiogenic therapies. Anti-VEGF therapy markedly increased the expression of hyaluronic acid in extracellular matrix and increases tumor stiffness [75]. Treatment-induced tumor hypoxia appeared to be the driving force for the remodeling of the extracellular matrix. The hypoxia-inducible factor 1α (HIF-1α) has been associated with the increased expression of basic fibroblast growth factor (bFGF) [76]. The bFGF release further augments these hypoxic inductions through the PI3K and MEK1/ERK pathway [77]. Further, activation of the PI3K/Akt pathway upregulates the expression of HIF-1α and promotes secretion of VEGF and eventually tumor angiogenesis [78]. A recent review indicated that targeting IL-17A/IL-17RA axis might improve efficiency of anti-VEGF therapy in colorectal cancer [79]. Understanding mechanisms and drivers of resistance to anti-angiogenic therapy is of paramount importance to design strategies to curtail the emergence of resistance.

14.13 Hepatocellular Carcinoma and TKIs

Hepatocellular carcinoma (HCC) is the sixth most commonly occurring cancer and the third largest cause of cancer deaths globally [80]. Molecular targeted therapies hold great promise in the management of HCC [81]. Several studies evaluated the effects of TKIs in unresectable HCC [82]. The multi-targeted tyrosine kinase receptor inhibitor (TKI) sorafenib (antiangiogenic and antiproliferative agent) is the first-line systemic treatment for advanced HCC [83]. Recently many new targeted agents have been explored in clinical studies, some available for medical treatment. However, sunitinib, brivanib, linifanib, and TSU-68 have all had disappointing results in advanced-stage HCC [84]. Hepatocellular carcinoma is a highly vascular tumor, and the overexpression of both VEGF and VEGFR has been reported [85]. Hence the success seen with sorafenib may be attributed to its inhibition of VEGF intracellular kinase pathway as well as to the inhibition of the RAS/RAF/MEK/ERK mitogen-activated protein kinases at the level of RAF [82]. Sudden upregulation of eIF5A2 expression level in cetuximab-treated cells indicates that eIF5A2 is an alternative pathway for cell proliferation in epithelial HCC cells escaping from the cytotoxicity of cetuximab [86]. The cetuximab also synergized the eIF5A inhibitor (GC7) to inhibit cell proliferation in epithelial cell lines. This suggests that the eIF5A inhibitor GC7 might be a potent agent that promotes the cytotoxicity of cetuximab on epithelial HCC cells [86].

14.14 Hepatocellular Carcinoma and Resistance to TKIs

Although sorafenib is the first-line systemic therapy for hepatocellular carcinoma (HCC), unfortunately most patients are highly refractory to this therapy. Hence a growing body of studies has been focusing on identification of resistant factors and methods to overcome it or substitute sorafenib [87]. Abnormal activation of PI3K/Akt and JAK-STAT pathways, the activation of hypoxia-inducible pathways, and epithelial-mesenchymal transition (EMT) are some of the important mechanisms that lead to resistance of sorafenib during HCC treatment [88]. Further, EMT correlated with aggressiveness of tumors and poor survival. In consistent to this, frequent amplification of FGF19 and its specific receptor FGFR4 in HCC cells was observed [89]. A recent study demonstrated that the elevated FGF19 expression or hyperactivation of FGF19/FGFR4 signaling in HCC cells is one of the main mechanisms of sorafenib resistance and its inhibition significantly overcomes sorafenib resistance by enhancing reactive oxygen species-associated apoptosis [90]. Epithelial-mesenchymal transition cell phenotype significantly contributes to the increased metastases occurrence but also causes drug resistance (acquired resistance) [91]. Although TKIs are one of the promising agents in HCC treatment, acquired resistance is a major limitation of their efficacy [92]. Sorafenib metabolism is significantly altered in the liver tumor tissue of HCC patient, due to a remarkable decrease of the expression level of CYP3A4 and UGT1A9 [93]. A study using pharmacokinetic model revealed the substrate specificity of sorafenib and its

metabolite sorafenib-glucuronide to the influx (Oatp) and efflux (Abcc2 and Abcc3) transporters [94]. Reductive carboxylation is a novel pathway of glutamine metabolism that supports the growth of tumor. Sorafenib-resistant HCC cells showed markedly higher glutamine metabolism and reductive glutamine carboxylation. Therefore, targeting compensatory metabolic reprogramming of glutamine metabolism by inhibiting PPARdelta constitutes a potential therapeutic strategy for overcoming sorafenib resistance in HCC [95].

14.15 Pancreatic Cancer and TKIs

Pancreatic cancer is the fourth leading cause of cancer-related deaths in men and women of the Western world [96]. Because of the lack of specific symptoms and effective screening methods, these pancreatic cancers are found incidentally or in more advanced stages of the disease [97]. Overall survival remains poor either in metastatic disease or in patients with early-stage disease [98]. Pancreatic cancers show high resistance to traditional chemotherapy and radiation therapy [99]. Overexpression of EGFR signaling is believed to induce increased survival and invasiveness of pancreatic tumors [100]. Further, EGFR inhibition has shown encouraging antitumor activity in preclinical pancreatic tumor models [101]. In the late 1990s, adjuvant gemcitabine chemotherapy has been considered as the standard of care for pancreatic cancer [102, 103]. In pancreatic cancer patients, erlotinib in combination with gemcitabine showed a statistically significant prolongation of overall survival [43, 104]. A phase III randomized trial demonstrated that there was no statistically significant difference with regard to overall survival in advanced pancreatic cancer patients receiving gemcitabine with or without erlotinib. Further, in patients with progression-free disease for 4 months, the overall survival is not significantly different between chemoradiotherapy and chemotherapy [105]. Combining gemcitabine-based chemotherapy with anti-VEGF mAb (bevacizumab) [106, 107] and anti- EGFR mAb (cetuximab) [108, 109] showed modest benefit. A study with metastatic pancreatic cancer patients found that the addition of bevacizumab to gemcitabine plus erlotinib did not improve overall survival [110]. Hence anti-VEGF mAb and anti-EGFR mAbs were not universally considered sufficient to declare the drugs worthy of further investigation in phase III trials [111]. Axitinib is an oral TKI selective for VEGF receptors and to a lesser extent for PDGFRs [112]. In phase II clinical trials, axitinib improved survival by the addition of gemcitabine [113]. However, phase III clinical trial did not ratify its improved survival rates for axitinib [114].

14.16 Pancreatic Cancer and Resistance to TKIs

The KRAS point mutations present early in 85% of pancreatic cancer patients and can be used as diagnostic markers to detect curable pancreatic neoplasia [115]. Patients with KRAS mutations showed a worse response than those with wild-type

KRAS [116]. A biomarker identification trial in patients with inoperable PC, the KRAS mutation status did not predict any subgroup of a detrimental effect or a strong PFS benefit with erlotinib [117]. EGFR expression and EGFR mutations do not predict the survival benefit of anti-EGFR drugs in pancreatic cancer patients [118]. As amphiregulin suppresses the activities of EGFR, HER3, and Akt pathways, combination chemotherapy of conventional anticancer drugs plus an inhibitor for amphiregulin would provide more favorable clinical outcomes for patients with pancreatic cancer [119]. Further, inhibition of STAT3 can resensitize cells that have acquired resistance to EGFR inhibitors or chemotherapeutics [120, 121]. Axitinib treatment is associated with increased glucose metabolism and increased cell surface expression of Glut-1 in pancreatic adenocarcinoma. Further, blocking pAkt with a PI3K inhibitor reversed the Glut-1 translocation and restored sensitivity to axitinib treatment [122]. The therapeutic resistance to anticancer medications is mainly determined by the tumor microenvironment, drug availability, and honing issues [123, 124]. Under hypoxic tumor environment, the prodrug undergoes reduction and preferentially releases the active drug [125]. Hence, targeting tumor hypoxia may be a viable approach to overcome resistance in pancreatic cancer.

14.17 Gastric Cancer and TKIs

Gastric cancer is the second most common cause of cancer-related death globally [126]. Expression of HER2, EGFR, MET, and FGFR2 is associated with gastric cancer progression [127]. Trastuzumab is a recombinant humanized mAb that binds to the extracellular domain IV of the HER2 protein. ToGA trial demonstrated the survival benefits of trastuzumab in HER2-overexpressing gastric cancer patients [128]. Pertuzumab is a new mAb that specifically binds to the extracellular domain II of the HER2 protein [129]. Addition of pertuzumab to trastuzumab and chemotherapy combination provided clinical benefit in HER2-positive gastric cancer patients [130]. Lapatinib, another HER2 blocking TKI, failed to increase median OS in first-line therapy when combined with capecitabine plus oxaliplatin in HER2-positive GC patients [131]. Small-molecule inhibitor saracatinib combined with trastuzumab resulted in a significant benefit over either agent alone, which demonstrated its potential use for treating ErbB2-overexpressing gastric cancer [132].

EGFR overexpression is relatively high in gastric cancers [133]. Inhibition of EGFR signaling prohibits metastasis of gastric cancer. EGFR-specific mAbs and TKIs neutralize apoptosis promoted by EGFR or arrest growth of tumor cells merely by binding their target. Cetuximab an anti-EGFR mAb, evaluated in a phase II EXPAND trial revealed that the addition of cetuximab to capecitabine-cisplatin did not improve median PFS compared to chemotherapy alone in the first-line treatment of gastric cancer [134]. Panitumumab, the first fully human anti-EGFR mAb, did not show any benefit in a randomized open-label phase III trial (REAL3) [135]. Nimotuzumab, a novel recombinant anti-EGFR mAb, showed beneficial effects while minimizing dermatological toxicity [136]. The inhibitory effect of EGFR-TKIs gefitinib and erlotinib exerts potential therapeutic effects. Inhibition of

SN38-triggered epidermal growth factor signals and interleukin-8 synthesis by gefitinib in gastric cancer cells suggested that it could be used in the treatment of certain gastric cancers [137, 138]. A phase II trial of erlotinib in patients of adenocarcinoma of the gastroesophageal junction and stomach revealed that the erlotinib was active in adenocarcinoma of gastroesophageal junction but not in the stomach [139]. Hence, we can say that anti-EGFR mAbs or TKIs are not effective in treating the metastasis gastric cancers.

Several novel anti-VEGF agents have been evaluated in phase II trials to inhibit the proangiogenic effects of VEGF. Bevacizumab, an anti-VEGF mAb that is used in combination with cisplatin-based first-line chemotherapy (AVAGAST trial), significantly improved median PFS and overall response rate in gastric cancer patients [140]. Another phase II study, AVATAR, in Chinese gastric cancer patients did not demonstrate overall survival benefit with the addition of bevacizumab to capecitabine-cisplatin chemotherapy [141]. Ramucirumab, a mAb against VEGFR-2, has shown a survival benefit in gastric cancer patient progressed on fluoropyrimidine- or platinum-based first-line chemotherapy [142, 143]. In contrast to this, ramucirumab in combination with FOLFOX regimen did not show PFS in advanced gastric cancer [144].

Apatinib is an anti-VEGF receptor TKI that was independently developed in China [145]. Compared to the placebo group, apatinib significantly improved the OS and PFS with some adverse effects [146]. Further, a phase III trial of apatinib in chemotherapy refractory gastric cancer patients from 32 centers in China revealed that the apatinib treatment has significantly improved OS and PFS with an acceptable safety profile [147].

14.18 Gastric Cancer and Resistance to TKIs

Hepatocyte growth factor (HGF) is a ligand of MET proto-oncogene (c-Met). Increased expression of HGF/c-Met signal pathway is associated with poor prognosis in various cancer types [148]. Recently, it was reported that the expression of HGF induces resistance to TKIs targeting EGFR, HER2, and BRAF proto-oncogenes. Aberrant activation of c-MET during oncogenesis may occur due to *MET* gene overexpression or activating point mutations [149]. c-MET TKIs had inhibitory effects only on cells overexpressing c-MET [150]. Further, MET-independent HER kinase activation using EGF or heregulin-beta1 was able to overcome the growth-inhibitory effects of MET inhibition by restimulating MEK/MAPK and/or PI3K/AKT signaling, suggesting a possible escape mechanism [151]. Furthermore, overexpression of HGF resulted in resistance to c-MET TKIs through an autocrine manner in gastric cancer cells [152]. Acquired resistance to TKIs in gastric cancer cell line is mediated through the increased levels of Bcl-2 and Bcl-xL protein [153]. Further, inhibition of Bcl-2 and Bcl-xL using a specific inhibitor ABT-263 blunted the acquired resistance to TKIs in gastric cancer cell lines [153].

Many HER2-positive cancers initially do not respond to trastuzumab-based therapies even when combined with chemotherapy [154, 155]. Trastuzumab-resistant gastric cancer cells (NCI-N87/TR) showed activation of the PI3K-AKT signaling pathway as one of the major mechanisms of resistance. Further, downregulation PTEN gene and overexpression of IGF-1R signaling pathway were associated with resistance [156]. The HER2 Ile655Val polymorphism that affects the function of HER2 gene only restricted in HER2-positive breast cancers, and the Val carriers have an aggressive phenotype but are sensitive to trastuzumab treatment [157]. There is no association between G/G (Val/Val) genotype of HER2 Ile655Val polymorphism and gastric cancer in Mexican population [158]. Emerging opportunity for anti-HER2 targeted therapies and inherent resistance to anti-HER2 drugs lead to the development of second generation of anti-HER2 agents to combat resistance.

14.19 Conclusion

Gastrointestinal malignancies are manifested by the activation of multiple signaling pathways targeted by a number of molecular alterations. Thus, targeting any of the abnormal molecular pathways may not be potent enough to control gastrointestinal malignancies. Stressing the fact that a large proportion of cancer drugs are poorly selective, agents targeting multiple drivers, which could provide a wider therapeutic scope, are needed to be developed. As a consequence, different multi-targeted agents or the combination of single-targeted drugs was developed to inhibit multiple signaling pathways. Here we summarize the known resistant mechanisms to various TKIs used in gastrointestinal malignancies and molecular pathogenesis of resistance against these tyrosine kinase inhibitors. The mechanistic understanding may help to put forward new hypotheses on drug development and design better therapies to overcome resistance to TKI treatment in cancer patients.

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EGFR Role in Cancer: A Potential Therapeutic Target

15

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Abstract

Protein kinases play a vital role in the regulation of pathways that control cell growth, proliferation, survival, and differentiation. Epidermal growth factor receptor (EGFR) is a key protein kinase that when dysregulated, disrupts these pathways and, accordingly, is associated with several cancers. Thus, EGFR has been a focus of investigation as a therapeutic target for cancer treatment for the past several decades, with fair success. Despite this success, EGFR-targeted therapies are not universally effective across cancers, and improving the specificity and efficiency of EGFR-targeted therapies is an area of continued investigation. This chapter discusses recent progress made in understanding the role of EGFR in cancer and how the knowledge have been used to develop more precise EGFR-based therapeutic regimens for cancer patients.

Keywords

EGFR · Protein kinases · Receptors · Therapy · Cancer

15.1 Introduction to Protein Kinases

Kinases catalyze the transfer of a phosphate moiety (PO43-) to a specific substrate via an enzymatic reaction known as phosphorylation. The phosphate donor molecule is typically ATP due to its high-energy phosphoanhydride bonds; however,

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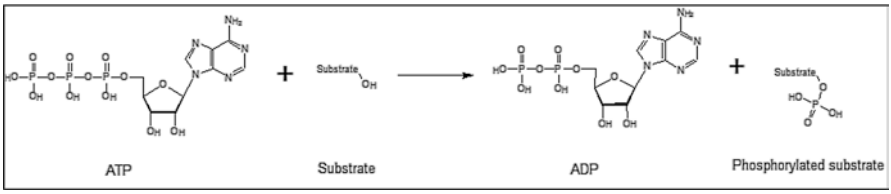
other high-energy molecules such as GTP can be used. Phosphorylation is an esterification reaction. Thus, for this reaction to occur, the substrate must have an available hydroxyl group to act as a nucleophile. The three major kinase substrates are carbohydrates, lipids, and proteins. The overall reaction catalyzed by kinases is depicted below. Protein kinases are classified based on the three amino acids they phosphorylate: serine, threonine, and tyrosine. There are serine/threonine kinases, which phosphorylate both of these residues, tyrosine kinases, and dual-specificity kinases, which can act on all three amino acids [1]. Overall, the protein kinase gene family has over 500 members, and at least 244 of these genes have been mapped to known disease loci or cancer amplicons [2]. Protein phosphorylation has been known to alter protein function in a variety of ways and is the most common reversible posttranslational modification that occurs in eukaryotes [3]. Phosphorylation can be both stimulatory and inhibitory [4]. Consequentially, protein kinases play an essential role in almost all facets of cellular function. Metabolism, the regulation of transcription and translation, cell division, and apoptosis are all influenced and tightly regulated by the function of protein kinases.



Protein kinases primarily exist in their inactive form and are activated by a change in their regulatory stimuli. When this happens, a signal transduction cascade can occur in which a cellular signal can be amplified when one kinase phosphorylates several others. The repetition of this process many times forms a chain reaction of phosphorylation events. Ultimately, a single protein kinase can potentially catalyze the phosphorylation of over one million substrates and have global physiological effects. Mutations that lead to the dysregulation of protein kinases have been linked to a number of different diseases including cancer. The epidermal growth factor receptor, a family of transmembrane receptor protein tyrosine kinases, is particularly of interest due to its overexpression in lung, breast, colorectal, pancreatic, and other cancer types [3].

Protein kinases (PKs) are a group of enzymes that add phosphate groups to proteins in a process known as phosphorylation. This phosphorylation results in changes to the target protein including alterations in its conformation, its cellular location, and/or its association with other proteins. Protein phosphorylation plays a vital role in numerous important cellular processes and is one of the most crucial signal transduction mechanisms in promoting regulated intracellular processes such as ion transport, cellular proliferation and differentiation, and hormone responses [5]. Consequently, disruptions in phosphorylation can impair cellular function resulting in human disease [6]. A variety of human cancers are associated with a deregulation in PKs [7]. The ability of protein kinases (PK) to regulate the biological activity of proteins via phosphorylation makes them an interesting target in cancer research [8].

15.2 EGFR Function and Role in Cancer

Epidermal growth factor receptor (EGFR) belongs to an ERBB family of receptor tyrosine kinases (RTK), which includes ERBB1 (EGFR), ERBB2, ERBB3, and ERBB4. EGFR mediates important cellular signaling pathways that control cell growth, proliferation, survival, and differentiation. This has made EGFR the target of extensive investigation aimed at understanding its expression, upstream regulation, downstream effects, and clinical relevance, particularly in cancer.

EGFR is a transmembrane receptor with an extracellular, transmembrane, and cytoplasmic region [9, 10]. The extracellular region is made up of four domains, I, II, III, and IV (Fig. 15.1). Domains I and III are compact with ~37% amino acid similarity and contain a β-helical fold. The cysteine-rich domains have made EGFR the target of extensive investigation aimed at understanding its expression, upstream regulation, downstream effects, and clinical relevance, particularly in cancer.

EGFR is a transmembrane receptor with an extracellular, transmembrane, and cytoplasmic region [9, 10]. The extracellular region is made up of four domains, I, II, III, and IV (Fig. 15.1). Domains I and III are compact with ~37% amino acid similarity and contain a β-helical fold. The cysteine-rich domains II and IV are homologous. The extracellular region forms a ligand-binding pocket and is connected to the hydrophobic transmembrane region, which is associated with the intracellular region. The intracellular region consists of a tyrosine kinase domain and a regulatory region [9]. Binding of ligands such as EGF and transforming growth factor alpha (TGF-α) to the extracellular region initiates EGFR activation.

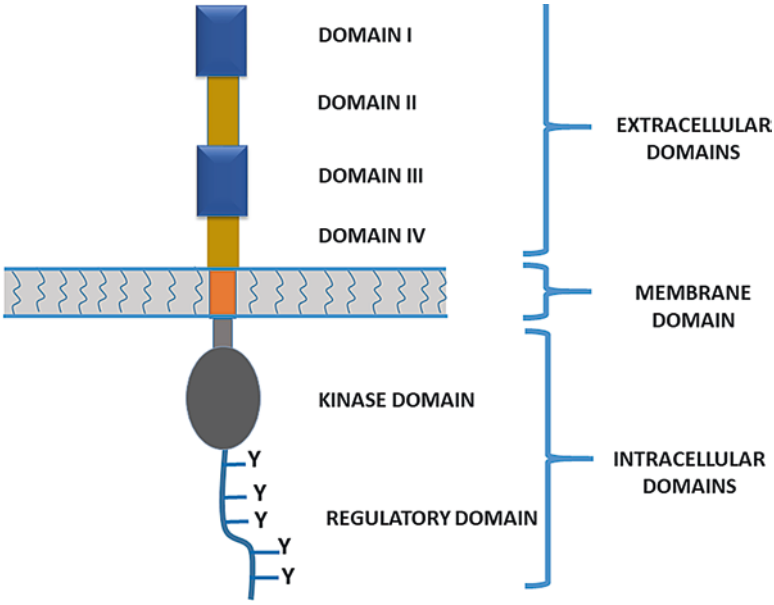


Fig. 15.1 A schematic representation of EGFR domains

However, it has been shown that EGFR can possibly be activated independent of ligand binding due to genetic mutations or EGFR overexpression, which often results in constitutive activation [11, 12]. In addition, interaction between membrane-associated genes such as urokinase-type plasminogen activator receptor has been implicated in ligand-independent EGFR activation [13].

EGFR canonical activation is initiated following the binding of a ligand to the external region triggering a conformational change that leads to dimerization of monomer EGFRs [14]. This will bring cytoplasmic regions in a close proximity facilitating autophosphorylation at tyrosine residues located in the regulatory region [15]. Autophosphorylated tyrosine residues serve as anchors for several genes such as GRB2, SHC, SRC, and PI3K which recruit and activate RAS, AKT, and STAT pathways facilitating a signal transduction that initiates critical cellular processes. For example, the RAS pathway, which is activated upon GRB2 and SOS binding to EGFR through adopter protein SHC, controls cell proliferation and survival. Docking of PI3K to autophosphorylated EGFR intracellular region activates the AKT pathway and promotes cell survival, growth, and invasion. The STAT pathway is initiated following direct interaction of JAK to EGFR, which in turn activates STAT and promotes cell survival. Besides the above well-established functions, EGFR involvement in autophagy and metabolic regulation has been demonstrated recently [16] (Tan 2016; Stress-induced EGFR trafficking).

While its kinase-dependent role has been well established, EGFR possesses a kinase-independent function mainly through interaction with other TKs. For instance, a loss of function due to kinase-dead EGFR expression can be rescued by overexpression of other TK, HER2 [17] (Deb TB: 2001 receptor kinase-independent signaling). In addition, despite losing ligand-mediated stimulation ability, an EGFR mutant retained its pro-survival capability [18] (Ewald JA, 2003 cell survival mediated by the epidermal growth factor receptor). Thus, tight regulation of EGFR-mediated pathways is extremely important since slight irregularity may result in uncontrolled cellular growth processes, a major cause of cancer.

Irregularity in EGFR-mediated cellular processes has been implicated in several cancers such as breast cancer, lung cancer, pancreatic cancer, colorectal cancer, and glioblastoma. The irregularities in EGFR function mainly arise from mutation, gene amplification, and protein overexpression, which result in constitutive activation. Constitutive activation of EGFR leads to major hallmarks of cancers, cell survival, proliferation, and metastasis. For instance, in lung cancer, particularly non-small cell lung cancer (NSCLC), EGFR is mutated in a high frequency and is suspected to be one of underlying causes involved in the early development of NSCLC. Most of EGFR mutations in NSCLC are single-nucleotide substitutions, in-frame deletions, and in-frame duplications which account for 51%, 44%, and 5% of all activating mutations, respectively. These mutations have been shown to be important to treatment response as well as to cancer progression [19, 20]. Constitutively active mutant EGFR has been shown to be expressed in frequency of ~50% in glial tumors and is associated with poor outcome in glioblastoma patients [21, 22].

While constitutively active EGFR plays an important role in cancer development and progression, its irregular expression equally contributes to carcinogenesis.

Accumulation of EGFR because of overexpression promotes ligand-independent dimerization leading to abnormal activation [23]. EGFR is overexpressed in GBM in high frequency and is associated with poor overall survival [24]. Similarly, EGFR overexpression correlates with tumor growth and poor outcome in breast cancer patients [25, 26]. Due to its importance in wide range of cancer, EGFR has been extensively investigated as potential therapeutic target, and two types of major inhibitors have been developed, monoclonal antibody and small molecules.

15.3 EGFR as Therapeutic Target for Cancer

Therapies designed to target specific molecules either alone or in combination with the more conventional approaches of surgery, radiation, and chemotherapy are emerging as one of the most promising strategies for treating patients with cancer. Given the multifaceted functional role of EGFR in cancer, it has emerged as a key molecule to target, for improved tumor specificity. Furthermore, EGFR is frequently amplified, overexpressed, or aberrantly activated in cancers, increasing the likelihood of a strong therapeutic index in most patients. Indeed, targeting EGFR has been shown to provide therapeutic benefit across multiple cancers, increasing overall and progression-free survival in colorectal cancer, head and neck cancer, NSCLC, pancreatic cancer, and breast cancer.

To date, EGFR inhibitors fall into two main categories, including monoclonal antibodies and small molecule inhibitors. Each of these approaches has a distinct mechanism of action for targeting EGFR, which provides clinicians with multiple avenues for treating EGFR-dependent tumors.

Monoclonal antibodies targeting EGFR are generally unable to permeate the cell membrane and thus are limited to targeting protein domains expressed on the cell surface (Fig. 15.2). Thus, the mechanism of action of monoclonal antibodies against EGFR is binding to the extracellular domain of the protein. Upon binding, monoclonal antibodies prevent the binding of EGFR-specific ligands, through competitive inhibition. Antibody-mediated cross-linking of EGFR further triggers EGFR endocytosis, ubiquitination, and degradation, resulting in reduced levels of EGFR that are stable over time [27]. Loss of EGFR can also result in enhanced apoptotic signaling in the target cell [28]. In addition, the binding of monoclonal antibodies to EGFR can activate the complement cascade of the immune system, resulting in the immune-mediated killing of target cells [29]. One drawback of monoclonal antibodies is that they rely on the presence of an intact extracellular domain.

EGFR is frequently mutated in tumors, and some of these mutations result in the loss of all or part of the extracellular domain. Consequently, monoclonal antibodies will have limited efficacy in these types of tumors. Additionally, monoclonal antibodies must be administered intravenously, or they will be degraded by the digestive system. They can, however, be administered with less frequency than small molecule inhibitors, with longer half-lives lasting up to around 1 week.

There are currently two monoclonal antibodies, panitumumab and cetuximab, that are FDA approved for the treatment of metastatic colorectal cancer.

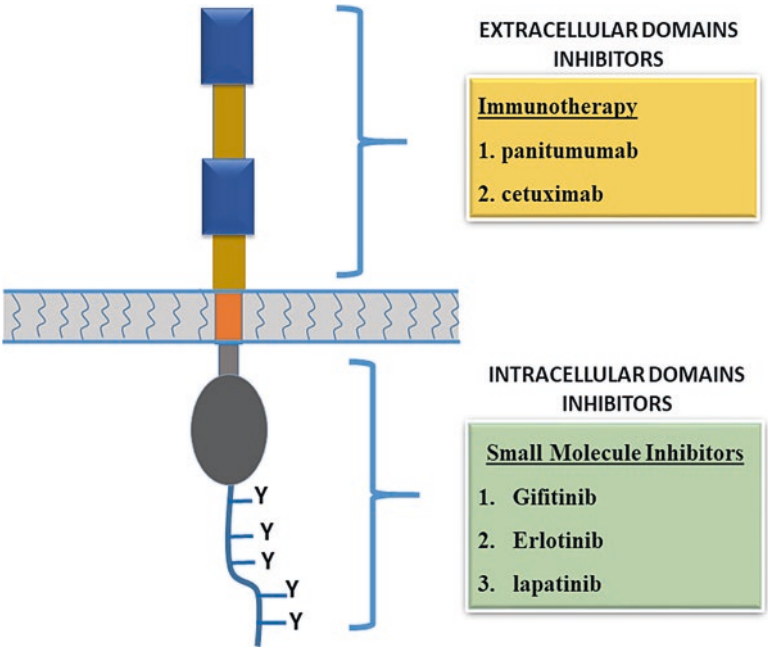


Fig. 15.2 A schematic description of EGFR inhibitors and a region of their target

Panitumumab, a human monoclonal antibody, is also being examined in a number of ongoing clinical trials, including in metastatic head and neck cancer, urothelial carcinoma, pancreatic cancer, and esophageal cancer. Although panitumumab is routinely tolerated, side effects include skin toxicities, as well as fatigue, nausea, diarrhea, and fever. Cetuximab is a chimeric antibody that is FDA approved for use alone or in combination with irinotecan for metastatic colorectal cancer and also is FDA approved in combination with radiation for metastatic head and neck squamous cell carcinoma. Cetuximab has been investigated in the context of NSCLC across several clinical trials [30], with limited improvements in patient survival when compared to the standard of care. However, there is evidence that certain subgroups of NSCLC patients may benefit from EGFR-targeted therapy [31]. Side effects of cetuximab include dermatological toxicities, fever, hypotension, nausea, and cardiac arrest. EGFR-based immunotherapy is also under investigation in clinical trials in rectal cancer, locally advanced skin cancer, and thymic carcinoma, among others.

Unlike monoclonal antibodies, receptor tyrosine kinase small molecule inhibitors targeting EGFR (gefitinib, erlotinib, lapatinib, and other derivatives) are usually able to permeate the cell membrane and thus typically function by targeting the intracellular domain of EGFR (Fig. 15.2). Thus, cancer cells carrying a mutated or truncated version of EGFR lacking an extracellular domain can still be targeted. Small molecule EGFR inhibitors typically are ATP analogues that function by binding within the kinase domain, within the ATP-binding pocket, which is located in

the intracellular portion of the protein. Inhibition of ATP binding impairs EGFR autophosphorylation and downstream signaling. Gefitinib is FDA approved for NSCLC as a first- or second-line therapy, and erlotinib is approved for patients with relapsed or advanced NSCLC. Erlotinib is also FDA approved for use in conjugation with gemcitabine in advanced-stage pancreatic cancer. Unfortunately, in clinical trials, it was found to have a statistically significant but disappointingly mild improvement in progression-free survival [32]. Gefitinib is also under clinical investigation for treatment of esophageal cancer. Erlotinib is under continued investigation for glioblastoma, where it shows promise in improving progression-free survival, but unfortunately does not appear to impact overall survival [33].

Specific limitations exist for small molecule inhibitors of EGFR. Due to their ATP-mimetic structural design, these inhibitors often lack specificity to EGFR alone and can target other RTK family members, which can result in elevated toxicity in patients. Lapatinib, for example, targets a number of RTKs beyond EGFR. This can, however, be uniquely beneficial in the case of a tumor that harbors mutations across multiple RTKs. Lapatinib is another derived small molecule inhibitor of EGFR that is FDA approved for use in HER2-amplified breast cancer. Similar to monoclonal antibodies, inherent or acquired point mutations within EGFR may also limit the efficacy of small molecule inhibitors, particularly if a mutation occurs within the catalytic site of EGFR. Unlike monoclonal antibodies, small molecule inhibitors to EGFR can be administered orally. However, these inhibitors need to be taken daily, due to their shorter half-lives [34]. Like immunotherapy-based treatment, the main side effect of small molecule EGFR inhibitors is their associated dermatological toxicities.

In sum, there is great potential in targeting EGFR in in patients with several types of solid tumors. However, for a therapeutic regimen to be effective, it will be important to understand if and how a tumor is dependent on EGFR. Indeed, immunohistochemical staining for EGFR levels in tumors is not predictive of response to EGFR inhibitors [35]. Sequencing EGFR itself can provide better insight into how EGFR is functioning in a tumor, defining whether EGFR is simply overexpressed, or if there are existing point mutations that predict changes in EGFR structure and thus the efficacy of existing inhibitors. This in turn can help guide the development of an optimized therapeutic plan on a patient-by-patient basis. This precision-based approach is not without its drawbacks. The cost associated with sequencing a biopsy remains high. EGFR also has the demonstrated potential to acquire point mutations over time, some of which can render inhibitors ineffective [36]. Routinely monitoring EGFR status in patient biopsies can help mitigate these effects.

In addition to EGFR status itself, K-ras is considered a critical biomarker, known to predict patient response to EGFR inhibitors. Both panitumumab and cetuximab were found to only have demonstrated efficacy in patients with non-mutated k-ras. Indeed, gain of function mutations in k-ras can activate MAPK and downstream signaling independently of EGFR. The FDA has since approved a commercially available diagnostic sequencing kit for k-ras, to be used in conjunction with EGFR-targeted therapies [37]. In addition to K-ras, a study in metastatic colorectal cancer recently revealed mutations in NRAS, BRAF, and PIK3CA and nonfunctional

PTEN can also predict resistance to EGFR therapies in metastatic colorectal cancer [38]. Accordingly, it will be necessary to continue the investigation of the complex signaling network of cancers, to determine if a particular tumor is truly EGFR driven and in turn if that tumor will respond to EGFR-targeted therapy.

15.4 Conclusion

The protein kinase enzymes play vital roles in many important cellular processes. A disruption of their normal function can have deleterious effects on cellular function, often ultimately leading to disease. Among many significant PKs, the receptor protein kinase EGFR mediates important cellular signaling pathways that control cell growth, proliferation, survival, and differentiation. Mutations in EGFR are implicated in several human cancers. Therefore, EGFR is the subject of ongoing research as a therapeutic target in cancer. Several successful therapies targeting EGFR currently exist, with more being developed. The understanding of the normal functions of protein kinases, as well as the study of the effects of deviations from these normal functions, continues to be an important pursuit in the study of cancer.

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Nanomaterials: Diagnosis and Therapeutic Properties

16

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Abstract

Therapeutic strategies toward the treatment of gastrointestinal (GI) malignancies frequently involve the administration of increased dosage of chemotherapeutic drugs, often resulting in nonspecific toxicities. Although conventional radio- and chemotherapy have been the gold standard of cancer therapy for decades, these approaches are not optimal and can lead to resistance to these and other therapies. Effectiveness of GI malignancy therapies depends on fine-tuning of eradication of cancer cells with minimal or ideally no toxic effect on normal cells. Nanomaterials (NMs) offer a solution for targeted killing of cancerous cells without causing damage to the healthy host cells. NMs are appealing drug carriers based on their high tissue permeability, high colloidal stability, small size in the nanometer range, high surface-to-volume ratio (large amount of drug can be loaded), aqueous solubility, ease of characterization, and surface modification. The enhanced permeability and retention (EPR) effect of NMs permit accumulation at the tumor site. Apart from the passive accumulation of nanoparticles at tumor sites, NMs actively delivered the drug at tumor sites by loading with various growth factor receptors, peptides, shRNA, and small molecules. In this chapter, we will discuss the impact of NMs on tyrosine kinases associated with growth and metastasis of selected GI malignancies.

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16.1 Introduction

Nanotechnology is the unification of physics, chemistry, biology, as well as engineering sciences. An excellent speech by physicist Richard Feynman, *There's Plenty of Room at the Bottom*, could be considered revolutionary in the field of nanotechnology because this speech for the very first time enabled people to think and imagine at nanoscale. Although, when Feynman delivered this lecture in 1959, there was no access to sophisticated imaging techniques like SEM, TEM, STM, and AFM, his interpretation about the concept of "plenty of room at the bottom" was entirely based on his vivid imagination. Nanomaterials are wonder materials due to their distinct properties and behavior which are so different from the bulk material. The most promising factors contributing to its unique properties are high aspect ratio (ratio of length to radius of the material) and high surface-to-volume ratio, i.e., higher number of surface atoms, contributing to its highly sensitive surface properties and quantum confinement effects due to the increase in the band gap, i.e., energy gap between valence band and conduction band is high; hence electron is confined within the space [1].

16.2 Nanotechnology Incorporated Therapies of GI Cancer

Our main goal is to focus on the variety of nanomaterials that has been used in combating gastrointestinal (GI) cancers. Nanomaterials have gained much attention due to their various therapeutic advantages, specifically targeting killing of cancerous cells without causing much damage to the healthy host cells and overcoming the shortcoming of anticancer drugs as well as conventional radio- and chemotherapy. Once a drug molecule is introduced into the system, its absorption cannot be controlled. Hence, when a small amount of drug reaches the cancerous cells, it is not sufficient enough to kill them since the dose is saturated and most of the drug molecules are absorbed in the bloodstream and end up in the healthy cells, finally producing a lethal and highly toxic environment finally killing them [2]. Subjecting the cancerous cells to conventional radio- and chemotherapy often leads to development of resistance, and hence when the radiation is subjected against the resistant cells, they are not damaged [3]. Moreover nanomaterials are used to treat malignant cells owing to their high permeability into the tissues, enhanced colloidal stability, and enhanced permeability and retention (EPR), an effect due to which nanomaterials accumulate in the cancerous cells [4, 5]. Nanomaterials further have excellent aqueous

solubility, facile surface modification or functionalization with various organic moieties with groups which directly get attached to cancerous cells, and easy characterization [6, 7].

16.3 Types of Nanoparticles

Out of the vast number of options available to be incorporated in preparing nanomaterials for therapeutic purposes, the most studied elements are gold and silver. Gold and silver are noble metals and have their own set of unique properties, which make them extremely toxic toward malignant cells without damaging the healthy cells [8, 9]. Gold nanoparticles act as excellent theranostics. Theranostics is the combination of therapeutic and diagnostic and exhibits a dual property of diagnosing as well as delivering the drug to cancerous cells [10]. When a light of certain frequency falls on the surface of the gold nanoparticles and it matches with the surface plasmon frequency of the surface electrons of the gold, then these electrons start to resonate or vibrate collectively. A process resulting in the collective vibrations of the surface electrons creating a large amount of thermal energy responsible for the killing of the cancerous cells is called photothermal therapy (PTT) [11]. Surface-enhanced plasmon resonance is observed mostly in gold and silver. A second mechanism for the death of cancerous cells has been reported by Akhter et al. which states that when light falls on a gold nanoparticle, then the surface electrons get excited and jump to a higher level of energy also known as the excited state and show strong resonance. When these electrons return to their native state, they liberate heat energy increasing the temperature of the tissues adjacent to it [12]. Groups have also reported killing of the cancerous cells by hyperthermia [13]. Organs are generally heated to a temperature between 41 and 45 °C which amplifies the damage caused by radiation [14]. Hyperthermia acts as a boon for cancer therapy as it regulates higher permeability and blood flow to localized cells [15]. Magnetic fluid hyperthermia is a novel field dealing with the application of heat generated by the high-frequency oscillation of magnetic nanomaterials in the presence of high magnetic field. Iron oxide-based magnetic nanoparticles incorporated in organic shells have been extensively studied for killing cancerous cells. The common systems are biocompatible polymers like polyethylene glycol and chitosan [16]. Iron oxide-based magnetic nanoparticles possess special characteristics like converting magnetic field energy thermal energy which is exerted on biological structure and biocompatibility [14, 17].

Groups have reported the study of gold nanoparticles functionalized with drug molecules which are released into the tissues which are at a higher temperature than normal cells. The drug molecules are attached with the gold nanoparticles with heat-sensitive chemical bonds [18]. Nanoprobes provide us effective way of targeting imaging. Folic acid (FA)-conjugated silica-coated gold nanoclusters have been used as nanoprobes for targeted gastric cancer cell imaging [19]. Chitosan-coated gold nanoparticles when irradiated with IR frequency absorb it and get excited to

higher energy levels and while returning to the ground state give out heat energy which kills the cancerous cells, since the nanoparticles get accumulated in the cancerous cells (EPR effect) [20]. Groups have reported work on core-shell nanoparticles with an iron core and shell consisting of noble metals and biopolymer-coated noble metals like gold, silver, and platinum [21–23]. Silver nanoparticle biosynthesis has been reported from honey bee extract which was found suitable for colon cancer treatment [24]. Silver nanoparticles are extensively studied species in the field of nanomedicine owing to its antibacterial properties. There are reported literatures about biosynthesis of silver nanoparticles using coriander leaf, henna leaf, and edible mushroom [25]. Many groups have also focused on the use of gadolinium and modified gadolinium nanoparticles as contrast agents in magnetic resonance imaging (MRI) [26]. Mostly conventional contrast agents show low sensitivity at lower concentration; hence to overcome the sensitivity issue, mostly magnetic nanomaterials have been studied like superparamagnetic iron oxide particles and fluorescent quantum dots [27].

Nanomaterials have been extensively studied for application in the field of gastrointestinal cancer therapeutics, but there is still a long way to go to implement the materials as drugs because of many major issues, namely, low biocompatibility, toxicity, and risk of accumulation within the body [28]. So future work could be the incorporation of specific properties within the nanomaterial entity such as biodegradability, property of forming self-assembled nanoparticles and nanogels.

16.4 Tyrosine Kinase in Gastrointestinal Malignancies

Tyrosine kinases (TKs) play an important part in intercellular signal transduction and in controlling the critical cellular process such as migrations, adhesion, proliferation, invasion, angiogenesis, and apoptosis [29]. TK is an enzyme capable of transferring a γ-phosphate group from ATP to the hydroxyl group of tyrosine residues on signal transduction proteins [30]. Receptor tyrosine kinase (RTK) is a class of tyrosine kinases, which are extensively studied for their potential involvement in various gastrointestinal cancers including tumors of the pancreas, colon, esophagus, and liver. The approaches for targeting RTKs include blocking the extracellular receptor domains on tumor cells and inhibiting the enzyme's ATP binding site [31]. Tyrosine kinase inhibitors (TKIs) have also been established as a promising target toward the treatment of many cancers with enhanced potency, specificity, and efficiency. Currently, more than half of the available TKIs are undergoing clinical trials for targeting RTKs, specifically cetuximab, which is a monoclonal antibody against EGFR-1, and erlotinib, which a TKI has shown promising results. Combination of these inhibitors with gemcitabine has led to a synergistic antitumor activity. A target drug delivery system based on nanoparticles that comprises of cetuximab (an anti-EGFR antibody) as a targeting agent and gemcitabine as an antitumor drug, as well as gold nanoparticles as delivery vehicle, was established by a group of researchers [32]. Administration of this delivery method is known to significantly inhibit many

GI malignancies including the proliferation of pancreatic tumor in vitro and in vivo [32]. Consequently, this novel strategy may perhaps become a comprehensive approach in treating a variety of GI tumors in the future.

16.5 Conclusion and Future Directions

The generalized use of gold and silver nanoparticles emerges due to the fact that both have the unique property of surface-enhanced plasmon resonance. Moreover, both can be used as optical contrast agents and also possess properties of being functionalized with groups which add to the overall imaging ability of cancerous cells. In addition to the above properties, nanomaterials are helpful in targeted drug delivery by encapsulating drug molecules on the surface of nanomaterials. They help in acquiring localized accumulation of drugs and contrast reagents. The shape and size dependence of nanomaterials on its properties has further triggered an intense interest in their research related to gastrointestinal cancers. Not only can the nanomaterials be functionalized but also can be synthesized to obtain various shapes, i.e., nanoshells, nanoeggs, nanocups, and core-shell structures [33]. The variations that can be carried out with the nanomaterials and their size-dependent properties make them wonder materials. Nanotechnology is a vast field of research giving opportunity to precisely fabricate our material by selecting the desired size and shape targeting a special property. For instance, nanoeggs are analogous to optical lenses that help us to view our target molecule. Hence, we try to fabricate the nanomaterials by changing various parameters and incorporating the essential functionality so that we can look at certain targeted cancerous cells. Medical imaging for gastrointestinal cancer tissues requires the nanomaterials to possess scattering characteristics. Hence the study of scattering properties of the various nanostructures can be studied and tested for their imaging efficiency of cancerous cells. Similar studies can be conducted to test their activity, i.e., drug delivering capacity in hyperthermic tissues and their localization or accumulation.

There is still a long way to go in terms of synthesizing biocompatible nontoxic nanomaterials for the use in therapeutic and biological imaging application. There lies a lot of scope in natural materials if we are concerned with biocompatibility. Hence focus must shift toward isolating capping agents, stabilizers, and functional groups from natural plant or animal extract and incorporating biocompatible polymers like biomolecules on the nanomaterials, hence making them less toxic and biocompatible.

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