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Preface

Recent biomedical advancement and discovery of unique epigenetic modifications to target different cancer types has revolutionized the cancer treatment regime. In recent years, the roles of epigenetic regulators have taken forefront in discovering the target molecules to treat cancer. According to the American Cancer Society, in 2014, approximately 1,665,540 new cancer cases and 585,720 cancer deaths were reported in the USA only, which accounts for one of every four deaths. This projection is same for coming years too.

Cancer refers to a group of diseases that share a common phenotype such as uncontrollable cell growth and proliferation. During the multistep process of carcinogenesis, cells acquire a series of genetic changes that lead to unrestrained cell growth and division, inhibition of cell differentiation, and evasion of cell death. However, these genetic changes alone are not sufficient enough to explain the phenotype of cancer cells. Concepts of "epigenetics" offer a partial but crucial explanation of carcinogenesis. The initiation and progression of cancer, traditionally considered to be a result of genetic alterations, is now realized to involve epigenetic abnormalities too. Recent advancements in the rapidly evolving field of cancer epigenetics have shown extensive reprogramming of every component of the epigenetic machinery in cancer. The reversible nature of these epigenetic alterations has led to the emergence of the promising field of epigenetic therapy. Growing evidences support the role of epigenetic modifications in the initiation and progression of various cancers, and failures of current cancer therapy are in part due to the lack of understanding of epigenetic changes in cancer cells. Recent advancements in cancer research suggested epigenetic regulation of DNA methylation, histone modification, nucleosome positioning, and noncoding RNA expression to be central in cancer growth and development. However, several important questions remain unanswered including: (a) How many genes undergo epigenetic alteration in a tumor? (b) Do these changes differ between distinct types of cancer cells? (c) What are the molecular and genetic mechanisms that underlie these altered epigenetic profiles? Recent technological advances allow epigenetic alterations in cancer to be

studied across the whole genome. These approaches are being used not only to answer key outstanding questions about cancer biology but also to provide new avenues for diagnostics, prognostics, and therapy.

The fundamental roles of epigenetics in carcinogenesis make an understanding of mechanisms such as DNA methylation, histone modification, nucleosome positioning, and noncoding RNA expression an essential goal both for basic and applied research. Targeting epigenetics will not only target cancer cells but also the tumor microenvironment more likely the entire host for achieving better treatment outcomes of cancer patients.

Although the term "epigenetics" literally means "above the genetics," it is now generally used to refer to the changes in gene expression that take place without changing the DNA sequence. Epigenetic changes encompass an array of molecular modifications to both DNA and chromatin; the most investigated of them are DNA methylation and histone modifications. Other epigenetic mechanisms of regulating gene expression include regulation by noncoding RNAs, such as microRNAs, and mechanisms that control the higher-level organizations of chromatin within the nucleus and have a broad range of effects on gene expression.

The chapters of this book are aimed to explore epigenetic alterations associated with various human cancers. Detailed information of epigenetic changes in various cancers will provide a better understanding of cancer growth and will help the scientific community to provide new avenues for diagnostics, prognostics, and therapy of this highly fatal human disease. Additionally, individual chapters of this book focus on exploring the current understanding of alterations in the epigenetic landscape that occur in various cancer cells, the roles of these changes in cancer initiation and progression, and the potential use of this knowledge in designing more effective strategies of cancer treatments.

In *Epigenetic Advancement in Cancer*, a team of skilled scientists with extensive expertise in epigenetic cancer biology presents the recent advancement in the field. This book covers various topics including breast, prostate, colorectal, and pancreatic cancers and the roles of different epigenetic regulators in cancer development and progression. Thus, this book presents a unique source of understanding for researchers interested in epigenetics and cancer.

We would like to thank Springer and their staff for the invitation and editorial support during the preparation of this book. We are thankful to experts who accepted our invitation to share their knowledge and expertise and contributed significantly to compile this book. We are sure that this book will be a valuable resource for cancer biologists, immunologists, cell biologists, clinicians, faculty, and students who are working for their research endeavors in their respective research areas.

We would like to thank our family members for their continued support.

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Chapter 1
Epigenetic Control of Genes Involved in Cancer Initiation and Progression

Sabita N. Saldanha and Shivani Soni

Abbreviations

ADAM19	A Disintegrin And Metalloproteinase Domain 19
ADAM12-L	A Disintegrin And Metalloproteinase Domain 12
AICDA	Activation-Induced Cytidine Deaminase
APC	Adenomatous polyposis coli
AKT	v-akt murine thymoma
ALX4	Aristaless-Like Homeobox 4
ATG7	Autophagy 7-Like
BCL2L11	BCL2-Like 11
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
CRC	Colorectal cancer
c-Myc	V-myc avian myelocytomatosis
CDKN2A	Cyclin-dependent kinase inhibitor 2A
DNMT1	DNA methyltransferase 1
DNMT3A	DNA methyltransferase 3A
DNMT3B	DNA methyltransferase 3B
EpCAM	Epithelial cell adhesion molecule
ER	Estrogen receptor
E2F1	E2F transcription factor 1
ERG	ETS-related gene
EOC	Epithelial ovarian cancers
ERK	Extracellular signal-regulated kinases

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ESCC	Esophageal squamous cell carcinomas
ERK5	Extracellular-signal-regulated kinase 5
Gli3	GLI Family Zinc Finger 3
hTERT	Human telomerase reverse transcriptase
HATs	Histone acetylases
HCC	Hepatocellular carcinomas
HNF4 γ	Hepatocyte nuclear factor 4 receptor γ
HDACs	Histone deacetylases
H3-K9	Histone H3 Lysine 9
H3-K27	Histone H3 lysine 27
INK4A	Inhibitor of Kinases
IGFBP2	Insulin-Like Growth Factor Binding Protein 2
ITCH	Itchy E3 Ubiquitin Protein Ligase
KPNB1	Karyopherin Subunit Beta-1
LIN28	Lin-28 homolog A
MMP3	Matrix Metallopeptidase 3
mTOR	Mechanistic Target Of Rapamycin
MERTK	MER Receptor Tyrosine Kinase
MSP	Methyl sensitive PCR
MAPK	Mitogen activated protein kinases
Mekk2	Mitogen-Activated Protein Kinase Kinase 2
Mekk5	Mitogen-Activated Protein Kinase Kinase 5
Mad1	Mitotic arrest deficient 1
MDR-1	Multidrug resistance-1
MiRs	Micro RNAs
MTS-1	Multiple tumor suppressor
NPC	Nasopharyngeal carcinomas
NSCLC	Non-small cell lung carcinoma
Notch 1	Notch Homolog 1
NR2F2	Nuclear Receptor Subfamily 2 Group F, Member 2
oncomiRs	Oncogene
PI3K	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase
PTEN	Phosphatase and Tensin homologue deleted from chromosome 10
PCa	Prostate cancer
PIPNC1	PTEN Induced Putative Kinase 1
PAK4	P21 (CDKN1A)-Activated Kinase 4
RASSF	Ras-association domain family
Rb	Retinoblastoma protein
RECK	Reversion-Inducing-Cysteine-Rich Protein with Kazal Motifs
SPARC	Secreted Protein Acidic, Cysteine-Rich
SEPT9	Septin 9
SMAD4	SMAD Family Member 4
SNAI1	Snail Family Zinc Finger
TIMP	Tissue Inhibitor of Metalloproteinases
TP53	Tumor protein p53

tsmiRs	Tumor suppressor
ZEB 1	zinc finger E-box binding homeobox 1
ZEB 2	zinc finger E-box binding homeobox 1

1.1 Introduction

In many cancers, the expression of key genes, for example *p21, p16; Ras; c-Myc; p53* that regulate the cell cycle, division, proliferation and death, respectively, are frequently altered. [1–6]. Tumor biogenesis stems from sequential aberrant changes in such genes that regulate cellular processes, which determine the outcome of cell fate and thus, cell numbers (Fig. 1.1). Over a decade or so epigenetics, a process beyond genetic control has gained prominence in the role of gene regulation. Microarray and promoter analysis of genes mentioned above, in addition to some others, have shown that genes are prone to epigenetic alteration by DNA methylation, histone modifications and miRNA regulation as well [7–10].

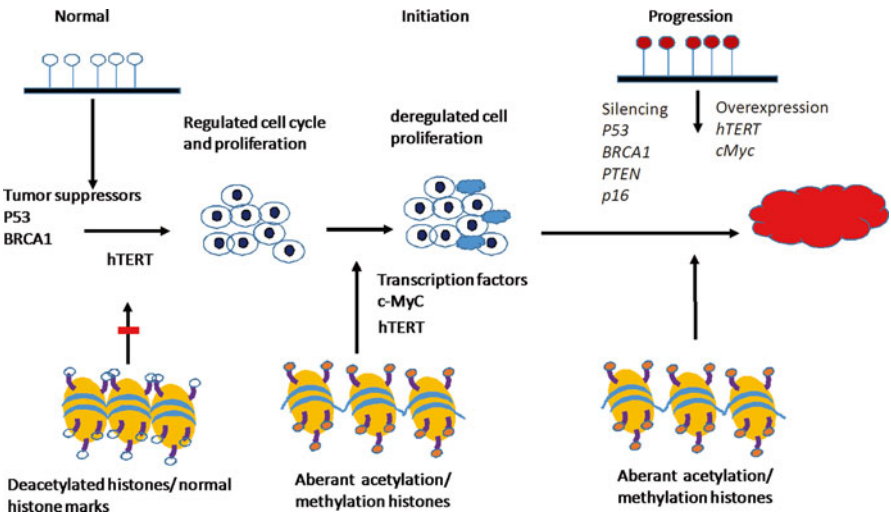

Fig. 1.1 Epigenetics of cancer initiation and progression. This figure illustrates the genesis of tumor formation by deregulation of genes controlling cell division and cell proliferation. In normal cell, the controlled functions of tumor suppressors such as p53 and BRCA1 correct abnormal changes in DNA through repair processes. Absence of *hTERT*, ensures that cells reach their replicative potential and undergo senescence preventing abnormal cells from entering the cell cycle. Deregulation of genes by histone modifications often precedes DNA methylation and encourages the initiation of tumors by the increase in expression of genes potentiating cell proliferation such as *cMyc* and *hTERT*. Furthermore, increased degrees of methylation in conjunction with histone modifications can lock genes in either a continued state of expression such as *hTERT* or inhibition or loss of tumor suppressor genes such as BRCA1. Cells expressing such altered epigenetic states promote the progression of the disease as seen in many cancers

DNA methylation constitutes the addition of methyl moieties to cytosine residues that exists in the CpG dinucleotide configuration [11, 12]. Promoters of genes containing stretches of CpG islands are subjected to methylation mediated gene regulation. DNA methylation is catalyzed by three major enzymes, DNA methyltransferase 1 (DNMT1), DNA methyltransferase 3A (DNMT3A) and DNA methyltransferase 3B (DNMT3B). DNMT1, a maintenance methylation enzyme, requires a premethylated DNA template strand [13–15] and ensures that methylation patterns are faithfully copied allowing for methylated signatures to be inherited in subsequent generations [15]. DNMT3A and DNMT3B are *de novo* methylating enzymes and act on newly formed DNA strands [14]. There exists an inverse relationship between promoter methylation and gene expression with the exception of a few genes such as the human telomerase reverse transcriptase (*hTERT*) where hypermethylation is associated with induced gene expression state and hypomethylation a silenced state [16–19]. Reversing promoter-methylated configurations and thus deregulated epigenetic patterns through an enzyme-based targeted approach presents as an excellent chemotherapeutic strategy.

Chromatin consists of repeating nucleosomal units that assists with higher order chromosomal organization and allows for accessibility of DNA to the transcriptome machinery [20]. Each nucleosomal is composed of individual histone subunits (H2A, H2B, H3 and H4) that are basic proteins and exists as dimers in the complex to which 147-bp of DNA is wrapped [21]. The linker histone H1 tethers individual nucleosomal units and assists with the condensation and decondensation states of the chromosome [21]. The amino tails of histones that extend from the nucleosomal complex are subjected to reversible enzymatic modifications. Several histone enzymatic modifications such as acetylation, phosphorylation, sumoylation, ubiquitinylation exists which dictate specific outcomes of gene expression and is residue-specific [22]. For example, acetylation of lysine residues is associated with gene expression and deacetylation with gene repression [23]. The enzymes histone acetylases (HATs) and histone deacetylases (HDACs) that catalyze the reactions are well characterized and are involved in inducing altered epigenetic changes in various cancers [23–25].

The 22 nucleotide long non-coding RNAs, miRs play a significant role in the etiology of cancers [26]. MiRs target genes involved in various facets of cell processes such as DNA repair, proliferation, cell-cycle and cell death [26]. The overexpression of miRs such as miR-182-5p, 96 repress genes necessary in the DNA repair processes inducing DNA damage [27, 28]. In addition, the over expression of miRs (miR-34 family, miR34a/b/c) strongly repress genes essential to apoptosis and the cell cycle resulting in the unified initiation of a neoplastic phenotype [29]. Targeting miRs thus serve as important cancer preventive and therapeutic tools. In addition to their important role in cancer therapeutics, the expression profile of miRs present as possible noninvasive tools in the diagnosis and prognosis of different cancers.

The chapter will focus on tumor suppressor genes associated at various levels of cell cycle regulatory control and include *p16*, *BRCA1*, *RASSF*, *PTEN* and *APC*. The expression of *hTERT*, the catalytic component of telomerase that is upregulated in a majority of tumors by altered methylation and *MDR-1*, a gene responsible for drug-resistance in most cancers will also be discussed. The aberrant epigenetic alteration

of these genes that occur in a majority of cancers and which are associated with the onset and progression of tumors will be covered. The significance of the epigenetic marks of these genes in the prognostics and diagnostics of various cancers will be highlighted.

1.2 Tumor Suppressors

1.2.1 P16

The p16 (INK4A/MTS-1/CDKN2A) tumor suppressor gene functions as a cell cycle regulator by inhibiting cyclin-dependent kinases [30]. Decades of research have provided solid evidence of its importance in carcinogenesis [31, 32] and its role in the cell cycle has been well elucidated [33]. The p16 protein binds to CDK-4/6 protein complex inactivating it [33]. The inactivated CDK4/6 furthermore prevents the activation of the retinoblastoma protein (Rb) inhibiting the transcription of cell-cycle inducing genes initiating cell-cycle arrest [33]. P16 is therefore an essential molecule in preventing the development of neoplastic phenotypes by encouraging cells to undergo senescence [34].

In many primary tumors, *p16* mutations are frequent and the gene is silenced either by homozygous deletions, promoter methylation and point mutations [30]. Of these alterations, inactivation of *p16* is governed by the former two mechanisms in a majority of primary tumors where, homozygous deletion are considered as events involved in premalignant lesions followed by loss of p16 through promoter methylation leading to cancer progression [35]. Evidence from many reports strongly supports the epigenetic regulation of p16 in many cancers where the promoter of the gene is hypermethylated (Table 1.1) [36, 37]. CpG island methylation mediated inactivation of p16 is also associated with neoplastic progression [38] and treatment with demethylating agents restores expression of functional p16 which induces cell cycle arrest through the activation of phosphorylated rentinoblastoma pathway propitiating cellular differentiation [39]. Aberrant *p16*INK4A transcripts have been observed in hepatocellular carcinomas (HCC) by hypermethylation of *p16* promoter [40]. Distinct epigenetic signatures of DNA hypermethylation, H3-K9 hypoacetylation and H3-K9 dimethylation at the promoter region have been shown to silence *p16* in gastric cancers [41]. The correlation of clinicopathological characteristics with *p16* hypermethylation is significant in gastric cancers and has been supported by meta-analysis studies and has potential to serve as a biomarker in the diagnosis of gastric cancers [42, 43].

In colon cancers, aberrant methylation of the p16INK4A gene is commonly observed [44, 45]. Nevertheless, conflicting reports regarding methylated states and *p16* expression exists in colon cancer. In a report by Yoruker et al., 53 % (n = 71) *p16* promoter hypermethylation was observed with increase in *p16* expression in tumor samples over normal matched controls [44]. Contradictory to this finding, the Malhotra study reported 40 % (n = 30) *p16* promoter methylation and the methylated state had a significant effect on the loss of *p16* expression encouraging cancer

Table 1.1 P16 promoter methylation status in various cancers

Tumor type	Analysis	Percent methylation (%)	Method	Reference
Oral squamous cell carcinoma	Cell lines SCC-15	Complete methylation	Bisulfite genomic sequencing	[157]
Oral squamous cell carcinoma	Tissue	64.70	MSP	[158]
	Serum of same patients	54.50		
Oral squamous cell carcinoma	Tissue	28	Bisulfite sequencing (Pyrosequencing)	[159]
Head and neck cancer	Tissue	67.50	Bisulfite modification/MSP	[160]
Primary lung carcinoma	Tumor tissue		MSP	[161]
• Nonsmall cell lung cancer (NSCLC)		41		
• Large cell carcinoma		71		
• Adenocarcinoma		36		
• Squamous cell carcinoma		33		
Nonsmall cell lung cancer	Tissue	51	MSP	[162]
Non-small cell lung cancer	Tissue	36.70	MSP	[53]
Lung cancer	Tissue biopsies	82.85	Two-stage MSP	[163]
Non-small cell lung carcinoma	Bronchial washings	80.35		
Small cell lung carcinoma		16.66		
Invasive breast cancer	Tumor tissue	24.20	MSP	[164]
Bladder cancer	Tissue biopsies	26	MSP	[165]
	Serum	21		
Cervical cancer	Tissue	57	MSP	[166]
Cervical cancer	Tissue	36	MSP	[167]
Recurrent colorectal cancer	Tumor DNA in the serum	69	MSP	[168]
Colorectal cancer	Tissue	26.90	MSP	[53]
Colorectal cancer	Tissue	66	MSP	[169]
Colorectal cancer	Tumor tissue	47	MSP	[170]

progression [46]. The disparity in observation may be due to the type and location of tissue sample analyzed and the influence of the ethnicity of the patient on the sample genomes analyzed.

Modification of histone residues by methylation has also been found to potentially regulate the expression of p16 in cancers in conjunction with promoter DNA methylation [41]. Methylation of lysine and arginine residues of histones H3 and H4 mediate transcription functions [47]. Histone 3 lysine 4, H3K4; Histone 3 arginine 17, H3R17 and histone 4 arginine 3, H4R3 methylation is associated with the activation of transcription [48–50]. By contrast, methylation of histone H3 lysine 9 H3K9 signals gene silencing [51]. Histone residue mono-, di- and tri-methylated states are differentially regulated and exert various functional outcomes in cancers. In breast cancers, *p16* hypermethylation serves as a precursor to epigenetic silencing mediated by the histone H3K27 trimethylation; H3K9 acetylation and the H3K9 dimethylation signatures. Histone H3K9 methylation of p16 is thought to precede DNA methylation and induce p16 gene silencing in colon cancers [52]. Evidence supporting the idea requires further verification as the study by Yoruker did not show a correlation between DNA methylation and H3K9 methylation and clinical parameters [44]. As previously thought, aberrant methylation of *p16* in colorectal cancers may not be sufficient enough to initiate the disease but rather deregulated other genetic events which together have a causative role in colon carcinogenesis.

The role of p16 promoter hypermethylation and its effect on p16 expression in CRC (colorectal cancer) is rather controversial. In vitro clinical studies analyzing promoter methylation and p16 expression have come up with inconclusive results as to the significance of *p16* promoter hypermethylation, its expression and clinicopathological characteristics [46, 53]. However, meta-analyses of 27 clinical cohorts which included 3311 CRC patients revealed that promoter methylation of the p16 gene strongly correlates with the clinicopathologic features of CRC and serves as a favorable marker in the diagnosis of the disease [54].

*1.2.2 Breast Cancer 1 (*BRCA1*)*

BRCA1 is an important tumor suppressor that confers genomic stability by fixing double-strand breaks through homologus recombination [55, 56]. The ability of DNA repair mechanisms to fix DNA damage is important in preventing the development of a cancerous phenotype. The promoter of *BRCA1* is CpG-rich and is frequently methylated in the genesis of breast cancer and the sensitivity to DNA-damaging agents; length of time of relapse and overall survival is associated with the extant of *BRCA1* promoter hypermethylation [57]. The epigenetic control of *BRCA1* has been observed in sporadic breast cancers as well [58]. DNMT1, a methyltransferase enzyme, is a transcriptional target of *BRCA1* and is affected by the loss of BRCA1 [59]. The binding of functional BRCA1 to the *DNMT1* gene enforces an open active configuration positively regulating *DNMT1* expression [59]. The loss of BRCA1 followed by DNMT1 down regulation induces global DNA hypomethylation at CpG islands coupled with site-specific hypermethylation and is

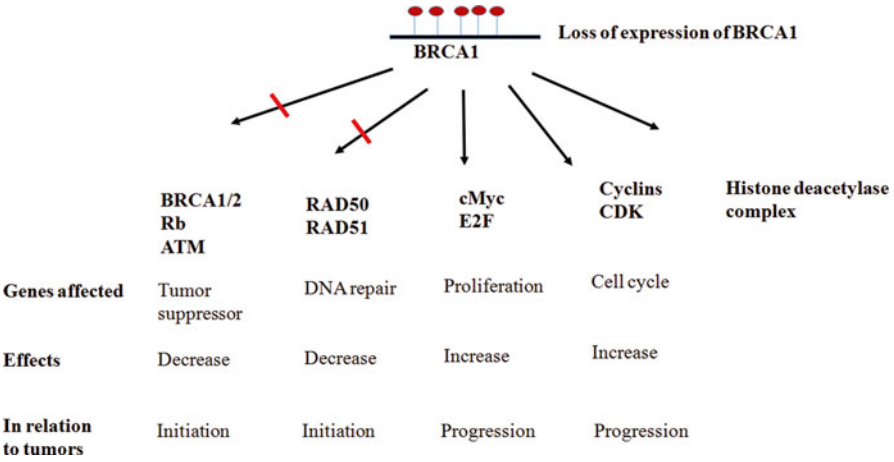

Fig. 1.2 Effects of hypermethylated BRCA1 on downstream genes and functions. BRCA1 tumor suppressor regulates several processes and has a major impact on tumor pathobiology. The protein is central to the control of downstream effectors that are important to the cell cycle, cell proliferation and directly effects the tethering of the HDAC complex to promoter sites to inhibit gene expression. Therefore loss of BRCA1 through the hypermethylation of its promoter region increases the expression of genes such as *cMYC* and *E2F* both of which are central to inducing a proliferative phenotype in cells. Tethered with increased expression of cell proliferative genes, the increased expression of cyclins, factors that regulate the cell cycle and thus cell division are further enhanced. Simultaneously, decrease in tumor suppressor functions of genes such as *p53*, *BRCA2* and *ATM* encourages a proliferative phenotype. Together, the defects in several of these pathways that are otherwise tightly controlled by functional *BRCA1* allows for tumorigenic phenotypes to develop

a hallmark for breast cancer [59]. BRCA1 regulates several downstream genes that affect the cell cycle and proliferation pathways and the loss of BRCA1 expression upregulates protooncogenes including *c-Fos, Ha-Ras, and c-Myc* [59] (Fig. 1.2). In addition to breast cancer, methylation-mediated loss of *BRCA1* and breast cancer 2 (*BRCA2*) is observed in ovarian cancer [60].

Tissue sample assessments and population-based epidemiological studies suggest that the methylation status of *BRCA1* can serve as a prognostic tool and determine survival [61]. Clinical assessments of breast and ovarian tumor tissue samples have shown an 82.1 % frequency of *BRCA1* methylation [62, 63]. However, 59 % *BRCA1* promoter methylation has been observed in a population-based study analyzing tumor tissue from invasive or in situ breast cancer [64]. Ethnic differences in *BRCA1* percent methylation have also been detected (24 %, 11/45 in Indian; 17.04 % 23/135 Bulgarian) [65, 66]. Patients with lobular type breast tumors with hypermethylation in *BRCA1* without p53 mutations display a favorable clinical status [65]. Therefore, methylation status of *BRCA1* has promising potential as a biomarker in the diagnosis and prognosis of the disease and demethylating agents (drug-based and natural bioactive molecules) targeting the gene provides potential treatment value.

1.2.3 *Ras-Association Domain Family (*RASSF*)*

RASSF are considered as effector/tumor suppressors and contain domains that associate with Ras [67]. Ras is important to many cellular processes and is involved in cell signaling events through the RAS-Mitogen activated protein kinases (RAS-MAPK) pathway [68]. RASSF family members are epigenetically silenced during tumorigenesis and many members have been identified that are involved in the biogenesis of tumors of specific tissues [69]. In primary lung and breast tumors high frequency of promoter methylation is observed in *RASSF1A and RASSF2* where as *RASSF6* is epigenetically silenced by promoter methylation in melanomas [70–72]. The loss of RASSF6 prevents the inhibition of MAPK activation and AKT that regulate cell proliferation [72] encouraging the development of melanomas.

Various RASSF proteins are epigenetic biomarkers and the promoter methylation status of the genes provides excellent prognostic information. Tumor-tissue *RASSF* promoter methylation studies have shown that based on the tissue analyzed the methylation status is associated with poor survival-rate. In salivary adenoid cystic carcinoma based on sample size (n = 167) 35 % of *RASSFA1* was hypermethylated [73]. The hypermethylation of *RASSF1* is also a clearly important predictor of gastric cancer risk (OR, 12.67; 95 % CI, 8.12–19.78; $p < 0.001$) supported by a meta-analysis study [74]. Several studies have examined the role of methylation of *RASSFA1* and prostate cancer (PCa) risk but most results have been conflicting and inconclusive [75–77]. However, a meta-analysis study, which included 19 studies, reported a strong association between *RASSFA1* promoter methylation and PCa risk [77]. The *RASSF1A* methylation status in tissue appears to be more concise in determining tumor stage and can be utilized for the early detection and prognosis prediction of PCa [78]. A report assessing the methylation frequencies of *RASSF1A* in breast cancer tissue revealed that 70 % of the tissues assessed contained methylated residues, where invasive carcinomas and advanced stage breast cancer showed the highest methylation frequencies of *RASSF1A* [79].

RASSF2A negatively regulates Ras and is epigenetically silenced in epithelial ovarian cancers (EOC) and cervical cancers. Methylation of the promoter is not observed in normal EOC or cervical tissues. Studies examining the methylation profiles of the RASSF2A promoter have shown a 51.1 and 56.5 % methylation in EOC and cervical cancer tissue, respectively [80]. The methylation of RASSF2A gene in plasma samples can be detected and serve as tools for the early diagnosis of EOC [80, 81].

The examination of esophageal squamous cell carcinomas (ESCC) have shown a 44.3 % methylation of *RASS10*, another RASSF family member [82]. This member of the RASSF family inhibits cell proliferation and induces G2/M arrest in ESCC [82]. *RASSF10* is highly methylated with a frequency of 61.6 % in primary gastric tissues [83]. The frequency of methylation is also associated with the degree of metastases and invasion (87.5 %). In lung cancers, RASSF10 has been shown to induce cellular apoptosis and thus exhibits tumor suppressor functions [84]. A study assessing the methylation of RASSF10 promoter in lung cancer cell lines showed

that *RASSF10* was methylated in 4/6 cell lines [84]. Taken together these findings suggest that RASSF family members have varying roles in tumor pathogenesis and are differentially regulated by methylation in various cancers, but individually are extremely important as epigenetic biomarkers.

*1.2.4 Phosphatase and Tensin Homologue Deleted from Chromosome 10 (*PTEN*)*

PTEN is functionally involved in inhibiting the AKT-pathway through its phosphatase activity and plays an important role in regulating the cell cycle [85]. However, in many cancers, a loss of PTEN expression is observed and the loss is attributed to the promoter methylation of its CpG residues [86, 87]. Promoter hypermethylation of *PTEN* is not a universal determinant of tumor development for all tissues but rather is tissue specific. The loss of PTEN expression is considered important in tumor initiation and progression and development of acquired drug resistant as determined in some cancers [87]. Aberrant methylation of *PTEN* also alters the Phosphatidylinositol-3 kinases-Protein kinase B (PI3-AKT) pathway and is a key determinant to the etiology of rare melanomas, example sinonasal mucosal melanomas [88–90]. Although loss of PTEN is observed in esophageal squamous cell carcinoma (ESCC), the frequency of methylation is low (18.9 %) and is considered not the only contributing factor to disease development [91]. Supporting the observation was a study conducted in a Chinese Kazakh population where the incidence of ESCC was shown to be high and significant when compared to normal esophageal tissue [92]. However, the frequency of methylation was not high when compared to the methylation status of *PTEN* in other cancers. Nonetheless, the study was able to determine its merit as an indicator of tumor metastasis. Different from ESCC, in prostate cancer *PTEN* promoter is 70 % methylated.

In lung cancers, hypermethylation of *PTEN* promoter followed by loss of PTEN expression contributes to the drug resistance phenotype against certain treatments [93]. Studies in non-small cell lung carcinoma have shown a 69 % methylation of *PTEN* promoter in various NSCLC cell lines and 35 % in NSCLC tissue samples and are hypothesized to be an event in the early-stage of the disease [94]. In cervical malignancies, downregulation of *PTEN* expression due to increased promoter methylation (62 %) contributes to the pathogenesis, invasion and metastasis of the disease and significantly correlates with the clinicopathological characteristics of the disease [95]. The role of *PTEN* in the pathogenesis of CRCs is thought to occur through *PTEN* downregulation. A combination of alterations such as mutations, loss of heterozygosity or promoter methylation of *PTEN* is believed to mediate the loss of *PTEN* expression in CRCs. The proposed two-hit gene hypothesis governing PTEN gene regulation have shown that the downregulation of *PTEN* although frequent in CRCs is not the direct result of a two-hit gene event as previously hypothesized [96]. Thus further investigations in the area of *PTEN* epigenetic regulation in CRCs are necessary.

PTEN hypermethylation is considered to be an early event in the etiology of nasopharyngeal carcinomas (NPC). A study investigating the methylation of *PTEN* in NPC showed a significantly higher level of *PTEN* promoter methylation (82.2 %) in NPCs specimens as compared to non-tumor nasopharyngeal epithelial tissues (5.3 %) [97]. The methylation status of *PTEN* is considered to be an excellent epigenetic biomarker in the detection of early-stage NPCs.

*1.2.5 Adenomatous Polyposis Coli (***APC***)*

APC is a protein encoded by the *APC* gene and is frequently altered in colorectal cancers [98]. The tumor suppressor gene regulates Beta-catenin levels and interacts with a cell adhesion molecule E-cadherin [99]. APC plays a very important role in controlling the frequency of cell division and ensures correct copy of the chromosomal complement in the daughter cells from cell division [100]. These controlled cell-regulatory functions by APC are accomplished through its ability to regulate the activity of B-catenin, which in turn affects the expression of downstream genes involved in the stimulation of cell division, inhibiting cell overgrowth. In addition to mutations, promoter methylation is a frequent mechanism by which APC is silenced and is linked to early development of CRCs [98, 101]. However, in colorectal metastasis there does not exist a significant correlation between APC protein levels and promoter methylation [98]. In contrast to the role of APC downregulation as an early event in CRCs, APC promoter methylation does not relate to breast cancer progression as determined in an MCF10 breast cancer model system [102]. Contradictory to this report, a study analyzing APC methylation in sporadic breast carcinomas revealed a significant association between APC methylation and tumor size but lacked a correlation with the clinicopathological characteristics [103].

Although a very prominent alteration in CRCs, APC promoter methylation is also observed in other cancers and serves as a determinant in terms of disease recurrence and poor survival [104]. In liver malignancies, the pathophysiological role of APC hypermethylation is unclear. APC hypermethylation is observed in both hepatocellular carcinomas as well non-cancerous liver tissue but the degree of methylation is higher in HCC (63.1 %) than non-cancerous liver tissue samples (24.98 %) [105]. Promoter methylation of APC is observed in ovarian carcinomas in conjunction with RASSF1A and is hypothesized to be an epigenetic associated trigger in the development of ovarian carcinomas [106]. Data from a study assessing 19 benign cystadenomas, 14 low malignant potential (LMP) tumors, and 86 carcinomas found both RASSFA1 and APC methylated with the following frequencies: 37 % and 16 %; 50 % and 28 %; and 58 % and 29 %, respectively [106]. Epigenetic insults of these specific genes as a combination can induce the transformation of benign and LMP tumors to carcinomas.

1.3 Human Telomerase Reverse Transcriptase (*hTERT*)

The expression of telomerase, a reverse transcriptase enzyme is required for telomere length maintenance and imparts cell immortality and tissue longevity [107]. Telomerase is absent in normal somatic cells, with the exception of a few, primarily stem cells, and is significantly upregulated in 90 % tumors and contributes to the immortality phenotype [108]. The human telomerase reverse transcriptase gene (*hTERT*) is the catalytic component of telomerase and the expression of hTERT is rate limiting for telomerase activity [108]. *hTERT* is therefore looked at as a favorable biomarker in the diagnosis, therapeutics and prognosis of various cancers [109–111]. Understanding the mechanistic regulation of *hTERT* is important and has received considerable attention. Since the characterization of the *hTERT* promoter, several transcription factors have been identified to be involved in the transcriptional regulation of *hTERT*, of which *cMyc* and *Mad1* are key activator and repressor, respectively [112]. Various other activators and repressors of *hTERT* have been identified and are well reviewed elsewhere [113] In addition to genetic regulation, the *hTERT* core promoter is subjected to epigenetic control where the dense CpG residues within the promoter are subjected to methylation [114].

Unlike most genes, methylation-mediated transcriptional regulation of *hTERT* does not follow the norm, where hypermethylation results in gene silencing. *hTERT* expression is governed by hypermethylation with region/residual specific hypomethylation, in that, the *hTERT* minimal promoter must be hypomethylated [19]. The observation has been supported by the study of Renuaud et al. which showed that the binding of CCCTC-binding factor (CTCF) a transcriptional repressor of *hTERT* is sensitive to the methylation status of the promoter at residues 31 and 33 [115]. The use of demethylating agents targeting *DNMT1* has been shown to downregulate *hTERT* where the CpG residues become hypomethylated [116]. Treatment of cancer cells to histone deacetylase inhibitors facilitates the binding of transcriptional repressors to the *TERT* promoter inducing repression [116]. Since histone modifications are various, histone methylation also affects *TERT* expression where trimethylation of specific histone residues induce expression and have been reported in tumor cells [117]. Transactivation of cMyc by class III HDACs facilitates *hTERT* expression and has been reported to be seen in human leukemic cells [118]. The re-expression of telomerase is essential to tumor initiation and progression and controlling *hTERT* would serve as a promising tool in cancer therapeutics.

1.4 Multidrug Resistance-1 (*MDR-1*)

MDR-1 gene responsible for inducing drug resistant phenotypes against chemotherapeutic treatment in various cancers is regulated by epigenetic mechanisms [119]. The presence of CpG islands in the promoter of this gene makes it a likely candidate for methylation-mediated regulation. The functional roles of MDR-1 in

cancer are not well understood. Apart from it canonical function of inducing drug resistant phenotypes, the product encoded by the gene, P-glycoprotein is also thought to regulate the progression of certain tumors when silenced or downregulated [120, 121]. The possibility that MDR-1 can function in pathways that regulate apoptosis is encouraging. It is plausible that MDR-1 may have an important role in balancing cell proliferation and cell death through apoptosis [122]. However investigations in this area are limited and further studies will be required to support its role in apoptosis.

Studies of *MDR-1* expression and its role in prostate cancer have resulted in controversial findings. Although a majority of studies support the finding of acquired drug resistance with increased *MDR-1* gene expression, a fairly recent study showed that the down regulation of *MDR-1* induced PCa progression [123]. Interestingly, although significantly higher promoter methylation has been observed in high-grade prostate intraepithelial neoplasia and prostate cancer as compared to normal prostate tissue (NPT) samples, promoter methylation alone is not responsible for reduced *MDR-1* gene expression [123]. Overall, histone signatures, H3 and H4 deacetylation, H3K9 deacetylation, decreased di- and trimethylation of H3K4 at the promoter are essential for the observed concomitant decrease [123]. Thus, *MDR-1* is regulated through dual epigenetic mechanisms in prostate cancer. In bladder cancers, *MDR-1* promoter methylation inversely correlates with gene expression and the overexpression of the gene can be a useful prognostic tool for determining recurrence of the disease and efficacy of the drug treatment [124]. In bladder cancer promoter methylation of the MDR-1 gene presents an opportunity for targeted therapy by employing drugs that target epigenetic enzymes that alters the methylation status of the gene reducing its expression. In CRCs, both *p16* methylation (29 %) and *MDR-1* methylation (28 %) are found in tumors with microsatellite instabilities and is associated with poor histopathological differentiation [125]. In leukemic cell lines opposing effects of MDR-1 expression have been observed indicating the functional role of methylation mediated epigenetic regulation of *MDR-1*. The K562 leukemic cell lines that respond to drug treatment have hypermethylated promoters in contrast to K562/DNR which are drug resistant and thus hypomethylated [126]. Similar findings have been determined in breast cancer cell lines in association with chromatin modifications [127].

1.5 Micro RNAs (MiRs)

MiRs are small non-coding RNAs of approximately 21–25 nucleotides (nt), which function as post transcriptional regulators of gene expression. MiRs regulate all mRNA transcripts involved in broad array of functions such as cell proliferation, apoptosis, growth and differentiation [128]. Deregulation of miR expression and consequently the target gene has been attributed to development of cancer phenotype. MiRs are considered as candidate tumor suppressor (tsmiRs) or oncogene (oncomiRs) and are involved in pathways which are dysregulated during tumor

Table 1.2 Dysregulation of miRs, their targets genes, and effects in different types of cancers

Micro RNA	Expression	Gene regulated	Cancer	Reference
miR-20a	Up-regulated	*TIMP and ATG7*	Cervical Cancer	[171]
miR-506	Down-regulated	*Transcription factor Gli3*	Cervical Cancer	[172]
miR 30c	Down-regulated	*ADAM19*	Colon Cancer	[142]
miR 330	Down-regulated	*E2F1*	Prostate Cancer	[173]
Mi R 30a/d	Up-regulated	*KPNB1*	Malignant peripheral nerve sheath tumor	[174]
miR-214	Up-regulated	*PTEN*	Breast Cancer	[175]
MiR-29 and MiR-200	Downregulated	*ADAM12-L*	Breast Cancer	[176]
MiR-122	Up-regulated	*P27 and p57*	Hepatic Cancer	[177]
Mi R-199a/b-3p	Down-regulated	*PAK4/Raf/MEK/ERK*	Hepatic Cancer	[178]
Mi R-126	Downregulated	*IGFBP2, MERTK, PIPNC1*	Breast Cancer	[179]
Mi R-15A16-1	Downregulated	*TP53*	CLL	[180]
Mi R-155	Up regulated	*AICDA*	Burkitt Lymphoma	[181]
Mi R-224	Downregulated	*SMAD4*	Colorectal Cancer	[182]
Mi R-411	Upregulated	*ITCH*	Hepatic Cancer	[183]

progression and cancer metastasis [129]. MiRs have multiple target genes and each gene has 3′UTR region for different miR. OncomiRs are up-regulated in cancer cells and negatively control tumor suppressor genes and thus are related to poor prognosis. Alternatively, tsmiR are downregulated and negatively regulate oncogenes. Some of the important miRs, their expression profiles, target genes and their role in specific type of cancers are listed in Table 1.2.

1.5.1 miR-17-92 Clusters

Of the oncogenic miRs, miR-17-92, a polycistronic cluster located at 13q31.3 is well characterized and encodes six different miRs [130, 131]. This region is amplified in various hematopoietic malignancies such as B cell lymphoma, follicular lymphomas, Burkitts lymphoma as well as various solid tumors including lung carcinoma [131]. A recent study has shown that miR17/20a acts as a tumor suppressor in colon and breast cancer cells leading to tumor cell reprogramming for NK cell mediated cytotoxicity. High expression of this cluster enhances NK cells recognition by inhibiting MHC class1 expression through *Mekk2-Mekk5-Erk5* pathway [132].

Additionally, miR 17-92 cluster has a crucial role in lymphocyte development as target deletion of this cluster leads to increased expression of BCL2L11 (pro-apoptotic protein) and prevents pro to pre B cell development. Overexpression of this cluster leads to increase proliferation and decreased cell death resulting in lymphoproliferative disease. These effects can directly be linked to down-regulation of anti proliferative PTEN protein along with BCL2L11 [133]. Moreover, miR17-92 cluster overexpressed with Myc leads to an enhanced chance of developing lymphoid malignancies and as well high tumor aggressiveness, attributed to Myc/ miR17-92 cluster/ E2F1 circuit [134].

Investigations assessing the functional role of the Oncomir in colon cancers have shown that increased expression of this cluster due to 13q31 locus gain has a direct correlation with the progression of colorectal adenocarcinoma [135]. Increased c-Myc expression and over expression of miR-17-92 cluster has been shown to initiate the transition from colorectal adenoma to adenocarcinomas [136]. Similarly, 13q31 amplification and thus consequently miR 17-92 cluster over expression has been considered a contributing factor to retinoblastoma (Rb) oncogenesis. Epithelial cell adhesion molecule (EpCAM) regulates the expression of miR-17-92 cluster, which plays a crucial role in Rb cell proliferation and invasion [137].

1.5.2 MiR-30 Family

MiR-30 family is characterized as tumor suppressor miRs and is down regulated in many cancers. Five mature miR sequences: miR30a/miR-30c-2, miR-30d/miR-30b and miR-30e/miRMiR30c-1 belongs to this family [138]. miR-30 is considered as the landmark for miR oncogenesis signal network in cancer and its dysregulation has drastic effect on tumorigenesis [138]. miR-30 has a myriad tumor suppressor functional role. The molecule induces cellular senescence by negative regulation of B-MYB, represses stem cell and cancer phenotype through LIN28 regulation, and suppresses epithelial-mesenchymal transition to reduce invasive potential of anaplastic thyroid cancer and prostate cancer [139]. Down regulation of *ERG, SNAI1, SPARC, MMP3, Notch 1, LIN28 and ZEB* 1/2 are observed to be demodulated in response to the overexpression of miR-30b in prostate cancer [140]. miR-30 has been observed as a metastatic signature for breast, bladder, colon and lung cancer [141]. Interestingly, miR-30c has been down regulated in NSCLC, PCa, leukemia, endometrial cancer, breast and colorectal cancer. Alternately, some studies have demonstrated the up-regulation of miR-30c in breast cancer and renal cell carcinoma [142]. Hormones such as estrogen and progesterone regulate miR-30 expression and in tumors that are devoid of estrogen and progesterone receptors show decreased miR-30 levels Thus, miR-30c expression is considered to be a predictor of endocrine therapy in ER+ (estrogen receptor positive) breast cancer cells [143]. A recent publication has shown that miR-30 down-regulation and miR-194 up-regulation effects the expression of transcription factor HNF4 γ and NR2F2, respectively which has been attributed to stomach to intestinal metaplasia progression, the initial stage of gastric cancer [144].

1.5.3 miR-21

miR-21 is a well-studied oncogenic miR and has been observed to be up-regulated in various cancers such as lung, ovarian, colon and astrocytoma [145]. miR-21 up-regulation and targeting of reversion-inducing-cysteine-rich protein with kazal motifs [68] gene leads to tumor cell invasiveness and correlates to prostate cancer aggressiveness and recurrence [145]. Role of miR-21 is well studied in hepatocellular carcinoma (HCC) where its upregulations is significant when compared to normal tissue. MiR-21 enhances HCC migration and invasion through target gene programmed cell death 4 (PCCD4) following the miR-21-PCCD4-AP-1 loop [146]. The role of miR-21 in HCC was further supported by the Zou L et al. investigation, suggesting that miR-21 helps in tumor dissemination through target genes PTEN, RECK and PCCD4 [147].

Studies have shown a significant correlation between increased expression of miR-21 and clinico-pathological conditions along with patient survival. This single stranded molecule is upregulated in breast cancers with lymph node metastasis and may have significant role in acquiring a metastatic potential related to poor patient prognosis and survival chance [148]. Additionally, dysregulation of miR-21 is also implicated in doxorubicin resistance in breast cancer via target gene PTEN [149]. Furthermore, miR-21 is also believed to be responsible for doxorubicin and taxol resistance in glioblastoma cells [150]. Thus miR-21 plays a crucial role in regulating drug sensitivity in cancer cells and miR-21 inhibitors can be potential candidates in reversing drug resistance. Findings of a study by Abue et al. showed a significant association between serum level of miR-21 and pancreatic ductal adenocarcinoma patients clinical outcome. Higher expression levels of miR-21 in the plasma of patients correlated with the advance stage; metastasis to lymph nodes and liver, as well as shorter survival [151]. Additionally, circulating miR-21 levels have been considered as potential biomarker for early detection of CRCs and high expression at tissue levels are candidate biomarker for poor prognosis [152].

1.6 Epigenetic Biomarkers

The etiology of cancer is complex. It involves deregulation of genes and epigenetic signatures that control pathways regulating cell division altering the balance between cell proliferation and cell death [153]. Epigenetic changes influence each step of tumor development and include initiation though promoter specific hypermethylation, progression and metastasis. Thus, epigenetic changes in the form of potential biomarkers can be advantageous for early diagnosis and cancer risk management [154]. Efficacious, sensitive and cheaper screening of cancer using epigenetic markers in serum/ plasma or other bodily fluids is the current goal for research

in the field of cancer biomarkers. For example, Vatandoosi et al. proposed the use of several serum and fecal biomarkers in addition to conventional sigmoidscopy and colonoscopy, to enhance the reliability of colorectal cancer diagnosis, preventing invasive methods for early screening. DNA methylation markers (*SEPT9, SFRP2, ALX4*) and miRs (miR-21) can be potential biomarkers for predicting CRCs and polyps [155]. In breast cancer, dysregulation of p53, PI3K/Akt/mTOR and Ras/MAPK pathways due to epigenetic alterations (histone methylation and acetylation, DNA methylation) and miR expression provides prognostic and predictive values as biomarkers for the diagnosis, therapeutic plans and determination of drug-resistant phenotypes [156].

An ideal biomarker should be sensitive to detect all the types of tumors, easily accessible and produce reduced number of false negatives. Early diagnosis is of utmost importance to control the outcome of cancer progression and reduce cancer related mortality. Therefore quest for diagnostic markers is the primary objective in this area of research. Predictive epigenetic markers such as miRs have potential in personalized therapy, correlating tumor regression and the treatment regimen to determine therapy benefit. Drug sensitivity varies from individual to individual, thus epigenetic biomarkers can also be used to determine beneficial treatment and avoid toxicity due to resistant drugs (Fig. 1.3).

1.7 Conclusion

Tumorigenesis is a complex process and does not always stem from single-hit events.

Focusing on the mechanistic pathways deregulated in the process due to genes that control the processes can provide valuable information and guide treatment outcomes. Investigations have shown that in many different cancers tumor suppressor genes, which are epigenetically silenced, are central to process further downstream. Analysis of the extant of epigenetic changes for example DNA methylation status of promoters is suitable for providing excellent diagnostic and prognostic information and enable clinicians in the control of the disease. We have not yet reached the ability of determining with accuracy that epigenetic changes can effectively predict disease or treatment outcomes. This is because analyses of epigenetic changes vary among studies assessing the same tissue, show variation among different ethnicities and vary greatly in different cancers. It is therefore very imperative to assess if there are a combination of epigenetic factors that can truly define an epigenetic signature of the disease. Nonetheless, epigenetic biomarkers provide an excellent opportunity in predicting and determining disease outcomes by noninvasive means and further studies in this direction are warranted.

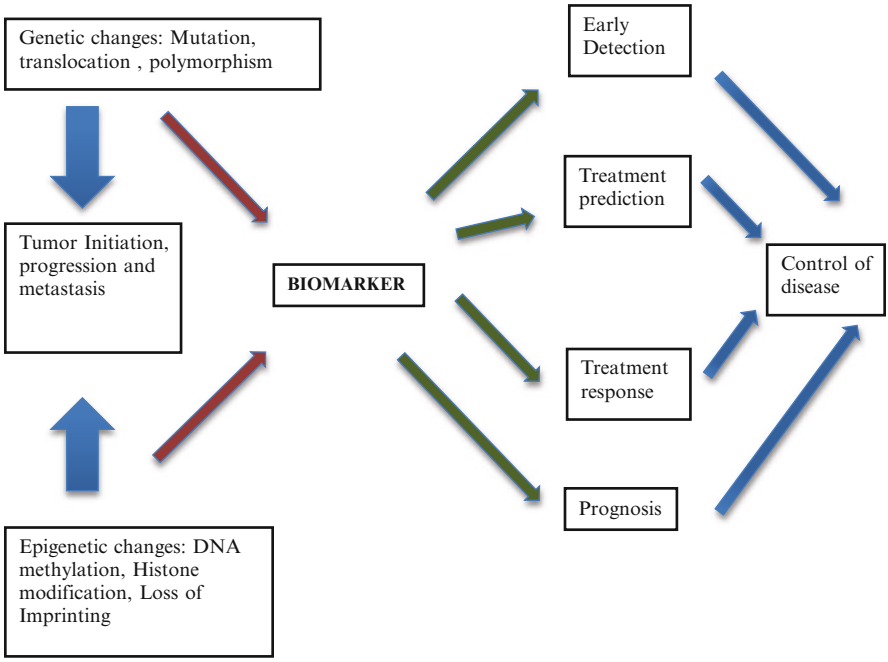

Fig. 1.3 The role of Epigenetic biomarkers in cancer diagnosis and therapy: tumorigenic control. Genetic and epigenetic changes influence tumor initiation, development and metastasis. However, unlike genetic changes, targeting epigenetic alterations are not only effective but also plausible as they are reversible. However, a potential problem with such an approach is that epigenetic biomarkers are not always altered, repressed or over expressed to the same degree in all cancers and therefore, a targeted combinatorial regimen is better suited. As an example, not all cancers contain hypermethylated *p16* promoters but in those cancers that do and where the hypermethylation status correlates with altered protein levels and clinicopathological characteristics, p16 can serve an excellent tool in the diagnosis, prognosis and treatment of the disease. As shown in the figure, various epigenetic biomarkers as discussed in the chapter can be used at different levels of management and control of many different cancers

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Chapter 2
Epigenetic Changes and Potential Targets in Pancreatic Cancer

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2.1 Introduction

Pancreatic cancer has the lowest survival rate than any solid cancer and is the fourth most common cause of cancer related death in the USA. It is estimated that 24,120 Americans will be diagnosed and 19,850 will die due to pancreatic cancer in 2015 [1]. One-year survival rate of patients diagnosed with pancreatic cancer is 50 % and 5-year survival is 6 % from date of diagnosis and late diagnosis is primarily associated with this poor survival. In addition to this, 85 % patients are diagnosed sate unresectable at. Furthermore, the 5-year survival of patients who undergo surgical resection with curative intent is only 23 % [2]. Other forms of treatment have little success in halting or slowing the progression of metastatic disease. Hence, restricting the mortality of pancreatic cancer requires identification of early diagnostic and prognostic markers to detect the disease at earlier stage to prolong intervention window.

Cancer has long been perceived as a genetic disease, but the past decade has seen a dramatic shift in our understanding of the disease and potential association of the epigenetics in cancer incidence and outcome [3]. Epigenetic changes are defined as any heritable changes to gene expression that are not accompanied by changes in DNA sequence [4]. In normal cells, epigenetic mechanisms are employed during development to silence and activate expression of specific genes when required. However, in cancer cells, epigenetic abnormalities contribute to the overexpression of oncogenes and suppression of tumor suppressor genes. They are conserved and

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frequently observed in both adenocarcinomas as well as in precursor lesions. Since cancer is a disease dictated by the multiple pathways, hence it is not surprising that, epigenetic and genetic abnormalities in pancreatic cancers are mostly non-overlapping with respect to the genes that are targeted. There are many examples of genes that are rarely mutated, but frequently silenced epigenetically and vice versa [5, 6]. These facts, emphasize the need for an integrative approach to studying cancer: one that explores both genetic and epigenetic aberrations and how they are coordinated in tumorigenesis shown Table 2.1.

To this end, the use of next-generation sequencing techniques has dramatically expanded our knowledge about epigenetic abnormalities in cancer. High throughput sequencing has made it possible to map genome-wide chromatin states [7, 8] and explore methylation maps with single base resolution [9]. These developments offer powerful tools to dissect some of the complex interplay and complementation between genomic and epigenomic factors. In this chapter, we review recent research efforts in finding into the epigenetic hallmarks of pancreatic cancer and their potential role in advancing diagnosis and treatment.

Although investigations of the epigenetic abnormalities of pancreatic neoplasms have focused on pancreatic ductal adenocarcinoma (PDAC) and its precursors, novel epigenetic abnormalities have also been uncovered in neuroendocrine tumors. Cancer is traditionally viewed as a genetic disease, and pancreatic cancer is no exception [10]. Only a concise overview of the most common genetic alterations found in pancreatic cancers alongside epigenetic changes can be given due to limitations of space; for more comprehensive reviews of molecular genetics of pancreatic cancer see [11, 12]. Activating mutations of KRAS2 on chromosome 12p represent the most common oncogene mutations in pancreatic cancer and are found in 80–100 % cases, usually point mutations in codon 12, 13 or 61, which abolish the intrinsic GTPase activity of its encoded product, and lead to constitutive activation of the intracellular signaling cascade [13, 14]. The most well-described downstream effector pathways of oncogenic KRAS2 are the RAF/MEK/ERK, PI3K/AKT and RALGDS/RAL signaling axes [11].

KRAS2 mutations are not only among the most frequently observed genetic alterations in pancreatic cancer, but also among those occurring extremely early during the multistep progression model, which leads to development of a fully invasive cancer phenotype, and can already be found in approximately 30 % of the earliest pancreatic intraepithelial neoplasia (PanIN-1) [15, 16]. The key importance of oncogenic KRAS2 signaling is further underscored by the fact that among all genetically engineered mouse models of pancreatic cancer, development of murine PanIN (mPanIN) lesions [17], reminiscent of human PanINs, was observed only in those models including pancreatic expression of a mutated KRAS allele, regardless of whether pancreatic precursor or adult differentiated cell compartments were targeted [18–21]. In fact, pancreas-specific expression of an oncogenic Kras2 allele was sufficient to induce formation of mPanIN lesions with high penetrance, which progressed to fully invasive pancreatic cancer in ~10 % of cases after 6–8 months, although this relatively long latency suggests that additional genetic alterations have to be acquired for the mPanIN lesions to progress towards a fully malignant cancer phenotype [22].

Table 2.1 List of selected genes that are genetically altered in pancreatic cancer

Gene symbol	Gene name	Epigenetic alteration	Chromosome site	Known or predicted function	Methylation in pancreatic cancer cell lines, no. (%)	Methylation in primary or xenografted pancreatic cancer, no. (%)	Reference
PENK	Preproenkephalin	Hypermethylation	8q23-q24	Neuropeptide precursor	11/11 (100)	43/47 (91)	[105]
UCHL1	Ubiquitin carboxylterminal esterase L1 (ubiquitin thiolesterase)	Hypermethylation	4p14	Ubiquitin hydroxylase	22/22 (100)	42/42 (100)	[187]
MDF-1	MAD (yeast Mitosis Arrest DeFicient) related	Hypermethylation	11q13	Glycogen metabolism	45/47 (96)	Not determined	[129]
NPTX2	Neuronal pentraxin II	Hypermethylation	7q21.3-q22.1	Neuronal transport	21/22 (95)	20/20 (100)	[187]
SPARC/ON	Secreted protein, acidic, cysteine-rich (osteonectin)	Hypermethylation	5q31.3-q32	5q31.3-q32 Cell-cycle progression inhibition, cell–matrix interaction	16/17 (94)	21/24 (88)	[126]
RPRM	Reprimo, TP53-dependent G2 arrest mediator candidate	Hypermethylation	2q23.3	P53-induced G2/M cell-cycle arrest	20/22 (91)	16/20 (80)	[109]
BNIP3	BCL2/adenovirus E1B 19 kDa interacting protein 3	Hypermethylation	10q26.3	Hypoxia-induced cell death	9/10 (90)	8/10 (80)	[121]
miR9-1	MicroRNA 9-1	Hypermethylation	1q22	miRNA translation control	42/47 (89)	Not determined	[129]
SERPINB5	Serpin peptidase inhibitor, clade B, member 5 (maspin)	Hypomethylation	18q21.3	Regulation of cell motility and cell death	20/23 (87)	32/34 (94)	[129]
CCND2	Cyclin D2	Hypermethylation	12p13	Cell-cycle control	19/22 (86)	71/109 (65)	[188]
ZNF415	Zinc finger protein 415	Hypermethylation	19q13.42		40/47 (86)	Not determined	
CLDN4	Claudin-4	Hypomethylation	7q11.23	Cell adhesion/invasion	17/20 (85)	33/37 (89)	[187]

(continued)

Table 2.1 (continued)

Gene symbol	Gene name	Epigenetic alteration	Chromosome site	Known or predicted function	Methylation in pancreatic cancer cell lines, no. (%)	Methylation in primary or xenografted pancreatic cancer, no. (%)	Reference
SFN	Stratifin (14-3-3 sigma)	Hypomethylation	1p35	P53-induced G2/M cell-cycle arrest	17/20 (85)	36/37 (97)	[187]
LCN2	Lipocalin-2	Hypomethylation	9q34	Epithelial differentiation	17/20 (85)	34/37 (92)	[187]
TFPI2	Tissue factor pathway inhibitor 2	Hypermethylation	7q22	Serine protease inhibitor	14/17 (82)	102/140 (73)	[187]
CNTNAP2	Contactin-associated protein-like 2	Hypermethylation	7q35-q36	Higher cortical function	39/47 (82)	Not determined	[129]
CDKN1C/p57	Cyclin-dependent kinase inhibitor 1C	Hypermethylation	11p15.5	Cyclin-dependent kinase inhibitor	7/9 (78)	Not determined	[187]
SIP1	Survival of motor neuron protein interacting protein 1	Hypermethylation	14q13-q21	Assembly of spliceosomal snRNP	11/15 (73)	34/35 (97)	[189]
ELOVL4	Elongation of very-long-chain fatty acids (FEN1/Elo2, SUR4/Elo3, Yeast like 4)	Hypermethylation	6q14	Fatty acid synthesis	32/47 (68)	Not determined	[129]
TFF2	Trefoil factor 2	Hypomethylation	21q22.3	Secretory polypeptide/epithelial repair	13/20 (65)	31/37 (84)	[187]
FOXE1	Forkhead box E1 (thyroid transcription factor 2)	Hypermethylation	9q22	Thyroid transcription factor	14/22 (64)	15/20 (75)	[187]

S100P	S100 calcium-binding protein P	Hypomethylation	4p16	Cell-cycle progression and differentiation	13/23 (57)	30/34 (88)	[187]
RARB	Retinoic acid receptor, Î 2	Hypermethylation	3p24	Cell-growth control	5/9 (56)	4/36 (11)	[105]
S100A4	S100 calcium-binding protein A4	Hypomethylation	1q21	Motility, invasion, tubulin polymerization	10/20 (50)	28/37 (76)	[190]
CDKN2A/p16	Cyclin-dependent kinase inhibitor 2A	Hypermethylation	9P21	Cyclin-dependent kinase inhibitor	3/9 (33)	5/36 (14)	[187]
MSLN	Mesothelin	Hypomethylation	16p13.3	Cell surface antigen/cell adhesion	8/20 (40)	34/37 (29)	[105]
SOCS1	Suppressor of cytokine signaling 1	Hypomethylation	16p13.13	Inhibitor of JAK/STAT pathway	6/19 (32)	13/60 (22)	[130]
PSCA	Prostate stem cell antigen	Hypomethylation	8q24.2	Cell surface antigen/cell differentiation	6/20 (30)	20/37 (54)	[187]
CADM1/TSLC1	Cell adhesion molecule 1	Hypermethylation	11q23.2	Cell–cell, cell–matrix interaction	4/17 (24)	25/91 (27)	[191]
MLH1	MutL homolog 1	Hypermethylation		DNA repair	2/36 (6)	0/9 (0)	[105]
NDRG1	N-myc downstream regulated	Hypermethylation	8q24.3	Hormone responses, cell growth, differentiation	0/6[a]	Not determined	[192]
CDH1	Epithelial cadherin	Hypermethylation	16q22.1	Cell adhesion/invasion	1/36 (3)	2/9 (22)	[105]

Moreover, functional studies by several independent groups have clearly shown that oncogenic KRAS2 signaling is not only involved in pancreatic carcinogenesis, but also required for maintenance of pancreatic cancer cell growth [23]. Amplifications of *CMYC* and c-Myc protein overexpression are found in about 50–60 % of pancreatic cancers, suggesting that oncogenic Myc signaling also plays a crucial role in this disease [24].

The product of the tumor suppressor gene cyclin-dependent kinase 2A (CDKN2A/p16) on chromosome 9p inhibits cell-cycle progression through the G1-S checkpoint and is inactivated in more than 90 % of pancreatic cancers, most commonly either through homozygous deletions (in about 40 % of cases) or by intragenic mutations and loss of the second allele (in another ~40 %) [25]. Of note, in 10–15 % of cases, p16 function is silenced by transcriptional inhibition due to promoter hypermethylation. Loss of nuclear p16 expression seems to increase with progression of PanIN precursor lesions towards fully invasive pancreatic cancer, and was observed by immunohistochemistry in 30 % of PanIN-1, 55 % of PanIN-2 and 71 % of PanIN-3 precursor lesions [26]. TP53 on chromosome 17p, which mediates cell-cycle arrest and induces apoptosis upon DNA damage, is inactivated in 50–75 % of pancreatic cancers, which is almost always caused by intragenic mutations in combination with loss of the second allele. Nuclear accumulation of the mutated protein, which can be detected by immunohistochemistry, has been used as a surrogate marker for loss of TP53 function and is usually found only in advanced PanIN-3 lesions and invasive cancers, suggesting that TP53 inactivation represents a later event in the multistep progression cascade towards pancreatic cancer [27]. Deleted in pancreatic carcinoma 4 (DPC4/SMAD4/MADH4) is a gene whose function is lost in about 55 % of pancreatic cancers [28]. This is due to somatic mutations and loss of the second allele in about 25 %, and to homozygous deletions in about another 30 % cases. Loss of Dpc4 protein function leads to increased proliferation and reduced growth inhibition. Interestingly, loss of Dpc4 function appears to be fairly specific to pancreatic cancer and is rarely seen in other malignancies [29]. Dpc4 loss also represents a relatively late event in pancreatic carcinogenesis, found in only a minority of PanIN-3 lesions [27].

2.2 Epigenetic Alterations in Pancreatic Cancer

Although pancreatic cancer is traditionally viewed as a genetic disease, there is increasing evidence suggesting that it is equally justifiable to call it a disease of epigenetic abnormalities. It is well understood that epigenetic alterations provide an additional mechanism through which alterations in gene expression patterns can be governed, and which can contribute to the development of a malignant phenotype. In pancreatic cancer, two major forms of epigenetic alterations are thought to play important roles: first, histone modifications, mediated most commonly through acetylation or methylation of histone proteins or through the incorporation of alternative histone proteins, and second, direct methylation of cytosine groups within genomic DNA molecules.

2.2.1 Histone Modifications

Nucleosomes represent the basic constituents of chromatin, in which a section of a double-stranded DNA molecule, 146 bp in length, is wrapped approximately 1¾ times around an octamer of histone proteins, consisting of two copies each of histone proteins H2A, H2B, H3 and H4 [30]. Each nucleosome is separated from its neighbors by a DNA sequence of around 50 bp in length, which is packaged by the linker protein H1. All four histone proteins found in the cores of nucleosomes have positively charged lysine-rich amino terminal tails that protrude from the cores and that are prone to post-translational modifications. A variety of different forms of post-translational modifications at over 50 known sites have been described, including methylation, acetylation, citrullination, phosphorylation, SUMOylation and ADP-ribosylation [31–33]. The most commonly observed modifications are methylation, acetylation and ATP dependent chromatin remodeling, which play central roles in regulating the condensation status of chromatin, i.e. how loosely or densely certain chromatin regions are packed, and thus influence expression of genes encoded within these regions.

The importance of histone modifications as epigenetic characteristics, contributing to maintenance of a malignant cancer phenotype has become increasingly evident in recent years, with acetylation and methylation of lysine residues within the N terminal tails of histone proteins being the best-studied changes [33–35].

2.2.2 Alterations in Histone Methylation

Histone methylation is most commonly observed in histone proteins, H3 and H4. Methylation at certain positions within these proteins is correlated with activation of chromatin and enhanced gene transcription, for example H3-K4, H3-K36, or H3-K79, whereas others have been linked to gene silencing, like H3-K9, H3-K27 or H4-K20 [36–38]. Of note, different methylation states exist for each lysine residue, i.e. lysine can be methylated by replacement of one, two or all three N-standing protons to mono-, di- or trimethyllysine. In pericentromeric regions, enrichment of trimethylated H3-K9 is found as compared to mono- and dimethylated forms. The trimethylated form of H3-K27 occurs more frequently in the inactivated X-chromosome [39].

Methylation of histone proteins is mediated by histone methyltransferases (HMTs). There are at least 17 different HMTs known to date, all of them share a common evolutionaly conserved (Su (var)3-9, enhancer-of-zeste, trithorax) motif. HMTs are not specific to histone proteins but are known to methylate other targets as well [33, 40–42]. Histone methylation has been directly linked to DNA methylation, and both processes share some of the involved regulatory protein machinery, such as DNA methyltransferases (DNMT) and methyl binding proteins. Therefore it has been suggested that histone methylation might also mediate its effects on differential gene expression at least in part via associated alterations in the DNA methylation status at the respective sites [43].

HMTs methylating H3-K4 and H3-K27 are frequently overexpressed in malignant disease. Further evidence supporting the hypothesis of a causal contribution of HMTs in carcinogenesis comes from the discovery of genetic alterations within HMTs in malignant tumors. In Sotos syndrome, which is caused by germline mutations of the histone methylase nuclear receptor binding SET domain protein 1 (NSD1), a ~170-fold increased risk of developing cancers is documented, including a propensity for developing Wilms tumors, neuroblastomas, acute lymphatic leukemia, hepatocellular carcinomas, and small cell lung cancers. Moreover, the translocation t(5;11)(q35;p15.5) found in childhood acute myelogenous leukemia often incorporates NSD1 [44]. The H3-K27 methyltransferase enhancer of zeste homolog 2 (EZH2), a member of the polycomb group complexes PRC2 and PRC3, is required for cell proliferation, and is frequently found to be overexpressed, amplified or rearranged in several malignant tumors. In some cases, the extent of EZH2 overexpression has been linked to disease aggressiveness [45–49]. Suppressor of variegation 3–9 homolog 1 (SUV39H1), a H3-K9 methyltransferase predominantly targeting pericentromeric chromatin regions involved in regulating cell-cycle progression and genomic stability, has been linked to cancer development [50, 51]. Another example of an HMT thought to be involved in cancer development is PR domain-containing protein 2 (PRDM2/RIZ1), a H3-K9 methyltransferase that was originally recognized due to its interaction with retinoblastoma (RB), which is found to be, inactivated by promoter hypermethylation in several malignancies [33, 52–54]. Its probable functional relevance is further supported by the occurrence of inactivating somatic RIZ1 mutations in cancer cells, as well as by the finding that RIZ1 knockout mice carry an increased risk of developing B-cell lymphomas [55, 56]. Most importantly, it is now known that histone methylation is not irreversible, as had originally been proposed. Lysine-specific demethylase 1 (LSD1), a recently identified enzyme is capable of demethylating mono- or dimethylated forms of H3-K4, while it is unable to catalyze demethylation of trimethylated forms [57]. Given the dynamic nature of regulation of gene transcription, it is not beyond the realm of speculation to believe that additional histone demethylating enzymes are going to be discovered in the future [33].

A recent report described nuclear overexpression of the histone methyltransferase enhancer of zeste homolog 2 (EZH2) in 71/104 (68 %) pancreatic cancer tissue specimens [58]. Moreover, RNAi-mediated knockdown of EZH2 led to decreased pancreatic cancer cell proliferation and enhanced sensitivity against commonly offered therapeutic options i.e. Doxorubicin and Gemcitabine.

In another recent study, the histone methyltransferase genes MLL and MLL3, which methylate at H3-K4, were found to be mutated in subsets of pancreatic cancers [14]. A total of seven intragenic somatic mutations within MLL3 were found in 4/24 (17 %) of pancreatic cancer samples screened, including two nonsense, four missense and one synonymous mutation. In a subsequent prevalence screen on a larger panel of pancreatic cancers, another five somatic MLL3 mutations were discovered in 5/90 (6 %) cases. The same paper reported two MLL missense mutations within 1/24 (4 %) cases of pancreatic cancer as well as one synonymous mutation of MLL2 within this panel.

2.2.3 *Alterations in Histone Acetylation*

Acetylation of histone proteins H3 and H4 causes more loose packing of chromatin, which in turn facilitates transcription factor binding and enhances gene transcription. This is believed to be due to neutralization of the positive charges of lysine residues within the N-terminal regions of H3 and H4, through which the electrophysical interaction with the negatively charged phosphate backbone of the associated DNA strands is weakened [59].

Histone acetylation states can change relatively rapidly and acetylation changes are thought to be a dynamic process, as opposed to methylation, which usually tends to accumulate more slowly and more stable over time [60]. The acetylation state of histone proteins is governed by two classes of enzymes: histone acetyltransferases (HATs), which acetylate lysine residues within aminoterminal tails of histone proteins, and histone deacetylases (HDAC), which catalyze the opposite reaction, i.e. deacetylation of lysine residues, thereby reconstituting positive charges and generally leading to a more condensed chromatin state and inhibiting gene transcription [61]. Histone acetyl-transferases comprise members of the GNAT (Gen5-related N-acetyl transferase), MYST and p300/CBP families [33, 61]. Interestingly, different HATs show differential affinity towards N-terminal tails of specific histone proteins, and some can also acetylate other non-histone proteins, for example p53, E2F1, or GATA1 [62–64]. HDACs, on the other hand, are subdivided into three classes according to their similarity to the yeast homologs Rpd3 (class 1), Hda1 (class 2) or Sir2 (class 3). As observed with HATs, HDACs are also not entirely specific for histone proteins, but can also deacetylate other protein targets, including p53, E2F1, GATA1, TFIIE, TFIIF, or glucocorticoid receptors [65, 66]. Mutations of both HAT and HDAC family members have been linked to carcinogenesis. For example, a subset of cases of Rubinstein–Taybi syndrome are caused by mutations within CBP (germline mutation of one allele and inactivation of the second allele by somatic mutation) and carry a more than 300-fold increased risk of developing cancer [67]. Furthermore, somatic mutations have also been found in sporadic gastric and microsatellite instability-positive colorectal cancers, mostly nonsense or missense mutations, in combination with loss of the second allele [42, 68]. In colorectal cancers, truncating HDAC2 mutations have also been described [69].

In pancreatic cancer, HDAC7 was recently shown to be overexpressed at the mRNA level in 9/11 (82 %) cases [70]. Especially, a variety of histone deacetylase inhibitors have been developed over recent years and proven to be valid candidate drugs for a wide spectrum of malignant tumors including pancreatic cancer [71–73].

2.3 DNA Hypomethylation in Pancreatic Cancer

DNA hypermethylation is considered to be involved in maintaining DNA integrity by causing a more dense packing and functional inactivation of affected chromatin regions [33]. Therefore it is not surprising that in non-neoplastic cells, areas of significant hypermethylation are frequently found in highly repetitive regions, many

of which are thought to represent retroviruses that have been trapped in the genome, functionally inactivated by hypermethylation and hence been passed on in a methylated state [74, 75].

A possible mechanism that could explain the contribution of global methylation to carcinogenesis is that demethylation/hypomethylation of methylated, evolutionarily conserved latent viral sequences re-expresses these sequences which in turn confer oncogenic stimuli. A well-documented example for this process in which re-expression of a latent virus can be linked to malignant tumor formation is human papillomavirus 16 (HPV16), where hypomethylation has been linked to activation of the HPV16 genome and progression of cervical dysplasia [76]. Similar observations have been made for Epstein–Barr virus (EBV) in EBV-associated lymphomas [77].

An alternative mechanism might be that genes that are usually expressed at defined stages during embryogenesis and organ development, but at later stages are silenced by means of promoter methylation, can become re-expressed due to hypomethylation and might thus exhibit oncogenic properties when aberrantly expressed in differentiated adult tissues. There are several examples of genes where this mechanism has been proposed. Cancer/testis antigen (CAGE), has been shown to be reactivated due to promoter hypomethylation in gastric and cervical cancers [78, 79], as has CD30 in Hodgkin disease and anaplastic large cell lymphomas [80]. Promoter hypomethylation and overexpression has also been shown for *N-acetyltransferase-1* (NAT1) in breast cancer [81], as well as for CD133 in gliomas [82]. In the case of pancreatic cancer, genes found to be overexpressed in neoplastic cells as compared to surrounding non-neoplastic tissues due to cancer-related promoter-hypomethylation and subsequent transcriptional re-expression include maspin (SERPINB5), S100A4, mesothelin (MSLN), prostate stem cell antigen (PSCA), claudin-4 (CLDN4), lipocalin-2 (LCN2), 14-3-3 sigma/stratifin, trefoil factor 2 (TFF2), S100 calcium binding protein P (S100P), and guanine nucleotide exchange factor (VAV1) [83, 84]. Functional studies, including RNAi-mediated knockdown, have shown a requirement of sustained VAV1 function to maintain the malignant phenotype of pancreatic cancer cells [83, 84], underscoring the importance of hypomethylation-dependent overexpression. Some of these hypomethylated genes (e.g. PSCA, mesothelin) have emerged as important targets for therapy, imaging, and for diagnosing pancreatic cancer [85, 86]. Thirdly, increasing evidence suggests that global hypomethylation might contribute to increased genomic instability, a hallmark of pancreatic as well as other cancers. In cancer cells, hypomethylated sequences are frequently found in pericentromeric regions, for example on chromosomes-1 and -16, and hypomethylation might thus predispose to recombination events [33, 87–89]. Repetitive unbalanced chromosomal translocations encompassing these areas have been linked to the formation of Wilms tumors, breast and ovarian cancers [87, 88]. In another study, loss of the ability to methylate ectopically expressed DNA molecules was correlated to the appearance of gross chromosomal aberrations in colon cancer cells with microsatellite instability [90]. Perhaps even more strikingly, similar observations could be

made using *in vivo* model systems: for example, several groups have reported a correlation between global hypomethylation and increased frequencies of spontaneous tumor formation in mouse models [91–93], and a similar correlation was observed in resected breast as well as in ovarian cancer tissue samples [87, 94]. Moreover, certain inheritable genetic syndromes provide another line of evidence demonstrating a functional relationship between global DNA hypomethylation and genomic instability. The ICF syndrome is caused by loss of function mutation in DNMT3B, as indicated by its name, it is characterized by immunodeficiency in combination with chromosomal instability and facial abnormalities [89, 95].

The underlying cause of global hypomethylation found in cancer is yet to be defined. One hypothesis suggests that hypomethylation could be caused by nutritional deficiency. Specifically, lack of folate or vitamin B12 leads to methyl group deficiency due to decreased *S*-adenosylmethionine levels and is thought to contribute to hypomethylation and genomic instability [96, 97]. Low intake of vitamin B12 or folate has been suggested to be a risk factor for pancreatic cancer [98]. Therefore, it seems possible that DNA hypomethylation due to malnutrition could be an underlying cause of enhanced accumulation of genomic alterations and increased cancer risk in a subset of cases [99].

2.4 DNA Hypermethylation in Pancreatic Cancer

Although the link between carcinogenesis and global hypomethylation was established relatively early on, it is now apparent that such hypomethylation is typically accompanied by hypermethylation at specific gene promoters, particularly the genes involved in tumor suppression [33, 100, 101]. Aberrant promoter hypermethylation seems to be an alternative mechanism, which can lead to silencing of tumor suppressor genes even in the absence of, or in addition to, intragenic mutations. One of the first examples linking promoter hypermethylation to repressed gene expression was reported by Baylin and co-workers in the calcitonin gene [102]. The first observations showing inhibition of a tumor suppressor gene due to promoter hypermethylation in cancer tissues were made studying the retinoblastoma (RB) gene [103]. Many other examples have followed since, linking silencing of an ever-expanding number of tumor suppressor genes to promoter hypermethylation, in sporadic as well as in inheritable forms of cancer. In fact, it is presently assumed that around 50 % of genes causing inheritable cancer through germline mutation or deletion are silenced by promoter hypermethylation in the sporadic counterparts of these malignancies, and promoter hypermethylation has, to date, been documented in a plethora of different genes [33, 104].

In pancreatic cancer, genes that act as tumor suppressor are frequently found to be repressed by promoter hypermethylation and subsets of cases includes p16/CDKN2A, E-cadherin, retinoic acid, suppressor of cytokine signaling-1 (SOCS1), tumor suppressor in lung cancer (TSLC1), C-X-C chemokine receptor type 4 (CXCR4), mucin 2 (MUC2) and reprimo [25, 105–108]. Reelin (RELN) is

Table 2.2 DNA hyper- and hypo-methylation involved in pancreatic cancer

	Material			Gene affected	Reference
DNA modification	Cancer samples	Cell lines	Others		
DNA Hypermethylation	✓			p16	[25]
	✓			RASSF1A	[226]
	✓			MDFI, hsa-miR-9-1, ZNF415, CNTNAP2, ELOVL4	[129]
	✓			SOX15	[227]
	✓			HOP hoemobox (HOPX)	[228]
	✓			KLF10	[229]
	✓			hMLH1	[230]
	✓			miR-34a/b/c	[231]
	✓			SPARC	[232]
	✓			FoxE1, NPTX2, CLDN5, P16, TFPI-2, SPARC, ppENK	[233]
	✓			SFRP	[234]
	✓	AsPC1, Hs766T, MiaPaCa2, Panc1		UCHL1, NPTX2, SARP2, CLDN5, reprimo, LHX1, WNT7A, FOXE1, TJP2, CDH3, ST14	[235]
	✓	AsPC1, BxPC3, CFPAC1, Panc1		NPTX2	[236]
	✓	Panc1, SW1990		miR-132	[153]
	✓	BxPC3, Capan2, CFPAC1, HPACI, HPAFII, MiaPaCa2, Panc1, PL45		FOXA1/2	[237]
		MiaPaCa2		ARID1B	[238]
		Panc1		NPTX2	[239]
		AsPC1, BxPC3, Panc1, MIA PaCa-2		Dkk3	[240]
		BXPC3, HPAFII, HPAC, hTERTHPDE, Panc1		Cldn18	[241]

		BxPC3, CFPAC1, Panc1, SW1990		TNFRSF10C	[242]
			Pancreatic juice	Neuronal pentraxin II (NPTX2)	[243]
			Pancreatobiliary fluid	UCHL1, RUNX3	[244]
			PanIN	p16	[190]
			IPMNs	BNIP3, PTCHD2, SOX17, NXPH1, EBF3, SPARC, SARP2, TSLC1, RELN, TFPI2, CLDN5, UCHL1	[245]
			Blood, brush cytology	NPTX2	[197]
DNA Hypo methylation					
	✓			VAV1	[84]
				Claudin4, lipocalin2, 14-3-3 sigma, trefoil factor 2, S100A4, mesothelin, prostate stem cell antigen	[187]
	✓			MUC4	[246]
		SW1990		ABCB1/MDR1, ABCC1/MRP1, ABCG2/BCRP	[247]

frequently downregulated due to promoter hypermethylation in pancreatic cancer, and its repression has been shown to mediate increased motility, invasiveness and colony formation, whereas opposite effects were observed upon re-expression of RELN [109]. Promoter hypermethylation of Hedgehog interacting protein (HHIP) was recently found in the majority of examined cases [110], including pancreatic cancer cell lines as well as primary tumor tissue samples, in line with observed aberrant re-activation of Hedgehog signaling [111–113]. A small subset of pancreatic cancers shows hypermethylation and silencing of MutL protein homolog 1 (hMLH1) [105, 114, 115], which has been associated with microsatellite instability and medullary histology in a fraction of cases [116, 117]. Prepro-enkephalin (ppENK) encodes a native opioid peptide, which can mediate growth suppression and is methylated in the majority of pancreatic cancers [105]. The Kunitz-type serine protease inhibitor tissue factor pathway inhibitor 2 (TFPI-2), which is thought to be involved in protecting surrounding matrix from degradation and thereby inhibiting cancer invasion and metastasis [118, 119], was recently shown to be silenced due to aberrant hypermethylation in 102/140 (73 %) of pancreatic cancers—in xenografts as well as primary tumor tissue samples were examined [120]. Of interest, re-expression of TFPI-2 in pancreatic cancer cells leads to increased proliferation, invasion and migration in vitro. BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3) is another example of a commonly silenced gene by hypermethylation in pancreatic cancer. Using RNAi techniques, inhibition of BNIP3 has been shown to contribute to resistance to 5-fluorouracil and gemcitabine in vitro. Moreover, hypoxic BNIP3 expression could by readily restored by 5-Aza-dC treatment, suggesting this might be a potential therapeutic approach for treating drug-resistant pancreatic cancers [121–123]. Within the GATA gene family, GATA-5 was shown to be frequently methylated, while GATA-4 was infrequently methylated in pancreatic cancer cells [124]. Secreted protein acidic and rich in cysteine (SPARC) or osteonectin/BM40 is a calcium-binding protein, which is involved in various cellular functions, including migration, proliferation, interaction with surrounding matrix, adhesion, angiogenesis and tissue remodeling [125, 126]. SPARC is frequently silenced in pancreatic cancer cells by aberrant DNA methylation [126], and SPARC knockout causes enhanced tumor growth and invasiveness in mice [127, 128].

An unbiased way to screen for genes that might be silenced in pancreatic cancer cell lines due to promoter hypermethylation is to look for re-expression upon treatment with demethylating agents, e.g. the DNA-methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-Aza-dC), as compared to mock-treated control cells. Goggins and co-workers used this approach to screen for genes silenced due to hypermethylation in pancreatic cancer using Affymetrix oligonucleotide microarrays [107]. In these studies, 475 candidate genes were identified as re-expressed in four pancreatic cancer cell lines but not in a non-neoplastic ductal epithelial cell line used as control. Subsequent experiments successfully confirmed aberrant hypermethylation of several of these candidates in primary pancreatic cancer tissue samples. The same group recently described another high-throughput approach by application

of a methylated CpG island amplification (MCA) technique to 44K Agilent promoter microarrays. This strategy was exploited to identify 606 differentially methylated genes in one pancreatic cancer cell line [129].

2.5 Gene Promoter Hypermethylation in Precursor Lesions of Pancreatic Cancer

Promoter-specific hypermethylation and inhibition of gene expression can be observed in all three known precursor lesions of pancreatic cancer—pancreatic intraepithelial neoplasias (PanINs), intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs), suggesting that promoter hypermethylation represents an early event during the multistep progression cascade of pancreatic cancer [12, 107, 130]. Sato and colleagues have compared the differences in global gene expression profiles in IPMNs as compared to normal pancreatic ductal epithelium and found under expression of CDKN1C/p57KIP2, which in some cases, was found to be due to partial promoter hypermethylation [99, 120]. CDKN1C on chromosome 11p15.5 is a known tumor suppressor, which inhibits cell proliferation and its inactivation is involved in formation of Wilms tumor and Beckwith–Wiedemann syndrome [131, 132].

Other genes found to be silenced as a result of DNA hypermethylation in PanIN or IPMN precursor lesions include CDKN2A/p16, RLN, TFPI-2 and ppENK [99, 133]. Of note, the prevalence of methylation seems to increase from low to high grades precursor lesions [134]. Similarly, progressively aberrant methylation patterns have been described in MCNs as well [56].

2.6 Epigenetics in Pancreatic Cancer Stem Cells

An interesting and possibly therapeutically relevant aspect is the role of EZH2 in maintenance of stemness characteristics in cancer, particular its role in maintaining the self-renewal capabilities of cancer stem cells (CSC) [135–141]. This subpopulation of cancer cells has been characterized in pancreatic cancer by surface markers CD44, CD24, CD133, ESA (EpCAM, epithelial cell adhesion molecule) [142, 143] and is thought to represent the population of cancer cells responsible for tumor maintenance, tumorigenicity, metastasis, and resistance to conventional chemotherapeutic drugs, as well as recurrence [142, 144]. Similar to studies with CSC derived from hepatocellular carcinoma and acute myeloid leukemia [137, 144], recent studies have demonstrated the therapeutic potential of epidrugs to directly target this tumorigenic subpopulation of pancreatic cancer cells, Avan et al. [135] used an inhibitor of the EZH2 methyltransferase (DZNep, deazaneplanocin-A) and showed that treatment with DZNep reduced spheroid formation of pancreatic cancer cells and decreased the $CD133^{+}$ subpopulation. Furthermore,

the combination of DZNep and gemcitabine was shown to be highly synergistic and was accompanied by a reduced percentage of G2/M cells, reduced migration, increased E-cadherin expression and increased apoptosis [135]. The level of EZH2 has furthermore been suggested as an assay to effectively measure changes in the CSC subpopulation: using pancreatic and breast cancer cell lines, knockdown of EZH2 by RNA interference decreased the CSC subpopulation, confirming its role in CSC maintenance, and genes affected by EZH2 knockdown were inversely correlated with their expression in enriched CSC subpopulations [141]. The Hedgehog pathway has also been implicated in the maintenance of CSCs in various models [145]; interestingly a combination of Hedgehog inhibition (SANT-1) and SAHA (a pan-HDAC inhibitor; suberanilohydroxamic acid, Vorinostat) synergistically suppressed proliferation and colony formation in gemcitabine-resistant pancreatic adenocarcinoma cell lines by increased Bax expression, activation of caspase-3/7, increased p21 and p27 and reduced cyclin D1 expression. This study suggests that combined inhibition of stem cell-associated pathways (Hedgehog) and epigenetic drugs could be efficient in targeting the CSC subpopulation in pancreatic cancer [146].

A study by Nalls et al. [147] could demonstrate that demethylating agents (5-aza-dC, 5-aza-2′-deoxycytidine) and the HDAC inhibitor SAHA restored the expression levels of miR-34a, which is reduced in pancreatic CSCs. These inhibitors caused a reduction in the EMT-related ZEB1, Snail, and Slug transcription factors, increased epithelial marker expression (E-cadherin) and, most importantly, reduced the number of viable pancreatic CSC, accompanied by reduced migration, colony formation and invasion of these cells. Based on the above-mentioned functions and properties of CSC, which is critical for tumor initiation, progression, metastasis and therapeutic resistance. These findings are of central importance and warrant further investigation to hopefully develop (epigenetics-based) therapeutic regimens specifically targeting this tumorigenic subpopulation in pancreatic cancer.

2.7 miRNA-Based Epigenetics in Pancreatic Cancer

Some reporters [148–151] and Park et al. [152] have reviewed the publications available on differential miRNA expression in pancreatic cancer vs. normal tissue culminating in a list of 64, partly overlapping individual miRNAs which were found to be deregulated in pancreatic cancer. Of these miRNAs, overexpression of miR-21, -155, -196a-2, -203, -210 and -222 was associated with poor outcome [152]. Table 2.3 provides an update (based on Park et al. [152]) of the currently available literature on the specific role of individual miRNAs in pancreatic cancer. All of these studies investigated the cellular/molecular mechanisms of the oncogenic or tumor-suppressive action of miRNAs, mainly by forced overexpression or knockdown of the respective miRNAs. An example of how epigenetic mechanisms are employed in regulating the expression of tumor-suppressive miRNAs is shown in the study of Zhang et al. [153]. Out off 12 miRNAs differentially expressed in

Table 2.3 Summary of miRNAs associated with specific targets/functions in pancreatic cancer

miRNA/function		Cell lines	Target gene(s)	Cellular effects	Reference
Function as oncogene	-10a	AsPC1, Capan1, Capan2, MiaPaCa2, Panc1, Patu8988T, Patu8988S, Patu8902	HOXB1, 3	Metastasis ↑	[193]
	-21	AsPC1, BxPC3, Capan1, Capan2, CFPAC1, Hs776T, H48N, KP-1N, KP-2, KP-3, MiaPaCa2, NOR-P1, Panc1, SUIT-2, SW1990	HOXA1	Invasion ↑	[194]
		AsPC1, Capan1, Capan2, CFPAC1, H48N, HS766T, KP-1N, KP-2, KP-3, MiaPaCa2, NOR-P1, Panc1, SUIT-2, SW1990		Proliferation ↑, invasion↑, chemoresistance ↑	[195]
		BxPC3		Proliferation ↑	[196]
		Capan1, HS766T, MiaPaCa2, MPanc96, Panc1, PL45, SW1990	PTEN, RECK	After miRNA inhibition: cell cycle arrest ↑, apoptosis ↑	[197]
	-132, -212	Panc1	Rb1	Proliferation ↑	[198]
	-155	Capan2, MiaPaCa2, MCF7, MEFs, 293T	TP53INP1	Apoptosis ↓	[199]
	-194, -200b, -200c, -429	AsPC1, A818, BxPC3, Capan1, Capan2, HPAFII, MiaPaCa2, MPanc96, Panc1, Patu8902, Patu8988T, Patu8988S, PT45, Suit 007, Su.86.86, Sut00281	EP300	Metastasis ↑	[200]
	-197	AsPC1, Panc1	p120 catenin	EMT ↑	[201]
	-210	Panc1, MiaPaCa2, SUIT-2		Migration ↓, vimentin ↓, snai-1 ↓, membraneous β-catenin ↑	[202]
	-221	Capan1, HS766T, MiaPaCa2, MPanc96, Panc1, PL45, SW1990	p27	Chemosensitivity ↑	[203]
	-224, -486	AsPC1, A818, BxPC3, Capan1, Capan2, HPAFII, Su 86.86, MPanc96, MiaPaCa2, Panc1, Patu8902, Patu8988T, PT45, Patu8988S, Suit 007, Suit 00281	CD40	Invasion ↑, metastasis ↑	[204]

(continued)

Table 2.3 (continued)

miRNA/function		Cell lines	Target gene(s)	Cellular effects	Reference
Function as tumor suppressor	-301a	BxPC3, Hs766T	Bim	Proliferation ↑	[205]
	-320c	AsPC1, Panc12	SMARCC1	Chemoresistance ↑	[206]
	-421	SW1990, Panc1	DPC4/Smad4	Proliferation ↑, colony formation ↑	[207]
	-491-5p	AsPC1, Capan1, MiaPaCa2, SW1990	Bcl-XL, TP53	Proliferation ↓, apoptosis ↑, STAT3 ↓, PI-3K/Akt ↓	[208]
	let-7	BxPC3, Capan1, Capan2, human HPNE (human pancreatic nestin-positive) cells		Proliferation ↓, K-RAS ↓	[209]
	let-7a	MiaPaCa2, Panc1		MAPK ↓	[210]
		AsPC1	RAS	K-RAS ↓, radiosensitivity ↑	[211]
	-22	BxPC3	SP1, ESR1	Tumourigenesis ↓	[212]
	-26a	SW1990, Panc1	HMGA1	Proliferation ↓, invasion ↓, migration ↓, apoptosis ↑	[213]
	-34	BxPC3, MiaPaCa2	Bcl-2, Notch-1/2	Clonogenicity ↓, invasion ↓, apoptosis ↑, cell cycle arrest ↑, chemosensitivity ↑, radiosensitivity ↑, CSC ↓	[214]
	-34a	Panc1		Cell cycle arrest ↑, apoptosis ↑, migration ↓, E2F3 ↓, Bcl-2 ↓, c-myc ↓, cyclin D1 ↓	
	-34b	AsPC1, MiaPaCa2	Notch-1	Proliferation, apoptosis	[215]
			Smad3	Progression in vivo ↑	[216]
	-107	MiaPaCa2, Panc1	CDK6	Proliferation ↓	[217]

	-126	AsPC1, BxPC3, KLM-1, MiaPaCa2, Panc1	ADAM9	Migration ↓, invasion ↓, E-cadherin ↑	[218]
	-132	BxPC3, HPAFII, HPAC, Panc1		Proliferation ↓, colony formation ↓, Akt ↓	[153]
	-143	AsPC1, BxPC3, Capan2, HPAFII, MiaPaCa2, Panc1	COX-2	Proliferation ↓, MEK/MAPK ↓	[219]
		Panc1	ARHGEF1 (GEF1), ARHGEF2 (GEF2), K-RAS	Migration ↓, invasion ↓, metastasis ↓, E-cadherin ↑	[220]
	-148a	IMIM-PC2	CDC25B	Proliferation ↓, colony formation ↓	[221]
	-148b	AsPC1, BxPC3, MiaPaCa2, Panc1, SW1990	AMPKα1	Proliferation ↓, apoptosis ↑, cell cycle arrest ↑, invasion ↓, chemosensitivity ↑, tumourigenicity ↓	[222]
	-150	Colo357, HPAF, Panc10.05	MUC4	Proliferation ↓, clonogenicity ↓, migration ↓, invasion ↓, cellular adhesion ↑	[223]
	-200	AsPC1, BxPC3, Colo357, HPAC, MiaPaCa2, L3.6pl, Panc12		EMT ↓ (ZEB1 ↓, slug ↓, vimentin ↓)	[224]
	-375			Proliferation ↓, cell cycle arrest ↑, apoptosis ↑	[225]
	-548d	Panc1		Proliferation ↓, apoptosis ↑, cell cycle arrest ↑	

pancreatic cancers vs adjacent normal tissue, miR-132 was downregulated in 16/20 pancreatic carcinomas accompanied by methylation of its promoter, as shown both in cell lines and tumor tissue. Sp-1 expression was correlated with miR-132 expression, and its binding affinity to miR-132 promoter was significantly lower in pancreatic tumors relative to non-tumor samples. As recently discussed [152, 154], epigenetic features and especially miRNAs could also serve as biomarkers to allow specific and sensitive diagnosis of pancreatic cancer—an important approach as most patients with this disease remain without symptoms until the lesion has progressed to an advanced or metastatic stage. In this context, Habbe et al. [155] has analyzed miR-155, which is upregulated in most IPMNs (83 % of cases) in pancreatic juice. The authors confirmed upregulation of the miR-155 transcript in 60 % (6/10) of IPMN associated pancreatic juice of cancer patients but in none of the five control cases. Wang et al. [156] profiled four miRNAs (miR-21, -210, -155, and -196a) in heparin-treated blood samples and found a sensitivity of 64 % and a specificity of 89 % to distinguish pancreatic cancer patients from healthy controls using this panel of miRNAs, thus proving the feasibility of plasma-based miRNA profiling as a potential biomarker for pancreatic cancer. Furthermore, Kawaguchi et al. [157] investigated the utility of plasma miR-221 as a biomarker for cancer detection and monitoring tumor dynamics in 47 consecutive pancreatic cancer patients: similar to cancer tissue, plasma miR-221 levels were significantly higher in pancreatic cancer patients and correlated with distant metastasis and non-resectable status. Also, miR-21 serum levels were shown to be associated with overall survival of pancreatic cancer patients, and, in combination with six other miRNAs, allowed for correct classification of clinically suspected pancreatic cancer with a rate of 84 % [158]. Similar results were obtained for miR-18a in plasma samples of patients with pancreatic cancer: miR-18a levels were significantly higher in 36 cancer patients compared with 30 healthy volunteers [159]. Kong et al. [160] investigated the utility of several miRNAs as serum markers: while miR-21 distinguished pancreatic ductal adenocarcinoma patients from chronic pancreatitis and controls, miR-196a could distinguish resectable (stages I and II) and unresectable pancreatic ductal adenocarcinoma (III and IV) as well as predict median survival time of pancreatic ductal adenocarcinoma patients (6.1 months vs. 12.0 months for high vs. low level miR-196a). Recently, miR-21 from pancreatic cyst fluid was investigated as a potential biomarker and could differentiate between benign, premalignant and malignant pancreatic cyst neoplasms [161].

2.8 Potential Targets for Epigenetic Therapy in Pancreatic Cancer

The classic cancer progression model from PanIN to invasive carcinoma highlights genetic alterations in several oncogenes and tumor suppressor genes [162]. Hanahan et al. [163] characterized additional distinct features of malignant tumor cells in their outstanding reviews on hallmarks of cancer that have also been identified in

pancreatic cancer: sustaining proliferative signaling (e.g., activating mutations of K-ras [164], evading growth suppressors (e.g., deletions or mutations of CDKN2A/p16Ink4A [165]), activating invasion and metastasis (e.g., expression of CXCL12/CXCR4 [chemokine (C-X-C motif) ligand 12/(CXC receptor 4)] [166]), enabling replicative immortality (e.g., telomerase activation via loss of ATRX in pancreatic neuroendocrine tumors [167]), inducing angiogenesis (e.g., increase in serum vascular endothelial growth factor (VEGF) [168]) and resisting cell death (e.g., overexpression of anti-apoptotic Bcl-2 [169])). Many of these alterations have been explored as targets for novel therapies (e.g., anti-angiogenesis using the anti-VEGF antibody bevacizumab or anti-epidermal growth factor receptor directed therapies using erlotinib or cetuximab) but achieved only marginal survival benefits in pancreatic cancer patients compared with standard therapy [170–172]. As outlined above, recent data also suggest strong roles for non-genetic events in pancreatic carcinogenesis and resistance to current therapies [173], e.g., by modulating ABC drug transporters or interfering with cell death pathways (see Table 2.3 for details). Consequently, these regulatory mechanisms could represent interesting and potent novel targets for therapy to overcome resistance and to improve treatment outcome [174, 175]. Inhibitors of DNMT are nucleoside analogues of cytidine and currently azacytidine and decitabine are available for clinical use, although the number of current trials is very limited. Zebularine is in preclinical development [176] with promising experimental data in pancreatic cancer [177]. Inhibitors of protein and histone deacetylases have been established as a novel approach to target hematologic and solid tumors [178]. Several phase-I studies using the first-in-class molecule vorinostat (SAHA) are currently ongoing, especially in combination with cytotoxic agents or radiotherapy. Other agents like belinostat (PXD-101), entinostat (MS-275) or panobinostat (LBH-589) are at various stages of early clinical development, with progression-free survival or maximum tolerated dose as study endpoints. As described above, in addition to deacetylases, HATs can also regulate gene transcription. Here, curcumin (derived from the South Asian plant turmeric) has been demonstrated to effectively inhibit the activity of the HAT p300/CBP in cancer cells [179, 180]. Although its pharmacokinetic properties are unsatisfying so far, it demonstrated early signs of clinical efficacy in pancreatic cancer patients in a phase-II setting [181]. Other epigenetic modifiers besides DNMT, HAT or HDAC have been identified and the first lead compounds are currently being extensively studied in preclinical or are in early clinical phases. However, clinical data for pancreatic cancer is not available [182]. While miRNAs are considered useful tools for diagnosis, prognosis and possibly patient stratification [183], miRNA-based therapeutics is currently not available. Although preclinical data suggests that antagomiRs or miRNA replacement therapy is promising for pancreatic cancer models, clinical use is hampered by unresolved drug delivery and the fact that one miRNA also has several target mRNAs, thus possibly being too unspecific [184, 185]. Overall, as most of the agents highlighted above are currently in early phases of clinical development, no clear data on efficacy of epigenetic agents in pancreatic cancer are available, but promising preclinical [186] and early clinical data warrant further development.

2.9 Conclusion

High mortality due to lack of early diagnostic tool and poor prognosis because of undefined therapeutic target in pancreatic cancer, understanding the molecular events driving this devastating tumor crucial for development of alternative and more effective treatment strategies and reliable diagnostic markers. Recent research on epigenetic mechanisms have greatly enriched our knowledge about the regulatory traits involved in initiation, progression and metastasis of pancreatic cancer. As shown in this book chapter, DNA-, histone- and miRNA based epigenetic events have been demonstrated to play a role in pancreatic cancer and could serve as future therapeutic targets aiming at reversing the epigenetic deregulation of the cellular machinery. Initial clinical trials at stages I–III using inhibitors of DNMTs, HDACs and HATs are currently under way, which also opens the door for the development of novel and hopefully more effective 'epidrugs' to treat pancreatic cancer patients.

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Chapter 3
Epigenetic and Cancer: An Evaluation of the Impact of Dietary Components

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3.1 Introduction

Natural dietary compounds isolated from fruits, vegetables, and spices have shown great potential in the prevention and treatment of various diseases such as cancer [1–12]. These compounds contain several bioactive properties that are ubiquitous in plants, many of which have been used in ancient traditional medicines. Herbs, fruits, and veggies are not only a good source of fiber, vitamins and minerals, but also consist of constituents like resveratrol (RES), curcumin, genistein, polyphenols, alkaloids, phenolics and sulforaphane. Evidence indicates that these compounds may serve more than a basic nutritional function; thereby, effectively mediating the regression of multiple debilitating diseases including cancer. In addition to the compounds listed above, other polyphenols such as isothiocynates, silymarin, dialyl sulfide, lycopene, rosmarinic acid, apigenin and gingerol have demonstrated their potency against cancer [1–12]. Interestingly, these compounds have shown the ability to inhibit cancer via the facilitation of various epigenetic processes. Therefore, this chapter will focus on the epigenetic targets of these compounds, which are heavily involved in cancer prevention and therapy.

The study of epigenetics is comprehensive and includes all intracellular and extracellular interactions that may affect the expression of specific genes without directly altering nucleotide sequences [11–25]. Epigenetics can best be defined as the study of the mechanisms affecting temporal and spatial control of gene activity during the development of complex organisms [26]. Perhaps one of the best

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examples of this is the epigenetic modification of chromatin during embryonic development after the fertilization of eukaryotic eggs. In fact, epigenetic changes are so wide-ranging that they can be used as molecular tools in the screening and treatment of various diseases including cancer. Cancer is the result of genetic mutations and/or epigenetic modifications stemming from the exposure to various adverse environmental factors [27–29]. Studies have shown that exposure to environmental toxins, the quality of nutrition and other factors including physical and chemical pollutants can alter gene expression and modulate individual genetic susceptibility to changes within the epigenome [17, 30]. To this end, there are several known mechanisms that are capable of altering the epigenome, which include DNA methylation, histone acetylation, chromatin remodeling and RNA-interference/interaction.

Epigenetic mechanisms often regulate the transcription of genes that facilitate cellular proliferation, differentiation, and survival. These mechanisms have also been linked with tumorigenesis. Aberrant chromatin modifications such as DNA methylation and histone acetylation are the main processes studied in cancer epigenetics [17, 31, 32]. Recent studies have demonstrated that during cancer development, approximately 50 % of all tumor suppressor genes are most likely inactivated by epigenetic rather than genetic, mechanisms [33]. Reports also suggest that bioactive dietary compounds can often restore the function of tumor suppressor genes, increase survival, and under certain circumstances induce apoptosis in many kinds of cancers [34, 35]. In addition to the transcriptional silencing of tumor suppressor genes, non-coding micro-RNAs (miRNAs) can be used to affect mRNA stability and subsequent translation by epigenetic processes during cancer progression [29, 32]. More interestingly, these miRNAs can regulate the expression of various epigenetic modifying enzymes such as methyltransferases (DNMTs), histone methyltransfereases (HMTs), and histone deacetylases (HDACs), which historically have been documented to participate in tumorigenesis [36, 37]. Recent studies also suggest that bioactive dietary compounds may target different tumor suppressor miRNAs to change the function(s) of genes that are being used to classify human cancers [38, 39]. Furthermore, miRNAs either directly or indirectly regulate cancer progression by acting as a tumor suppressor or epigenetically modifying enzyme. In a recent study, *miRNA-221* and *miRNA-222* inhibit the oncogene *KIT*, and therefore functions as a tumor suppressor in erythroblastic cells and other solid tumors of human origin [40]. Conversely, the *miRNA-29* family can directly control the expression of DNMTs and enhance the expression of both *DNMT-3a* and *DNMT-3b* causing genomic hypermethylation and the silencing of sensitive tumor suppressor genes: *FHIT* and *WWOX* [41].

3.2 Mechanism of Epigenetic: DNA Methylation

DNA methylation has been observed in many different types of organisms including mammals, plants and bacteria [42, 43]. DNA methylation occurs during DNA replication and is considered a stable gene-silencing mechanism. During this process

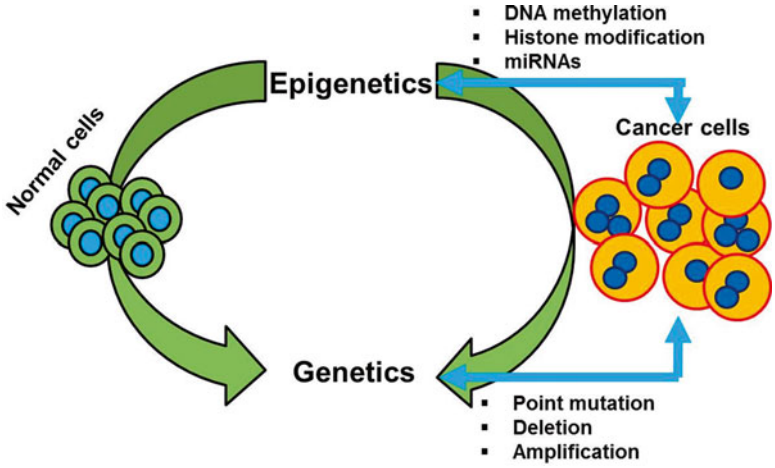

Fig. 3.1 Mutations in epigenetic modifiers not only induce cancer formation, but also induce epigenetic changes like DNA methylation, histone modification, and microRNAs, which lead to abnormal gene expression and genomic instability [52, 53]

DNMTs add methyl groups to the 5′ end of the DNA molecule, thus inactivating the affected gene by directly interfering with the assembly of transcription factors essential for gene expression. These enzymes use *S*-adneylmethionine (SAMs) to transfer methyl groups to cytosine-phosphate-guanine (CpG) sites along the DNA. However, CpG sites are not randomly distributed in the genome, but are concentrated in short CpG-rich DNA fragments commonly referred to as CpG islands [33, 44–46]. Additionally, the majority of CpG sites (except the nucleotide cytosine) are methylated, during development and differentiation in normal cells. Certain subsets of CpG islands at promoter regions may be methylated leading to long term inactivation of target genes, which can be seen in the CpG islands of tumor suppressor genes [47–51]. DNA methylation patterns are formed during cell proliferation, and can disrupt cellular division. DNA methylation is tissue specific, and distinct methylation patterns have been observed across various tissue types. Evidence indicates that the hypermethylation of genes often facilitates conditions that are conducive to carcinogenesis (Fig. 3.1) [33, 44–48, 54–59].

3.3 Histone Modification

The basic structure of the nucleosome consists of the histone octamer, which includes two molecules of each H2A, H2B, H3 and H4 proteins. The N-terminal of these proteins extends from the nucleosome core and the exposed amino acids undergo a series of covalent modifications including methylation, acetylation, phosphorylation, ubiquitinization and sumolization [11, 18, 32, 60]. Singular occurrence

or a combination of these modifying events are believed to cause inheritable epigenetic programs that facilitate different nucleosome functions such as gene transcription, the inactivation of the X-chromosome, formation of heterochromatin, mitosis and DNA repair and replication [10, 36, 57, 61, 62]. Direct interaction between the chromodomain of *Tip60* and histone H3 trimethylized on lysine 9 (H3K9me3) at double-strand breaks (DSBs) activate acetyltransferase. H3K9me3 deletion inhibits acetyltransferase activation of *Tip60*, resulting in defective ATM activation that leads to defective DSB repair. These functions are induced either by altered nucleosome interactions with chromatin or by recruiting effector proteins that possess modules that recognize specific histone modifications in a sequence specific manner. The epigenetic codes reside in the substrate specificity of the enzymes that catalyzes the various covalent modifications as well as the enzyme that reverses these modifications.

Chromatin is the template for DNA mediated processes; therefore, it might be worthy to note that histone modifications are an important component in controlling the structure and/or function of the chromatin, which often produces functional consequences. Previous reports suggest that site-specific histone modification can be linked with gene transcription [33, 63, 64]. For instance, histone H3, lysine 9 acetylation (H3K9ac), H3 serine 10 (H3S10) phosphorylation and H3 lysine 4 trimethylation (H3K4me3) are found to be associated with transcriptional activation [33, 64–67]. However, hypomethylation of H3 and H4 have shown to suppress transcription. In brief, the importance of histone modification is highlighted after the revelation that transcription apparatuses often recognize and respond to histone modifying activity [44, 58, 68]. Studies have also shown that histone H3S10 phosphorylation is catalyzed by mitogen and stress activated protein kinase 1 (MSK1). H3S10 phosphorylation is also recognized by a 14-3-3e/14-3-3y heterodimer through its interaction with H3K4 trimethyltransferase (SMYD3) and the *p52* subunit of FIIH (Fig. 3.2) [64].

3.4 microRNAs Interaction

MicroRNAs are evolutionarily conserved endogenous non-coding RNAs. MiRNAs are typically 19–25 nucleotides long, which partially or completely match the 3′ untranslated regions (3′UTR) of target RNAs. The hybridization of miRNAs to target RNAs controls gene expression by post-translational modification, silencing, and degradation mechanisms [21, 38, 40, 41, 68, 71–73]. Previous reports suggests that more than 30 % of human genes are controlled by miRNAs which suggests that these small non-coding RNAs play important roles in many biological processes including cell cycle regulation, cell growth, apoptosis, cell differentiation and stress reactions [42, 43, 74–78].

In recent studies, increased detection of miRNA among clinical samples clearly suggests that regulatory functions involve miRNAs [12, 16, 18, 21, 73, 79, 80]. According to data retrieved from the Sanger miRNA Registry in 2013, more than 800 or 1000 human miRNAs have been recorded however; many more miRNAs are

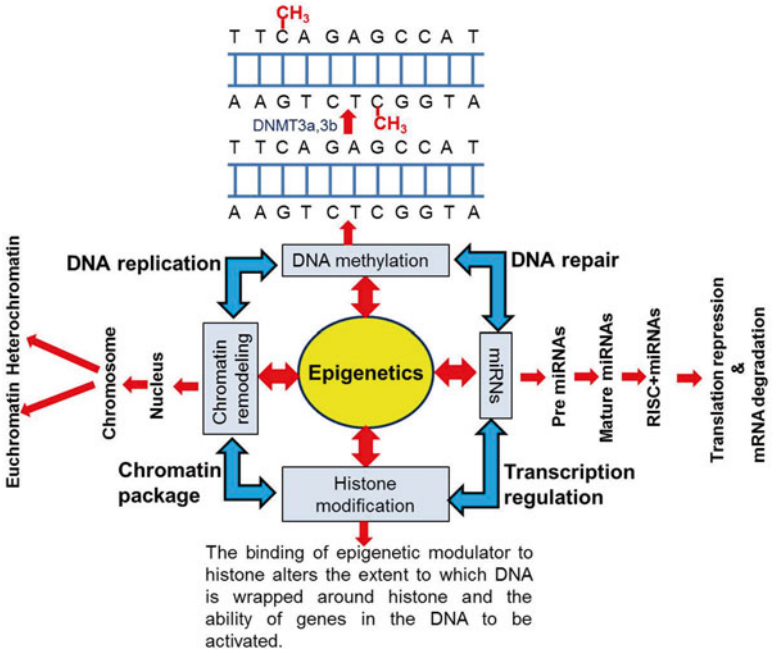

Fig. 3.2 Epigenetic mechanisms of gene regulation [69, 70]

expected to be discovered in the future [81]. miRNA control is very similar to the regulation of tightly controlled protein encoding genes. However, during cases of cancer proliferation miRNAs have been found to be greatly deregulated [42, 43, 74–78, 82–85].

Epigenetic manipulation of miRNAs is believed to be highly complex [4, 18, 21, 22, 68, 73]. Additionally, tissue specific expression of miRNAs is tightly regulated by epigenetic mechanisms such as DNA methylation and histone modification; however, miRNAs themselves can also affect epigenetic mechanisms and regulate gene transcription via post-translational gene silencing [16, 37, 41, 73]. In addition to these important biochemical pathways miRNAs can also be regulated by dietary supplements such as RES. Research shows that oncomirs such as *miR-21* are upregulated during the manifestation of various types of cancers. RES is an effective regulator of these [86–89].

3.5 Epigenetic and Carcinogenesis

Epigenetic mechanisms help to maintain cellular homeostasis during normal physiological conditions [5, 10, 13, 20, 21, 23, 24, 30, 48]. However, alterations in epigenetic regulation may lead to aberrant gene expression, which can result in the

Table 3.1 Lysine methylation pattern during cancer progression

Histone modification	Expression during cancer progression	Cancer types
H3K4me1	↑	Unknown
H3K4me2	↑	Prostate [95, 96]
H3K4me3	↓	Bladder cancer [95]
H3K9me2	↑	Gastric adenocarcinomas [95, 97]
H3K9me3	↓	Prostate [95, 97]
H3K27me3	↑	Paragangliomas [77]
H4K20me1	↓	Bladder cancer, Lymphomas, colorectal adenocarcinomas [77, 78], breast carcinomas, bladder cancer, liver cancer, non-small cell lung cancer
H4K20me3	↓	Lymphomas, colorectal adenocarcinomas [77, 78], breast carcinomas, bladder cancer, liver cancer, non-small cell lung cancer

development of cancer. Cancer development is typically associated with genetic mutation and the subsequent improper unregulated functioning of genes [9, 15, 40, 44, 54, 90–94]. However, our understanding shows that carcinogenesis cannot be the result of genetic alterations alone, but also involve epigenetic changes such as DNA methylation, histone modifications and microRNAs (Fig. 3.2). The level of lysine methylation varies and depends upon cell type. Data suggests that these molecular changes are associated with different types of cancers (Table 3.1).

Additionally, the deregulation of lysine methyltransferase and demethylases has been found in a variety of cancers as shown in Tables 3.2 and 3.3.

These changes lead to stable alterations in the pattern of gene expression that control the neoplastic phenotype, such as cellular growth and invasiveness. At this point, we focused on epigenetic targets of the bioactive compound resveratrol (RES) and its role in cancer prevention and therapy.

RES is a dietary polyphenol obtained from grapes, berries, peanuts, and other plant sources. RES shows a wide range of anti-cancer benefits such as modulating signal transduction pathways that regulate growth, differentiation, apoptosis, inflammation, angiogenesis, and metastasis [117–122]. Studies also suggest that treatment with RES inhibits the proliferation of various human cancers such as skin, breast, prostate, lung and colon [123–127]. The success of RES has led to the development of preclinical animal studies in an effort to determine the potential of this agent for cancer chemotherapeutics. Furthermore, RES has shown remarkable effects against cancer cells at both the biochemical and molecular levels [128].

RES has weaker DNMT inhibitory activity as compared to other bioactive compounds such as epigallocatechin-3-gallate (EGCG). In addition, RES inhibits epigenetic silencing of *BRCA-1* induced by aromatic hydrogen receptor (AhR) in MCF-7 cells [129]. Studies show that treatment with RES results in AhR-mediated enrichment of mono-methylated-H3K9, DNMT1, and methyl-binding domain protein-2 at the *BRCA-1* promoter, which was associated with *BRCA-1* reactivation in MCF-7 cells [129]. Conversely, it has also been reported that RES induces retinoic

Table 3.2 Histone lysine methyltransferases implications in cancer

Histone modifier	Changes during cancer	Cancer types
MLL1	Translocation, amplification, duplication	Human lymphoid and myloid leukemia [98, 99]
MENIN	Mutated	Multiple endocrine neoplastia type-1 [94, 100]
Ash2L	Increase expression	Squamous cell carcinoma of cervix and lyrix, melanoma, rhabdomyosarcoma, breast and colon carcinoma, pancreatic ductal adenocarcinoma and gastric carcinoma [101, 102]
	Low level	Hepatocellular carcinoma [103]
Ezh2	Over expression	Prostate neuroblastoma, breast cancer [51, 104]
	Mutation	B cell lymphoma, gallbladder adenocarcinoma [105]
Suv39H1	Over expression	Colon
SMYD3	Over expression	Colon, breast, hepatocellular carcinoma [105]
RIZ1	Mutation/down regulation	Liver breast and gastric cancer [105]
NSD1	Translocation	acute myeloid leukemia [14, 106]
	Mutation	Soto's syndrome [14]
	Silencing by promotor	Neuroblastoma and gliomas
	Hyper mutation	
NSD2	Translocation	Multiple myeloma
	Over expression	Multiple tumors
NSD3	Translocation	Leukaemia
	Amplification	Breast cancer [107, 108]
G9a	Over expression	Hepatocellular carcinoma [107, 108]
	Hypoxia mediated upregulation	Gastric, lung cancer [109]

Table 3.3 Histone lysine demethylase implicated in cancer

Histone activator	Changes during cancer	Cancer types
LSD1	Over expression	Prostate, neuroblastoma, breast cancer [67, 110]
	Low level	Hepatocellular carcinoma [67, 110]
FBXL10	Mutation	Lymphoma [111]
	Decrease	Brain glioblastoma
JMJD2C	Over expression	Prostate, oesophageal squamous cell carcinoma, desmoplastic medulloblastoma, MALT lymphoma [103, 111–113]
RBP2	Over expression	Gastric cancer [114]
PLU-1	Over expression	Breast, prostate, testis, ovary, lung, bladder cancer [115]
UTX	Mutations	Multiple myeloma, renal cell carcinoma [116]
JMJD3	Over expression	Prostate, pancreatic cancer, lymphoma [116]

acid receptor beta 2 (RARbeta2) expressions by blocking RARbeta2 promoter methylation in MCF-7 cells as compared to other adenosine analogs [130]. Furthermore, RES induced activation of the type III HDAC inhibitors, *sitrin 1* (*SIRT1*) and *p300*, in several *in vitro* and *in vivo* models [131]. However, activated *SIRT1* negatively down regulated the expression of survivin by deacetylase activity [132–135]. Human *BRCA-1* breast cancer cells showed decrease expression of *SIRT1* [132–135]. RES has been shown to induce the activation of *SIRT1* by altering H3 acetylation. This proved to be a useful approach for target therapy for *BRCA-1* mediated breast cancer [136]. Furthermore, *SIRT1* associated *BRCA1* signaling is important for targeting tumorigenesis by activating oncoproteins in human breast cancer [136]. It has been shown that *SIRT1*-encoded proteins are needed for RES-induced chemotherapy in APC/+ and APC/− mice [137]. *SIRT1* also play an important role in aging, since *SIRT1* null mice are unable to tolerate caloric restriction and fail to extend their life duration [137]. This demonstrates RES's ability to modulate epigenetic processes via the activation of expressed HDAC inhibitors [138].

3.6 Conclusion and Future Prospects

The emerging field that involves nutritional genomics to target nutrient related genetic and epigenetic alterations for cancer therapeutics is unique and timely. The bioactive dietary compound (RES) holds great potential not only in the prevention, but also in the therapy of a wide range of cancers by inducing epigenetic modifications. Cancer is a highly resistant disease and uses several survival pathways to prevail over normal cells. RES can act at several levels to inhibit multiple cellular pathways (for instance the induction of *SIRT1* and the inhibition of NFkB) and can be developed as a potential therapeutic agent. Many bioactive dietary compounds have shown great promise in targeting many cellular pathways involved in carcinogenesis as compared to other traditional therapies. However, further research is needed to assess organ specificity, bioavailability and general safety of these dietary compounds for any prudent conclusions. Empirical evidence of the healing powers of ancient medicines strongly supports the use of RES for cancer therapy.

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Chapter 4
Gene Expression, Epigenetic Regulation, and Cancer

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4.1 The Principles of Gene Expression

DNA (deoxyribonucleic acid) serves as a blueprint for the manufacture of different proteins that are critical in coordinating virtually every biological function performed in a cell. Genes are constructed of sequences of DNA nucleotides (each composed of a phosphate group, a five-carbon sugar, and a nitrogenous base) positioned on chromosomes that exist in the nucleus of a cell. Each gene will dictate the order of amino acids needed to make a particular protein, and differences in the sequences of amino acids contribute to the shape and function of the protein. The process by which a gene is turned on to construct a specific protein is called gene expression [1].

Proteins are important molecules that perform a variety of functions. For example, some are structural proteins, while others play enzymatic roles, serve as hormones, or protect the body against infections. Structural proteins provide the body with support; keratin, for instance, is a component of nails and hair, and collagen gives strength to tendons, ligaments, and skin. Enzymes are involved in catalyzing metabolic pathways whereby a chemical reaction can occur within a second, whereas hours may be required to complete the same reaction without enzymes. Some proteins serve as hormones, which control the cellular activities of target organs. Antibodies are plasma proteins that bind with antigens, thereby preventing foreign substances from injuring cells and causing homeostatic disruption. These and other operations make proteins essential to life [2].

Transcription and translation are the two major stages associated with gene expression. Transcription takes place in the nucleus and entails a gene operating as a template for the construction of an RNA molecule. Though most genes are

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transcribed into messenger RNA (mRNA), which contains instructions that will eventually produce proteins, some genes are transcribed into two other classes of RNA: (1) ribosomal RNA (rRNA), a chief component of ribosomes—organelles on which proteins are synthesized and (2) transfer RNA (tRNA)—molecules that carry amino acids, which are subunits of proteins, to ribosomes [3].

In eukaryotes, the production of an mRNA molecule is initiated with the binding of proteins called transcription factors to the promoter, a DNA sequence situated just upstream of a gene. Subsequently, the transcription factors recruit to the promoter the enzyme RNA polymerase [1]. As RNA polymerase travels along the DNA helix associated with the gene, the enzyme unfastens the two strands of DNA and constructs together RNA nucleotides complementary to one of the DNA strands that is serving as a template; that is, when DNA bases adenine (A), thymine (T), cytosine (C), or guanine (G) are located in the DNA template, bases uracil (U), adenine (A), guanine (G), or cytosine (C) are respectively positioned into the RNA molecule. This process eventually produces a single-stranded RNA molecule that has corresponding nucleotides of the DNA sequence for the gene in question. The complementary RNA strand of the gene is referred to as messenger RNA, given that it possesses the "message" or genetic material of the gene which will be expressed [4].

The resulting strand of mRNA is then processed to remove introns, sequences of nucleotides that will not be translated into a sequence of amino acids for a protein. Once introns are deleted, the remaining nucleotide sequences—exons—are spliced together. The resulting mRNA strand is then modified with the addition of a guanine-nucleotide cap at the 5′ end of the strand and the inclusion of a tail consisting of 30–100 adenine-nucleotides (poly-A tail) at the 3′ end. The modified mRNA strand will then leave the nucleus through the nuclear envelope and then enter the cytoplasm, where the process of translation will proceed [5]. Though ordinarily the mRNA strand that is produced after transcription is composed of a sequence of all of the exons of the gene, alternative splicing may occur whereby only certain exons are used. Hence alternative splicing allows for the manufacture of different variations of a protein. For example, white blood cells are able to construct particular antibodies based on the type of antigen that enters the body [2].

The second major stage pertaining to gene expression is called translation and occurs in the cytoplasm. Translation involves the conversion of the sequence of mRNA nucleotides into a sequence of amino acids associated with a protein. Along with mRNA and amino acids, ribosomes and tRNA are some of the other components that play important roles in translation [3].

The ribosome is the organelle on which protein synthesis takes place and is constructed of two subunits, each consisting of rRNA enfolded around various proteins; the ribosomal ensemble allows for the binding of mRNA and its passage through the ribosome. A complex series of interactions ensues once mRNA is bound to the ribosome. First, small mRNA sequences called codons are exposed. A codon is three nucleotides long and typically encodes for a particular amino acid. (Sixty-one codons will encode for the 20 different amino acids; therefore, most amino acids are designated by more than one codon. The diversity of codons allows for some degree of protection against mutations that alter the sequence of bases.)

Next, a tRNA molecule physically transports an amino acid to the ribosome, which possesses three binding sites constructed for three incoming tRNA molecules. An amino acid is attached to one end of the incoming tRNA, and a sequence of nucleotides complementary to the codon, identified as the anticodon, is located at the other end of the tRNA molecule. The tRNA anticodon binds to its complementary mRNA codon that is positioned on the ribosome (e.g., codon AUG pairs with anticodon UAC). Once a second tRNA molecule docks at the adjacent codon, this newly-arriving tRNA receives the amino acid from the initial tRNA, thereby forming a peptide bond between the first amino acid and the one already coupled with the second tRNA. The initial tRNA molecule eventually leaves the ribosome once it is freed of its amino acid. Afterwards, the ribosome moves sideways across the strand of mRNA, enabling the next codon to bind to an incoming tRNA. Thus, the chain of amino acids (polypeptide) elongates one amino acid at a time. The ribosome will eventually encounter a codon (UAA, UAG, or UGA) that will not encode for an amino acid but will instead signify the termination of polypeptide synthesis. Afterwards, the polypeptide chain is released into the cytoplasm, where it will conform to the final three-dimensional shape associated with a protein, and the two ribosomal subunits will dissociate. Hence during transcription, a sequence of DNA nucleotide bases determines the sequence of mRNA nucleotide bases, and during translation, the mRNA sequence then directs the order of amino acids bonded to form the desired protein [4].

Though produced in the cytoplasm, proteins can be found in all parts of the cell. Proteins manufactured by ribosomes that float freeing in the cytoplasm remain and operate in the cytoplasm. However, proteins produced by ribosomes that are fastened to the rough endoplasmic reticulum have chemical signals that direct the proteins to their appropriate destinations within the cell, and, interestingly, these sorting signals are also encoded in the genome and are transcribed and translated [5].

Every organism has the capacity to control which genes will be expressed at any time. Organisms have to continually turn on and off genes in response to internal and external environmental signals. The regulation of gene expression is particularly important for multicellular organisms since they are composed of different types of cells, each carrying out its own unique role; some genes are turned on while others are turned off. In fact, only about 20 % of genes in a typical human cell are expressed to ultimately produce proteins required for distinctive functions, and an even smaller percentage of genes are expressed in highly specialized cells like nerve or muscle cells [6].

In eukaryotes, gene regulation can occur at different levels, ranging from pretranscriptional to posttranslational control. The regulatory mechanisms employed by cells can affect a number of operations, such as dictating whether a gene will be turned on and moderating the speed and duration at which a gene is expressed [2]. Transcription, for example, can be regulated in response to the way in which chromatin—DNA and protein—is structured in the nucleus, in that genes are typically silenced, or turned off, when chromatin is tightly packed; however, genes are more likely to be expressed when chromatin is less condensed. Histone modification and DNA methylation are two epigenetic mechanisms that have been extensively studied with regard to their

influence on chromatin structure and, subsequently, gene expression [7]. In general, epigenetics refers to any mechanism that influences gene expression without changing the nucleotide sequence of the DNA of a gene [1]. While stable epigenetic switches are critical for cell differentiation and normal development, research has shown that disruptions in normal epigenetic processes are linked to disease, including the initiation and progression of different types of cancers [8].

4.2 Histone Modification

The nucleus of a human somatic cell possesses 46 chromosomes, and when fully extended, the DNA within the nucleus measures at least 2 m. However, DNA is wrapped around proteins called histones, forming nucleosome complexes, which enable DNA to fit within the nucleus [2]. Nucleosomes can suppress transcription if they are positioned over the promoter region; the enzyme RNA polymerase and accompanying regulatory proteins will not be able to bind to the promoter. As noted earlier, RNA polymerase is essential to transcription in that it catalyzes the production of mRNA from the DNA template. However, nucleosomes can become displaced because of structural changes in the chromatin, thereby allowing room for the binding of the transcriptional proteins and hence the expression of a gene [1].

Histone modification and DNA methylation are two major types of acquired or inherited epigenetic mechanisms that can affect transcriptional activity by regulating the access of transcriptional proteins to gene promoters without altering the DNA base sequence. When observed under an electron microscope, chromatin has been described as resembling an array of beads on a string, whereby the beads represent nucleosomes, separated from one another by 10–60 base pairs of DNA. Each nucleosome contains a core of 8 histone proteins wrapped approximately 1.7 times with DNA of 147 base pairs. The histone core of a nucleosome has two copies of the following histone proteins: H2A, H2B, H3, and H4. Each of the eight histones possesses regions for histone-DNA and histone-histone interactions [9]. In addition, a histone carries an extension called a tail that is rich in positively charged amino acids, like lysine. The positive charge of the amino acids enables them to have a strong attraction to DNA, which is negatively charged because of its phosphate groups [10]. Histones H3 and H4 have been identified in particular to possess certain amino acids that are vulnerable to epigenetic modification [11]. Protein H1 serves as a linker histone that binds to DNA positioned outside the histone octamer [9]. Histone H1 is needed to create the secondary level of chromatin organization, which consists of condensed 30-nm chromatin fibers composed of several nucleosome beads; the fibers will then join to produce a tertiary structure of radial loops [12]. Histone tails are necessary for chromatin to condense into the 30-nm fibers. For instance, lysine 16 of the N-terminal tail of H4 has been shown to be very important in compacting chromatin, in that the positively charged lysine is attracted to a negatively charged area at the H2A-H2B interface of an adjacent nucleosome that is part of the condensed 30-nm fiber form [10].

Histones are subjected to a number of modifications, like acetylation, methylation, phosphorylation, and ubiquitination, and these covalent modifications can work together to alter the chromatin structure, thereby regulating transcriptional activity by controlling access to gene promoters. As noted, the positive charge of histone amino acids and the negative charge of DNA form a strong attraction. However, the addition of certain chemicals has the potential to alter this attraction. Of the various histone modifications, acetylation has been investigated most extensively. Histone acetyltransferases (HATs) are enzymes that catalyze the addition of acetyl groups to the positively charged amino acids located on the tails of the histone octamer. The addition of acetyl groups will reduce the positive charges of the histone tails, hence easing the hold of the histones to DNA and subsequently exposing the once compact nucleosome to additional proteins that serve to modify chromatin. Exposed genes can then be expressed when chromatin is loosely packed [13]. Lysine 16 in histone H4 was previously noted as a critical component in the process of condensing chromatin, in that the positively charged amino acid is attracted to a negatively charged area of a neighboring nucleosome in the 30-nm fiber form; however, the acetylation of lysine 16 tends to decrease the attraction between the amino acid and its adjacent nucleosome. Therefore, chromatin becomes less compact, allowing more space between one nucleosome bead and the next, thus enabling transcription to take place [10].

Conversely, histone deacetylases (HDACs) remove acetyl groups from histones, thereby restoring the positive charge to histone tails that contributes to the high-level compaction of chromatin and thus the repression of transcription. Furthermore, certain proteins can recruit HDACs. For example, the retinoblastoma protein (Rb), a tumor suppressor, blocks the production of molecules that are critical for numerous biological processes, including DNA replication and mitosis [14], and the influence of Rb is associated, in part, with its recruitment of HDACs to certain gene promoters [9]. HDACs have been utilized in certain treatments for cancer. In some forms of cancer, genes that normally produce proteins that inhibit cell division are not as active, and the histones close to these genes exhibit high levels of deacetylation. Therefore, drugs that function as histone deacetylase inhibitors have the potential to activate genes that typically suppress cell division, thus arresting the growth of cancer cells [13].

Along with acetylation, there are other types of histone modifications—all of which are reversible. Histone methylation, for example, may either repress or activate transcription, depending on the amino acid that is methylated and on the extent of methylation. Different classes of enzymes called histone methyltransferases (HMTs) add methyl groups to particular arginine and lysine amino acids on histone tails, while histone demethylases (HDMs) remove methyl groups. The methylation of arginine is linked with transcriptional activation, whereas the methylation of lysine is usually associated with transcriptional repression [11]. When lysine is methylated, acetylation normally does not take place, and the positive charge is maintained. In addition, lysine may be methylated more than once [10]; however, the location of the modified lysine can also determine whether transcription will occur. For instance, transcriptional inactivation is linked to the methylation of lysine 9 in histone H3, but transcriptional activation accompanies the methylation of lysine 4 in H3 [12].

Histone phosphorylation is accomplished with the assistance of kinases and involves the targeting of amino acids serine and threonine, and all four core histones can be affected. Phosphorylation takes place at the N-terminus of the histone near methylated and acetylated lysines. For example, the phosphorylation of serine 10 on histone H3 is linked with acetylation of the nearby lysine 14. The added phosphate group to serine 10 contributes a negative charge, thereby partially neutralizing the net positive charge of the modified histones and thus altering their capacity to interact with DNA, which is also negatively charged. As a result, the repellent force contributes to a less compacted chromatin state, which may allow for the transcription of genes [15]. Conversely, the phosphorylation of both serines 10 and 28 on H3 has been found to correlate with chromosome condensation during mitosis [16]. Moreover, the close association of different modifications can promote further epigenetic changes. In fact, the removal of a methyl group that is attached to lysine 9 on H3 promotes the phosphorylation of serine 10 and the acetylation of lysines 9 and 14 [15].

Another important process by which histones can be modified is ubiquitination. The polypeptide ubiquitin is composed of 76 amino acids and can be enzymatically coupled to different types of proteins. In most cells, ubiquitin is primarily used to tag damaged or old proteins for degradation by proteasomes. In fact, proteasome inhibitors have been shown to be effective in certain types of cancer therapy whereby cancer cells eventually die when proteasomal activity is suppressed. Controlled administration of proteasome inhibitors has been shown to be effective against multiple myeloma in that cancer cells are destroyed but not normal cells. Though ubiquitin may serve as a tag for the destruction of some proteins, it does not damage other proteins and can function in epigenetic genome control. For example, with regard to histone modification, ubiquitination takes place only on H2A and H2B, and only one lysine is targeted in each histone: lysine 119 in H2A and lysine 120 in H2B. Ubiquitin possesses a number of amino acids that are negatively charged, and when bound to histone tails, ubiquitin relaxes the chromatin structure by neutralizing the positively charged amino acids in the histone and thus activating transcriptional activity. Furthermore, ubiquitin can influence other modifications. For example, the ubiquitination of H2B promotes the methylation of lysines 4 and 79 on H3 [10].

The examples above illustrate some of the many complexities associated with the different types of histone modifications and their roles in altering chromatin structure, which influences the accessibility of genes for transcription.

4.3 DNA Methylation

Along with histones, the DNA itself can be tagged with external chemicals. Genes can be silenced when methyl groups are enzymatically attached to certain nucleotide bases. The methylated DNA influences the interaction of the DNA with proteins, including histones. Gene regulation involving DNA methylation is

characterized as epigenetic in that gene expression is altered without affecting the sequence of DNA. Highly methylated regions of a gene are normally associated with tightly packed DNA wrapped around histones, resulting in transcriptional inactivation [1]. DNA methylation is also heritable. Not all eukaryotes exhibit both histone modification and DNA methylation. Fruit flies and yeasts, for instance, display only histone modification [16].

During DNA methylation, the methyl group is covalently added to a cytosine situated 5′ to a guanine (CpG), forming 5-methylcytosine, and the reaction is catalyzed by DNA methyltransferase (DNMT). Methylation normally takes place in only 3–4 % of cytosines. Methylcytosines occur primarily in repetitive sequences and in the CpG islands (GpC and CpG-rich regions) of the promoters of silent genes like X-chromosome inactivated genes and imprinted genes, both of which will be discussed later in this section. During transcription and DNA synthesis, 5-methylcytosine pairs normally with guanine; however, the methylation of cytosine is reversible with the aid of the enzyme demethylase, which removes the methyl group [9].

DNA methylation may cause the cell to incur risks in some cases. Cytosines that are methylated are chemically less stable than those that are not. As a result, a methylated cytosine may undergo deamination, whereby a thymine is generated in its place, i.e., a methylated CpG is converted to TpG. Unfortunately, this error in the base sequence may not be identified by the DNA repair machinery before replication takes place [16].

Typically genes that are heavily methylated are inactive. Processes by which DNA methylation silences genes involve complex interactions with various proteins. This form of epigenetic regulation of transcription interlinks histone deacetylases and chromatin-remodeling enzymes [9]. Specialized proteins that attach to methylated cytosines are called DNA methyl-binding domains, which are usually subunits of larger complexes like histone deacetylases or methyltransferases. These enzymes then alter histone proteins located near the methylated DNA. For instance, methyl groups attached to DNA can recruit methyl-binding proteins that then recruit histone deacetylases, which change chromatin by detaching acetyl groups from histone tails. As noted in the previous section, deacetylation induces chromatin condensation by restoring the positive charge to histones. In addition, methylated DNA may be bound to proteins possessing histone methyltransferase activity; this interaction causes histone proteins in the vicinity to be methylated, resulting in chromatin condensation and, consequently, the silencing of genes. Conversely, specialized proteins that bind to histones can set in motion a chain of reactions that eventually methylates DNA with the assistance of DNA methyltransferases. For example, methylated histone tails can recruit chromodomain-containing proteins that then attract DNA methyltransferases that bind methyl groups to neighboring DNA [16].

A good illustration of how DNA methylation normally operates in compacting chromatin in a formation that represses genes is X-chromosome inactivation. A normal human somatic cell consists of two sex chromosomes, each copy inherited from a parent. The normal complement of sex chromosomes for a human female is XX, while the complement for a human male is XY. The Y chromosome is smaller than the X chromosome and does not possess all of the genes located on the X

chromosome. In theory, since the female has two X chromosomes, she should have the capacity to produce substantially more protein from her sex chromosomes than the male can produce from his. However, the female normally has only one X chromosome in the cell that contains genes that are active, and the genes of the remaining X chromosome are primarily inactive. The determination of whether an X chromosome will be inactive is random; hence within the body of a female, the maternal X chromosome may be inactive in one cell, but the paternal X chromosome may be active in a nearby cell. The inactivation of one of the two X chromosomes in a female allows the female and the male to produce relatively the same amount of X chromosome-encoded protein. And the silencing of genes on the inactive X chromosome is primarily attributed to heavy DNA methylation [17].

Genomic imprinting is a form of gene inactivation that is also associated with DNA methylation. A somatic cell possesses two copies of each gene—one copy (allele) inherited from the mother and the other allele inherited from the father. And typically a gene is expressed from both copies; however, with some genes, transcription is limited to either the maternal or the paternal copy of the gene, not both. This condition is referred to as genomic imprinting, and the repression of a parental copy of a gene can be replicated during cell division and passed on from one generation to the next [18].

Imprinting usually occurs in a limited number of genes in the mammalian body, but it is important in regulating embryonic development and growth. In some genes, the inherited maternal allele is silenced while the inherited paternal allele is expressed. In other genes, the paternal copy is silenced while the maternal copy is expressed. The silenced copy of a gene is typically highly methylated [16]. For example, *IGF2* is an imprinted gene located on chromosome 11 in humans that encodes for a protein hormone called insulin-like growth factor 2. This hormone is important for placental and fetal growth. Normally the maternal allele is repressed because of DNA methylation, but the paternal copy of the gene is actively expressed, and the resulting protein product is essential for development. Research utilizing mice as subjects has shown that when the paternal allele of *IGF2* is deleted, the placenta will be small in size, and the young will exhibit low-birth weight [19].

Prader-Willi syndrome and Angelman syndrome are examples of developmental disorders in humans related to genomic imprinting, and both diseases involve deletions in chromosome 15 that result in the silencing of one or more genes. In Prader-Willi syndrome, a small region of the paternally inherited chromosome is missing, and an equivalent region on the maternal chromosome is silent. Clinical symptoms include mental retardation, short stature, and small feet and hands. In contrast, with Angelman syndrome deletions are found in a region of the maternal chromosome, but the paternal chromosome has a silent region. Seizures and uncontrolled muscle movements are some characteristics exhibited by those inflicted with Angelman syndrome [18].

Modifications to normal patterns of DNA methylation have also been implicated in the initiation of certain cancers. Promoters of tumor suppressor genes have been found to be inactive because of the addition of methyl groups, while promoters of proto-oncogenes, which normally stimulate cells to divide, may become demethylated to the point of causing cells to multiply uncontrollably [13].

4.4 Environmental Influences on Gene Expression

Proposed in 1809 by Jean-Baptiste Lamarck, the concept of inheritance of acquired characteristics argues that environmental pressures and personal necessities can result in beneficial changes in physical appearance and body function and that eventually offspring inherit these characteristics. Lamarck employed his concept to describe why giraffes exhibit long necks. He suggested that giraffes originally possessed short necks, but in an effort to procure food located in higher areas of trees, the giraffes stretch their necks in order to reach leaves. The necks are lengthened with the aid of a nerve "fluida," and longer necks are then passed on to offspring [3]. By contrast, Charles Darwin's theory of natural selection would presume that early giraffe populations had individuals with necks of varied lengths. Natural selection due to competition caused those with longer necks to acquire more food for survival, live longer, and have more offspring. Eventually, after generations, the population would consist of giraffes with long necks because of the beneficial adaptation to the environment [2]. Darwin's approach to evolution is, of course, widely accepted today: natural selection dictates that beneficial traits are acquired by chance and are inherited through generations because of their selective advantage. However, with the discovery of epigenetic regulation, scientists are forced to take a closer look at the role of environmental influences on gene expression and how epigenetic factors are passed on to future generations [20].

Gene expression is tightly regulated in the body in order to ensure that cells manufacture the appropriate level of proteins needed for a given period and for a specific function. Cell specialization is closely tied to epigenetic regulation. Cells differentiate into various types, each performing distinctive functions, and epigenetic factors regulate how and when particular genes are turned on and off to assist the body with growth and development [21]. Liver and pancreatic cells, for instance, share the same set of genes; however, cells of the liver access the genes associated with the production of enzymes used to neutralize particular toxins but not the genes required to synthesize glucagon and insulin, glucose regulating hormones made in pancreatic alpha and beta cells, respectively [3].

In eukaryotes, gene expression can be regulated at different levels—pretranscription, transcription, posttranscription, translation, and posttranslation. And studies have shown that a variety of mechanisms used to control gene expression operate before transcription and after translation [6]. Sections above that pertain to histone modification and DNA methylation illustrate some of the epigenetic mechanisms that manipulate the structure of chromatin and thus the onset of gene expression. Furthermore, external signals, including environmental chemicals, aging, and diet, have a major impact on the operation of these epigenetic mechanisms and, ultimately, the health of an individual [1].

Both genetic mutations and epigenetic changes can be linked to the onset of disease. Some diseases are the result of genetic mutations, whereby changes exist in the sequences of nucleotides of the genome, and these mutations can develop from environmental factors and can run in families. And many diseases are influenced by

epigenetic modifications. The epigenome of an individual can continually change throughout life and can be influenced by environmental factors, including chemical cues and social interactions, which may have positive or negative effects. An accumulation of epigenetic changes is normally associated with aging; however, these changes can alter the standard operations of certain genes and lead to particular age-related diseases, like diabetes and cancer [21].

The health effects of environmental factors have been studied with monozygotic (identical) twins, which develop from one egg fertilized by a single sperm. Though identical twins share the same DNA sequences, they typically differ to some degree in appearance, behavior, and health. A thorough explanation of these differences has not been developed; however, recent studies suggest that epigenetics appears to be the cause of some of these phenotypic differences. Fraga and his associates [22], for example, found that monozygotic twins are not only identical genetically, but they are similar epigenetically with regard to DNA methylation and acetylation of histones H3 and H4. Nonetheless, the content and distribution of these epigenetic factors change considerably as twins get older, and the degree of epigenetic differences has been shown to correlate with the length of time twins live apart and variations in lifestyles, such as smoking habits, food choices, and physical activity. This strongly suggests that environmental cues play critical roles in altering one's epigenetic profile and, subsequently, the expression of genes. Epigenetic differences might explain why one identical twin is diagnosed with a disease like schizophrenia, which is genetically based, but the other twin is not [6].

Environmental exposure to certain chemicals has the capability to alter the epigenome. In fact, early exposure to certain chemicals *in utero* has been linked to epigenetic changes. Waterland and Jirtle [23] famously demonstrated in pregnant Agouti mice that dietary supplements can result in phenotypic changes in offspring. The *Agouti* gene controls the amount and distribution of pheomelanin (yellow and red) and eumelanin (brown and black) pigmentation in the coat of a mammal. A mouse carrying an *Agouti* gene with the lethal yellow mutation has a distinctive yellow coat, is obese, and is prone to develop diabetes and cancer [24]. The promoter of the mutated gene has been found to be hypomethylated, unlike the promoter of the normal *Agouti* gene [25]. In an experiment where pregnant yellow mice were fed methyl-rich supplements—folic acid, vitamin B12, betaine, and choline—most of the pups were of normal weight, displayed a brown coat, and lacked the predisposition to develop diabetes and cancer. However, pregnant yellow mice that were not given methyl supplements primarily produced pups that were also yellow and unhealthy. These findings suggest that the diet of mothers can influence the health of the young. In this example, the methyl donors supplemented in the food of pregnant mice with a mutated gene were able to make their way onto the chromosomes of the developing embryos and methylate the critical gene, hence counteracting the negative effects of the faulty gene [23]. This and many other examples show correlations between the exposure to certain chemicals and epigenetic changes. With regard to adverse health effects, prenatal introduction as well as postnatal exposure to deleterious chemicals can have a major impact on development and the susceptibility to chronic diseases [26].

If acquired environmental cues modify the chromosomes of eggs and sperm, these external influences have the potential to have long lasting effects through generations [27]. Sollars and his colleagues [28] presented a dramatic example of transgenerational inheritance involving fruit flies with a defective *Krüppel* gene, which causes abnormally small eyes. In addition, unusual outgrowths appear on the eyes under certain conditions. When the antibiotic geldanamycin was added to the food of the flies, the eye outgrowths increased substantially. It was reported that although only the first generation was directly exposed to geldanamycin, the effects of the drug were noticeable in future generations. In fact, the eye anomaly endured through 13 generations. The antibiotic was suspected of altering histone proteins since histone deacetylase inhibitors were found to suppress the development of the eye outgrowths.

In another example, Guerrero-Bosagna et al. [29] found that rats exposed *in utero* to the fungicide vinclozolin had negative reproductive consequences that lasted through future generations. Vinclozolin is an endocrine disruptor, which can lead to certain cancers and reproductive defects. When pregnant rats were injected daily with the fungicide, the resulting male offspring were found to display a decrease in sperm production. This suppression was also discovered in several generations that were not exposed to vinclozolin. Increased DNA methylation was reported in rats that were exposed to the fungicide from daily injections as well as in those of subsequent generations.

In the case of tobacco, diseases such as lung cancer, asthma, and chronic obstructive pulmonary disease have been linked to long term exposure to tobacco smoke, and substantial evidence suggests that epigenetic alterations are responsible for inducing these chronic disorders [30]. It has been proposed that epigenetic effects cause an imbalance in histone acetyltransferases and histone deacetylases of the airway immune cells. For example, studies reported by Ito and his colleagues [31] involving alveolar macrophages and bronchial biopsies from smokers and nonsmokers of similar age disclosed that in smokers, *HDAC2* gene expression is suppressed, along with general HDAC protein activity; however, the expression of inflammatory mediators *GM-CSF*, *TNF-α*, and *IL*-8 are enhanced. Furthermore, Launay et al. [32] found that tobacco smoke alters DNA methylation in a number of genes. For instance, in platelets, hypomethylation exists on the promoter of the gene that encodes for the enzyme monoamine oxidase (MAO) type B, suggesting that tobacco smoke overstimulates the expression of the gene for MAO, which has been linked to heart failure, mitochondrial dysfunction, and other abnormalities when present at elevated levels in the body [33].

The adverse effects of tobacco smoke can be reflected in the health of offspring and even subsequent generations. The respiratory function of the fetus is compromised when its mother is exposed to cigarette smoke. In fact, studies have shown that children as young as 1 year of age may exhibit asthma-like symptoms if their mothers smoked during the third trimester [34]. Furthermore, a child's increased risk of developing asthma is linked to a grandmother who smoked during pregnancy, suggesting that environmental factors can have long lasting consequences [35]. When comparing the buccal cells of children exposed *in utero* to tobacco

smoke from their mothers and those who had no such exposure, Breton et al. [36] reported that global DNA hypomethylation existed in children exposed to tobacco. It has been proposed that this occurrence may be the result of tobacco-induced oxidative stress to DNA that hinders the binding of DNA methyltransferase, thereby preventing methyl groups from binding to DNA. Another outcome of this form of early exposure to tobacco smoke was shown to be hypermethylation of certain genes. For example, the genes *AXL* and *PTPRO* were found to have an unusually high degree of methylation, though the significance of this discovery with regard to asthma pathogenesis is not yet fully understood.

These and many other studies show how the environment can have a major influence on development and on disease susceptibility. Epigenetic marks can be altered over time, and if these acquired chemical tags are passed on to sex cells, epigenetic influences can therefore be transferred from parents to offspring and even future generations. Hence chemical tags acquired from life experiences have the potential to be passed from one generation to the next.

4.5 Epigenetics and Cancer

Disruptions in normal cellular processes can be attributed to mutations, changes to the nucleotide sequences of genes, and some mutations may set the stage for the development of certain cancers. Moreover, there is increasing evidence that supports the importance of epigenetic control of gene expression with regard to carcinogenesis. DNA methylation aberrations, either as hypo- or hypermethylation, have been shown to be common in a variety of carcinogenic tumors. Chromosomal instability and oncogene activation are two processes typically associated with cancer that involve DNA hypomethylation, and tumor suppressor gene inactivation is often linked with DNA hypermethylation. Irregularities in histone modifications can also lead to notable interruptions in gene regulation. Modifications made to histone proteins can contribute to the development of disease, histone acetylation or deacetylation being the most commonly observed alterations [8].

The disruption of normal gene expression has the potential to lead to cancer. Normal cell activities are altered when genes are silenced or overly stimulated. A gene that is not typically expressed in a cell can be turned on, and abnormally high levels of a particular protein may be produced. This disruption can be the result of mutations and/or changes in gene control. Epigenetic influences on the regulation of genes have been detected in various cancer cells. Researchers are trying to understand the common changes that prompt the development of particular types of cancers and how these changes can be manipulated to ultimately destroy cancer cells [1].

There are over 100 different types of cancers, which generally comprise three main groups: carcinomas, sarcomas, and leukemias/lymphomas [37]. About 85 % of cancers are carcinomas and involve malignances of epithelial cells [9]. Sarcomas are tumors of connective tissues, like muscle and bone. Leukemia is a

malignancy of the blood cells in the bone marrow, while lymphoma is a cancer of the lymphocytes, typically in the lymph nodes. The tissue of origin (e.g., lung carcinoma) and the cell type (e.g., erythroid leukemia) can be used to further classify malignant growths [37].

Environment and heredity are the primary factors contributing to the onset of cancer, with environmental influences accounting for the vast majority of cancers [38]. Environmental factors include diet, tobacco, radiation, and infectious organisms [39].

Cancer arises from the accumulation of multiple mutations [40], and abnormalities in proto-oncogenes and tumor suppressor genes have been identified in many malignant cells. Over 100 proto-oncogenes have been identified, and at least 15 tumor suppressor genes have been discovered [9]. Proto-oncogenes normally promote cell division; however, once they mutate to become cancer-causing genes, oncogenes, excessive cell proliferation can occur. Tumor suppressor genes produce proteins that normally inhibit cell division; however, mutated tumor suppressors encode for proteins that directly or indirectly allow cells to divide uncontrollably [41].

The suppression of gene expression through epigenetic regulation has been identified in certain cancers. Both DNA methylation and histone modification have been found to silence genes. In some malignancies, cells may exhibit abnormally high levels of methyl groups on cytosines in CpG islands of promoter regions of suppressed genes. Also, histones that surround promoter regions may not be acetylated as in nonmalignant cells. Therefore, a combination of DNA methylation and histone deacetylation mechanisms may result in the suppression of gene expression [1].

Both DNA hypermethylation and hypomethylation are mechanisms that occur in healthy cells under certain conditions, but both mechanisms have also been identified in cancer. Of the two processes, hypermethylation has received more attention with regard to its role in cancer, primarily because of its occurrence on certain cancer-associated gene promoter sites. Nonetheless, global DNA hypomethylation is reported to be prominent in highly repeated DNA sequences in cells of certain cancers, like breast, colorectal, and gastric cancers. Chromosomal instability and oncogene activation have been linked with hypomethylation. The degree of demethylation varies within and between different cancer types. However, hypomethylation has the potential to be used as a biomarker for detecting the early stage and progression of certain malignancies [42].

Gene inactivation by aberrant DNA hypermethylation in promoter regions has been shown to be an important procedure in carcinogenesis, whereby key genes that normally inhibit cell division are turned off. And regions that are frequently targeted by hypermethylation are CpG islands. Normal ovarian epithelial cells, for example, possess an estrogen receptor protein, but this protein is not often present in ovarian cancer. The promoter of the *estrogen receptor-α* gene is typically hypermethylated in malignant ovarian cells, suggesting that hypermethylation is a contributing factor to the absence of the receptor protein [43]. In another example, the tumor suppressor gene *BRCA1*, which participates in DNA repair, is often mutated in inherited breast cancer. Though a mutated *BRCA1* gene is usually not identified

in non-inherited breast cancer, hypermethylation of the gene has been observed. Therefore, both epigenetic mediation and DNA mutation appear to function in the development of breast cancer [9].

Strong evidence suggests that aberrant DNA methylation patterns are responsible for adversely altering certain biological pathways. For instance, *p53*, which functions as a tumor suppressor gene and is commonly mutated in cancer cells, can be silenced indirectly through epigenetic operations. Under normal circumstances, the tumor suppressor gene *p14ARF* inhibits MDM2, an oncogenic protein that assists with the degradation of p53 protein; however, *p14ARF* is found to be inactivated through methylation. Furthermore, in leukemia, hypermethylation has been observed in gene *p73*, which is a *p53* homolog [44]. Another example of how pathways may be affected by methylation involves the inactivation of the *Rb* gene. In its active state, the Rb protein binds to the transcription factor E2F, which controls the expression of several genes necessary for the transition of the cell into the S (DNA synthesis) phase of the cell cycle. DNA synthesis cannot take place as long as Rb and E2F are bound [45]. However, hypermethylation appears to participate in the suppression of the *Rb* gene in some cancers, thereby silencing its production of protein product [46]. Also, DNA repair pathways can be thwarted when *hMLH1* is methylated. The suppression of this gene can result in microsatellite instability in gastric, endometrial, and colorectal tumors [47]. Additional examples of well-known tumor suppressor genes that have been reported to carry out methylation-mediated silencing in cancer are *APC*, *p16INK4a*, and *VHL*. These and other discoveries have encouraged the continuation of research related to DNA methylation and its influence in initiating cancer. Specialized techniques for studying methylation have also been developed, in particular, sodium bisulfate treatment and methylation-specific PCR [9].

Though not investigated as extensively as DNA methylation, histone modification has been implicated in some cancers. The amino acids of the histone tails are subject to chemical alterations—i.e., acetylation, methylation, etc. These chemical tags influence chromatin structure and function by changing the properties of the tail itself and by offering binding sites for non-histone proteins [48]. The histone is continually modified with the addition and removal of different chemical tags. Enzymes critical to these processes are HDACs, HATs, HMTs, HDMs, etc. However, irregular patterns of histone tags have been found in cancer. For example, it has been reported that genes that encode HDACs are usually over-expressed in gastric and prostate cancers [49]. In addition, aberrant deacetylation of histones has been found in certain types of leukemia, whereby gene translocations abnormally produce fusion proteins that recruit HDACs to promoters that subsequently suppress genes associated with cell differentiation [9]. The loss of both histone acetylation and histone methylation has been strongly linked with malignancy, in which the losses take place primarily at the acetylated lysine 16 and the trimethylated lysine 20 of H4. These losses were also linked to hypomethylation of repeated DNA sequences [50].

A combination of epigenetic procedures can be involved in altering gene expression. Another look at the tumor suppressor gene *Rb*, for instance, reveals that its suppression during tumorigenesis may be associated with DNA hypermethylation [46], as noted above, as well as the inactivation of HDAC1. Under normal conditions, the cell cycle is halted during the G1 (Gap 1) phase in order for the cell to make any necessary DNA repairs before the cell enters the S phase [41]. This pause in the cell cycle is accomplished, in part, when the Rb protein recruits an HDAC complex composed of HDAC1, HDAC2, or HDAC3 to the promoter-bound E2F transcription factor. HDAC activity is needed for the suppression of E2F target genes, notably the gene that encodes the cyclin E protein. This process is carried out when the enzyme deacetylates histone tails, causing chromatin to condense and, subsequently, genes to be silenced [51]. However, in cancer, a mutated Rb protein is inactivated, thus preventing its binding with HDAC so that chromatin can transform to a condensed state. As a result, the cell is no longer halted and progresses prematurely into the S phase. Hence the cell cycle is unregulated, resulting in uncontrolled proliferation of abnormal cells [52].

Research has shed light on ways in which some epigenetic pathways may be thwarted, and this knowledge has been translated into the development of novel cancer drugs that are designed to counteract negative epigenetic changes. These therapeutic strategies take into account that epigenetic modifications are potentially reversible. Scientists are examining how epigenetically inactive tumor suppressor genes and major signaling pathways can be reactivated [53].

Many genes identified as playing critical roles in carcinogenesis have been found to exhibit hypermethylation in their promoter regions; therefore, the use of DNA methylation inhibitors has the potential to become a promising treatment against tumor formation. The DNMT inhibitors 5-azacytidine and 5-aza-2′-deoxycytidine have been studied extensively. However, results have been mixed regarding their effectiveness. It has been reported that both drugs exhibit anti-leukemic activity in clinical trials, but positive results were not noted in solid tumors. A potentially adverse reaction to these drugs includes DNA instability. And, there is the possibility that abnormal methylation may return once treatment has been discontinued [54].

Inhibitors of histone deacetylases have also been examined as possible cancer drugs. HDACs typically suppress gene expression, and the abnormal recruitment of these enzymes has been found in certain cancers, like leukemia. In clinical trials, several drugs have been developed to block the binding of HDACs to their substrates. These drugs include the following: butyrates, valproic acid, depsipeptide (FR901228), and hydroxamic acid-based compounds SAHA and pyroxamide. Though these drugs have been reported to be well tolerated, alteration of gene expression has been found to be selective [9]. Therefore, further research is required to identify substrate specificities of different HDACs and drugs that are more appropriate under certain conditions [55]. Detailed understanding of the numerous and complex mechanisms involved in aberrant epigenetic regulation may lead to more effective treatments against different cancers as well as other diseases.

4.6 Conclusion

The information acquired from epigenetic studies provides a better understanding of how genes are turned on or off and the means by which epigenetic chemicals can be acquired. Though epigenetic chemicals are important for normal growth and development, they may also induce certain diseases, including cancer. Histone modification and DNA methylation are two epigenetic mechanisms that have been studied extensively, but the complexities of these processes still challenge our comprehension of their many intricacies. The study of epigenetics has not only enhanced our understanding of what makes each of us unique, but it has advanced the possibility of developing more effective ways of diagnosing and treating diseases.

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Chapter 5
Epigenetics of Colorectal Cancer

Kumar S. Bishnupuri and Manoj K. Mishra

Abbreviations

CCND1	Cyclin D1
CGI	CpG Island
CIN	Chromosomal Instability
CIMP	CpG Island Methylator Phenotype
COX-2	Cyclooxygenase 2
CRC	Colorectal Cancer
DNMT	DNA methyltransferase
DTL	Denticleless Homolog
FAP	Familial Adenomatous Polyposis
GI	Gastrointestinal
GIST	Gastrointestinal stromal tumor
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HDM	Histone Demethylase
HMT	Histone Methyltransferase
HNPCC	Hereditary Nonpolyposis Colorectal Cancer
ICC	Interstitial cells of Cajal
LINE	Long Interspersed Elements
LncRNA	Long Non-Coding RNA

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LOI	Loss of Imprinting
MIF	Macrophage Migration Factor
miRNA	Micro-RNA
MSI	Microsatellite Instability
ncRNA	Non-Coding RNA
NHL	Non-Hodgkin lymphoma
RB	Retinoblastoma
siRNA	Small Interfering RNA
SNX1	Sorting Nexin 1
TSS	Transcription Start Site
UC	Ulcerative Colitis

5.1 Introduction

Common gastrointestinal (GI) cancers including those of colon/rectum, stomach, pancreas, liver and esophagus account for more than half of the worldwide cancer related deaths. Colorectal cancer (CRC) is the most common GI malignancy and third most commonly diagnosed cancer in both men and women. It is a major cause of cancer death worldwide with an estimated 50,000 deaths per year in the United States only [1]. Even with the development of successful screening programs in last decade, no significant decline in CRC incidence and deaths is reported or expected in coming years. Although, a significant advancement has occurred towards understanding the multistep process of colorectal carcinogenesis, most of studies and existing therapeutic approaches are primarily based on genetic alterations of the disease. Growing evidences in recent years have now realized the involvement of epigenetic abnormalities along with genetic alterations to be crucial for growth and development of CRC, and failures of current cancer therapy are in part due to lack of understanding epigenetic changes in CRC cells. Epigenetic changes in CRC cells include alterations in DNA methylation, histone modification, nucleosome positioning and non-coding RNAs expression, which provide a base for multistep process of colorectal carcinogenesis. This chapter is focused on providing basics of human CRC and enumerating epigenetic changes associated with its growth and development, hence may provide a better understanding for improving therapeutic approach of CRC treatments.

5.2 Colorectal Cancer

Colorectal cancer (CRC) is a term used for cancer that starts either in the colon or the rectum. These cancers can also be referred separately as colon cancer or rectal cancer, depending on where they start. Though both colon cancer and rectal cancer can be referred to as CRC, the difference lies in where the cancer actually began.

If the cancer began in the colon, which is the upper part of the large intestine, it may be referred to as colon cancer. If the cancer began in the rectum, which is the later part of the large intestine leading to the anus, it is called rectal cancer. Both colon cancer and rectal cancer share many common features, and develop slowly over several years. Before a cancer develops, a growth of tissue or tumor usually begins as a non-cancerous *polyp* on the inner lining of the colon or rectum. These polyps are benign, non-cancerous tumors. Some polyps may change into cancer but not all. The chance of changing into a cancerous (malignant) polyp depends on its kind. The two main types of polyps are:

- **Neoplastic polyps** (adenomatous polyps and adenomas) are polyps that can change into cancer, and because of this, these adenomas represent a *pre-cancerous condition*.
- **Nonneoplastic polyps** (hyperplastic, juvenile, hamartomatous, inflammatory, and lymphoid polyps) in general, are not pre-cancerous. But some hyperplastic polyps can become pre-cancerous or might be a sign of having a greater risk of developing adenomas and cancer, particularly when these polyps grow in the ascending colon.

Another kind of pre-cancerous condition is called *dysplasia*. Dysplasia is an area in the lining of the colon or rectum where the cells look abnormal (but not like true cancer cells) when viewed under a microscope. These cells can change into cancer over time. Dysplasia is usually seen in people who have had diseases such as ulcerative colitis or Crohn's disease for many years, which cause chronic inflammation of the colon.

If cancer forms in a polyp, it can eventually begin to grow into the wall of the colon or rectum. When cancer cells are in the wall, they can then grow into blood vessels or lymph vessels. Lymph vessels are thin, tiny channels that carry away waste and fluid. They first drain into nearby lymph nodes, which are bean-shaped structures containing immune cells that help fight against infections. Once cancer cells spread into blood or lymph vessels, they can travel to nearby lymph nodes or to distant parts of the body, such as the liver, and acquire the form of *metastatic cancer*.

Several types of cancer can start in the colon or rectum. Most common among them is adenocarcinoma.

Adenocarcinomas: These cancers start in intestinal gland cells that make mucus to lubricate the inside of the colon and rectum. Adenocarcinomas are the most common type of colorectal cancer, which represent more than 95 % of colon and rectal cancers. "Adeno" is the prefix for gland, and adenocarcinomas typically start within the intestinal gland cells that line the inside of the colon and/or rectum. They tend to start in the inner layer and then spread deeper to other layers. There are two main subtypes of adenocarcinoma:

- **Mucinous adenocarcinoma** is made up of approximately 60 % mucus. The mucus can cause cancer cells to spread faster and become more aggressive than typical adenocarcinomas. Mucinous adenocarcinomas account for 10–15 % of all colon and rectal adenocarcinomas.

- **Signet ring cell adenocarcinoma** accounts for less than 1 % of adenocarcinomas. Named for its appearance under a microscope, signet ring cell adenocarcinoma is typically aggressive and may be more difficult to treat.

There are many other types of rare CRCs, and combined these types account for just 5 % of all cases. Below are examples of other colorectal types:

- **Gastrointestinal carcinoid tumors:** This slow-growing cancer forms in the neuroendocrine cell (a nerve cell that also creates hormones) in the lining of the gastrointestinal tract. These tumors account for just 1 % of all colorectal cancers, but half of all of the cancers found in the small intestine.
- **Primary colorectal lymphomas:** It is a type of non-Hodgkin lymphoma (NHL). These lymphomas are cancers that develop in the lymphatic system from cells called lymphocytes, a type of white blood cell that helps the body fight infections. NHL can develop in many parts of the body, including the lymph nodes, bone marrow, spleen, thymus and the digestive tract. Primary colorectal lymphomas account for just 0.5 % of all colorectal cancers, and about 5 % of all lymphomas. The disease usually occurs later in life, and is more common in men than women.
- **Gastrointestinal stromal tumors (GISTs):** It is a rare type of CRC that starts in a special cell found in the lining of the gastrointestinal tract called interstitial cells of Cajal (ICCs). More than 50 % of GISTs start in the stomach. While most of the others start in the small intestine, the rectum is the third most common location. GISTs are classified as sarcomas, a type of cancers that begin in the connective tissues, which include fat, muscle, blood vessels, deep skin tissues, nerves, bones and cartilage.
- **Leiomyosarcomas:** Another form of sarcoma, leiomyosarcomas essentially means "cancer of smooth muscle." The colon and rectum have three layers of muscle that can be affected, which all work together to guide waste through the digestive tract. This rare type of colorectal cancer accounts for about 0.1 % of all colorectal cases.
- **Melanomas:** Though most commonly associated with the skin, melanomas can occur anywhere, including the colon or rectum.
- **Squamous cell carcinomas:** Some parts of the gastrointestinal tract, like the upper part of the esophagus and the end of the anus, are lined with flat cells called squamous cells. These are the same type of cells that are found on the surface of the skin. Cancers starting in these cells are called squamous cell carcinoma.

About 75 % of CRC patients have sporadic disease with no apparent evidence of inheriting the disorder. The remaining 25 % of patients have a family history of CRC, and commonly referred familial colorectal cancer. A single gene, a combination of genes, or a combination of genetic and environmental factors can cause familial colorectal cancer. Typically these families have one or two members with a history of colorectal cancer or precancerous polyps. This type of CRC is also called as hereditary colorectal cancer as the exact gene that causes the disease is known. Inherited colorectal cancers are associated with a genetic mutation in a cancer

susceptibility gene. Everyone inherits one susceptibility gene from each of their parents, making a total of two working copies of each gene. If a mutation in one copy of a cancer susceptibility gene is passed from the parent to their child, the child is predisposed (or has the potential) to develop cancer. The genetic causes of two hereditary colorectal cancer syndromes, Familial Adenomatous Polyposis (FAP) and Hereditary Nonpolyposis Colorectal Cancer (HNPCC) have been identified. Familial adenomatous polyposis is a disorder that leads to hundreds, even thousands, of polyps in the colon and rectum at a young age, usually as a teenager or young adult. Other names for this condition are hereditary polyposis of the colorectum, familial polyposis, and Gardner's syndrome. This condition is inherited and primarily affects the gastrointestinal tract, commonly the colon and less often the stomach and small intestine. Hereditary nonpolyposis colorectal cancer is also known as Lynch syndrome or cancer family syndrome. It is a condition in which the tendency to develop colorectal cancer is inherited. People with HNPCC have a 50 % chance of passing the HNPCC gene to each of their children. A mutation in the genes (hMLH1 and hMSH2), that when functioning normally would protect against colon cancer, is the cause of HNPCC. People affected with this type of colorectal cancer do not develop large numbers of polyps (only a small number may be present or none at all). In families with HNPCC, cancer usually occurs on the right side of the colon. It often occurs at a younger age than colon cancer that is not inherited.

In addition to the genetic regulations of the human CRC, growing evidences in recent years have now realized the involvement of epigenetic abnormalities to be crucial for growth and development of CRC. The next part of this chapter is primarily focused on enumerating epigenetic changes in CRC cells including alterations in DNA methylation, histone modification, nucleosome positioning and non-coding RNAs expression, and their association with growth and development of human CRC.

5.3 Epigenetics of Colorectal Cancer

Epigenetic dysregulation is a common feature across all cancer types including CRC. Epigenetic changes differ from genetic changes mainly in that they occur at a higher frequency than do genetic changes, are reversible upon treatment with pharmacological agents and occur at defined regions in a gene. Epigenetic mechanisms, from DNA methylation to histone modifications, allow for a vast number of cellular phenotypes to be created from the same genetic material. Just as certain genetic changes play a key role in tumor initiation and progression, epigenetic changes may also set the course of tumor development and be required for malignant transformation. In recent years it has become clear that there is a synergy between genetic and epigenetic changes and that Knudson's two-hit hypothesis needs to be revised: instead of only two possibilities (loss of heterozygosity or homozygous deletion), there is also a third possibility of transcriptional silencing by DNA methylation of promoters, which constitute the most common mechanism of epigenetic alteration associated with a large number of cancer phenotypes including human CRC.

5.3.1 Methylation of DNA

Most of CRC cases demonstrate chromosomal instability characterized by alterations in tumor suppressor genes and oncogenes, including APC, P53, and K-RAS [2, 3]. However, in addition to these genetic alterations, epigenetic mechanisms including abnormal DNA methylation is frequently observed in cancers, and now is growing as a potential tumor marker. Cytosine (C) methylation occurs after DNA synthesis by enzymatic transfer of a methyl group from the methyl donor *S*-adenosylmethionine to the carbon-5 position of cytosine (m^5C). Cytosines are methylated in the human genome mostly when located 5′ to a guanosine. Hypermethylation of DNA sequences of promoters of tumor suppressor genes and homeobox genes has been reported to be one of the most constant features of the cancer genome [4–9]. The most frequently studied epigenetic changes investigated so far are global genomic DNA hypomethylation along with specific hypermethylation, predominantly at promoter CpG islands (CGI) of tumor suppressor genes. CpG islands are defined as a 500-base pair window with a G:C content of at least 55 % and an observed overexpected frequency of at least 0.65. Computational analysis of the human genome sequence predicts 29,000 CpG islands. It has been increasingly recognized over the past years that the CpG islands of a large number of genes, which are mostly unmethylated in normal tissue, are methylated to varying degrees in human cancers. Methylation of some CpG islands in non-malignant tissue also increases with age, whereas the total genomic content of m^5C declines. The same is true during carcinogenesis of several tumors (e.g. adenoma-carcinoma sequence), where methylation takes place at specific promoter regions, followed by general hypomethylation of the whole genome, and this is thought to induce a higher rate of chromosomal instability (CIN). Post-synthetic covalent addition of a methyl group to cytosine is mediated by the three known active DNA cytosine methyltransferases (DNMT1, 3a, and 3b). When DNA containing a symmetrically methylated CpG dinucleotide is replicated, the result is two double-stranded DNA molecules, each containing a methylated CpG dinucleotide on the parental strand, but also containing an unmethylated CpG dinucleotide on the newly synthesized strand. The methylated state of the site in the parent molecule is maintained in the daughter molecules when a maintenance methyltransferase recognizes the hemimethylated site and methylates the unmethylated cytosine, restoring the symmetrically methylated CpG dinucleotide pair. DNMT1 is mainly responsible for maintenance of DNA methylation, whereas DNMT3a and DNMT3b have been shown to methylate hemimethylated and unmethylated DNA with equal efficiency. Overexpression of both DNMT1 and DNMT3 mRNAs has been reported in human tumors. The reciprocal relationship between the density of methylated cytosine residues and the transcriptional activity of a gene has been widely documented. However, this inverse correlation has been demonstrated conclusively only for methylation in the promoter regions and not in the transcribed parts of a gene. Several tumor-suppressor genes contain CpG islands in their promoters, and many of them show evidence of methylation silencing (reviewed in references [10–12]). Advances in the technology of DNA methylation analysis have spurred the

discovery of numerous cases of hypermethylation of tumor-suppressor gene promoters in human tumors including human CRC.

Hypermethylation of CpG Island

Promoter hypermethylation is frequently observed in colorectal carcinomas, but is rare in adenomas [13]. Hypermethylation of CGI constitutes one of the most common epigenetic alterations involved in colorectal carcinogenesis. The presence of CpG island methylator phenotype (CIMP) in CRCs has been supported by the fact that one group of CRCs has few methylated promoter CGIs and another group harbors simultaneous aberrant methylation of multiple promoter CGIs [14, 15]. CIMP is initially defined using cancer-specific CIMP markers (*CDKN2A, MINT1, MINT2, MINT31*and *MLH1*) in CRCs [15], but in 2006, Weisenberger *et al.* [16] challenged the application of these classic CIMP markers and insisted upon the efficacy of novel marker panels to endorse the CIMP as a distinctive molecular feature of CRCs. Although based on a systematic analysis of a large number of CRCs with aberrant methylation of numerous promoter CGIs, later studies failed to emulate the original results using the same markers selected by Weisenberger *et al.* [17, 18]. No matter how the markers are selected, CIMP is certain to be involved in CRC development as the third molecular pathway, following CIN and microsatellite instability (MSI). Hypermethylation of promoter CGIs can prevent transcription of tumor suppressor or mismatch repair genes, such as MutL homolog 1 (*MLH1*), and occurs at an early stage of colorectal carcinogenesis. Methylation of promoter CGIs followed by transcriptional silencing of *MLH1* is present in ~70 % of sporadic MSI CRCs [19–21]. However, *MLH1* is usually included in CIMP marker sets of promoter CGIs, and up to 60 % of CIMP-positive CRCs have aberrant methylation of *MLH1* [22]. This may be one of the reasons for the clinical and pathological resemblance between CIMP-positive and MSI CRCs. The high frequency of serrated polyps with *MLH1* gene promoter methylation in individuals with MSI CRC suggests the presence of a serrated pathway in colorectal carcinogenesis [23]. More recently, genetic and epigenetic profiles of a variety of colorectal polyps have demonstrated that sessile serrated adenomas/polyps may be precursor lesions for MSI CRCs and follow the CIMP pathway [24]. Little is known about the CRCs that are without methylation of any promoter CGIs. The absence of aberrant methylation of any promoter CGIs in these patients confers possible global hypomethylation, which has been often associated with CIN in CRC [25, 26]. Cancer-specific methylation of CGIs and subsequent loss of expression of associated genes in CRC cell lines that had hypermethylation of these promoter CGIs suggested possible involvement of promoter methylation of these genes in colorectal carcinogenesis. For example, *SLC13A5*, a member of the solute carrier (SLC) families and a Na^+/sulfate/selenate/thiosulfate/carboxylate symporter [27] is one of the hallmarks of CIMP in renal cell carcinoma [28]. Certain SLC family members increase chemosensitivity against anticancer drugs by mediating the cellular uptake of hydrophilic drugs [29]. One of the sodium transporter families also has tumor suppressor activity, and aberrant methylation of promoter CGI is detected in aberrant crypt foci, which is considered

to be the initial lesion of the serrated adenoma-carcinoma pathway [30]. Just as there are cancer-type specific differences in DNA hypermethylation patterns [6], some DNA sequences are more or less susceptible to DNA hypomethylation depending on the kind of cancer [31]. Another risk factor for CRC is ulcerative colitis (UC) and Crohn's disease. Specific hypermethylation was seen to be a very early event in UC-associated carcinogenesis, thus indicating the possibility that hypermethylation might serve as a biomarker for early detection of cancer or dysplasia in UC. In addition, age is the principal function of CRC incidence, and age-related methylation changes are well documented for CRC [32].

Hypomethylation of DNA

DNA hypomethylation was the initial epigenetic abnormality recognized in human tumors. The first-described epigenetic changes in human cancer, reported in 1883, were losses in DNA methylation (m^5C residues replaced by unmethylated C residues) [33] and later Feinberg and Vogelstein described hypomethylation of DNA in few cancer-irrelevant gene regions in colon adenocarcinomas versus normal colonic epithelium [34]. Many subsequent reports have later confirmed the frequent overall genomic hypomethylation in other types of cancers relative to their respective control tissues [35–37]. However, for several decades after its independent discovery, it was often ignored as an unwelcome complication, and almost all of the attention was given to the hypermethylation of promoters of genes that are silenced in cancers (e.g. tumor suppressor genes). Because it was subsequently shown that global hypomethylation of DNA in cancer was most closely associated with repeated DNA elements, cancer-linked DNA hypomethylation continued to receive little attention. However, along with modern technological development, recent high-resolution genome-wide studies confirmed that DNA hypomethylation is the almost constant companion to hypermethylation of the genome in cancer, usually but not always in different sequences.

DNA hypomethylation occurs in many tumors, particularly in advanced stages, and is generally assumed to be a genomewide event [35, 38]. Hypomethylation of highly repeated DNA sequences [36, 39–41], which comprise approximately half of the genome, is largely responsible for the global DNA hypomethylation that is observed quite frequently in cancers. Tandem centromeric satellite α, juxtacentromeric (centromere-adjacent) satellite 2, the interspersed *Alu* and long interspersed elements (LINE)-1 repeats are the most frequently studied DNA cancer-hypomethylated repeats [39–43]. In contrast with normal cells, hypomethylation in tumor cells typically occurs at the repetitive sequences residing in satellite or pericentromeric regions. The pattern of hypomethylation may make chromosomes more susceptible to breakage and, therefore, is thought to predispose to chromosomal instability (CIN) and aneuploidy [44]. Global DNA hypomethylation, which can lead to activation of previously silenced genes, is generally considered to be a genome-wide event [33, 35, 45]. In colorectal neoplasia, it is associated with an increased risk of colorectal carcinogenesis [46, 47] and has been observed in advanced, metastatic stages of colon cancer [48, 49]. Recently, hypomethylation of

the CDH3 (P-cadherin) promoter was found in ACF and CRC with a potential "field effect" of CDH3 hypomethylation in the normal epithelium adjacent to cancer [50]. In another study, a significant correlation between the aberrant demethylation of the CDH3 gene and the tumor site and Dukes' stage was observed [49]. The hypomethylation of the gene is associated with induction of CDH3 expression in CRC, and epigenetic demethylation of the CDH3 promoter causes its ectopic expression early in the colorectal adenoma–carcinoma sequence, which persists during invasive cancer [50]. In addition, a small population of undifferentiated CD133+ has been reported to create and propagate colorectal carcinoma [51], and the CD133 expression is directly regulated by epigenetic modifications [52]. In primary tissue, demethylation of the CD133 gene was observed at 40 % of CRC (19 out of 48 cases) and more frequently in advanced CRC with a trend toward preferentially developing lymph node metastasis [48]. These results demonstrate that CDH3 and CD133 genes are more frequently demethylated in advanced colorectal carcinomas [49]. LINE-1 is an emerging marker for global demethylation. Most carcinomas including breast, lung, head and neck, bladder, esophagus, liver, prostate, and stomach reveal a greater percentage of LINE-1 hypomethylation than their normal tissue counterparts, though normal tissues from different organs show tissue-specific levels of methylated LINE-1 [53]. Greater hypomethylation of LINE-1 is also observed in colon carcinoma than those of dysplastic polyp and histological normal colonic epithelium [53]. DNA derived from sera of patients with carcinoma display more LINE-1 hypomethylation than those of noncarcinoma individuals [53]. LINE-1 hypomethylation is partially reversed in cancers with MSI [54] and inversely correlated with methylation of CIMP-H genes in CRC [55]. LINE-1 hypomethylation is associated with an increase in colon cancer-specific mortality and overall mortality [56]. In normal colon mucosa, the LINE-1 methylation level is inversely correlated with methylation of CpG island loci (MLH1, CDKN2A/p16, TIMP3, APC, ESR1, and MYOD), though no associations in colon cancer were observed [57].

So far, three types of altered DNA methylation patterns have been known in human cancer: hypermethylation, hypomethylation and loss of imprinting (LOI) [58]. The LOI at the IGF2/H19 region as a result of hypomethylation is a clear example for this phenomenon. LOI is seen in about 40 % of CRC tissue [59]. In addition to DNA methylation changes, there is an abundance of other epigenetic alterations occurring within cancer cells including DNA methylation alterations outside of CpG islands, non-CpG methylation, changes in cytosine oxidative species (hydroxymethylcytosine, formylcytosine, carboxylcytosine) levels, and histone modifications.

5.3.2 Histone Modification

In addition to altered DNA methylation, post-translational histone modifications play an important role in gene regulation and carcinogenesis. The coiling of DNA around core histone proteins (H2A, H2B, H3 and H4) forms nucleosomes that are

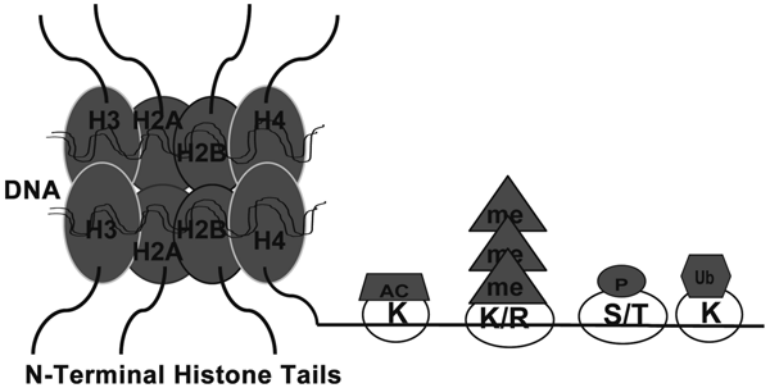

Fig. 5.1 N-terminal histone tails protruding from the nucleosomal units of DNA can be post-translationally modified by acetyl (Ac), methyl (me), phosphate (P), ubiquitin (Ub) and other groups at the basic amino acids including lysine (K), arginine (R), serine (S) and threonine (T). These post-translation modifications in DNA are achieved histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), histone demethylases (HDMs), histone ubiquitinating/deubiquitinating enzymes

the basic units of eukaryotic chromatin packaging. The core histones display highly dynamic N-terminal amino acid tails of 20–35 residues in length extending from the surface of nucleosome. Histone proteins can be chemically modified by the addition of residues on these tails, and can become post-translationally methylated (me), phosphorylated (P), acetylated (Ac), sumolyated (Sum), ubiquitinated (Ub) and ADP-ribosylated. Lysine residues (K) can either be mono-, di, or trimethylated, while arginine residues (R) can be monomethylated and symmetrically or asymmetrically dimethylated (Fig. 5.1). The addition or removal of post-translational modifications from histone tails is dynamic and achieved by a number of different histone-modifying enzymes. These include histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), histone demethylases (HDMs), histone ubiquitinating enzymes as well as deubiquitinating enzymes. They can be either specific (i.e. HMTs and HDMs) or general (i.e. HATs and HDACs) in their ability to recognize and alter the amino acid residues of histone tails [60, 61].

Post-translational modifications of histones can regulate the accessibility of chromatin to the transcriptional machinery. Generally, acetylation and phosphorylation are thought to change chromatin structure by altering the net positive charge of the histone proteins, thereby rendering DNA sequence information more accessible [62]. Acetylation of histone tails is typically associated with transcriptional activation of genes, while the functional consequences of methylation depend mainly on the number of methyl groups and their location within the histone tail [63]. Examples for modifications that are associated with open chromatin and active gene expression include histone 3 lysine 4 di- and trimethylation (H3K4me2 and H3K4me3, respectively) and histone 3 lysine 9 monomethylation (H3K9me1). Histone 3 lysine

27 di- and trimethylation (H3K27me2 and H3K27me3, respectively) and histone 3 lysine 9 di- and trimethylation (H3K9me2 and H3K9me3, respectively) are associated with inactive chromatin and repression of gene expression [64]. The high combinatorial potential of different modifications has been described as the 'histone code' [63] and a multitude of different post-translational modifications play an important role in eukaryotic gene regulation and in fine-folding of nucleosomes into higher-order chromatin [65]. Distinct modifications at specific histone tail residues serve as domains for interaction with specific proteins, and such interactions compartmentalize chromatin into heterochromatin and euchromatin as illustrated by recent genome-wide chromatin modification mapping studies [66]. Distinct histone modifications correlate with distinct genomic regions; for example, H3K4me3 with promoters; H3K39me1 with enhancers; H3K9 and H3K27 acetylation (H3K9ac, H3K27ac) with active regulatory regions; H3K36me3, H3K79me2 and H4K20me1 with transcribed regions and intron/exon usage; H3K27me3 with polycomb-repressed regions; and H3K9me3 with pericentromeric heterochromatin [66, 67]. As histone modifications play fundamental roles in gene regulation and expression, it is not surprising that aberrant patterns of histone marks are found in cancer. Dysregulation of histone-modifying enzymes, such as HDACs, HATs, HMTs and HDMs, is often responsible for these aberrant histone modifications. Genetic, cytogenetic and molecular approaches have identified many chromosomal translocations, deletions, and amplification events that link histone-modifying enzymes to cancer [68]. HDACs, for example, are often overexpressed in multiple types of cancer [69]. Dysregulation of HMTs and HDMs in cancer cells also contributes to aberrant histone modification patterns [70]. Advances in high throughput techniques enable genome-wide mapping of chromatin changes that occur during carcinogenesis [71]. Several studies linked global changes of PTMs to prognosis of patients with different types of cancer (reviewed in reference [72]).

Knowledge regarding the patterns of histone modification alterations in CRC is accumulating. Fraga et al. published the first report on a global change of histone modification in CRC in 2005 [73]. By immunodetection, high performance capillary electrophoresis and mass spectrometry, they found global loss of H4K16ac and H4K20me3 in cancer cells and primary tumors, including colonic tumors. Subsequent studies investigated the global pattern of individual histone marks, mainly by immunohistochemistry. Two different studies reported that global levels of H4K12ac and H3K18ac increased in adenocarcinomas in respect to normal tissue or adenoma [74, 75]. A recent report from Stypula-Cyrus et al. found upregulation of HDACs (HDAC1, HDAC2, HDAC3, HDAC5, and HDAC7) in human CRC [76]. Lysine methylation is one of the most prominent post-translational histone modifications that regulate chromatin structure. Changes in histone lysine methylation status have been observed during cancer formation, which is thought to be a consequence of dysregulation of histone lysine methyltransferases or the opposing demethylases [70]. KDM4/JMJD2 proteins, which are demethylases targeting histone H3K9 and H3K36 and histone H1.4K26 were found to be overexpressed in CRC [77, 78]. Moreover, the presence of H3K9me3 positively correlated with lymph node metastasis in patients with CRC. Methylation of histone H3K9 is associated

with gene repression [79]. Nakazawa et al. observed a gradual increase in global level of histone 3 (H3K9me2) in neoplastic cells, in the adenomas, in the nuclei of adenocarcinomas and suggested its association with cancer progression from adenoma to adenocarcinoma [75]. In view of gene repressive effect of methylated histone 3 (H3K9), it is suggested that the increased H3K9me2 level repress transcriptional activity of certain genes that function as tumor suppressors and/or carcinostasis promoters in colorectal tumors. Dimethylation of H3K4 (H3K4me2) and acetylation of H3K9 (H3K9ac) correlated with the tumor histological type. In addition, lower levels of H3K4me2 correlated with a poor survival rate. The multivariate survival analysis showed that H3K4me2 status is an independent prognostic factor for patients with CRC [80]. In addition, it has been found that the methylation level of H3K27me2 detected with immunohistochemistry is an independent prognostic factor for metachronous liver metastasis of colorectal carcinomas [81]. The global level of H3K9me2 was distinctly higher in neoplastic cells (adenoma and adenocarcinoma) than in normal glandular cells; in addition, it was significantly higher in adenocarcinoma than in adenoma. Aberration of the global H3K9me2 level is an important epigenetic event in colorectal tumorigenesis and carcinogenesis involving gene regulation in neoplastic cells through chromatin remodeling [75]. Furthermore, a group of researchers also reported an increase in global levels of H3K18ac and H4K12ac in adenocarcinomas in comparison with those in normal tissue and adenomas, and demonstrated that HDAC2 and H4K12ac expressions in adenocarcinoma were higher than in adenoma, implying that these epigenetic changes also have a role in the progression from adenoma to adenocarcinoma [74].

5.3.3 Nucleosome Positioning

In addition to altered DNA methylation and histone modifications, gene expression can also be regulated by the positioning and occupancy of nucleosomes at promoter regions [82]. Altered promoter nucleosome positioning is an early event in gene silencing [83]. The term *positioning* describes the precise location of a given nucleosome, whereas *occupancy* describes the proportion of molecules bearing a nucleosome at a specific location, at any given instant [84]. The positioning of nucleosomes at promoters regulates gene expression by demarcating the promoter region and transcription start site (TSS) [85]. At gene promoter regions, nucleosomes can be held at specific positions by DNA-binding proteins such as transcription factor complexes [86]. While activation of gene expression correlates with nucleosome depletion at promoters, nucleosomes have been shown to rapidly reform when transcription ceases [87, 88]. In cancer, many genes critical to tumor development are known to undergo epigenetic silencing. Typically, this silencing occurs in association with hypermethylation and dense nucleosome occupancy across the CpG island (CGI) promoter region [89]. However, the majority of genes that are hypermethylated in cancer are also silenced in normal precursor cells despite no evidence of promoter methylation [90, 91]. These studies support the view that hypermethylation serves to consolidate a transcriptionally silent state rather than initiate it [92].

Nucleosomes are released by apoptotic and necrotic cells into the blood circulation. Although macrophages efficiently clear dead cells by phagocytosis [93], nucleosomes can enter the circulation in certain diseases, reflecting either increased production or impaired clearance. In addition to apoptotic and necrotic processes, the active release of DNA from all living normal and diseased cells into the bloodstream has also been described [94]. In patient with cancer, the release of nucleosomes and DNA is elevated due to the increasing cell turnover [95]. Many studies have investigated circulating nucleosomes for their potential as diagnostic and prognostic biomarkers or their usefulness in therapy monitoring (reviewed in reference [96]). The prognostic value of the pre-therapeutic nucleosome concentration has been demonstrated in different types of cancer [97]. As nucleosomes are stable structures in the circulation [98], they could be a valuable source of novel biomarkers. Two histone methylation marks, H3K9me3 and H4K20me3, the hallmarks of pericentric heterochromatin [99], were investigated in circulating nucleosomes. H3K9me3 and H4K20me3 have been found to be lower at the pericentromeric satellite II repeat in patients with CRC when compared with healthy controls or patients with multiple myeloma. Recently, through next-generation sequencing of immunoprecipitated plasma DNA, reduced levels of H3K9me3 and H4K20me3-related repetitive sequences in circulation of patients with CRC was confirmed [100]. These data suggested the biomarker potential of H3K9me3 and H4K20me3-related nucleosomes in CRC. Since histone modification alterations can be detected in nucleosomes circulating in the blood of patients with cancer, it offers the possibility of using them as biomarkers in CRC and other types of cancer.

5.3.4 Non-coding RNAs

High throughput genome-scale studies have demonstrated that more than 93 % of the DNA sequences in the human genome are actively transcribed [101]. However, only approximately 5–10 % of the sequences are stably transcribed into mRNA or non-coding RNA (ncRNA). Genome tiling arrays have revealed that the amount of non-coding sequence is at least four times larger than the amount of coding sequence, which indicates that only 1 % of the human genome is composed of protein-coding genes and the remaining 4–9 % is transcribed into ncRNAs [102]. Therefore, ncRNAs constitute a very large proportion of the total RNA molecules. According to their transcript size, ncRNAs are grouped into two major classes: (a) small ncRNAs with transcripts <200 nucleotides (e.g. siRNAs and miRNAs, Piwi-interacting RNAs, and some retrotransposon-derived RNAs) and (b) long non-coding RNAs (LncRNAs) ranging in length from 200 nucleotides to ~100 kilobases (kb) that lack significant protein-coding abilities [102, 103]. This class includes five broad categories: sense, antisense, bidirectional, intronic, and intergenic, based on the proximity between neighboring transcripts [104]. The function and clinical significance of short regulatory ncRNAs, such as microRNAs (miRNAs) and small interfering RNAs (siRNAs), were elucidated first [105], and the regulatory roles of

miRNAs have been broadly recognized in almost all physiological and pathological processes in the body, including carcinogenesis [106]. For example, MIR95 promotes cell proliferation and targets sorting Nexin 1 in human colorectal carcinoma [107]; moreover, in CRC patients, the plasma levels of MIR29a and MIR92a are significantly upregulated and the plasma levels of MIR601 and MIR760 are significantly downregulated; thus the levels of these miRNAs have good diagnostic value for CRC screening [108, 109].

Long Non-coding RNAs (LncRNAs) in Human CRC

Emerging studies have revealed that particular LncRNAs are involved in diverse physiological and pathological processes, such as cell growth, apoptosis, stem cell pluripotency, and development, by acting as transcriptional, post-transcriptional, or epigenetic regulators. Notably, observations of a few known LncRNAs have suggested that their dysregulation is linked to tumor pathogenesis, and these molecules perform essential regulatory functions by acting on cellular proliferation, apoptosis, or metastasis by participating in a variety of key signaling pathways [110–113]. Recently, the roles of dysregulated functional LncRNAs in human cancers have received considerable attention [102, 111, 113–115]. Increasing evidence suggests that these LncRNAs are frequently aberrantly expressed in cancers, and some of them have been implicated in diagnosis and prognostication [116]. As LncRNAs do not encode proteins, their functions are closely associated with their transcript abundance [117]. It has been reported that LncRNAs demonstrated higher specificity than protein-coding mRNAs [111, 118], and had the advantages of being detectable in the blood [119] and urine [118, 120] of cancer patients by conventional PCR methods. The significance of LncRNAs in human CRC was realized in 2001 when Tanaka *et al.* [121] determined that a loss of imprinting of long QT intronic transcript 1 (LIT1/KCNQ1OT1) was frequently observed in CRC patients, suggesting a link between LncRNAs and CRC. Following this research, several studies focused on the aberrant expression of lncRNAs during colorectal carcinogenesis, and an accumulating number of studies indicated that specific LncRNAs had potential biological and clinical relevance in CRC. Table 5.1 below shows a list of LncRNAs that are linked to human CRC.

Accordingly, understanding the pathophysiological roles of LncRNAs in CRC undoubtedly represents an important aspect of current and future research, as these molecules may be the hallmark features of CRC. Furthermore, the detection and identification of potentially functional LncRNAs in CRC is an emerging avenue of LncRNA research, which will be necessary before the application of LncRNAs in cancer diagnosis and therapy.

Table 5.1 LncRNAs in human CRC

LncRNA	Size (bp)	Expression level	Potential function and mechanism	References
CCAT1	2407	Increased	NA	[122–124]
CCAT2	340	Increased	Mediates MYC and WNT signaling, promotes tumor growth, metastasis, and chromosomal instability	[125]
CRNDE	1070	Increased	Promotes growth and suppresses apoptosis	[126, 127]
E2F4 antisense	~5000	Increased	Induced by WNT/beta-catenin signaling, which leads to decreased levels of E2F4	[128]
HOTAIR	2158	Increased	Promotes cell invasion	[129]
HULC	500	Increased in liver metastatic nodules	NA	[130]
PCAT1	173,960	Increased	NA	[131]
MALAT1	8708	Increased	Promotes proliferation, invasion, and metastasis	[132]
H19	2322	Increased or LOL	The absence of the H19 locus increases the number of polyps in the APC murine model, H19-derived MIR675 regulates RB	[133–136]
uc.73a	201	Increased	Promotes proliferation and suppresses apoptosis	[137, 138]
uc.388	590	Increased	NA	[138]
UCA1/CUDR	2314	Increased	NA	[139, 140]
XIST	19,296	Increased in MSI and Sporadic CRC	NA	[141]
BA318C17.1	673	Decreased	NA	[142]

(continued)

Table 5.1 (continued)

LncRNA	Size (bp)	Expression level	Potential function and mechanism	References
lncRNA-LET/NPTN-IT1	2606	Decreased	Hypoxia-induced histone deacetylase 3 represses lncRNA-LET by reducing the histone acetylation-mediated modulation of the lncRNA-LET promoter region, which leads to cancer cell invasion	[143]
LOC285194/TUSC7	2105	Decreased	A TP53-regulated tumor suppressor, inhibits growth through the repression of MIR211	[116, 144]
MEG3	1595	Decreased	Mediates TP53 signaling, inhibits cell proliferation in the absence of TP53	[145, 146]
PTENP1	3932	Decreased	A decoy of the PTEN-targeting microRNAs, inhibits cell growth	[147]
KCNQ1OT1/LIT1	59,461	LOI	NA	[121, 148]

MicroRNAs (miRNAs) in Human CRC

MicroRNAs are small, 18–24 nucleotide RNAs that regulate the translation and stability of specific target mRNAs. During the last decade, it has become clear that aberrant miRNA expression has a functional role in the initiation and progression of CRC. Specific miRNAs can act as either tumor suppressors or oncogenes depending on the cellular environment in which they are expressed. The expression of miRNAs is reproducibly altered in CRC, and their expression patterns are associated with diagnosis, prognosis, and therapeutic outcome in CRC. Extensive research is now aimed at determining if miRNAs can be used as diagnostic biomarkers and therapeutic targets for cancer.

To date, numerous studies have examined miRNA expression patterns in CRC and confirmed that miRNAs are consistently and reproducibly altered in this disease [149]. A recent review of 23 microRNA expression studies found that of the 164 microRNAs that are significantly altered in CRC, approximately 2/3 of them were elevated and 1/3 that were reduced in tumors [149], indicating that microRNAs may have more oncogenic than tumor suppressive functions in CRC. Regardless of these findings, it is clear from functional studies that certain miRNAs have important oncogenic functions while others have important tumor suppressor functions.

Michael et al. were the first to show that miRNA expression patterns were altered in CRC [150]. They reported reduced expression of miR-143 and miR-145 in CRC and suggested that these miRNAs were tumor suppressors. Multiple studies have since validated these findings and demonstrated that miR-143 and miR-145 indeed have tumor suppressive functions in CRC [151]. Another highly relevant miRNA in CRC is the oncogenic miRNA, miR-21. At least seven studies reported that miR-21 is elevated in CRC [149]. Furthermore, miR-21 has been found to be elevated in many other solid tumor types [152] and this miRNA has important roles in cancer initiation, progression and metastasis. Other miRNAs which have been found to be altered in CRC in multiple reports include the miR-17-92 cluster, miR-106a, miR-31, miR-181b, miR-183, miR-135a/b, the miR-200a/b/c family, miR-203 and miR-224 [149]. The causes of the altered expression of miRNAs in CRC are diverse and complex. Aberrant transcription of miRNAs in CRC can be the result of transcription factors that are activated through various oncogenic signaling cascades, the result of genomic amplification/loss, genotoxic stress or inflammatory stimuli. Epigenetic mechanisms also affect miRNA expression levels. Several miRNAs, including let-7 [153], miR-34 [154], miR-342 [155], miR-345 [155], miR-9 [156], miR-129 [156], and miR-137 [156] are frequently hypermethylated in colon tumors and this is thought to lead to their reduced expression. MicroRNAs can also contribute to global epigenetic regulation in CRC. For example, miR-143 is a tumor suppressor miRNA that directly targets DNA methyltransferase 3A (DNMT3A) and loss of miR-143 expression leads to increased DNMT3A expression in CRC tissues [157]. Similarly, loss of miR-342 leads to increased DNA methyltransferase 1 (DNMT1) and this contributes to the hypermethylation of several tumor suppressor genes in CRC [158]. Several other miRNAs have also been implicated in CRC. MiR-30a-5p is a tumor suppressor miRNA that targets denticleless homolog (DTL) to suppress tumor growth in CRC [159]. MiR-192 and miR-215 are both effectors and regulators of p53 function to suppress colon carcinogenesis [160]. Another p53 related miRNA, miR-34a, has been shown to inhibit cell invasion in colon cancer cell lines by targeting FRA1 [161]. Cyclooxygenase 2 (COX-2) can be negatively regulated by miR-101 [162] and this may contribute to the initiation and progression of colon tumors. MiR-451 overexpression in colon cancer cells leads to reduced cell proliferation through targeting of the oncogene macrophage migration factor (MIF) [163]. Over expression of miR-499-5p in CRC cell lines targets FOXO4 and PDCD4 to promote cell migration and invasion [164]. MiR-675 can target the retinoblastoma (RB) tumor suppressor gene to increase tumor growth [134]. MiR-365 acts as a tumor suppressor to inhibit cell cycle progression and promotes apoptosis of colon cancer cells by targeting Cyclin D1 (CCND1) and Bcl-2 [165]. Loss of miR-29 leads to increased expression of MMP2 to promote metastases in mouse models of colon cancer [166]. The oncogenic miR-95 promotes tumorigenicity by targeting sorting nexin 1 (SNX1) [107]. Furthermore, circulating microRNAs can be detected in blood serum, plasma or stool. Therefore, measuring microRNAs in blood serum, plasma or stool offers non-invasive approach to detect CRC. Because altered microRNA expression can influence the initiation and progression of colon cancer, it suggests that microRNAs have potential as therapeutic targets for CRC.

5.4 Summary

Cancer refers to a group of diseases that share a common overall phenotype: uncontrollable cell growth and proliferation. During multistep process of carcinogenesis, cells acquire a series of genetic changes that eventually lead to unrestrained cell growth and division, inhibition of cell differentiation, and evasion of cell death. However, these genetic changes alone cannot explain the overall phenotype of cancer cells. Concepts of 'epigenetics' offer a partial but crucial explanation of carcinogenesis. The initiation and progression of cancer, traditionally seen as a genetic disease, is now realized to involve epigenetic abnormalities along with genetic alterations. Recent advancements in the rapidly evolving field of cancer epigenetics have shown extensive reprogramming of every component of the epigenetic machinery in cancer including DNA methylation, histone modifications, nucleosome positioning and non-coding RNAs expression. The reversible nature of epigenetic aberrations has led to the emergence of the promising field of epigenetic therapy. As we continue improve our understanding of the biology and both genetic and epigenetic changes in CRC, we may be able to develop additional biomarkers and therapies to help treat and even prevent this disease.

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Chapter 6
Epigenesis in Colorectal Cancer: A Lethal Change in the Cell

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6.1 Introduction

Colorectal cancer (CRC) is a heterogeneous disease characterized by progressive aggregation of genetic mutations and epigenetic alterations of the genes involved in cell cycle regulation and cell differentiation [1]. These alterations provide growth advantage for clonal expansion of these altered colons epithelial cells to transform into colon adenocarcinomas. Colorectal cancer arises as a polyp outgrowth, called an adenoma, in the colon and/or rectum lining and undergoes a malignant transformation to cause cancer [2]. It has been widely observed that colorectal cancer is initiated due to dysfunction in the signaling elements of Wingless/Wnt-signaling pathway resulting in either activation of oncogenes or silencing of tumor suppressor genes [3]. About 70–85 % of colorectal cancers are sporadic in nature, i.e. arise from somatic gene alterations. However, the heritable colorectal cancers originating from germline mutations are either familial adenomatous polyposis or hereditary nonpolyposis colorectal cancer [4, 5]. Multiple molecular pathways have been identified for the development of colorectal cancers (CRCs) that comprised of both mutations and epigenetic alterations. For instance, tubular adenomas mostly arise in response

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to inactivated tumor suppressor gene, Adenomatous Polyposis Coli (APC) and concurrent genetic alterations via chromosomal instability while serrated polyps arise in response to micro satellite instability and aberrant DNA methylation [6].

Colorectal cancer is characterized by parallel histopathological, genetic and epigenetic alterations in cells where microscopic mucosal abnormality's, i.e. aberrant crypt foci (ACF) have been observed, which is among the earliest precursor lesion in colorectal cancer [7]. In humans these ACF are classified into two types, i.e. Dysplastic and Heteroplastic [8], among them dysplastic ACF are premalignant lesions commonly found in familial adenomatous polyposis (FAP) and are similar to adenomas due to their related genomic profile, clinical association and histological resemblance with adenomatous polyps with the absence of mucin production [9]. On the other hand, heteroplastic ACF arises from hyperplastic polyps lacking dysplasia that are majorly diagnosed in sporadic colorectal patients [10]. Moreover, hyperplastic ACF is significantly smaller and carries fewer apoptotic bodies than dysplastic ACF [9] which upon genetic and/or epigenetic alterations give rise to dysplastic ACF [11]. Although genetic and epigenetic alterations are attributed for CRC development, here we are mainly emphasizing on epigenetic causes of CRC.

6.2 Epigenetic Modifications and Underlying Mechanism Involved in Colorectal Cancer

Epigenetics provide insights to the cellular and physiological traits that are independent of the genetic changes at the sequence level but are known to affect transcriptional potential of cell via altering expression of genes. Such epigenetic changes include DNA methylation, covalent histone modifications, miRNA/Non coding RNA production and Nucleosome positioning/organization that ultimately affect the protein expression leading to colorectal cancer.

6.2.1 DNA Methylation

DNA methylation has been widely studied among the epigenetic modifications causing cancer whose prime function is to regulate the gene expression by switching it on/off. DNA methylation occur at the cytosine nucleotide (fifth position of the pyrimidine ring) present in the CpG islands, i.e. large clusters of CpG dinucleotide, which is also responsible for altering chromatin architecture by histone modifications [12]. DNA methyltransferases are the candidate enzymes responsible for carrying out DNA methylation whose hyper-and hypo-activity both are detrimental to the normal functioning of gene. Numerous studies have established the fact that a range of genes get inactivated by aberrant DNA methylation in various cancers [13].

Hypermethylation

The epigenetic basis of colorectal cancer is majorly attributed to CpG island methylator phenotype (CIMP) i.e. CpG islands of various genes across the genome were aberrantly methylated resulting in the silencing of the corresponding gene [14]. Although the mechanism leading to CIMP or selection of genes for promoter hyper-methylation remained elusive but numerous genes have been identified whose hypermethylation leads to cancer. Mutation in mismatch repair genes (MMR), such as MSH2, MLH1, MSH6 and PMS2, has been linked to colorectal cancer who rectified errors due to functional glitches in the proofreading mechanism of DNA polymerase and thus, restore genomic integrity [15]. Therefore, dysfunctional MMR enzymes cause microsatellites to acquire insertions or deletions in their sequences thereby increasing or decreasing the number of repeats in the microsatellite region, and this phenomenon is referred to as microsatellite instability MSI. Although these genes possess sizeable CpG islands but epigenetic gene silencing by methylation is only attributed to MLH1 gene [16], which was the first identified gene that was epigenetically silenced in colon cancer [17]. Hypermethylation of MLH1 promoter is found in 80 % of sporadic colorectal cancers and is usually accompanied by BRAFV600E mutations [18]. Thus, silencing of MLH1 gene promotes tumor formation by generating genomic instability.

MGMT (O^6-methylguanine-DNA methyltransferase) gene is another MMR gene whose epigenetic silencing is an early event in adenoma to colorectal carcinoma sequence that further enhances the chance of mutation in KRAS and p53 gene [19]. MGMT codes for O^6-alkylguanine DNA alkyltransferase protein, which cleaves a methyl group from O^6-methylguanine, added by alkylating agents such as temozolomide and dacarbazine thereby preventing the formation of a mismatch pair with thymine [20, 21]. Krakowczyk et al. observed methylated MGMT in nearly 59 % of colorectal adenocarcinoma samples obtained from patients aged between 23 and 80 years [11]. Various studies have identified numerous other genes that are methylated to govern the pathogenesis of colorectal cancer; for instance, RB (retinoblastoma), ERs (estrogen receptors), RARB (Retinoic acid receptor beta) and SFRP (Secreted frizzled-related protein) have been shown to undergo promoter methylation in colorectal cancer [22–25]. Epigenetic methylation inhibits the physical binding of regulatory enzymes to the promoter region thereby causing gene silencing; for instance, transcriptional factor AP-2 (activator protein-2), CREB (cAMP-response-element-binding protein), E2F and NF-κB (nuclear factor κB) are not being able to bind DNA due to gene methylation [3].

Hypomethylation

DNA hypomethylation is almost observed along with hypermethylation in the cancer genome and is a prognostic event in colorectal carcinogenesis. It is believed that the demethylation rate in colorectal prognosis is usually 8–10 % [26]. Hypomethylation of DNA is directly related to the degree of malignancy or tumor progression; for instance, transition of myeloid leukemia to the chronic blast crisis is associated with LINE-1 (long interspersed nucleotide element-1) promoter

hypomethylation [27]. Various studies have also shown that LINE-1 methylation level is an indicator of cellular global methylation levels [28, 29]. A loss in global DNA methylation has been associated with genomic instability through revival of transposable sequences in colorectal carcinogenesis [30–33]. Consequently, hypermethylation in LINE-1 is associated with poor survival among colorectal cancer patients in response to genetic instability [34]. Numerous genes have been identified to be affected in response to hypomethylation. For instance, aberrant promotor hypomethylation of TCF3 (transcription factor 3) gene up regulates its expression and contributes to cancer prognosis via upregulating Wnt signaling pathway [35]. Another gene IGF-2 (Insulin-Like growth factor Like growth factor 2) is also associated with colorectal cancer due to loss of methylation from CpG di-nucleotide in the differentially methylated regions (DMRs) of the IGF 2 genes [36–38]. Other genes that have been correlated with hypomethylation in colorectal cancer include CDH3 (P-cadherin) and CD133 [39].

6.2.2 Histone Modification

Histone proteins play an important role in the packaging of eukaryotic chromatin by forming nucleosomes. Histone proteins contain an N-terminal 20–35 amino acid residue tail which is post-translationally modified by methylation, acetylation, phosphorylation, sumoylation, ADP-ribosylation, ubiquitylation, etc. Upregulation of these histone modifying enzymes has been observed in various types of cancers [40]. The removal of post translational modification (PTMs) is controlled by many histone modifying enzymes, which includes histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), histone demethylases (HDMs), histone ubiquitinating and deubiquitinating enzymes [41, 42]. Post translational modifications of histone regulate gene expression by controlling chromatin accessibility to the transcriptional factors, and different PTMs follow different mechanisms for affecting chromatin architecture.

Histone Methylation

There are various modifications of histone proteins, which contrive their physiological functions. One of the modifications is methylation, which occurs only on arginine(R) and lysine (K) residues present on the histone tails [43, 44]. Methylation is carried out by histone methyl transferases (HMTs) by catalyzing the co-substrate *S*-adenosyl methionine (methyl group donor). The effect of methylation on gene expression varies with the number and location of methylation. For instance, methylation of H3K4 was stimulatory for transcription [45] while methylation of H3K9 serves as a recognition site for binding of heterochromatin protein-1 (HP-1) that maintain the heterochromatin state of chromatin [46]. Furthermore, it was observed that higher expression of trimethylated H3K4 led to aberrant gene expression. On the contrary, down regulation of H4K20me and H3K9me3 contributed to poor prognosis of colorectal cancer [47]. Moreover, it was observed that the promoter

Table 6.1 Histone methylation and their functional consequences

S. No.	Histone protein	Translational modifications	Functional consequences	References
1	Histone 3	Lysine 4 di- and trimethylation (H3K4me2 and H3K4me3)	Inactive chromatin repression of gene expression	Schneider et al. [126]
2	Histone 3	Lysine 9 monomethylation (H3K9me1)	Inactive chromatin repression of gene expression	Gupta et al. [127]
3	Histone 3	Lysine 27 di and trimethylation (H3K27me2 and H3K27me3)	Inactive chromatin repression of gene expression	Kondo and Issa [3]
4	Histone 3	Lysine 9 di- and trimethylation (H3K9me2 and H3K9me3)	Inactive chromatin repression of gene expression	Steen et al. [128]
5	Histone 4	Lysine 20 trimethylation	Worsen prognosis of CRC	Tryndyak et al. [129]

methylation of p16, MLH1 and MGMT tumor suppressor genes in colorectal cancer was directly correlated with H3K9 methylation status and lead to silencing of these genes [48]. Other methylated histones in colorectal cancer have been summarized in Table 6.1.

Histone Acetylation

Another type of epigenetic modification is acetylation and deacetylation that regulate the gene expression. Acetylation eliminates the positive charge on histones thereby hindering the interaction with negatively charged DNA that relaxes the chromatin. Therefore, recruitment of histone deacetylases (HDACs) aids in epigenetic silencing of genes by stabilizing condensed state of chromatin [49]. HDAC is a family of transcriptional co-repressors that play an important role in colon cell maturation and transformation, and they get their name due to their ability to catalyze the deacetylation of lysine residues within DNA bound, core histone proteins [50]. HDACs are overexpressed in CRC that may lead to the transcriptional repression of genes functioning in growth arrest, differentiation and apoptosis, by inducing histone hypoacetylation in core promoter regions. HDAC overexpression can also induce hypoacetylation thereby modifying the role of multiple non-histone proteins, including transcription factors and critical cytoplasmic proteins such as Hsp90. In higher eukaryotes, HDACs are classified into four classes based on their homology to a prototypical HDAC found in yeast, which has been illustrated in Table 6.2.

Class I and II HDACs regulate gene expression by two mechanisms. One is lysine deacetylation of DNA bound-core histone protein while other is deacetylation of DNA binding transcriptional factors such as p53, TIIF and cMyb that silence the transcription activation of their target genes [51, 52]. For instance, Class I-HDAC1, 2, 3 and class IIa-HDAC4 regulates transcriptional repression of p21 (cyclin dependent kinase inhibitor) by deacetylating the surrounding histone at the

Table 6.2 Classification of histone deacetylase enzymes and their role in CRC

Class	Yeast HDAC	HDACs included	Substrate	Role	References
I	rpd3	HDAC-1, HDAC-2, HDAC-3, HDAC-8	Androgen receptor, SHP, p53, MyoD, E2F1, Glucocorticoid receptor, YY1, BCL6, STAT3, SHP, YY1, GATA1, RELA, STAT3, MEF2D	p53 deacetylated by HDAC1, deacetylation of glucocorticoid receptor by HDAC2, MEF2 by HDAC3	Ito et al. [56, 130], Gregoire et al. [55]
II	had-1	HDAC-4, HDAC-5, HDAC-6, HDAC-7, HDAC-9, HDAC-10	GCMA, GATA1, HP1, SMAD7	p21 repression by HDAC-4 regulated by sp1/sp2	Paroni et al. [131]
III	sirt2	Sirt1, Sirt2, Sirt3, Sirt4, Sirt5, Sirt6, Sirt7	–	Aberrant deacetylation of specific lysine residues within histones (H1K26, H3K9, H3K14 and H4K16) and non-histone proteins p53, Ku70, FOXO, p300 and NFκB	Michan and Sinclair [132]
IV		HDAC-11	–	No role detected	Gao et al. [133]

promoter region [53] or by deacetylating Sp1/Sp3 transcription factors [54]. Other transcriptional factors p53, glucocorticoid receptor and MEF2 are also deaceytylated by HDAC1, HDAC2 and HDAC3 respectively [55, 56]. Moreover, Class III HDAC Sirt1 is highly expressed in the cytoplasm of colon cancer cell lines relative to normal colonic tissue [57].

Histone Phosphorylation

Like acetylation, phosphorylation also modifies the net positive charge on histone proteins thereby increasing the accessibility of DNA sequence to transcriptional activation of genes [3]. Histone phosphorylation plays an important role in cellular processes like mitosis, apoptosis and DNA repair in cell. For instance, instance, phosphorylation of histone3 at Ser-10 maintains the chromosomal dynamics during mitosis [58]. Thus, any alteration in histone phosphorylation level leads to abnormal cell proliferation and cancer [59]. Major family of kinases involved in phosphorylation of histones is Aurora kinases whose over expression leads to increased phosphorylation of histone 3 resulting in chromosomal instability in colorectal cancer [60].

Histone Ubiquitylation

Ubiquitylation is the covalent addition of ubiquitin (Ub) at the lysine residues on histone tails of H2A/H2B and is the least studied histone modifications in colorectal cancers [61]. It is involved in controlling a variety of cellular functions like cell-cycle regulation, protein trafficking, protein degradation, endocytosis signaling and stress response [62]. Target protein is ubiquitinated with an isopeptide bond between its ϵ-amino group of lysine residue and C-terminal glycine of ubiquitin, which is catalyzed by complex regulation of E1 activating, E2 conjugating and E3 ligase enzymes. Although histone ubiquitination is the least studied but one monoubiquitinated histone (H2Bub1) is identified where Ub is attached to the lysine 120 residue that is found to regulate various signaling process in transcription, DNA damage and histone crosstalk [63, 64]. Moreover, alteration or deletion of H2Bub1 is observed in colorectal cancer signifying their tumor suppressor activity [65, 66].

Histone Sumoylation

Histone sumoylation is an epigenetic event that was first observed by Shiibo and Eisenman in [67]. In cellular milieu, SUMO (small ubiquitin-like modifier) is a ~100 amino acids long polypeptide chain that is conjugated to numerous nucleoproteins to alter their functions [68]. SUMO polypeptide can be added to any of the four core histone proteins at certain lysine residues [69] that causes repression of transcription through recruitment of HDACs and HP-1 (Heterochromatin protein 1; [67]). It is a covalent modification of proteins involving an isopeptide bond formation between a glycine on UbLs (Ubiquitin like proteins) and lysine on the substrate protein. The E1-E2-E3 enzymatic cascade is similar to the ubiquitination but sumoylation is altogether a separate pathway involving a proteolytic cleavage of the C-terminal residues by SENP (Sentrin-specific peptidases) followed by E1-E2-E3 molecular events [70].

Sumoylation also competes with other lysine based histone modifications such as acetylation, ubiquitination and alters the transcription status from an active state to repressed state [70, 71]. Brandl et al. proposed that sumoylation of elevated HDAC2 triggers its attachment with p53 and deacetylate its promoter to inhibit p53-dependent gene expressions in cancer [72]. In another study it was shown that sumoylation of KLF5 (Kruppel-like factor 5; a zinc finger domain containing transcription factor) stimulated the anchorage independent growth of HCT116 colorectal cancer cells [73, 74]. Moreover, sumoylation of transducin β-like proteins TBL1-TBLR1 acts as a molecular switch of the Wnt/β-catenin oncogenic pathway that stimulate the recruitment of TBL1-TBLR1 to the promoter site of Wnt specific target genes and leads to activation of signaling pathway. However, desumoylation by SENP1 (sumo-specific protease I) reversed these events, suggesting that this sumoylation is reversible in nature [75].

6.2.3 Micro RNAs and Non coding RNAs

Micro RNAs (MiRNAs) are class of small non-coding RNA molecules, which are repeatedly modified in CRC and their schematic expression is associated with diagnosis, prognosis and therapeutics. The role of a specific miRNA is majorly studied using global miRNA expression profiling with microRNA microarrays [76]. Depending on the cellular environment, miRNA can function as either tumor suppressor or oncogenic summarized in Table 6.3. Michael et al. demonstrated that miR-143 and miR-145 are important tumor suppressors, and their expression is declined in CRC [77] while oncogenic miR-21 exhibited higher expression that played a major role in cancer initiation, advancement and metastasis [78]. Moreover, it has been observed that various micro RNAs are persistently hypermethylated in colorectal tumors such as let-7, miR-9, miR-34, miR-129, miR-137, miR-342 and miR-345 [79–81]. MicroRNA expression patterns can differentiate between normal colonic mucosa, colon adenomas and colon carcinomas. In this context mir-21 appears as an ideal biomarker whose expression is elevated in both adenomas and colon carcinoma. Higher expression of miR-21 in mice can induce malignancies depicting its efficiency to initiate malignancies and causing an increased cell proliferation, decreased apoptosis, increased cell migration, intravasation and metastasis by targeting several tumor suppressor genes [82].

6.2.4 Nucleosome Positioning

Nucleosomes are the fundamental repeating units of the chromatin in eukaryotes that are translocated with respect to genomic DNA in case of nucleosome positioning. They epigenetically regulate gene expression through non-covalent mechanisms such as nucleosome remodeling or substitution of specialized histone variants in place of canonical histone proteins in an ATP-dependent manner [83]. They achieve this regulation by altering the availability of the regulatory sequences to various transcription factors [84]. The 5′ and 3′ end regions of genes are usually nucleosome free regions (NFRs) to facilitate the assembly and the subsequent dismantling of the transcriptional machinery [85]. However, chromatin remodeling enzymes such as SWI/SNF, ISW, CHD and INO80 orchestrate the shifting or ejection of nucleosome to promoter regions to repress gene expression in an ATP-dependent manner [86, 87]. It has been shown that inappropriate expression of c-Myc gene through Wnt/ß-catenin signaling pathway leads to colorectal pathogenesis where nuclear ß-catenin recruits chromatin remodelers and histone modifiers that directly targets c-Myc gene for unrestricted transcription [88–90]. Hesson et al. investigated the epigenetic mechanism behind silencing of two genes CDH1 and CDK2NB involved in colorectal cancer. They reported that silencing of CDK2NB was due to loss of nucleosome from −2 position upstream from the transcription start site in contrast to wild-type tissues while CDH1 was silenced due to combined effects of promoter hyper methylation and a dense nucleosome occupancy [91].

Table 6.3 Micro RNAs and their role in colorectal cancer

S. No.	Micro-RNA	Type	Function	References
1	MiR-143	Tumor suppressor	Suppresses cell growth and proliferation by directly repressing the translation of KRAS, DNMT3A21 and extracellular signal-regulated kinase-5 (ERK5)	Ng et al. [134]
2	MiR-145	Tumor suppressor	Inhibition of the oncogenic insulin receptor substrate-1 (IRS-1), c-Myc, Yamaguchi sarcoma viral oncogene homolog 1 (YES1) signal transducer and activator of transcription 1 (STAT1) and Friend leukemia integration 1 (FLI1)	Shi et al. [135], Sachdeva et al. [136], Gregersen et al. [137]
3	MiR-21	Oncogenic	Targets the tumor suppressor genes: programmed cell death 4 (PDCD4), phosphatase and tensin homolog (PTEN), Cell division cycle 25 homolog A (Cdc25a), reversion-inducing-cysteine-rich protein with kazal motifs (RECK), TIMP3, maspin, nuclear factor 1 B-type (NFIB), tropomyosin 1 (TPM1), sprouty 2 (SPRY2), T-lymphoma invasion and metastasis-inducing protein 1 (TIAM1)	Lu et al. [138], Wang et al. [139], Gabriely et al. [140], Zhu et al. [141], Fujita et al. [142], Sayed et al. [143], Cottonham et al. [144]
4	MiR-30a-5p i	Tumor suppressor	Targets denticleless homolog (DTL) to suppress tumor growth	Baraniskin et al. [145]
5	MiR-34a	Tumor suppressor	Repressed by p53 isoform Δ133p53 and affects cellular senescence	Fujita et al. [142]
6	MiR-101	Tumor suppressor	Downregulates Cyclooxygenase 2 (COX-2)	Strillacci et al. [146]
7	MiR-451	Tumor suppressor	Reduces cell proliferation through targeting of the oncogene macrophage migration factor (MIF)	Bandres et al. [147]
8	MiR-675	Tumor suppressor	Silences retinoblastoma (RB) tumor suppressor gene to increase tumor growth	Tsang et al. [148]
9	MiR-365	Tumor suppressor	Inhibits cell cycle progression and promotes apoptosis of colon cancer cells by targeting Cyclin D1 (CCND1) and Bcl-2	Nie et al. [149]

These epigenetic modifications put a significant impact on the expression profile of genes inside a cell. They have the potential of reversing the functional state of genes that are important in governing the pathophysiology of a disease. Such epigenetically modified genes have been summarized in Table 6.4, which is involved in pathogenesis of colorectal cancer.

Table 6.4 Epigenetically modified genes and their role in colorectal cancer

Affected gene	Class of protein	Epigenetic modification	Functional role	References
MLH1	Mismatch repair	DNA hypermethylation	Mutation in oncogenes, TSG, MSG	Cunningham et al. [16]
MGMT				Esteller et al. [19]
RB (retinoblastoma)	Tumor suppressor			Greger et al. [23]
RARB (retinoic acid receptor beta)				Youssef et al. [25]
SFRP (secreted frizzled-related protein)				Dahl et al. [22]
Estrogen receptors				Issa et al. [24]
LINE-1	Retrotransposon	DNA hypomethylation	Activation of oncogenes	Murata et al. [150]
TCF3	Anti-apoptotic factor			Chunxiang et al. [35]
IGF-2				Bell and Felsenfeld [36]
CDH-3	Cell adhesion protein			Hibi et al. [39]
CD133	Organises cell membrane topology			
p16	Tumor suppressor	Histone methylation	Activation of oncogenes	Kondo et al. [48]
MLH1 MGMT	Mismatch repair			
p53	Tumor suppressor	Histone acetylation	Transcriptional activation of genes	Ito et al. [56]
MEF2	Cell differentiation regulator			Gregoire et al. [55]
p21	Tumor suppressor			Davie et al. [53]
H2Bub1	Tumor suppressor	Histone ubiquitination		Espinosa [65]
p53	Tumor suppressor	Histone sumoylation	Repression of transcription	Brandl et al. [72]
KLF5	Mediator of cell proliferation			McConnell et al. [73]
Myc	Transcription factor	Nucleosome positioning	Silencing of gene	Mosimann et al. [89]
CDH-1	Tumor suppressor			Hesson et al. [91]
CDK2NB				

6.3 Epigenetic Markers Regulating Cell Signals in Colorectal Cancer

Numerous epigenetic modifications evidenced that they are involved in altering signal transduction pathways in tumor development. These epigenetic changes are known to affect cell regulatory genes such as oncogenes, tumor suppressor genes, metastatic genes and angiogenic genes that favor cancer progression (Fig. 6.1). Such epigenetically modified genes in colorectal cancer have been demonstrated here.

6.3.1 Oncogenes

Various studies have shown that mutation in APC (Adenomatous Polyposis Coli) gene is an initial event in the progressive adenoma-carcinoma sequence in sporadic colorectal cancers. Genetic and/or epigenetic disruption of the APC gene activates the Wnt/β-catenin signaling pathway [92, 93]. The dysfunctional APC protein stabilizes β-catenin leading to cytoplasmic aggregation and its consequent localization to the nucleus that co-activates the T-cell factor/lymphoid enhancer factor family (TCF/LEF) transcription factors to trigger cell proliferation [94]. KRAS (Kirsten's rat sarcoma viral oncogene homolog) gene alteration is another early event in the pathogenesis of colorectal cancer that triggers G-protein signaling

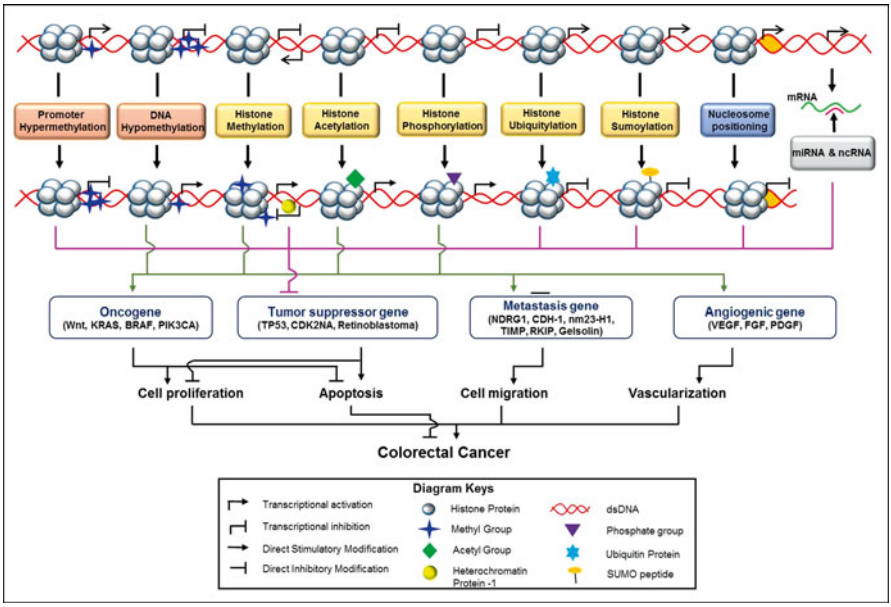

Fig. 6.1 Epigenetically modified genes mediated colorectal cancer pathogenesis

pathway, which regulates proliferation and differentiation of cells [95]. Mutant KRAS protein has a dysfunctional intrinsic GTPase activity, thus it remains in GTP-bound state and constitutively activates the proliferative downstream signals [96]. BRAF (V-raf murine sarcoma viral oncogenes homolog B1), a serine-threonine protein kinase is a downstream regulator of activated Ras pathway [97]. Mutations in these two oncogenes have been associated with activation of MAPK-ERK pathway, which causes uncontrolled proliferation and impaired differentiation leading to colorectal carcinogenesis [98, 99]. Various other genes have been implicated in colorectal tumorigenesis, such as PIK3CA gene encoding p110α catalytic subunit of PI3K (Phosphatidylinositide-3-kinase), a lipid kinase promotes cellular proliferation, and survival is linked with KRAS mutations, increased levels of CIMP and under expression of MGMT [100]. Moreover, dysfunctional PTEN (a tumor suppressor gene) is involved in PI3K/AKT signaling pathway and showed accelerated metastasis [101].

6.3.2 Tumor Suppressor Genes

Tumor can also develop by primary epigenetic silencing of tumor suppressor genes in colorectal cancer such as TP53, which is implicated in control of cell cycle progression and apoptosis in cell [102]. Another tumor suppressor CDK2NA/p16 (Cyclin-dependent kinase inhibitor 2A) gene has a significant role in cell cycle regulation that is silenced upon promoter methylation that lead to uncontrolled cell proliferation [103]. The p16 protein further inhibits the activity of CDK4, CDK6 and D-type CDKs that phosphorylate the retinoblastoma tumor suppressor protein and accelerates colorectal carcinogenesis [104]. Moreover, it has been found that in high serum folate/vit B12 environment, hypermethylation of p16 was associated with MLH1 promoter methylation that further promotes tumor formation [105].

6.3.3 Metastatic Genes

Metastasis is an inherent property of cancer cells by which primary tumors invade and proliferate at a distant site. The metastatic process involves the migration of primary tumors through the stroma followed by intravasation into the vasculature finally leading to “colonization” [106]. There are three key mechanisms for metastasis suppression that involves (a) alteration of cell adhesion, (b) induction of apoptosis and (c) regulation of transcription [107]. Metastasis suppressor genes include NDRG1 (known as cap43/rit42/RTP/Drg1/TDD5) which is epigenetically regulated by both DNA methylation and histone modifications in colorectal cancer [108]. Another metastasis gene CDH-1 encoding E-cadherin is responsible for maintaining tissue structure by regulating intercellular contacts in CRCs [109]. Further nm23-H1, a metastasis suppressor encodes the subunit A of nucleoside diphosphate

kinase, is found to act as a prognostic factor in CRC whose mRNA and protein expression was higher in early stages but diminish with the CRC progression [110]. Moreover, epigenetic silencing of nm23-H1 in response to methylation promotes ECM invasion, cellular scattering and motility by upregulating several matrix metalloproteinases and promoting invadopodia formation [111]. Other metastasis suppressors include TIMP (tissue inhibitor of metalloproteinases), RKIP (Raf kinase inhibitory protein) and Gelsolin that have also been correlated with advanced stages of CRC [112–114].

6.3.4 Angiogenic Genes

Angiogenic genes govern the process neovascularization that provides oxygen and nutrients to cell for growth. Although quiescent in normal adult cells, angiogenesis is highly active during tumorigenesis. This active state is a result of an equilibrium shift from a balanced expression of anti- and proangiogenic factors, to an increased expression of proangiogenic factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) etc. [115]. VEGF is the most frequent factor that is associated with angiogenesis and is upregulated in response to promoter methylation of miRNA-126 in cancer cells thereby increasing its ability to metastasize. This study suggests that in the non-cancerous milieu miRNA 126 co-regulates the expression of VEGF to desirable levels [116].

6.4 Therapeutic Strategies in Targeting Colorectal Cancer

Therapeutic strategies for epigenetically caused colorectal cancer primarily lie in reversing the effects of epigenetic modifications. Such drugs that have the potential to reverse the epigenetic modifications and are being used for targeting colorectal cancer have been demonstrated in Table 6.5.

6.4.1 Use of DNA Methylation Inhibitors

Therapeutic strategies for colorectal cancers can be hypomethylation based, which uses DNA methylation inhibitor like *5-azacytidine* and *5-aza-2′-deoxycytidine* (decitabine) as an anti-cancer agent to reverse the epigenetic alterations caused due to hypermethylation [117]. Although both are efficient drugs but display substantial cytotoxicity and loss of hypomethylation activity at higher doses. Other drug *Zebularine* [1-(β-D-ribo-furanosyl)-1,2-dihydropyrimidin-2-one] demethylates hypermethylated promoter region and prevent its re-methylation by a complete

Table 6.5 List of anti-epigenetic drugs implicated in colorectal cancer

S. no.	Drug	IUPAC name	Mechanism of action	References
1	Azacitidine	5-azacytidine	DNA methylation inhibitor	Ghoshal and Bai [151]
2	Decitabine	5-aza-2′-deoxycytidine		Jones and Taylor [117]
3	Suberanilohydroxamic acid (SAHA)	*N*-hydroxy-*N*′-phenyloctanediamide	Histone deacetylase inhibitor and antineoplastic agent	Marks et al. [49]
4	Trichostatin A (TSA)	(2E,4E,6R)-7-[4-(dimethylamino) phenyl]-*N*-hydroxy-4,6-dimethyl-7-oxohepta-2,4-dienamide	Histone deacetylase inhibitor	Minucci and Pelicci [122]
5	Zebularine	1-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl] pyrimidin-2-one	Inhibits DNA methylation and tumour growth	Cheng et al. [118]
6	Genistein	5,7-dihydroxy-3-(4-hydroxyphenyl) chromen-4-one	Mediates alterations of histone acetylation and DNA methylation	Wang and Chen [152], Majid et al. [153]
7	Nanaomycin A	2-(9-hydroxy-1-methyl-5,10-dioxo-3,4-dihydro-1H-benzo[g]isochromen-3-yl) acetic acid	Inhibits DNA methylation	Caulfield and Medina-Franco [154]
8	Romidepsin	(1S,4S,7Z,10S,16E,21R)-7-ethylidene-4,21-di(propan-2-yl)-2-oxa-12,13-dithia-5,8,20,23-tetrazabicyclo [8.7.6] tricos-16-ene-3,6,9,19,22-pentone	Histone deacetylase inhibitor	Thaler and Mercurio [155]
9	Belinostat	(E)-*N*-hydroxy-3-[3-(phenylsulfamoyl) phenyl] prop-2-enamide	Histone deacetylase inhibitor	Chun [156]
10	3-Deazaneplanocin A	(1S,2R,5R)-5-(4-aminoimidazo[4,5-c] pyridin-1-yl)-3-(hydroxymethyl) cyclopent-3-ene-1,2-diol	HMT inhibitors	Tam et al. [157]
11	Azobenzene	Diphenyldiazene	Targets miR-21	Gu et al. [158]
12	Panobinostat	(E)-*N*-hydroxy-3-[4-[[2-(2-methyl-1H-indol-3-yl)ethylamino]methyl]phenyl] prop-2-enamide	HDAC inhibitor	Lu et al. [159]

depletion of DNA methyltransferase I (DNMTI) and partial depletion of DNMT3a and DNMT3b [118]. Combination of drugs *5-aza-2′-deoxycytidine* and *Zebularine* can be used as efficient therapeutic strategy against colorectal cancers. Moreover, Tea polyphenols [119] and Procainamide [120] had been identified as the weak DNA methylation inhibitors in living cells [119–121]. Further, Histone methyltransferases can reactivate the silenced genes, thus they can be observed as potential drugs as single agent or with other epigenetic drugs having synergistic effects.

6.4.2 Use of Histone Deacetylase Inhibitors

Other approaches in this field include the use of histone deacetylase (HDAC) inhibitors as potential anti-cancer drugs. Human HDAC enzymes can be classified into three classes, where Class I-HDACs1, 2, 3, 8 and 11 are sensitive to suberoylanilidehydroxamic acid (*SAHA*), Class II-HDACs 4, 5, 6, 7, 9 and 10 are sensitive to trichostatin (*TSA*) and Class III-NAD-dependent HDAC (SIR-2) family of deacetylase are sensitive to both *SAHA* and *TSA*. *SAHA* and *TSA* are involved in upregulation of genes like p21, TATA binding protein-2 (TBP2), Bcl6 and cyclin-E while downregulation of genes like cyclin-D1, ErbB2, thymidylatesynthetase, cyclin A and vascular endothelial growth factor thereby inhibiting cancer progression [49, 122].

6.4.3 Micro-RNA Mediated Therapy

Researchers have found that miRNAs can also be implicated as a potential therapeutic target besides being a diagnostic biomarker. Therapies related to activation of silenced miRNAs can also be used in the treatment of cancer [123]. Generally, miRNA based therapies involve either inhibition of oncogenic miRNA or restoration of tumor suppressor miRNA. Direct inhibition of miRNAs can be achieved by using antisense oligonucleotides or miRNA sponges to bind and sequester the target miRNA. Similar strategies have been used to inhibit miR-122 expression in primates [124]. Indirect inhibition of miRNAs can be done through treatment with various chemical compounds; for instance, *azobenzene-2* may be used as a specific and efficient inhibitor of miR-21 expression [125]. However, research on miRNAs is still in its infancy and the biogenesis of many miRNAs is still not fully understood.

6.5 Conclusion

Research on epigenetic mechanisms is being done from several decades. Although recent years have seen an enormous increase in the amount of studies conducted, few questions still remain unanswered in regard to colorectal cancer. However, it has

become clear that in addition to genetic mutations, aberrant epigenetic modifications are also fundamental to the prognosis of cancer. Of all the modifications, aberrant DNA methylation has been recognized as critical biomarker of the disease. Most of the epigenetic changes are concerned with the change in expression profile of cancer critical genes like oncogenes (Wnt, KRAS, BRAF, PIK3CA) and tumor suppressor genes (TP53, CDK2NA, Retinoblastoma). Moreover, only few epigenetic modifications are reversible, for instance, DNA methylation and Histone deacetylation, but we still need to diagnose the reversibility of unidentified epigenetic modifications. Although drugs like *5-azacytidine* and *5-aza-2′-deoxycytidine* have attained success in reversal of DNA methylation, they have been accompanied by adverse effects for the cell. Moreover, the epigenetic drugs discovered so far are mostly nonspecific thus development of novel drugs specific for targets is a high priority. Similarly, different therapeutic strategies have been used with other modifications but a perfectly efficient cure has not been achieved, especially for later stages of cancer, which still remains a challenging task. Thus evaluation of the clinical utility of these assays as an early detection marker or potentially risk stratification markers remain under active investigation. Although, several cancer causing-aberrant alteration models have been developed but much more study and further insights are required to bring such models into clinical use.

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Chapter 7
Epigenetics and Angiogenesis in Cancer

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7.1 Introduction

Conrad Waddington was the first to coin the term "epigenetics" in 1939, and defined as "any heritable changes in a cellular phenotype without altering the DNA sequence" [1]. Epigenetic is the phenomenon which describes the event eventually involving chromatin mediated regulatory process of DNA-template. Moreover, highly regulated machineries are involved in the process of DNA and histones' modification and their removal by chromatin-modifying enzymes while, DNA modifications are of four different types [2, 3], histone modifications have 16 classes [4]. These modifications can alter non covalent interactions within and between nucleosomes and thus lead to modify the chromatin structure. These altered chromatin structures act as docking sites for particular proteins with unique domains that specifically identify these modifications.

Epigenetic modification plays an integral role in the regulation of all DNA-based processes, including DNA replication, transcription, and DNA repair. Furthermore, altered genome or irregular expression patterns of chromatin regulators may trigger different tumor cells to transform into malignant cells. Interestingly, epigenetics

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process like histone modification, DNA methylation, nucleosome remodeling, and RNA-mediated processing trigger various biological activities and play profound regulatory roles in mammalian gene expression. Normal methylation state in the promoter region is involved in the regulation of gene expression; however, altered promoter methylation levels are molecular hallmarks for severe conditions ranging from cancers to psychiatric disorders. Additionally, epigenetic process is also involved in the regulation of vascular genes, and growth factors mediated angiogenic process. Angiogenesis is responsible for new growth in the vascular network since the proliferation along with the metastatic spread, of cancer cells is governed by an adequate supply of oxygen and nutrients and the removal of waste products. Expression levels of angiogenic factors reveal the hostility of tumor cells. Epigenetic state of the vascular endothelial growth factor-A (VEGF-A) promoter activity can be changed by using small RNAs and this result in either increased or decreased VEGF-A expression.

This epigenetic change in VEGF-A expression could be possible through changes in the histone code. Further, DNA methylation has no major impact on the epigenetic alterations of VEGF-A gene [5]. Epigenetic alteration plays an important role in signal transduction and gene-gene interaction. For instance, fibroblast growth factor receptor-2b (FGFR-2b) and Estrogen controls the activity of pituitary tumors by the activity of melanoma-associated antigen (MAGE-A3). This regulatory role is played by both DNA and histone modification and therefore, making an association between epigenetic alterations in vascular cells to angiogenesis [6]. Further, there are several genes present in the epigenetic state of cancers and exert their role in cell-proliferation, migration, DNA repairing, and cell cycle regulation. A comprehensive list of genes and their associated functions in various cancers has been described in Table 7.1.

Table 7.1 Genes and their role in epigenetic mediated cancers

Genes	Mode of action	Cancers	References
APC	Involves in unbalanced regulation of cell proliferation, cell migration, cell adhesion, chromosomal stability, and cytoskeletal reorganization	Lung, Breast, and Esophageal Cancer	Virmani et al. [87], Kawakami et al. [88]
BRCA1	Associated with DNA repairing and transcriptional activation	Ovarian and Breast cancer	Dobrovic and Simpfendorfer [89], Chan et al. [90]
CDKN2A/p16	Acts as cyclin-dependent kinase inhibitor	Lung, Head, and Neck cancer	Sanchez-Cespedes et al. [91], Villuendas et al. [92]
Cyclin D2	Involves in cell-cycle regulation, differentiation, and malignant transformation	Breast Cancer	Evron et al. [93]

(continued)

Table 7.1 (continued)

Genes	Mode of action	Cancers	References
DAPK1	Involves in suppression of apoptosis	Lung Cancer	Harden et al. [94]
E-cadherin	Associated with increasing proliferation, invasion, and metastasis	Thyroid, Breast, and Gastric Cancer	Graff et al. [95], Waki et al. [96]
ER	Acts as hormone resistance	Prostate and Breast Cancer	Yang et al. [97], Li et al. [98]
GSTP1	Associated with loss of detoxification of active metabolites of several carcinogens	Breast, Prostate, and Renal Cancer	Lee et al. [99], Esteller et al. [100]
hMLH1	Involves in DNA mismatch repair and gene mutations	Ovarian, Colon, Gastric, and Endometrial Cancer	Waki et al. [96], Kondo et al. [101]
MGMT	It belongs to p53-related gene family and involves in DNA repairing and drug resistance	Lung and Brain Cancer	Harden et al. [94], Esteller et al. [102]
p14ARF	Involves in cell-cycle arrest	Bladder Cancer	Dominguez et al. [103]
p15	Assists in unrestrained entry of cells into activation and proliferation	Leukemia, Lymphoma, Squamous Cell Carcinoma, and Lung Cancer	Melki et al. [104], Garcia et al. [105]
p16	Inhibits the transcription of important cell-cycle regulatory protein which results in cell-cycle arrest	Head and Neck Cancer, Colorectal Cancer, and Lung Cancer	Sanchez-Cespedes et al. [91], Zou et al. [106]
p16ink4a	Acts as tumor suppressor gene which is mainly associated with senescence and tumors	Breast Cancer	Silva et al. [107]
RARβ	Blocks cell-migration and inhibits metastasis	Breast Cancer	Evron et al. [93]
RASSF1A	Loss of negative regulatory control of cell proliferation through inhibition of G1/S-phase progression	Lung Cancer, Breast Cancer, Ovarian Cancer, Kidney, and Nasopharyngeal Cancer	Morrissey et al. [108], Kwong et al. [109]
Rb	Involves in transcription of cellular genes required for DNA replication and cell division	Retinoblastoma and Oligodendroglioma	Gonzalez-Gomez et al. [110]
Twist Gene	Involves in angiogenesis development and stem cells phenotype formation	Breast Cancer	Evron et al. [93]
VHL	Alters RNA stability through erroneous degradation of RNA bound proteins	Renal Cancer	Morrissey et al. [108]

7.1.1 DNA Methylation, Acetylation and Histone Modification

The methylation of the 5-carbon on cytosine residues in CpG dinucleotides has been reported to be involved in covalent modification of DNA and the most extensive alteration of chromatin. DNA methylation is predominantly identified in centromeres, inactive X-chromosomes, telomeres, and repeated sequences. Furthermore, an epigenetic study in alterations of cancer cell due to methylation is mainly occurred within CpG island promoters. Methylation of CpG Island plays a central role in transcriptional regulation and is altered during malignant transformation. NGS (Next-Generation Generation Sequencing) has provided the genome maps of CpG methylation. NGS has recognized that between 5 and 10 % of normally, unmethylated CpG promoter islands become unusually methylated in several cancer genomes. It has also demonstrated that CpG hypermethylation of promoters not only changed the expression of mRNAs but also the expression of several noncoding RNAs, some of which in turn lead to progression of malignant transformation [2].

In higher eukaryotes, three well-known active DNA methyltransferases have been recognized viz. DNMT1 which is responsible for methyltransferase process identifies hemimethylated DNA during DNA replication [7], DNMT3a and DNMT3b, which are also able to methylate hemimethylated DNA [8]. DNA methylation makes a platform for various methyl-binding proteins, which contains different DNA binding domains such as MBD1, MBD2, MBD3, and MeCP2. These domains are responsible for recruiting histone-modifying enzymes to organize the chromatin-template processes [9]. Mutations in MDB proteins and DNA methyltransferases have been also well-known that contribute to the onset of cancers. Importantly, these mutations are always heterozygous and predicted to interrupt the catalytic activity of such enzymes and thus lead to growing abnormalities [10].

In 1964, Vincent Allfrey prophetically introduced that histone modifications have a functional impact on the regulation of transcription [11]. However, histone modifications occur in distinctive histone proteins, including histone residues such as arginine, lysine and serine and histone variants. These modifications as well involve several chemical groups, including phosphate, methyl and acetyl and have various degrees of methylation such as tri-methylation, di-methylation, and monomethylation. Methylation and acetylation of histones have direct impacts on a variety of nuclear processes, including DNA repair, DNA replication, gene transcription, and organization of chromosomes. Importantly, histone acetylation is often linked with transcriptional activation whereas histone methylation depends on amino acid sequence and its position in the histone tail [12, 13].

In the promoter regions of CpG islands, hypermethylation leads to progression of tumor-suppressor genes in tumor and is mainly associated with histone markers. It also promotes loss of H3K4 tri-methylation, deacetylation of histones H3, H4, and gain of H3K9 methylation [14, 15]. The occurrence of the hypermethylated and hypo-acetylated histones H3 and H4 silences certain genes, which lead to reducing progression of tumor. Interestingly, in human cancer cells, modifications of histone H4 require a reduced monoacetylated and trimethylated forms [16]. However, monoacetylated Lys16 and trimethylated Lys20 residues of histone H4 is also

Table 7.2 Histone modifying enzyme and their functional significance in different cancers

Histone modifying enzyme	Mode of action	Cancers	References
BMI1	Acts as oncogene	Lymphoma, Leukemia, Breast, and Colorectal Cancer	Pietersen et al. [111], Kim et al. [112]
DOT1	Involved in DNA damage repairing	Leukemia	Chang et al. [113], Tatum and Li [114]
EZH2	Involved in transcriptional repression and also associated with tumor aggressiveness	Lymphoma, Glioblastoma, Prostate, and Breast Cancer	Kleer et al. [115], Suvà et al. [116]
ING4	Acts as tumor suppressor	Glioma and Breast Cancer	Tapia et al. [117], Gunduz et al. [118]
JMJD2C	Involved in transcriptional activation	Lymphoma, Breast, and Esophageal Cancer	Liu et al. [119], Vinatzer et al. [120]
JMJD3	Involved in transcription activation	Prostate Cancer	Xiang et al. [121]
LSD1	Involved in transcriptional repression	Prostate Cancer	Lim et al. [122], Wang et al. [123]
MLL	Involved in transcriptional activation and gene fusions	Leukemia	Armstrong et al. [124], Corral et al. [125]
NSD1	Involved in transcriptional activation and gene fusions	Leukemia and Multiple Myeloma	Taketani et al. [126], Wang et al. [127]
SETDB1	Involved in transcriptional repression	Melenoma	Ceol et al. [128]

associated with hypomethylated repetitive DNA sequences, commonly found in breast and liver cancer [17, 18]. Table 7.2 shown below presents a comprehensive list of histone modifying enzyme and their associated functions in various cancers.

7.1.2 Interaction Between Epigenetics and miRNA

The miRNA is short, 22-nucleotide noncoding RNA, which plays a pivotal role in gene expression through sequence-specific base pairing in the 3′-untranslated region (3′-UTR) of the targeted mRNA. However, outcome of mRNA degradations is tightly regulated and simultaneously plays an essential role in cell proliferation, differentiation, and apoptosis [19]. Most of the human genes lose their binding activity of miRNA to the untranslated regions of the polypeptide chain that helps in quick growth mRNA. Recent studies have revealed that miRNA expression profile differs between normal tissues, cancerous tissues and among various types of tumor [20]. In tumor cell DNA hypermethylation in miRNA 5′-regulatory region causes down-regulation of miRNA expression [21]. However, in colon-cancer cells with damaged DNMTs, hypermethylation of the CpG Island does not occur in miRNAs. In other studies, it has been confirmed that methylation silencing of miR-124a also provokes the CDK6 activity in the cell-cycle which is the general feature of epigenetic wound in tumors [21] (Fig. 7.1).

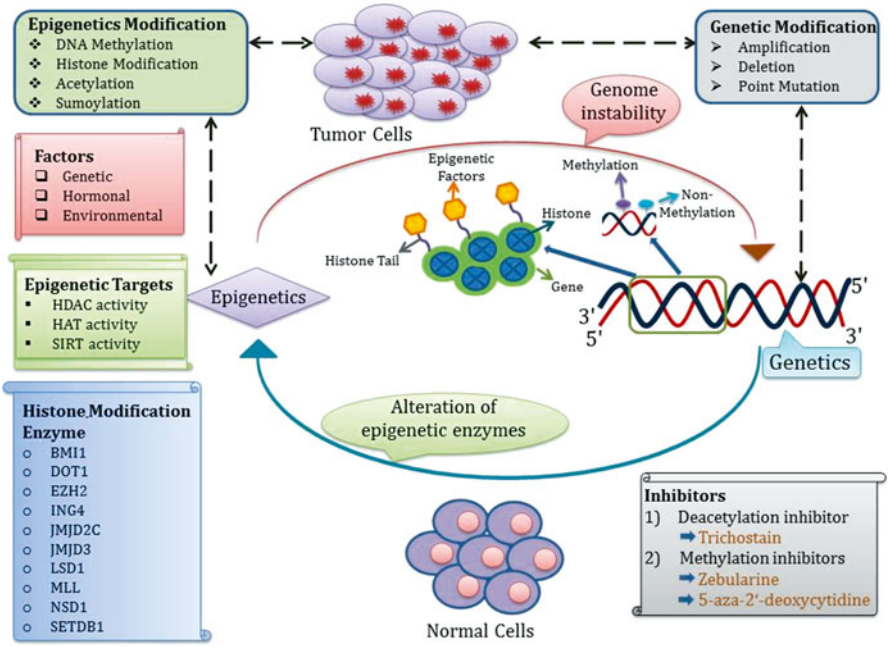

Fig. 7.1 Crosstalk between genetics and epigenetic modifications in cancer identification

7.1.3 Relation Between Epigenetics and Cancer

Gene expression and DNA methylation studies highlighted the link between epigenetic process and the pathology of cancer [22]. These early studies were purely correlative, which provided possible interaction between cancer and epigenetic cycle. However, these observations have been significantly highlighted by recent results from the International Cancer Genome Consortium (ICGC). The genome sequencing of tumor cells have also given a list of recurrent somatic mutations in several epigenetic regulators [23]. Moreover, the principle theory behind analyzing these cancer genome sequences is the recognition of "driver" mutations. These driver mutations are frequently found in a number of cancers, and they are often present at high primacy in a particular tumor type. For instance, in most of the cases follicular lymphoma is caused due to result of recurrent mutations occurring in histone methyltransferase MLL2, similarly another histone modifying enzyme histone demethylase UTX is mutated in nearly 12 different cancers [24, 25]. Accumulation of such mutated epigenetic regulators in tumor cells describe the involvement of two major epigenetic modifications such as histone methylation and acetylation, which widely affects the epigenetic cycle.

Mapping of Chromatin modifications by deep sequencing technologies have also started to focus in the beginning of epigenetic defects in cancer. Nevertheless, DNA methylation profiles in human cancer with different data, including CHIP-Seq, histone modification and binding of chromatin regulators have raised interesting

concerns between cancer associated hypermethylation and genes marked with "bivalent" histone modification in multipotent cells [26]. Although, these bivalent genes are highlighted by active and repressive histone modifications and often act as transcriptionally poised genes, which play a crucial role in development and lineage commitment [27].

In cancerous cells, Most of the genes are mainly targeted on DNA methylation process. In contrast, recent comparisons between normal tissues and malignant from of the same individuals are equally fascinating. These comparisons explain about wide domains in malignant cells that bring major changes in DNA methylation, which is mainly associated with late-replicating regions of the genome with the nuclear lamina [28]. Global alterations in the epigenetic landscape and genomic abrasions in chromatin modifiers show a vital role in cancer and also provide potentiate therapeutics against cancer. However, various numbers of small-molecule inhibitors have been already developed for chromatin regulators. They have various stages of development, which includes Janus kinase 2 (JAK2), histone deacetylase (HDACs), histone acetyl transferase (HAT), and DNA (cytosine-5-)-methyltransferase (DNMTs). These findings reveal that epigenetic pathways are crucial for drug discovery targets over the past decade and thus provide potential therapeutic approaches against a broad spectrum of cancers [29]. Table 7.3 shown below represents a comprehensive list of drugs that affects the acetylation process in various cancers.

Table 7.3 List of drugs that affect the acetylation process in various cancers

Drugs	Specific target	Cancers	References
Anacardic acid	HAT activity	Stomach Cancer	Balasubramanyam et al. [129], Sun et al. [130]
Butyrate	HDAC activity	Leukemia, Colon, and Prostate Cancer	Candido et al. [131], Sealy and Chalkley [132]
Cambinol	SIRT activity	Burkitt's Lymphoma Cancer	Heltweg et al. [133]
Curcumin	HAT activity	Pancreatic Cancer	Balasubramanyam et al. [134, 135], Kang et al. [136]
Diallyldisulfide	HDAC activity	Colon and Stomach Cancer	Bianchini and Vainio [137], Nian et al. [138]
Dihydrocoumarin	SIRT activity	Lymphoma Cancer	Olaharski et al. [139]
3,3′-Diindolylmethane	HDAC activity	Breast Cancer, Colon Cancer, and Prostate Cancer	Li et al. [140]
EGCG	HDAC activity	Skin and Breast Cancer	Nandakumar et al. [141], Li et al. [140]
Garcinol	HAT activity	Colon and Tongue Cancer	Padhye et al. [142], Balasubramanyam et al. [134, 135]
Genistein	HDAC and SIRT activity	Prostate Cancer	Kikuno et al. [143]
Sulforaphane	HDAC activity	Colon Cancer	Myzak et al. [144]

7.2 Angiogenesis and Epigenetics

Angiogenesis is a well-defined phenomenon of formation of new blood vessels from pre-existing ones while vasculogenesis is the de novo formation of endothelial cells from mesodermal progenitors. Bone marrow derived endothelial progenitor cells are the lead participants of angiogenesis. Angiogenesis is a crucial determinant of normal human physiology, which plays a central role during reproduction, embryonic growth, development, wound healing and tissue repair following trauma. It is a multistep process; mediated by "on" and "off" triggers, which are regulated by a sophisticated network of interaction between angiogenic factors, tumor cells, phagocytes and their secreted factors, stromal cell cancer stem cells, components of the extracellular matrix (ECM) and most importantly the endothelial cells in a spatio-temporal manner. These factors Angiogenesis is a well-defined phenomenon of formation of new blood vessels from pre-existing ones while vasculogenesis is the de novo formation of endothelial cells from mesodermal progenitors. Bone marrow derived endothelial progenitor cells are the lead participants of angiogenesis. Angiogenesis is a crucial determinant of normal human physiology, which plays a central role during reproduction, embryonic growth, development, wound healing and tissue repair following trauma. It is a multistep process; mediated by "on" and "off" triggers, which are regulated by a sophisticated network of interaction between angiogenic factors, tumor cells, phagocytes and their secreted factors, stromal cell cancer stem cells, components of the extracellular matrix (ECM) and most importantly the endothelial cells in a spatio-temporal manner. These factors taken together have a significant impact on vessel dynamics; and as a certain consequence, uncontrolled or aberrant angiogenesis leads to baffling angiogenic disorders such as vascular insufficiency (myocardial or critical limb ischemia) and vascular overgrowth (hemangiomas, vascularized tumors, and retinopathies), and inflammatory diseases. In addition, uncontrolled angiogenesis marks a significant event during tumor propagation, invasiveness and metastasis thus resulting in aggressive tumor behavior [30–36]. Since, various vascular genes and growth factors are involved in the angiogenic process and thus its regulation is an important for maintaining its normal functions. Epigenetic process is involved in the regulation of these vascular genes and growth factors. The expression of the VEGF gene can be changed by epigenetic processes using small RNAs. The role of small RNAs in epigenetic regulation is crucial for targeting the promoter of VEGF gene thereby leading to alteration of histone code. VEGF bears its effects, mostly via two receptors, VEGFR1 and VEGFR2, and their expression is also controlled by promoter DNA methylation in various cancer cells [5]. These findings advocate about the significance of epigenetic mechanisms in the regulation of vascular genes and growth factors involved in the angiogenic process. together have a significant impact on vessel dynamics; and as a certain consequence, uncontrolled or aberrant angiogenesis leads to baffling angiogenic disorders such as vascular insufficiency (myocardial or critical limb ischemia) and vascular overgrowth (hemangiomas, vascularized tumors, and

retinopathies), and inflammatory diseases. In addition, uncontrolled angiogenesis marks a significant event during tumor propagation, invasiveness and metastasis thus resulting in aggressive tumor behavior [30–36]. Since, various vascular genes and growth factors are involved in the angiogenic process and thus its regulation is an important for maintaining its normal functions. Epigenetic process is involved in the regulation of these vascular genes and growth factors. The expression of the VEGF gene can be changed by epigenetic processes using small RNAs. The role of small RNAs in epigenetic regulation is crucial for targeting the promoter of VEGF gene thereby leading to alteration of histone code. VEGF bears its effects, mostly via two receptors, VEGFR1 and VEGFR2, and their expression is also controlled by promoter DNA methylation in various cancer cells [5]. These findings advocate about the significance of epigenetic mechanisms in the regulation of vascular genes and growth factors involved in the angiogenic process.

7.2.1 Background of Angiogenesis

See Fig. 7.2

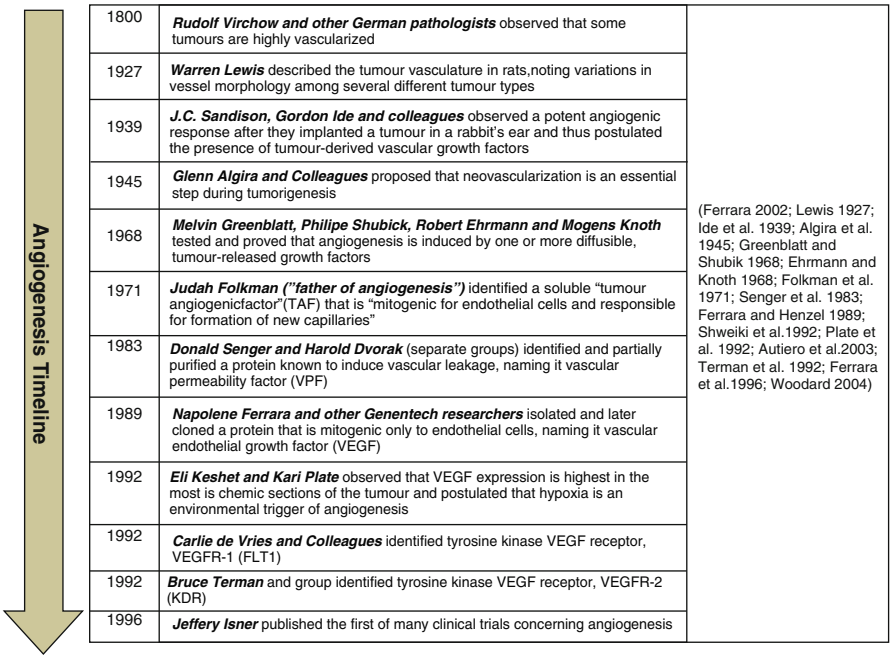

Fig. 7.2 Timeline depicting the progress of angiogenesis research over the years

7.2.2 Types of Angiogenesis?

Angiogenesis; the mechanism by which new blood vessels "sprout" from the pre-existing ones, occurs primarily by endothelial sprouting or via non-sprouting mechanisms.

Sprouting angiogenesis: It was the first identified form of angiogenesis. Initially, it is facilitated via local degradation of the basement membrane at the site of dilated peritumoral postcapillary venule that is at a close proximity to the site of the angiogenic stimulus. Later, the inter-endothelial contacts are loosened, and the endothelial cells (ECs) subsequently migrate into the connective tissue. A solid core of ECs is then formed, and finally; lumen formation takes place proximal to the migratory front and reconnection of the adjacent tubular sprouts occurs in order to form functional capillaries. Formation of basement membrane and recruitment of pericytes occurs simultaneously during the final step of the process [37, 38].

Intussusceptive (splitting) angiogenesis: It was first observed in neonatal rats. This mode of angiogenesis operates in order to expand the capillary bed in size and complexity (intussusceptive microvascular growth). The process starts with the initial formation of a zone of contact between two capillary walls. Following which endothelial cell junctions are reorganized, and the vessel bilayer is perforated in order to facilitate the entry of growth factors and cells into the capillary lumen. Then, a core is formed at the zone of contact between the two vessels that is charged with pericytes and myofibroblasts. Thereafter, these cells start laying collagen fibers in the core area to lay the platform for an extracellular matrix (ECM) necessary for the growth into the vessel lumen. It is interesting to mention here that intussusceptions allow a significant increase in capillaries without a proportional increase in the number of ECs [39, 40].

7.2.3 Mechanisms of Vessel Formation

Apart from sprouting and spitting angiogenesis, nascent blood vessels are formed through several alternative mechanisms under the control of distinct arterio-venous differentiation signals. Table 7.4 briefly summarizes mechanisms for blood vessel generation. It is to be noted here that vascular co-option and vascular mimicry are exclusively used by tumor cells in order to form capillaries.

7.2.4 Roles of Endothelial Cells (ECs) in Angiogenesis?

Endothelial cells play a central role in the control of vascular function. Endothelial cells (ECs) arise from the splanchnopleuric mesoderm and internally line the blood vessels thereby erecting an anticoagulant barrier between the capillary wall and

Table 7.4 Alternative modes of blood vessel generation

Modes	Brief description	References
Vasculogenesis	Differentiation of angioblasts into endothelial cells, which accumulates into a vascular labyrinth in the developing embryo. Vasculogenesis is initially responsible for the formation of the first blood vessels in an embryo and thereafter angiogenesis takes over to form majority, if not all, blood vessels during development, adulthood and in disease such as cancer	Flamme et al. [31], De Spiegelaere et al. [40], Carmeliet and Jain [46]
Arteriogenesis	Process of endothelial cell channel covering by pericytes or vascular smooth muscle cells (VSMCs) thus providing stability and control over perfusion dynamics	
Vascular co-option	Tumor cells can grow to a certain extent without triggering any angiogenic response by colonising the existing vessel architecture; as the tumor grows in size the blood vessels are completely engulfed by the tumor. It is mainly observed during initial tumor growth	
Vascular mimicry	Tumor cells acquire uptake an endothelial phenotype and are armed to form their own capillaries their own. As tumor cells mimic the behaviour of endothelial cells, the capillary networks are formed, comprising of tumor cells rather than the regular endothelial cells	
Intussusceptive arborization	Formation of highly structured pillars within the developing capillary network, which leads to the formation of vascular trees	
Intussusceptive branching remodeling	Pillars formed at the branching angle of the vessels either remodels the vascular geometry or leads to vascular pruning	

blood. It is known to have diverse functionalities comprising of both basal and inducible metabolic and synthetic functions. Indeed, ECs can respond to a wide repertoire of both physical and chemical stimuli and can critically modulate homeostasis, vasomotor tone, immune and inflammatory responses, cellular adhesion, thrombo resistance, smooth muscle cell proliferation. In addition, ECs are central to the process of angiogenesis and vasculogenesis and commencement of sprouting during vessel formation prerequisites the specification of ECs into tip and stalk cells with different morphological and functional parameters [38, 41–43].

Tip, Stalk and the Phalanx Phenotype

Tip endothelial cells are found to operate at the leading edge of an emerging vessel and are the first specialized ECs type within a sprouting vessel. In contrast, stalk cells generally trail behind the tip cells during the sprouting phenomenon and readily form the stalk of the sprout. Notch signals arising out of the tip cells readily attenuate the vascular endothelial growth factor (VEGF)-induced expression of Dll4 on stalk cells thus allowing the tip cells to maintain their forward post during active

sprouting. Tip cells are polarized and have the ability to migrate but proliferate minimally, in comparison to endothelial stalks, which has been optimal proliferating capacity. Tip cells have numerous filopodia that serve to guide the newly formed capillary toward the direction of an angiogenic stimulus in comparison to the stalk cells, which produce fewer filopodia, form tubes and branches and proliferate rapidly during the extension of the sprout in order to form the nascent vascular lumen cell. Tip cells express increased levels of Dll-4; platelet derived growth factor-b (PDGF-b), receptors for axon guidance cues, such as the Netrin receptor unc-5 homolog b (UNC5b), CXCR4, neuropilin-1 VEGF receptor-2 (VEGFR-2), and VEGFR-3/Flt-4, while maintaining minimal notch signaling activity. Ang-2 receptor and Tie-2 is expressed in endothelial stalks but not found to be expressed in tip cells. Interestingly, endothelial tip cells can readily adopt a unique "phalanx" phenotype that resembles the phalanx formation as used by the earlier century Greek soldiers. The "phalanx" phenotype is normally observed during the transition from conditions of active sprouting to quiescence tip cells. The cells displaying a "phalanx" phenotype (cobblestone-shaped morphology) are lumenized, non-proliferating, and immobile, which promotes vessel integrity and stabilizes the newly formed vasculature through increased cell adhesion and diminished response to VEGF. Phalanx cells display higher levels of soluble and membrane-bound Flt-1. Flt-1 is a well known to mitigate the pro-angiogenic signals of VEGF thus enabling the phalanx population to maintain a stable morphology. In addition, the expression of VE-cadherin, which tightens the EC-to-EC adhesions, also aids in the phalanx cells in adopting a more quiescent behavior [38, 44, 45].

7.2.5 Growth Factors Involved in Angiogenesis

VEGF (Vascular Endothelial Growth Factor)

Vascular endothelial growth factor (VEGF) also known as a vascular permeability factor (VPF) was originally reported as an endothelial cell-specific mitogen. It is secreted by a panel of cells such as tumor cells, macrophages, platelets, keratinocytes, and renal mesangial cells. VEGF also plays critical roles during bone formation, hematopoiesis, wound healing, and development. VEGF family comprises mainly of functionally non-redundant members; of which, the central component VEGF-A participates in the signal transduction and activates the phenomenon of angiogenesis by interaction with VEGFR-2 (FLK1). NRP1 and NRP2 operate independently and act as co-receptors of VEGF signalling. VEGF isoforms facilitates vessel enlargement, whereas matrix-bound isoforms stimulate branching. VEGF (autocrine) released by ECs maintains vascular homeostasis whereas VEGF (paracrine) secreted by tumors, myeloid or stromal cells lead to increased vessel branching and form vessels with a disturbed architecture and dynamics. VEGF-C, acts as a ligand for VEGFR-2 and VEGFR-3 activity thus initiates tip cells. It moderates the formation of the vasculature during early embryogenesis, and later acts as a

regulator of lymphamogenesis; the formation of new lymphatic vessels from pre-existing ones. VEGF-B has limited angiogenic capabilities in certain tissues such as the heart, but it can facilitate neuronal survival and induce metabolic effects. VEGF-B also has an articulate role in pathological angiogenesis, where it promotes the growth of cardiac vessels [46, 47].

VEGF receptors VEGFR-1, VEGFR-2 and VEGFR-3 belong to the tyrosine-kinase receptor family and are activated by all the VEGF isoforms. Both the receptors VEGFR-1 and VEGFR-2 are glycosylated. However, only the final glycosylated form of VEGFR-2 undergoes autophosphorylation in response to VEGF signals. These receptors form a subfamily characterized by the presence of seven immunoglobulin-like loops in their extracellular domain and a split tyrosine-kinase domain in their intracellular architecture VEGFR-2 and VEGFR-1 receptors are normally expressed in endothelial cells, but other cells could also express these receptors; VEGFR-1 is expressed in trophoblasts whereas VEGFR-2, in hematopoietic stem cells, megakaryocytes, and retinal progenitor cells. In addition, cancer cells also have the innate tendency to express VEGFR-1 or VEGFR-2. Both the receptors can participate in signal transduction mediated by other growth factors belonging to the VEGF family, but only the VEGF isoforms are equipped to bind to VEGFR-1 and VEGFR-2 [48–50].

PDGF (Platelet Derived Growth Factor)

PDGF, a 30 kDa dimer composed of an A- and/or B-chain, was initially extracted and studied as a potent mitogen and chemotactic factor for fibroblasts and all cells of mesenchymal origin, including chondrocytes and mesenchymal stem cells. Genes located on chromosomes 7, 22, 4, and 11 codes for each chain of PDGF. Interestingly, all four PDGF chains contain a highly conserved growth factor domain of approximately 100 amino acids in parallel to the VEGF family. PDGF can act as a mediator of meniscal cell proliferation and migration. It is secreted primarily by platelets but other cells like endothelium and smooth muscle also can act as PDGF sources. Human PDGF comprises of several dimeric forms that are produced from PDGF genes -A, -B, and most recently elucidated C and -D. PDGF plays critical roles during embryonic development and also acts as a stimulator of wound healing. The loss of any PDGF ligand or receptor gene is extremely lethal, and it has recently been reported that mice lacking PDGF receptor expression are vulnerable to traumatic defects in lungs, kidneys, vessels, placenta, brain, and skeleton. In addition, PDGF over expression can lead to several fibrotic disorders and malignancies. PDGF is produced in response to external stimuli like, exposure to low oxygen tension, thrombin, or stimulation by other cytokines and growth factors. PDGF can also function as an autocrine stimulator of tumor cells, a regulator of interstitial fluid pressure, and most importantly as a dynamic modulator of angiogenesis [51–55].

PDGF receptors belong to the type III receptor-tyrosine kinase (RTK) family of receptors. The receptor comprises of five extracellular immunoglobulin (Ig) loops

and a split intracellular tyrosine kinase (TK) domain. Other members of the family with a similar structural prototype include c-KIT, c-Fms, FLT3, receptors for CSF-1, SCF, and Flt3-ligand and the macrophage-colony-stimulating factor receptor. PDGF receptors α and β, are encoded from two highly homologous genes. These receptors have specific binding preferences; the isoform binds to all the ligands barring PDGF-D, whereas, PDGFRβ binds to PDGF-B and -D alone [52, 56].

FGF (Fibroblast Growth Factor)

The last of the major growth factors involved in angiogenesis is the fibroblast growth factor (FGF). Fibroblast growth factors (FGFs) belong to a family of structurally related polypeptides that are essential during embryonic development and which, operates postnatally as homoeostatic factors, which enables wound healing following trauma. In addition, FGFs also plays a critical role in cellular proliferation, survival, migration, and differentiation. FGFs have been identified in metazoans, but their presence in unicellular organism is a matter of debate. Human FGF comprises of approximately 22 members (Fgf-1-Fgf-23) except Fgf-15, which has not been characterized in humans. bFGF was among the first discovered angiogenic factors just like FGF1, can modulate angiogenesis and arteriogenesis. In addition, FGF9 can also stimulate angiogenesis during bone repair FGFs can have intracrine, paracrine and endocrine functionalities. The paracrine and endocrine functionalities are mediated via FGF receptors (FGFRs), which are expressed on the cell surface. However, the intracrine module of FGF operates independent of the receptors [46, 57, 58].

Besides these all growth factors, Epidermal Growth Factor (EGF) and Transforming Growth Factor-β1 (TGF-β1) also induce angiogenesis. EGF is responsible for Growth, migration, and tube formation to evaluate the direct and indirect effect in the angiogenic process. VEGF has protective role on endothelial cells from apoptosis; whereas, TGF-β1 induces apoptosis. Thus it signifies TGF-β1 has been opposing effect on endothelial cells. TGF-β1 mediated angiogenesis needs a rapid and transient apoptotic effect, facilitated by VEGF/VEGFR2. These major growth factors are involved in the angiogenesis process through different signalling cascade. The major signalling pathway and their associated growth factors have been listed in Table 7.5.

7.2.6 Molecular Signature of Angiogenesis

In addition to the growth factors discussed above, there is a participation of wide repertoire of molecules, which collaborate in order to accomplish the angiogenic program. Table 7.6 shown below presents a comprehensive list of those angiogenic determinants and their associated functionality.

Table 7.5 Major Angiogenic factors and their associated signaling cascade

Angiogenic factors	Cancers	Associated signaling pathway	References
VEGF	Breast	ERK and Akt	Presta et al. [145]
	Lung	PI3K/Akt, c-Jun and NF-κB	Zhang et al. [71, 72]
	Prostate	FAK/Akt/NF-κB	Chen et al. [146]
	Colon	PI3K/Akt	Jeong et al. [147]
FGF	Breast	AKT- mTOR, RAS-MAPK and Wnt	Abolhassani et al. [148]
	Lung	RAS-MAPK and PI3K/Akt	Salgia [149]
	Prostate	RAS-MAPK, PI3K and phospholipase Cγ	Corn et al. [150]
	Colon	RAS-MAPK and Akt	Schulze et al. [151]
TGF	Breast	ERK and PI3K/Akt	Ren et al. [152]
	Lung	Akt and p38	Wang et al. [153]
	Prostate	m-TOR, Ras, MAPK, PI3K/Akt, PP2A/ p70s6K, and JNK	Lee et al. [76, 77]
	Colon	Ras/MAPK, PI3K/Akt, TGF-β and JNK	Xu and Pasche [154]
EGF	Breast	PI3K/Akt/mTOR/MMP, RAS/MAPK, JNK, phospholipase Cγ, and LIMK/cofilin	Li et al. [155]
	Lung	PI3K/Akt and Ras/MAPK	Scagliotti et al. [156]
	Prostate	PI3K/Akt/ERK, mTOR, and PKC/GSK-3β	Bhat et al. [157]
	Colon	PI3K/Akt and NF-κB	Ien et al. [158]

7.2.7 Mechanism of Angiogenesis

As mentioned earlier, the developing embryo forms a primary vascular plexus initially by the mechanism of vasculogenesis, thereafter; both sprouting and non-sprouting angiogenesis becomes operational to generate blood vessels and the entire functional adult circulatory system [59]. Herein, we discuss the sequential steps involved during the phenomenon of vessel branching [46, 60–62].

Endothelial cells maintain the stage of quiescence: Quiescent endothelial cells are able to maintain longer half-lives in a healthy adult, owing to the operation of an autocrine signaling cascade involving VEGF, Notch, angiopoietin-1 (ANG-1), and fibroblast growth factors (FGFs). In addition, the endothelial mass express oxygen sensors and hypoxia-inducible factors viz. prolyl hydroxylase domain 2 (PHD2) and hypoxia-inducible factor-2α (HIF-2α), which respond to various signals (environmental and physiological) and accordingly allow the vessels to modify their architecture in order to maintain optimal blood flow. During the quiescent stage, streamlined ECs adopt a phalanx phenotype, maintain their pericytic coverage and are interconnected by junction adhesion molecules VE-cadherin and claudins. Pericytes retard EC proliferation, in addition, release cell-survival signals (VEGF and ANG-1), and together with the monolayer of ECs comprise the basement membrane during the stage of quiescence. The basement membrane is 100–200 μm thick and is located immediately below the monolayer of endothelial cells in the arterial

Table 7.6 Molecules involved in the process of angiogenesis and their functional significance

Tip cell specification	VE-cadherin	Loosening of endothelial cell-cell junctions	Carmeliet and Jain [46], Karamy sheva [159], Klagsbrun and Moses [160], Ucuzian et al. [62]
	MMPs	Reorganization of the matrix	
	VEGFR-2, Dll-4, JAG-1, NRP-1, integrins, HIF-1α, MT1-MMP, PGC-1α	Formation of the tip cell	
	VEGF, VEGF-C, FGFs, ANG-2, and Chemokines	Angiogenesis stimulating factors	
	ANG-2	Detachment of pericytes	
	VEGF	Permeability, vasodilation and extravasation	
Stock cell elongation and tip cell guidance	Semaphorins, ephrins, integrins	Guidance and adhesion of the tip cell	
	VEGFR-1, Notch, Wnt, NRARP, PIGF, FGFs, EGFL 7	Elongation of the stock	
	VEGF, FGFs	Release of angiogenic factors from the ECM	
	PDGF-B, ANG-1, Notch, ephrin-B2, FGF	Recruitment of pericytes	
	VE-cadherin, CD34, sialomucins, VEGF	Formation of the lumen	
	ANG-2, SDF-1α, PIGF	Recruitment of myeloid cells	
Quiescent phalanx resolution	VEGF-B	Transport of transendothelial lipids	
	TIMPs, PAI-1	Deposition of the basement membrane	
	VEGF, ANG-1, FGFs, Notch	Maintenance of the vasculature	
	VE-cadherin, ANG-1	Barrier formation	
	PDGF-B, PDGFR-β, ephrin-B2, ANG-1, Notch, TGF-β1	Maturation of pericytes	
	PHD2, HIF-2α, VE-cadherin, TIE-2	Phalanx phenotype	

intima. Major components of the basement membrane include laminins, type-IV collagen, type-VIII collagen, and proteoglycans.

Sensing the angiogenic signals and endothelial sprouting: Quiescent endothelial cells are capable of sensing a panel of factors (see Table 7.3), which act as angiogenic triggers and in response to those stimuli, ECs enter into a stage of active sprouting categorized by high mitotic index, increased migratory potential and matrix degradation. In addition, pericytes detach themselves from the basement

membrane (specifically in response to ANG-2) by proteolytic degradation assisted by matrix metalloproteinases (MMPs). Afterwards, the tight junctions, adherens junctions, and gap junctions present between the intimal ECs and perivascular cells are lost thus allowing the ECs to stalk into the basement membrane and the surrounding milieu. In fact, VEGF augments the permeability of the endothelial cell membrane, which leads to extravasation of several plasma proteins in order to erect a temporary extracellular matrix (ECM). Endothelial cells once liberated from the capillary intima, proliferate, migrate, and are routed in the direction of the angiogenic stimulus in a 3-D extracellular environment thereby giving rise to fresh angiogenic sprouts. Several proteases operate within the ambit of the ECM in order to facilitate the release of angiogenic variables, which are accountable for remodelling the matrix in order to provide an optimal angiogenic ambience.

Endothelial cells split into tip and stalk phenotypes: In order to form a perfused tube with appropriate vessel dynamics, it is therefore, critical for the endothelial mass not to progress the angiogenic signal in unison. However, to prevent this activity from occurring, one endothelial cell is chosen amid the populace to act as leading or tip cell. The cells bordering the tip cell assume secondary positions and are known as stalk cells; these cells divide repeatedly to elongate the stalk and thereby form the vessel lumen. Tip cells are directionally guided by ephrins and semaphorins whereas stalk cells release EGFL7, which is compulsory for stock elongation. Myeloid cells facilitate the fusion of the newly formed vessel with another branch vessel, which is necessary for initiation of blood flow.

Lumen and Tube formation: Lumenogenesis (formation of the lumen) and Tubulogenesis (formation of tubes) are significant phenomenon observed during the process of angiogenesis. The ECs are genetically capable of building luminal compartments, which allows the flow of blood from pre-existing to the newly formed vasculature. The most widely investigated mechanism for the same is intracellular vacuolization (or intracellular canalization); a phenomenon mediated via α2β1 integrin and members of the Rho GTPase family. ECs activate pinocytic mechanisms in order to form a number of intracellular vacuoles; these vacuoles fuse together to form one large intracellular lumen. The protein caveolin-1, which is known to be a key player during receptor-mediated endocytosis and patterning of the caveolae (invagination of the cell which regularly occurs before pinocytosis and subsequent vacuole formation).

Transition to quiescent state and adopting the phalanx phenotype: Once the condition of active sprouting is over and new vessels are formed, the endothelial cells driven by signals (see Table 7.3) revert to the phalanx stage. Platelet-derived growth factor B (PDGF-B), ANG-1, transforming growth factor-β (TGF-β), ephrin-B2 and NOTCH act together in order to render a pericytic covering to the endothelial cells, following which, tissue inhibitors of metalloproteinases (TIMPs) and plasminogen activator operate to lay the basement membrane and reestablish the dismantled junctions to ensure proper vessel dynamics and flow. Vessels, which lack proper perfusion, normally regress.

7.2.8 *Epigenetic Modifications of Major Angiogenic Growth Factors*

Vascular endothelial growth factor A (VEGF-A) is crucial for the differentiation of endothelial cells. Moreover, its importance in various pathological states such as cancer, retinopathies, inflammation, and arthritis is well documented. VEGF-A activities are mediated through two tyrosine kinase receptors, including VEGFR1 (Flt-1) and VEGFR2 (KDR). The expression of VEGF-A gene is firmly regulated at multiple levels. Recently, it has been revealed that VEGFR1 and VEGFR2 are regulated by an epigenetic mechanism in most of the cancer, including stomach, colon, and hepatocellular carcinoma. Epigenetic state of the VEGF-A promoter can be manipulated by using promoter-targeted small RNAs and this result in either increased or decreased VEGF-A expression. This epigenetic change in VEGF-A could be possible, mostly through changes in the histone code rather than DNA methylation. VEGF-A also induces epigenetic reprogramming of the promoter regions of Rex1 and Oct4 genes. Rex1 gene is important for proliferation, differentiation and exhibits gene control in developing embryos via its epigenetic control on genes, for instance, PEG3, which has been found to play a key role in fetal growth. On the other hand, Oct4 expression is associated with an undifferentiated phenotype and tumors. Upon treatment with VEGF-A, methylation patterns in promoter of both genes (Rex1 and Oct-4) were diminished in endothelial progenitor cells. VEGF-A expression can therefore lead to epigenetic modifications in promoters of these genes [5]. It has been revealed that VEGF-mediated reduction in miR-101 expression causes pro-angiogenic effects that are mediated through reduced repression by miR-101 of the histone-methyltransferase EZH2. This results into increasing methylation of histone H3 at lysine7 and transcriptome alterations. Furthermore, in the tumor vasculature, increase in endothelial histone-methyltransferase EZH2 is a direct result of VEGF stimulation by a paracrine circuit that stimulates angiogenic states by methylating and silencing vasohibin1 (vash1). This Vash1 gene expression is mainly associated with colorectal cancer [63].

Epigenetic alteration plays an important role in signal transduction and gene-gene interaction. For instance, FGFR2b and Estrogen regulate the activity of melanoma-associated antigen (MAGE-A3), which then controls p53 and p21 activity in pituitary tumors. This regulation is occurred via both DNA and histone modification. Further, FGFR2 allows fibroblast growth factors (FGFs) to transmit signals, which are involved in cell differentiation and proliferation, and is down-regulated by both epigenetic and genetic mechanisms in breast cancer. This is happened due to the presence of Loss of Heterozygosity (LOH) and DNA methylation in specific regions of FGFR2 which determine breast cancer progression by loss of the gene or by limiting transcriptional process. Importantly, epigenetic changes and genetic sequence alterations can simultaneously lead to cancer and other diseases. Fibroblast growth factor receptors (FGFRs) are also dysregulated in a number of developmental and neoplastic conditions. Genome-wide association studies have identified single nucleotide polymorphisms (SNPs) within intron 2 of FGFR2 as a locus is

associated with increased risk of breast cancer [6]. Recently, Rao and colleagues have reported that ischemic injury also provokes DNA methylation process in several genes, which is critical for angiogenesis and endothelial cell survival. This DNA methylation process is sensed by MBD2 protein, which mediates transcriptional repression of genes involved in angiogenesis and endothelial cell survival. This report makes a connection between epigenetic alterations in vascular cells to angiogenesis and paving the way for new therapies that could ameliorate perfusion in patients with vascular disease.

7.2.9 Major Signalling Pathways Involved in Angiogenesis

ANG/TIE Signaling

Angiopoietins are approximately 70 kDa-secreted secreted glycoproteins, which can be structurally characterized by the presence of an amino-terminal half with coiled. However, these coil domains are necessary for ligand oligomerization. The most widely studied angiopoietins are Ang-1 and Ang-2. Ang-1 and Ang-2 can bind to Tie-2 (for tyrosine kinase with immunoglobulin and EGF-like domains) receptors expressed on the EC surface; however, the binding of Ang-1 alone facilitates angiogenesis and induces the restructuring and stabilisation of blood vessels. In addition, Ang-1 critically modulates vessel maturation, migration, adhesion and survival of endothelial cells, in contrast; Ang-2 acts to destabilise the connections between the endothelium and perivascular cells and promotes cell death and vascular regression and acts as a natural antagonist to Ang-1. However, Ang-2 can also promote neo vascularisation in union with VEGF. Therefore, clearly angiopoietins exert dominant effects in the angiogenic switch and up regulated expression Ang-2 in comparison to Ang-1 has been shown to associate strongly with tumor progression and poor prognosis.

As mentioned earlier, angiopoietins bind to Tie receptors, which are specifically expressed on the vascular endothelial surface and on macrophages deployed during angiogenesis. Tie receptors (Tie-1 and Tie-2) are tyrosine kinase receptors, which have critical functionalities in vascular maturation during development, and both during physiological and pathological angiogenesis. Ang 1-4 are bonafide ligands of the Tie-2 receptor, as a subtle coincidence, Tie-1 remains an orphan receptor. Tie-2 contributes by heterodimerizing with Tie-2 and thereby facilitating Tie-2 signal transduction. Binding of angiopoietins to Tie-2 is facilitated by fibrinogen-related domain (FReD), which is located in close proximity to a 20 residues short linker sequence. FReD comprises of three sub domains namely A, B and P; of which, P mediates the interaction of FReD containing proteins viz. Fibrinogen, tachylectin 5A and angiopoietins with their respective ligands.

Angiopoietin-like proteins (Angptls) have structural similarity with angiopoietins and are coded from seven genes, Angptls 1-7, with all inheriting N-terminal coiled-coil domains and a C-terminal fibrinogen-like domain, which are characteristic

angiopoietin signatures. Interestingly, Angptls do not bind to either of the Tie receptors and therefore, remain orphan ligands. Angptls 1, 2, 3, 4, and Angptl6/angiopoietin-related growth factors have critical roles in modulating angiogenesis and regulating lipid, glucose, and energy metabolism independently of angiogenic effects [61, 64–66].

Notch Signalling

Notch signaling operates as a crucial angiogenic switch during development, wound healing and pregnancy (physiological angiogenesis), tumor angiogenesis (pathological angiogenesis), and in embryonic vascular development and tumor growth. Notch signalling cascade, therefore has been a subject of pristine interest in recent years towards formulating novel anti angiogenic cancer therapies. Delta-like ligand 4 (DLL4) is a ligand of the notch signalling cascade that operates as a negative regulator of tumor angiogenesis via the Notch-Dll-4 signalling axis. Notch-Dll4 axis serves as an intersection point between pro angiogenic and metabolic signalling prototypes in endothelial cells. Notch directly regulates the expression prototype of the VEGFR transcript and in EC cell lines with activated Notch signaling display a fall in expression level of VEGFR-2 (Kdr). In contrast, DLL-4 haploinsufficient neonatal mice display augmented levels of VEGFR-2 in the retinal vasculature. The angiogenic behavior of endothelial cells within developing blood vessel sprouts is limited by VEGF activity, which in turn is regulated via Notch. Furthermore, Notch signaling cascade is reiteratively utilized during the entire duration of angiogenesis. During the process of angiogenesis in tumors, DLL4 provides a negative feedback on VEGFR2 activity. Recent evidences gathered from various cancer studies predominantly highlight the role of Notch-1 in regulating tumor angiogenesis. However, Notch-3 also can mediate vascular development, remodeling and maturation and most recently it was observed that Notch-3 and the monomeric C-reactive protein (mCRP) together operate via the PI3K/Akt pathway in order to promote angiogenesis. Experiments on both zebrafish and mouse suggest that VEGF-2 and Notch-3 collaborate significantly in order to modulate cell behavior during the process of angiogenesis. In fact, this can be attributed to the ability of ECs within a developing blood vessel to sprout and utilize the Notch cascade in order to modulate cellular phenotypes during angiogenesis. Notch receptors are particularly expressed in “connector” cells and on cells lining the stable blood vessels such as the dorsal aorta. Notch cascade is triggered in these cells through interaction with DLL-4 expressed on the surface of tip cells. As a consequence, aberration in Notch signaling or loss in DLL-4 expression results in an increase in endothelial cells with tip cell signature phenotype in both mouse and zebrafish embryos. Interestingly, cells in zebrafish with activated Notch signaling reportedly do not contribute to vessel formation during angiogenesis. These observations indicates that cells with activated Notch signaling remain in the patent vessel, whereas, cells displaying a dampened Notch activity have the tendency to leave the patent vessel [33, 34, 67–72].

Interestingly, Notch functions as a "double faced angiogenic switch" and recent evidence strongly supports the hypothesis that the cascade has both the pro and anti angiogenic effects. Fatty acid binding protein 4 (FABP4) is a known mediator of VEGFA effects. Notch-DLL4 signalling can directly activate FABP4 independent of VEGFA participation and thereby display pro angiogenic effects. However, the activation of FABP4 following stimulation by VEGFA and/or Notch-Dll4 cascade, requires the involvement of the transcription factor FOXO1. It is therefore, evident that Notch signalling and FOXO1 mediated control of endothelial gene transcription are crucial determinants of angiogenesis. Notch signalling pathway also has the contrasting ability to limit sprouting angiogenesis by reducing the expression level of the pro angiogenic mediator Sox-17. Sox-17 is known to facilitate endothelial migration by destabilizing endothelial junctions and rearranging cytoskeletal structure and by upregulating several tip cell specific genes. The angiogenic effects of Notch signalling activation in GBM (Glioblastoma multiforms) stem cells is associated with a reduction in their growth and migratory potential, downregulation of neural stem cell transcription factors (ASCL1, OLIG2, SOX2) and upregulation of HEY1/2, KLF9, and SNAI2 transcription factors. Notch intracellular domain (NICD) expression in GBM stem cells facilitates the expression of pericyte cell markers NG2, PDGFRβ and α-smooth muscle actin (αSMA) and the induction of angiogenic factors such as HB-EGF, IL8, PLGF, MMP9, VCAM-1, ICAM-1, and ITGA9. Epidermal growth factor-like domain 7 (EGFL7) in parallel with secreted and crucial angiogenic factors such as VEGF and FGF-2, is suitably expressed in endothelial cells (ECs). Probable theories suggest that EGFL7 exerts its angiogenic functionalities by interfering with the Notch pathway and/or by discrete interaction with miR-126. DLL4 overexpression in human endothelial cells (ECs) ablates the effect of IFNγ in those cells. IFNγ is a cytokine and a key mediator of angiostatic response and the observed angiostatic effects of IFNγ could be due to dampening of the DLL4/Notch signal. Thus, the role of IFNγ in ECs is crucial for maintaining tumor angiostasis, which critically involves DLL4 down regulation [73–78]. Regulation of endothelial cell biology by the signaling pathway (Notch) is also necessary for vascular development, homeostasis, and sprouting angiogenesis. Krüppel-like factor (Klf) family of transcription factors act as modulators of endothelial cell biology. Krüppel-like factor 4 (KLF4) regulates sprouting angiogenesis via regulation of Notch activity by retarding the process of cleavage and subsequent Notch-1 activation and by differentially modulating the expression prototype of Notch components (receptor, ligands and target genes). In addition, Notch signaling necessarily mediates critical aspects of tumor angiogenesis as triggered by the vascular endothelial growth factor (VEGF). DLL4 has a major role in the process, and it was suggested that blocking DLL4 inhibits tumor growth and consequently, increases endothelial cell sprouting, but vessels formed lack perfusion. Indeed, this could be due to a significant reduction in the endothelial nitric oxide level caused primarily due to inhibition of the DLL4-Notch signalling module and can be a probable factor behind reduced vessel integrity [79, 80].

αv Integrin Signaling

Integrins are transmembrane receptors, which have the ability to bind to extracellular matrix proteins, or other adhesion receptors expressed on neighboring cells. Substrate binding specificity of integrin receptors is facilitated via heterodimeric pairing of α and β subunits. In fact, the αv subunit pairs with β1, β3, β5, β6, and β8. These pairings can preferentially bind a single ligand (αvβ5 for vitronectin) or recognize a number of ligands (αvβ3 binds vitronectin, fibronectin, vWF, tenascin, osteopontin, fibrillin, fibrinogen, and thrombospondin). Endothelial cells undergoing active angiogenesis and remodelling and pathological tissues most commonly display integrin αvβ3 on their surface. A transcriptional activator, Hox D3, mediates the expression of αvβ3 on these cells. However, the efficacy of αvβ3 as a marker of angiogenic endothelial cells is not limited to cancer as this is a common feature associated with the activation of ECs and therefore, activated EC population are highly sensitive to inhibition of αvβ3 most commonly during wound repair, arthritis and proliferative diabetic retinopathy. It is interesting to note here that despite the appearance of αvβ3 on all angiogenic endothelial cells, the participation of αvβ3 in other angiogenic signalling cross talk is not necessarily generic. Angiogenesis induced by basic fibroblast growth factor (bFGF) or tumor necrosis factor α (TNF-α) requires the involvement of αvβ3, whereas integrin αvβ5 is required for processes induced by vascular endothelial growth factor (VEGF) or transforming growth factor α (TGF-α). The striking difference in the functioning of β3 and β5 can be attributed to the ability of these integrins to differentially activate the Ras/Raf/MEK/Erk pathway in capillaries.

αvβ5 integrin pathway operating downstream of VEGF leads to the activation of focal adhesion kinase (FAK) and Src kinase, whereas αvβ3 pathway activates p21-activated kinase (PAK). αvβ5 also plays a major role in endothelial cell protection from apoptosis (intrinsic pathway), independently of MEK-1. This apoptotic protective module in EC results from αvβ5 mediated activation of Raf on serines 338/339, which leads to Raf-1 mitochondrial translocation. Conversely, αvβ3 protect the EC from extrinsic mediated apoptosis by activating Raf on tyrosines 340/341. However, αvβ3 signal activation requires the participation of MEK-1 [81].

Wnt Signaling

Cellular proliferation and polarity, apoptosis, branching morphogenesis, inductive processes, and the maintenance of stem cells in an undifferentiated, proliferative state are key cellular prototypes under the control of Wnt signaling. A Wnt/Frizzled signaling pathway also modulates vascular growth, endothelial proliferation, survival and migration in mammals and hence controls the phenomenon of angiogenesis. Wnt signals are propagated by the transcriptional activity of β-catenin and in the absence of ligand-receptor interaction; the cytoplasmic β-catenin is subjected to degradation by the phosphodestruction complex formed by adenomatous polyposis coli (APC), GSK3β and few other proteins thus resulting in repression of Wnt

downstream targets. However, in the event of Wnt binding to the receptor Frizzled, β-catenin is preserved from degradation by the complex. The stabilized β-catenin is trafficked into the nucleus where it interacts with T-cell factor (TCF)/Lef transcription factors and activates transcription of target genes. β-catenin can also stabilize cell-to-cell adhesion and tissue integrity by binding to VE- and N-cadherins at the cellular junctions.

β-catenin initiates the transcription of DLL4, which in turn, activates Notch-1 and -4; this interaction plays a central role during embryonic vascular development. Moreover, β-catenin overexpression is associated with alterations in vascular morphology characterized by endothelial cell arterialization and lack of venous specificity. Notch and Wnt pathway operate synergistically in regulating EC differentiation and vascular morphogenesis. This is facilitated via the formation of a transcription complex comprising of β-Catenin, RBP-J and intracellular domain of Notch (NICD); the complex in upregulates the expression of arterial genes. Nrarp is a transcription factor activated via Notch signaling in the retina vasculature. Nrarp can inhibit Notch activation and at the same time increase Wnt signaling by stimulating the transcription factor Lef-1. Th pro angiogenic transcription factor Sox-17 which is required for Norrin/Frizzled-4/Lrp-mediated angiogenesis in a 3D matrix gel is a target of both the Notch and Wnt signaling pathways. Gain-of-function mutation of Frizzled-4 results in diminished levels of Sox-17.

However, the exact mechanism of Sox-17-β-catenin mediated transcriptional activity during angiogenesis in vivo is still a subject of immense curiosity. HIF-1α is a potent angiogenic trigger of vascular endothelial growth factor (VEGF) activity. HIF-1α competes with TCF4 in order to bind to β-catenin. B-Catenin/HIF1α interaction occurs at the promoter regions of HIF1α target genes thereby increasing their expression. In addition, Rspo1-Wnt-Vegfc-Vegfr3 signaling pathway plays a central role in developmental angiogenesis. R-spondin1 (rspo1) is a Wnt signaling regulator and the catalogued pro-angiogenic effects of Rspo1/Wnt signaling are mediated by Vegfc/Vegfr3 (Flt4) signaling. Actually, Vegfc expression is dependent on Rspo1 and Wnt, and Vegfc and Vegfr3 acts as a mediator of angiogenesis downstream of Rspo1-Wnt axis. All these molecules are active during the dynamic stage of endothelial sprouting and Rspo1-Wnt-VegfC-Vegfr3 signaling cascade plays a crucial role as an endothelial-autonomous permissive cue for developmental angiogenesis. These observations altogether strongly suggest that Wnt transcriptional activity is controlled in a cell-context manner and the interaction of Wnt pathway components with other angiogenic transcriptional factors indeed determines the functional phenotype of the cell during the entire duration of neo-vasculation [82–84].

Shh Cascade as an Indirect Angiogenic Switch

The last of our discussion on major angiogenic signaling pathway focuses on the Shh (sonic hedgehog) cascade. The entire panel of Hh (hedgehog) proteins viz. Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh) act as morphogens for a wide range of cells during embryonic development. Shh cascade

is signalled via the interaction between Shh and the receptor Patched-1 (Ptch-1); on activation, Ptch-1 consequently, represses the Ptch-1-mediated inhibition of Smoothened (Smo). This leads to the activation of Gli transcription factors, which subsequently control the expression of Shh downstream targets. Shh cascade is known to act as an indirect activator of EC function's evidence supporting the direct involvement of the cascade in angiogenesis is lacking.

Nevertheless, the role of Shh in vaculogenesis has been investigated, and it was observed that transgenic over expression of Shh in the dorsal neural tube of mouse embryos leads to hypervascularization of the neuroectoderm. Zebrafish deficient in Shh levels, display muddled endothelial precursors, which are incompetent to form the dorsal aorta or axial vein, whereas, Shh deficient mice develop lungs with the improper vasculature. The embryonic Shh cascade becomes operational in adults in response to ischemic injury, including hind limb-ischemia and myocardial infarction, and it was most recently observed that delivery of Shh into the traumatized cells promote neovascularization of ischemic tissue both by promoting angiogenesis and by controlling endothelial progenitor cell adhesion, migration, and proliferation. Shh can facilitate the release of pro-angiogenic factors VEGF and ANG-1 from Shh induced fibroblasts and cardiomyocytes, thereby highlighting the indirect role of Shh on vascular cells. Ptch-1 is not over expressed in corneal neovessel ECs and human umbilical vein endothelial cells (HUVECs) on Shh mediated activation; however, Shh augments capillary morphogenesis in both HUVECs and murine brain capillary ECs. Shh can activate the PI3K/Akt pathway in ECs, and in turn, cyclic AMP/protein kinase A axis can modulate the activity of Shh downstream transcription factor Gli-1. In addition, Shh also interacts with other crucial signaling components such as chicken ovalbumin upstream promoter transcription factor II (COUP TFII) in mouse embryonic carcinoma cells, extracellular signal-regulated kinases 1/2 (ERK 1/2) and protein kinase Cδ (PKCδ) in fibroblasts, Rho/Rho kinase (ROCK) pathway in neuronal cells, matrix metalloproteinase 9 (MMP-9), and osteopontin (OPN). Taken together, the function of Shh activity in mature endothelial cells and the intricate role of non-classical signalling pathways in Shh-mediated angiogenesis remain an obscure subject for active debate in future [85, 86].

7.3 Conclusion

The future of cancer therapies depends on the researchers' ability to adjust actions to circumstances and has a clear projection relating to the aberrant mechanisms that ultimately decide the fate of the pathogenesis and henceforth degeneration through various processes. Despite great advancement in the field of cancer therapies, still the future of such therapies hangs on morbid conjecture and fragile hopes. The distinct signalling axis presents us an interesting opportunity to exploit in pathology of cancers; furthermore, interrupting critical interactions of the pathways by using different epigenetic regulators can solve the "targeted therapy crisis" problem in cancer pathogenesis.

Angiogenesis plays a crucial role in cell proliferation, metastasis and thus leads to cancer pathogenesis. Since, epigenetic modification plays a key role in the regulation of all DNA-based processes, including DNA replication, transcription, and DNA repair. Thus, altered genome or irregular expression patterns in chromatin regulators can have intense results, which may lead to the trigger various tumor cells and also maintain the tumor cells. Here in this book chapter, we have also discussed about various genes which is mainly involved in epigenetic mediated regulatory process and also highlighted about several histone modifying enzymes, which have a potent role in transcription repression, DNA damage repairing, and also have a tumor suppressor like activity. At last, we have focused on various drugs such as butyrate, curcumin, and several others, which are critical for acetylation process in epigenetics. They mainly target HAT, HDAC, and SIRT activity in different cancers. However, further research is needed in order to make drugs/compounds in cancer therapeutics a blatant reality.

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Chapter 8
Epigenetic Post transcriptional Mutation in Neuro-Oncology

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8.1 Introduction

The term epigenetics was coined by Conrad Waddington (1942) as the branch of biology, which studies the causal interactions between genes and their products, and thus affecting the phenotype. It also describes changes in gene expression, which could not be attributed to DNA sequence [1]. These alterations are heritable mitotic changes and are reversible in nature [2]. Our body contains thirty to forty thousands of functional genes, and each cell of the body has same genetic information and despite this; different organ systems are regulated by specialized cells and tissues. For instance, the nervous system is characterized by neurons and glial cells; parietal and oxyntic cells control digestion, nephrons being the office bearers in excretory system, B and T-cells for expedition of inflammatory response through the immune system and myocytes for the production and transmission of signals in the body. This diversity and dynamicity are brought about by the expression or 'turning-on' and silencing or 'turning-off' of a set of genes, which are transcribed in the production or inhibition of particular proteins. In short, what we understand now is the

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differential expression, both within the different systems of the body as well as between individuals are the outcome of epigenetic manifestations. Epigenetic modifications can be brought about by the interaction of three systems.

8.1.1 DNA Methylation

Addition of a methyl group to specific sites of DNA is termed DNA methylation. A Cytosine nucleotide adjacent to Guanine and joined by a phosphate are called the CpG site and a hub of such CpG sites in the genome constitutes CpG Island (CPI). DNA methylation occurs on CPI [3, 4] and leads to altered shape and structure of the DNA, resulting in modified gene interaction and expression. The process ultimately accomplishes gene silencing in mammals. An enzyme DNA methyltransferases (DNMTs) is responsible for catalyzing methylation [5, 6]. In mammals, the pattern of DNA methylation during replication is dictated by 'maintenance of DNMTs' through addition of the methyl group at the hemi-methylated region, while, '*de novo* DNMTs' transfer methyl group involves in post-replication. DNA methylation can further be outlined as hypermethylation, hypomethylation and hemimethylation. Moreover, DNA methylation is responsible for the gene silencing on inactive X-Chromosome in mammalian females [7, 8], silencing of transposable elements and to maintain the genome stability [9, 10]. Interestingly, imprinting or differentiation between maternal and paternal genes can also be manifested through DNA methylation.

8.1.2 Histone Modifications

The DNA protein complex is primarily composed of histone protein where DNA reel around histones and post-translationally modifications in histones regulates transcription of chromosomal DNA. The decondensed chromatin is active and allows DNA transcription, whereas, heterochromatin or condensed chromatin being the dormant state inhibits DNA transcription. Histone can be modified by two phenomena such as methylation and acetylation.

As discussed earlier, attachment of one methyl group at lysine residue of histone is called histone methylation, whereas, histone acetylation refers to attachment of an acetyl group to the same lysine residue. Methylation can attribute both active and inactive states of chromatin, for instance, K9 methylation on histone H3 silenced DNA whereas, on the same H3 histone, when a different K4 lysine residue is methylated, genes are transcribed [11]. Histone methylation similar to DNA methylation can be mono, di- or tri-methylation at single lysine residue such as H3K4. Multiple modifications can be done on single histone protein by the involvement of phosphatases or kinases and some coactivators like CREB binding protein (CBP) that determine the pattern of gene expression. The active stage of chromatin or euchromatin is due to histone acetylation while deacetylation contributes to heterochromatin organization. Histone acetylation is controlled by Histone Acetylase Transferases (HAT) and Histone Deacetylases (HDAC; [12]). Post-translational

modifications at the N-terminal tail of histone can also be accounted to other mechanisms like acetylation, phosphorylation, ubiquitylation, sumoylation and ADP ribosylation [13] whereas involvement of specific enzyme catalyzes such as post-translational modifications including acetyl transferases, deacetylases.

8.1.3 RNA Associated Gene Silencing

Gene's transcription can be silenced with the help of different methods such as RNA interference, antisense strand or through non-coding RNA sequences called micro RNA. RNA can be silenced by triggering any of the modifications in histone, DNA methylation and DNA condensation. Micro RNA destabilizes mRNA entity and thus making it unavailable for translation and thereby controlling gene expression [14].

8.2 Epigenetic Modifications Alter Gene Expression in the Brain

Much evidence suggested that during the developmental process in the brain, variations occur at the level of DNA methylation of different genes [15] and different cell types of CNS may be marked distinctly by DNA methylation pattern [16]. One such enzyme, DNMT1, a type of DNA methyltransferase is highly expressed in human brain, including post-mitotic neurons [17–19]. The conditional DNMT1 deletion in 12th day of embryonic neuroblasts led to DNA hypomethylation associated lethality. On contrary, conditional deletion of DNMT1 in postmitotic neurons imparted no overall changes in DNA methylation levels in mouse [20, 21]. Several studies conducted on mice confirmed that DNMT3B is responsible for neuronal development [22, 23], which if inherent, may lead to premature death, hypo-activity, weaker neuro-muscular function and motor coordination [24]. Further, it has been established that DNA demethylation and polycomb-mediated histone H3K27 had been shown to be one of the regulators of neuronal differentiation [25], whereas, DNA methylation down regulates the pluripotency and differentiation [26]. Mutations in the genes encoding proteins that are required for epigenetic mechanism may also be one of the major causes of neuronal development associated disorders. Rett-syndrome results from mutation in the MECPZ gene that code for the protein which binds and regulates the gene expression of methylated DNA [27] while ICF syndrome is caused by mutation in the DNMT3B, a *de novo* DNA methyltransferase [28–30].

8.3 Initiation of Glioma by Epigenetic Variations

Hypermethylation of DNA can change the differentiation properties of glioma cells, which are considered as putative stem cells of cancer and oftenly called as "Tumor Initiating Cells" (TIC). The differentiation of normal astroglial cells is regulated by

two major ligands of the JAK/STAT pathway viz. Bone Morphogenetic Proteins (BMP) and Ciliary Neurotrophic Factor (CNTF).

When BMP receptor is silenced epigenetically, it leads to the inhibition of JAK/STAT pathway through the enhancer of Histone Methyltransferase [31]. Gene that encodes CD133 a cell-surface marker and helps in identifying and upregulating TICs can also be regulated epigenetically while methylation of CD133 was rather observed in case of tumorous conditions in the brain compared to the normal brain [32].

8.4 Types of Brain Tumors

Based on the origin, brain tumors are classified as:

Astrocytoma: Star-shaped structure with occurrence in cerebellum, cerebrum, brainstem. This can be low grade (benign in nature) or high grade (malignant) astrocytomas.

***Atypical Teratoid Rhaboid Tumor (ATRT)*:** Considered as high-grade tumors, mostly reported in children of age 3 years or less, occurs in the cerebellum.

***Chondrosarcoma*:** A malignant form of chondroma arising from the skull base, specifically from the pituitary. The different types include conventional, mesenchymal, clear cell and de-differentiated. It can be of Grade, I, II, III and IV.

***Choroid Plexus*:** Choroid Plexus Papilloma is a non-cancerous and rarely occurring form of tumor, and it are located in the outer lining of ventricles of brain and CSF. Choroid Plexus plexus is the malignant form of this tumor affecting mostly children.

***Craniopharyngioma*:** It is the benign form of tumor and is reported to arise from the mass of cells at or near the pituitary stalk. It is located in the cellar portion of the brain, and or near the pituitary, optic nerve and ventricles.

***Cysts*:** Although cysts reside in the brain but do not originate from brain tissue and are non-cancerous in nature. Cysts can be of four types: (a) Arachnoid cyst or Leptomeningial cyst—it constitutes an area filled with fluid in the subarachnoid space, in children as well as in adults (b) Colloid cyst—it arises during the development of CNS and is usually benign in nature (c) Dermoid Cyst—it is formed at early stages of fetal development, but symptoms arise years later and (d) Epidermoid cyst—it is also known as epidermoid tumor and develops during early weeks of fetal development.

***Ependymoma*:** It originates from ependymal cells that cover the ventricles of brain and spinal cord. It is subdivided into four types: (a) Subependymomas—Grade, I tumor growing slowly (b) Myxopapillary ependymomas—Grade, I tumor with slow growth (c) Ependymomas—Grade II tumor is the most common form of ependymal tumors and (d) Anaplastic ependymomas—Grade III tumor growing rapidly.

***Glioblastoma*:** Grade IV tumor originating from astrocytes. It has a very high potential of malignancy and arises in the cerebral hemisphere of the brain.

***Germ cell tumor*:** The source of origin of this type of tumor is germ cell in the brain. It originates from the suprasellar and/or pineal part of the brain. It can either be benign or malignant.

***Glioma*:** The tumor originating from tissue that supports the brain called 'Glia', which is responsible for positioning and functioning of neurons.

***Hemangioma*:** The aberrant growth of blood vessels in the skin or internal organs of the body is Hemangioma. It can be of two types: (a) Hemangioblastoma—

non-cancerous tumors growing slowly and (b) Humangiopericytoma—these are uncommon, Grade II or III type of tumors. They do not enter the brain instead they can prevail elsewhere like bones, lungs and liver.

***Lymphoma*:** This tumor originates from the lymphatic system cells and is called Primary CNS lymphoma (PCNSL) in the brain.

***Lipoma*:** These are uncommon, benign brain tumors of fatty tissue associated with congenital disorders of nervous system.

***Medulloblastoma*:** This is Grade IV tumor that grows rapidly. It can be categorized as: classic medulloblastoma, desmoplastic nodular medulloblastoma, anaplastic or large cell medulloblastoma, medulloblastoma with glial cell differentiation, medullomyloblastoma, medulloblastoma with neuroblastic differentiation and melanotic medulloblastoma.

***Meningioma*:** Benign tumors that originate from spinal cord or brain are called meningiomas. Although they are considered as brain tumors but tissues of the brain are not responsible for their growth. These tumors originate from meninges and occur mostly near the outer curve of brain and top of the brain, as well as in the basal region of a skull.

***Metastatic Brain Tumor*:** It is Grade II brain tumor originating from primary cancer cells, which can be anywhere in the body and reaching to the brain.

***Neuronal and Mixed Neuronal Glial Tumor*:** It is the rare form of non-cancerous tumors originating from ganglion like cells and appears to be a cluster of nerves.

***Oligoastrocytoma*:** This type of tumor originates from the glial cells of the brain. It is considered to be Grade II tumor, but one of its subtypes anaplastic oligoastrocytoma is a Grade III tumor.

***Oligodendrogliomas*:** This tumor originates from oligodendrocytes. It can below Grade II or high-Grade IV tumor.

***Pineal Tumor*:** as the name suggests, pineal tumor arises from cells of the pineal gland. Pineal tumors include germinoma, non-germinoma, meningioma, astrocytoma, dermoid cyst and ganglioglioma.

***Pituitary Tumor*:** These tumors originate in the pituitary glands as non-cancerous, slowly dividing masses.

***Primitive Neuro-ectodermal Tumor (PNET)*:** PNET resembles medulloblastoma when viewed under the microscope but mainly occurs in the cerebrum, though it can invade other parts such as brain and spine.

***Schwannoma*:** It is positioned between cerebellum and pons, at the posterior fossa, an area at the base of the skull. It is a benign form of tumor.

8.5 Classification of Epigenetic Variations on the Basis of Tumor Glioma

Different methylation patterns in the promoter region of RB1, TIMP-377, Methylguanine DNA methyltransferase 11 (MGMT 11), p14ARF, p16INK4a represent different types of GBMs [33]. Secondary GBMs exhibit higher level of promoter methylation as compared to primary GBMs [34]. Distinct methylation also

exists for CNS cancers other than gliomas, for instance, instance, p73, THBS1, TIMP3 is reported to be hypermethylated in Schwannomas [35]. NF2 is altered in Meningioma genetically as well as epigenetically [36]. Glioma progression with time can be marked by different epigenetic patterns. GBM can also be relapsed by hypermethylating CASPASE 8 [37].

8.6 Response of MGMT Methylation on Alkylating Agents of DNA

MGMT expression guards normal cells from cancerous products and oncogenes from alkylating agents at the time of chemotherapy. Methylation of MGMT promoter is associated with reducing the affinity of transcription factors from binding with GBM cell [38, 39]. Recently, it has been reported that MGMT methylation is associated positively with cancer cell line expression [40], whereas, silencing of gene expression in multiple types of human tumors such as glioma, retinoblastoma, lymphoma can be done by simply hypermethylating MGMT promoters [41]. A common example of epigenetic gene silencing in glioma where a gene is resistant to drug is hypermethylating the promoter by O6-methylation DNA methyltransferase (MGMT). Moreover, MGMT codes for a protein involved in DNA repair, which removes alkyl groups from the O6 position of guanine residue and thymine from the O4 position at times [42, 43]. Methylation of MGMT promoter in GBM leads to hypermutator phenotype [44] while hypermethylation of MGMT leads to longer survival of patients with GBM [45, 46]. Hypermethylation of MGMT can predict the outcome of false progression [47], and it has been varying prognostic parameters for oligodendromas than the gliomas [48].

8.7 CGI Hypermethylation and DNA Hypomethylation in Gliomas

Hypomethylation results in the large number of initial GBMs. Hypomethylation level varies between tumors of different types and single-copy loci as well as repetitive DNA sequences can be hypomethylated in GBM, for instance, MAGEA1, a gene of MAGE family, which is, in fact, an oncogene, when undergoes demethylation leads to the most potent genome-wide hypomethylated GBMs [49–52]. On contrary, hypermethylation at some specific loci of CGI promoters is very common in gliomas [53], whereas, hypermethylation of CGI promoters in case of gliomas occur at genes with a wide range of functions associated with tumor progression like regulation of cell cycle, DNA repair, and Angiogenesis. For example, CGI promoters affect RB, p53, PI3K pathways [54–59].

Another gene, EMP3 expression is silenced through hypermethylation in primary gliomas while hypermethylation of promoter in highly aggressive and

proliferative GBMs regulates the oncogenic and TGF-β signaling pathways and results into poor prognosis. However, silencing of PDGF-β gene expression epigenetically can attenuate the proliferative impacts of TGF-β signaling. Hence, the oncogenic effects of TGF-β signaling pathways can be inhibited by hypermethylation which stops PDGF-β gene expression by SMAD protein induced by TGF-β [60]. Hypermethylation of CGI promoters also affects the genes responsible for invasion and metastasis of cancer cells into the normal brain parenchyma in gliomas [61]. Promoter hypermethylation also makes impacts on radiotherapy and drug sensitivity in GBM, for instance, methylation of MGMT promoter and its response to alkylating agents [62, 63].

8.8 Hypermethylation of MGMT, DNMT1 and EGFR in the Proliferation of Glioma

Glioma can be categorized into two distinct classes: (a) Primary glioma that generates frequently without any kind of histopathological evidence of an initiator lesions with malignancy of low grade and (b) Secondary glioma, which is the last stage of proliferation of Astrocytoma of low grade.

WHO classifies Gliomas into four different grades: (a) Low Grade [Grade II as per WHO standards] representing benign condition, which is treatable while (b) High-grade [Grade III as per WHO standards] exhibits malignant tendencies and resulting into poor prognosis. Various studies reported abnormalities at the genetic and epigenetic level may lead to GBs development [64, 65] like heterozygosity loss, unstable microsatellites [66, 67]; with distinct mRNAs and protein expression [68]. Latest studies implicated CGI promoter hypermethylation as the central process in the inactivation of tumor-related genes [52]. DNMT adds up methyl group at the 5′ cytosine residue of CGI at the replication [69]. This process is mediated by hypermethylation event of tumor suppressor genes and oncogene's hypomethylation [70]. When this process takes place abnormally then it might lead to tumor formation [71–73]. Upregulation of the expression of Epidermal Growth Factor Receptor [EGFR] gene which is HER/ERB-B family member having tyrosine kinase activity is responsible for tumor formation, also regulates patient prognosis [74]. Various studies have demonstrated that primary GB is associated with the high expression and amplification of EGFR. Whereas this behavior of EGFR is least specific in secondary GB. However, EGFR up regulation is quite a delayed process in de-differentiation and gene inactivation in case of EGFR at the time of glial cell progression. MGMT resists the action of alkylating agents by eliminating the mutagenic precursors of DNA. It has been suggested that loss of MGMT may promote glioma formation [46]. Formation of oligodendromas and secondary GBs can take place through hypermethylation of MGMT promoter [58, 75]. Similarly, hypermethylation of DNMT1 is related with the development of secondary GBs and not with the low grade or primary gliomas.

8.9 Role of Notch Signaling Pathway in Cancer and Its Association with DNA Methylation

Notch signaling plays a pivotal role in the development of species, whether it is invertebrates or vertebrates. This signaling pathway involves four different types of Notch genes; Notch 1, Notch 2, Notch3 and Notch4. Basically, these ligands belong to the (Delta/Serrate/LAG-2) DLS family of single pass TMP. Its dysregulation plays the different role in different types of cancer like breast cancer, TALL, cervical cancer, prostate cancer and brain cancer. In brain tumors Notch1 acts as Tumor suppressor gene [76]. The hypermethylation, hypomethylation or demethylation of Notch signaling pathway may lead to distinct types of malignancies.

8.10 Strategies for Mapping the Global Methylation Pattern

The methods employed in discovering new epigenetic variations in brain tumors can be: (a) Silencing of normal gene functions by inactivation of other alleles due to DNA hypermethylation, candidate loci, which are affected by mutation on a single allele. This strategy is useful in discovering hypermethylation (b) DNA methylation immunoprecipitation (MeDICP) is a recent strategy and representation of reduced bisulfate sequencing in association with DNA microarrays [77, 78] (c) Parallel Sequencing and (d) Treating cells with 5-aza-2′-deoxycytidine (5-aza) a DNMT1 inhibitor or with Trichostatin A(TSA) a Histone Deacetylase inhibitor followed by microarray analysis of gene expression [79]. By using this approach, Brain Expressed X-linked 1 (BEX1) and Brain Expressed X-linked 2 (BEX2) genes could be identified, because they acted as tumor suppressors in GBM [80], while SPINT2 in case of Medulloblastoma [81]. Further, Runt-related Transcription Factor3 (RUHX3) and Testin (TES) were found after massive methylation by 5-aza activated many genes indirectly in primary Gliomas [82].

8.11 Histone Modifications in Glioma

Loss of Histone H3K9 acetylation and increased H3K9 methylation are characteristics of CGI promoters which are silenced. Silencing in case of stem cells of an embryo and gene expression activation in differentiated cells for those genes which are developmentally regulated can attain a very normal chromatin state by inactivating H3K4 methylation and H3K27 methylation. These are called as '*bivalent domains*'. These bivalent domains in addition to repressing marks such as dimethylated H3K9 and trimethylated H3K9 is observed in case of embryonic carcinoma cells that are more commonly silenced by DNA hypermethylation in adult carcinoma cells. Such histone modifications are supposed to remove tumor suppressing genes to DNA

hypermethylation and gene silencing inheritably [83]. The effect of H3K27 methylation by gene encoding BMI-1 is seen in gliomas. BMI-1 genes can alter the copy number in both low and high-grade gliomas, and the deletion of BMI-1 may become the cause of poor cure in patients [84]. INK4a/ARF loci is two important transcriptional repressors of BMI-1 in GBM. Up regulation of BMI-1 expression is commonly seen in case of gliomas [85]. In GBMs, Class II and IV HDACs exhibit reduced mRNA expression than that of Astrocytoma of low grade and normal brain while acetylation of histone H3 takes place in GBMs [86]. Massive sequencing of genes that encodes the protein in case of mutations of many genes that are not covered in case of gliomas include epigenetic regulations like Histone demethylases (JMJD1A) and (JMJD1B), Methyl CpG Binding Domain Protein1 (MBD1), Histone Deacetylases 2 and 9 [87].

8.12 Epigenetics Contributes to GBM

Genetic variation at micro and macro level in the genes affecting apoptosis, metastasis and angiogenesis is characteristic features of GBM. Epigenetic alterations are also responsible for the manipulation of expression level in cancer genes either alone or in coordination with genetic alterations. For instance, high frequency of genes undergoes the process of DNA hypermethylation at CGI promoters in the case of glioma. Apart from this, various other epigenetic alterations have also been reported in order to understand the pathology of glioma such as position change in histone variants and alterations in histone modification processes. Drugs useful in the clinical trials for gliomas target Histone Deacetylases [88] (Table 8.1).

8.13 Epigenetic Alterations in Drug Resistance Genes MGMT, ABCB1 in GBM

A major cause of worse patient prognosis suffering from GBM is showing resistance to drug therapy. From various studies, it has come into consideration that ABCB1 and ABCB2; the two efflux transporters apart from the methylation of O6MGMT gene may contribute to drug resistance.

8.14 GSCs in Concern with or in Association with miRNA Dysregulation

The generation of miRNA is a multistep process where genesis begins from nucleus and then translocates into the cytosol. Initially, it is transcribed into a primary transcript or pri-miRNAs, process that is transcribed by using enzymes such as RNAPol

Table 8.1 Epigenetic modifications contributing to different types of brain tumors

S. no.	Type of tumor	Epigenetics mechanism	References
1	Gliomas	(a) Abnormal methylation of promoters of CGI which were normally unmethylated (b) Gene silencing in context with CGI	Jones and Baylin [142], Baylin and Bestor [143], Costello [144]
2	Oligodendroglioma	Upregulation of V-erbB membrane of EGFR family	Weiss et al. [145], Dai et al. [128], Holland et al. [146, 147], Ding et al. [148]
3	Medulloblastoma	(a) Dysregulated Notch and Canonical pathways	Jenkins [149], Lauth and Toftgard [150], Morton and Lewis [151]
		(b) Sonic Hedge-hog pathway	Hatton et al. [152]
		(c) Aberrant changes in the methylation pattern on the CGI promoters related to few specific genes. This might change gene expression pattern	Fruhwald et al. [153], Lindsey et al. [154], Northcott et al. [155], Irizarry et al. [156]
		(d) Unregulated miRNA expression especially the expression of miR-199b-p upregulation or downregulation may lead to the development of Medulloblastoma	Croce [157], Davalos and Esteller [158], Garzon et al. [159], Ryan et al. [160], Waldman and Terzic [161], Tsang et al. [162]
4	GBM	(a) CGI hypermethylation	Martinez and Schackert [163]; Nagarajan and Costello [88]
		(b) Proteins involved in Chromatin modeling and remodeling when undergoes into dysregulation may lead to GBM development like BMT-1, EZH-2	Abdouh et al. [164]; Suva et al. [165]
		(c) Altered expression of Polycomb repressive (a) BMI-1, a PRC-1 component, which is concerned with compaction and ubiquitination of Chromatin and Histone-2A. (b) EZH -2, aPRC-2 component which is responsible for the H3 methylation to downregulate gene expression	Margueron and Reinberg [166], Schuettengruber and Cavalli [167], Simon and Kingston [168]

(continued)

Table 8.1 (continued)

5	Germ cell tumor	(a) Hypermethylation not only at the promoters of CGI, but also at the regions adjacent to CGI	Doi et al. [169]
		(b) Along with CGI hypermethylation, gene silencing by repression the transcription by inhibiting Transcription Factors binding	Baylin and Jones [170], Jones and Baylin [142], Park et al. [171]
		(c) Distinct subtypes of GCC have different types	Oosterhuis and Looijenga [172], Okamoto and Kawakami [173], Smiraglia et al. [174], Wermann et al. [175]
		(d) A wide range of Histone modification process for the chromatin such as acetylation, phosphorylation at the specific residues of amino acids are known. Any manipulation that may lead to the change in expression of germ cell and could result into GCC	Ernst and Kellis [176]
6	Pituitary gland tumor	Insufficient methylation at different gene locus	Farrell [177]
7	Astrocytoma	Enhanced expression of AKAP12 Protein A-Kinase Anchor Protein12 has been demonstrated into Pilocytic-Astrocytoma	Goeppert et al. [178]

II or RNAPol III, where a primary transcript undergoes capping and polyadenylation [89]. This primary transcript, by the use of RNAPol III Drosha along with its partner Pasha is further processed for the generation of pri-miRNA of size 70–100 nucleotide long, which has a hairpin like structure [90, 91]. Later on, this processed pre-miRNAs with the help of Exportin-5 protein expelled out from nucleus and translocate into the cytosol. Here dissociation occurs and double stranded; mature miRNA is generated. Lastly, the single-strand miRNA or Guide miRNA is incorporated into the RNA-induced silencing complex (RISC) enzyme complex, which includes DICER, TRBP and an Argonaute family protein molecule [92, 93].

It has been implicated from recent studies that miRNA dysregulation plays major role in the regulation of GSCs pathological conditions. The enhanced expression of miRNAs including miR-9, miR-10a, miR-10b, miR-17, miR-106 serves as cancer-causing miRNAs, resulting into increased invasion, progression, self-renewal and decreased apoptosis of glioblastoma cancer stem cells (GSCs). Enhanced expression of miR-10a and miR-10b in GSCs down regulated the CUB and SUSHI mul-

tiple domain 1 (CSMD1) expressions [94]. Contrary to that, enhanced expression of miR-10b was found in GSCs where miR-10b inhibition mediates the inhibition of GSC progression and migration, while miR-10b over expression stimulates cellular migration and invasion. Further, inhibition of miR-10b reduced the progression or proliferation of GBM xenograft, which is stem-cell derived [95]. miR-9 and/or miR-17 inhibition lead to cell differentiation induced by targeting Calmodulin Binding Transcription Activator1 (CAMTA1). It has also been proposed that CAMTA1 acts as tumor suppressor and CAMTA1 expression is associated with patient prognosis [96].

8.15 Epigenetics and Etiology of Neurodegenerative Disorders

Epigenetic modulations are instrumental and a key cellular processes inside the cell. However, when the harmony of any of the three epigenetic mechanisms is breached, abnormal gene repression or activation manifesting in cancer, chromosomal aberrations linked syndromes and neurodegenerative disorders including AD, PD, HD and ALS [97]. The onset, latency period and disease progression in NDD has been shown to be influenced by epigenetic modulations [98]. Heavy metals seem to exert epigenetic modifications through perturbed gene expression resulting in late-onset NDD [99]. Gene-Environment interactions are orchestrated through the concerted action of histone-modifications, DNA methylation and non-coding RNA [100]. NDDs are a complex interplay of genetic-environmental stimuli, therefore it becomes imperative to study and underline the epigenetic changes in such disorders [101–105]. DNA methylation is one of the most commonly recognized epigenetic mechanism regulating various neurogenesis and developmental processes including cognition and learning [106, 107]. Histone acetylation carried out by HAT have been shown to be involved in cognitive function and consequently, loss of HAT function led to impaired memory formation [108–116]. On the same lines, HDAC inhibitors improved cognitive function [117, 118]. Improved learning was reported in mice that lacked HDAC2 or HDAC3 compared to wild type [119, 120]. On contrary, aborted HDAC4 activity led to stunt synaptic plasticity and cognition in both, humans as well as mice [121–123]. The epigenetic modifications resulting into various NDD has been comprehended in Table 8.2.

8.16 Epigenetics Is a Two-Edge Sword: Implications in Therapeutics

GBMs exhibit high malignancy conditions and are known to be one of the most severe forms of primary brain tumors [124]. Patients suffering from GBM shows worst prognosis and tends to have high resistance towards radio-chemotherapies

Table 8.2 Epigenetics involved in various neurodegenerative disorders (NDD)

S. no.	NDD	Type of epigenetic modification	Epigenetic modifications	Reference
1	PD	Methylation	Decreased DNA methylation linked to γ-synuclein and DNMT1 interaction. Attenuated SNCA intron 1 methylation associated with γ-synuclein deregulation. Methylated short telomers in contrast to demethylate in age-related controls. SNCA methylation down-regulated in SNPC	Desplats et al. [179], Jowaed et al. [180], Matsumoto et al. [181], Maeda et al. [182]
		Histone	γ-synuclein interacted with and inhibited histone acetylation. HDAC6 enrichment in Lewy bodies	Goers et al. [183], Kontopoulos et al. [184]
		RNA	Attenuated miR-133b and decreased miR-34b/c led to a difference in DJ1 and Parkin's expression. Hypomethylation of vtRNA 2-1 MIR886. Upregulated vtRNA 2-1 led to neuronal dysfunction and marked early events in PD etiology	Kim et al. [185], Miñones-Moyano et al. [186, 187]
2	AD	Methylation	Increased level of DNA methylation intermediate 5-hydroxymethylcytosine in hippocampus/parahippocampal gyrus. APOE 3′-exon CGI hypermethylation in post-mortem brains. Soluble Aβ blocked EAAT3 regulated cysteine recruitment and resulted in attenuated DNA methylation	Bradley-Whitman and Lovell [188], Yu et al. [189], Hodgson et al. [190]
		Histone	Soluble Aβ impaired histone H3 activity led to altered DNA transcription. In SHSY5Y cells oxidative stress induced transcription of genes linked to AD and Aβ overproduction through histone acetylation and DNA demethylation. Enhanced histone H3 acetylation of BACE1 in peripheral blood mononuclear cells (PBMC) of AD patients as well as in triple transgenic mice model	Lithner et al. [191], Gu et al. [192], Marques et al. [193]
3	HD	Methylation	Downregulation in rDNA transcription due to UBF trimethylation at lysine 232/254 by histone H3K9 methyltransferase ESET	Hwang et al. [194]
		Histone	In mutant htt, Poly Q repeats associated physically with CBP and inhibited its HAT activity. Impaired UBF1 acetylation at K352 associated with retarded rDNA transcription. Attenuated CBP histone acetylation linked to stunted long term memory	Steffan et al. [195], Lee et al. [196, 197], Giralt et al. [198]

(continued)

Table 8.2 (continued)

S. no.	NDD	Type of epigenetic modification	Epigenetic modifications	Reference
4	ALS	Methylation	Upregulated Dnmt1 and Dnmt3a levels and 5-methylcytosine enrichment in ALS and Frontotemporal dementia (FTD). Hypermethylated CGI proximal to G4C2 repeat associated with small disease time span in fALS	Martin and Wong [199], Xi et al. [200]
		Histone	Histone H3 and H4 trimethylation at lysine residues H3K9, H3K27, H3K79 and H4K20 led to suppressed c90rf72 gene expression in ALS and FTD	Belzil et al. [201]
5	AT	Histone	HDAC4 inhibition by Trichostatin A or shRNA reverses neuronal cell death mediators, activated caspase-3 and ameliorates enhanced cell cycle proteins level. Restored optimal HDAC4 nuclear-cytoplasmic level rescued ATM deficient phenotypes. Attenuated H-acetylation in ATM deficient primary neurons and decreased CREB and MEF2 dependent transcription upon nuclear localization of HDAC4. Nuclear localization of HDAC4 promoted neurodegeneration in ATM deficient neurons	Herrup et al. [202], Li et al. [203]
6	Dementia with Lewy bodies (DLB)	Methylation	DNMT1 sequestration in the cytoplasm associated with attenuated DNA methylation	Desplats et al. [179]

[125, 126]. Cancer Stem Cells are responsible for the origination of GBMs [127–129]. Different groups have discovered that conventional tumor therapies such as radiotherapy may result into recurrence of tumors in GBM patients [130–132]. Hence new approaches of therapeutics in gliomas have been developed by targeting GSCs. GSCs are cancerous stem cells similar to the Normal Stem Cells (NSCs) which are contributors of primary gliomas and play the pivotal role in their malignancies after conventional therapy. It has been demonstrated by several studies that miRNAs play major role in glioma progression and most importantly in the origination and maintenance of GSCs [133–136]. There are several epigenetics mediated therapeutic interventions in brain tumor that has been enlisted in Table 8.3.

Table 8.3 Therapeutic potential of epigenetic interventions in neuro-oncology

S. no.	Type of tumor	Epigenetic intervention	Implication	References
1	GBM	SAHA (suberoylanilide hydroxamic acid)	Inhibits glioma cell growth, migration, and invasion in vitro and in vivo. Can cross the blood-brain barrier (BBB), and is tolerable to patients	Eyupoglu et al. [204], An et al. [205], Galanis et al. [206], Hockly et al. [207]
2	Neuro-blastoma	HDAC inhibitor	Shows anticancer effects on NB cells causing cell-cycle arrest, differentiation, apoptosis and better response to chemotherapy	Witt et al. [208]
		Epithelial Membrane Protein 3 (EMP3)	Re-introduction of EMP3 in methylation silenced EMP3 neuroblastoma cell lines introduced tumor suppression	Alaminos et al. [209]
3	Glioma	SAHA	Down-regulated elevated levels of EZH2	Orzan et al. [210]
		CpG island methylation	induces tumor suppressing (EMP3, VHL), cell cycle regulation (p16), invasion and metastasis (PCDH-gamma-A11), and DNA repair (MGMT)	Nagarajan and Costello [88], Martinez and Esteller [211]
		O6-Methylguanine-DNA Methyltransferase (MGMT)	By causing inhibition of AGT, MGMT promotes drug-selection at the time of Chemotherapy and leads to better patient prognosis. Prevents normal tissues against the adverse effects of chemotherapy	Gerson [42, 43]

(continued)

Table 8.3 (continued)

S. no.	Type of tumor	Epigenetic intervention	Implication	References
4	Cutaneous T cell lymphoma (CTCL)	SAHA	Therapeutic potential	Kazantsev and Thompson [212]
5	Medulloblastoma	Curcumin	Induces apoptosis, cell cycle arrested at G2/M phase in medulloblastoma cells. Reduced HDAC 4 expression and activity and increased tubulin acetylation, decreased tumor growth and increased vitality in the Smo/Smo transgenic mouse model	Seung Joon et al. [213]
		KDM1A knockdown	Induces apoptosis, suppresses proliferation and reduced tumor migration. NCL-1 which is KDM1A inhibitor ameliorated tumor growth in vitro	Kristian Pajtler et al. [214]

8.16.1 Epigenetic Therapeutics in Neurodegenerative Disorders

Providing *in vitro* the stimulus may develop epigenetic changes in neurons, which can modulate memory linked gene expression [107, 137–139]. Hence, epigenetic alterations in the neurons are involved partially in the origination and proliferation of NDD [140]. Targeting the epigenomes with specific compounds might lead to epigenetic alterations, and this approach can be used to develop therapeutics in NDD ([141]; Table 8.4).

8.17 Conclusion

Epigenetic is a two-edge sword. On one hand, it contributes to the onset and progression of fatal neurological aberrations such as cancer and various neurodegenerative disorders, whereas, on the other hand, it stands out as a promising therapeutic tool for the correction and amelioration of such disorders. Epigenetic therapies have gained momentum in recent years owing to their beneficial effects in terms of vitality. However, lots remained to be unveiled and explored, which might pave the way for a better survival rate in neurological diseases.

Table 8.4 Role of epigenetic modulations in neurodegenerative disorders

S. no.	NDD	Epigenetic intervention	Effects	References
1	AD	Folate and B group vitamins (B2, B6, and B12) and Homocysteine	Take part in one carbon metabolism, which is required for SAM production and shows impaired one-carbon metabolism and down regulation of DNA methylation potential	Coppedè [215]
		SAM supplementation	Restored PSEN1 methylation levels and reduced the progression of the AD like features induced by vitamin B deficiency in mice. Reduce oxidative stress, slows down Aβ and tau accumulation in transgenic AD mice	Fuso et al. [216–218], Tchantchou et al. [219], Lee et al. [220]
		Histone tail modifications	Decreased levels of H3 acetylation in the temporal lobe	Zhang et al. [135, 136]
		Histone acetylation with HDAC inhibitors	Delayed cognitive deficits and better memory recovery	Karagiannis and Ververis [141]
		Sodium butyrate	Promote learning and memory in transgenic mice. Decreased tau phosphorylation and restored dendritic spine density in hippocampal neurons. Improved associative memory by increasing both hippocampal histone acetylation and gene expression in associative learning	Fischer et al. [117], Ricobaraza et al. [221], Govindarajan et al. [222]
		Entinostat	Reduced neuro inflammation, amyloid plaque deposition and further improved behavioral impairment in APP PS1-21 mice	Zhang and Schluesener [223]
		Trichostatin A	Reduced acetylated H4 levels and contextual freezing performances to wild-type values	Francis et al. [224]
2	PD	Sodium butyrate or vorinostat	Reduced toxicity of α-synuclein	Kontopoulos et al. [184]
		Sirtuin2	Reduce α-synuclein-mediated toxicity	Outeiro et al. [225]
		Valproic acid	Mediates α-synuclein translocation into the nuclei	Monti et al. [226]
		Trichostatin A	Reduced mitochondrial fragmentation and cell death caused by MPP+ in human neuroblastoma cells	Zhu et al. [227]
		Sodium butyrate	Improved locomotor impairment and early mortality. Reduced cognitive deficits. Increment of DJ-1 protein expression and protection of neurons in cell cultures and in mice models against MPP+ toxicity	St Laurent et al. [228], Rane et al. [229], Zhou et al. [230]

(continued)

Table 8.4 (continued)

S. no.	NDD	Epigenetic intervention	Effects	References
3	ALS	SAM supplementation	Delayed onset of motor neuron pathology	Suchy et al. [231]
		Sodium phenylbutyrate	Increased vitality in G93A transgenic ALS mice, increased histone acetylation	Ryu et al. [232], Cudkowicz et al. [233]
		Phenylbutyrate and riluzole	Reduced gross lumbar and ventral horn atrophy, attenuated lumbar ventral horn neuronal cell death and decreased reactiveness in astrogliosis in ALSG93A mice	Del Signore et al. [234]
		lithium and valproate	Delayed disease onset, reduced neurological deficits and improved survival	Feng et al. [235]
		Trichostatin A, valproate	Delayed disease progression and increased lifespan in the SOD1-G93A mice	Yoo and Ko [236], Sugai et al. [237]
4	HD	HAT proteins	Altered histone acetylation	Steffan et al. [238], Jiang et al. [239]
		4b (HDAC1i and HDAC3i)	Improved motor functions and reduced cognitive decline	Jia et al. [240]
		AK-7 (sirtuin2 HDACi)	Improved motor functions, vitality and reduced mutant huntingtin aggregation	Chopra et al. [241]

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Chapter 9
Epigenetics of Breast Cancer: DNA Methylome and Global Histone Modifications

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9.1 Introduction

Breast cancer is the most frequently diagnosed cancer in women. In 2012, more than 1.6 million women were diagnosed with breast cancer worldwide. Despite the improvements in screening and therapeutic approaches, in 2012 more than half a million women died due to breast cancer, which is among the leading cause of cancer deaths in women [1].

Breast cancer is a heterogeneous disease comprised of tumors with different histological characteristics and clinical outcomes in terms of prognosis, drug response and metastatic potential. Heterogeneous nature of the breast cancer demands delicate approaches to diagnose and follow the most appropriate strategy for clinical management. Classical histological analysis including assessment of hormone receptor (HR) and receptor tyrosine-protein kinase erbB-2 (ERBB2) status, tumor size, histological grade and lymph node invasion was improved in the last decade with the gene expression profiling. Microarray analysis of mRNA expression revealed mainly four molecular subtypes of breast tumors: (a) luminal A, low grade estrogen receptor (ER)(+) tumors with good prognosis, (b) luminal B, high grade (ER)(+) tumors with poor prognosis, (c) basal-like, HR (−) and ERBB2(−), and (d) ERBB2(+), increased expression of several genes of ERBB2 amplicon [2–4]. Molecular subtypes not only provided additional significant information for better diagnosis, prognostic estimates and drug response predictions but also improved our understanding of breast tumor biology (reviewed in [5]). Effective therapeutic approaches could only be developed by unrevealing the mechanisms underlying tumorigenesis and metastasis. Despite the advancements due to molecular subtypes, there is still room for improvement for better diagnostic and therapeutic approaches.

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Cancer as a general was considered to be resulted from the accumulation of genetic mutations but numerous studies now implicate the contribution of abnormal epigenetic modifications in various cancer types including breast cancer. Contribution of epigenetic modifications in breast cancer development was further supported by the identification of several recurrent mutations in genome-wide sequencing analysis in the regulators of DNA methylation, post-translational histone modifications and chromatin remodeling in general [6–9]. Similar to genetic mutations, epigenetic alterations are also involved in abnormal regulation of oncogenes and tumor suppressors that play role in breast tumor development and progression. Further studies suggested global epigenetic alterations in breast tumors compared to normal samples that can affect the activities and/or regulation of hundreds to thousands genes involved in several cellular processes including DNA repair, signaling pathways, cell cycle, and transcription that are known to be important in tumorigenesis. In contrast to genetic mutations, epigenetic alterations are reversible that could provide an advantage for the correction of abnormalities in affected regions to revert to the normal genome state. Thus, epigenetic mechanisms are prominent candidates for the development of novel therapeutic approaches. Using both genetic and epigenetic factors would give a better definition of breast cancer subtypes that would increase the diagnostic and prognostic success for breast cancer patients with the development of better markers and treatment options.

In this chapter, we summarize the recent advances in global epigenetic changes, mainly DNA methylation and histone modification alterations, and their relevance to breast cancer classification and clinical observations.

9.1.1 DNA Methylome

Abnormal DNA methylation has long been observed in several cancer types including breast. Abnormalities include both hypermethylation and hypomethylation. Hypermethylation is described as gain of methyl residue at the 5′ position of cytosine nucleotide at regions that are normally not methylated, and mostly observed in promoter CpG islands. In general hypermethylation is associated with silencing of gene expression and observed in tumor suppressor genes (Reviewed in [10]). On the other hand, global hypomethylation is another characteristic property of cancer cells. Genome wide loss of methyl residues on cytosine nucleotides is observed usually in gene poor areas such as repetitive sequences and pericentromeric satellite DNA. Hypomethylation has long been known to be associated with genomic instability (Reviewed in [11]).

Analysis of individual genes or DNA sequences revealed many cancer-associated modifications in breast tumors. Pubmeth, a web based tool for automated text mining, lists 100 genes that are implicated as hypermethylated in breast cancer [12]. MeInfoText, another automated tool that extracts gene methylation and cancer relations from the literature, shows more than 150 genes that are supposedly hyper- or hypomethylated in breast cancer [13]. Both lists contain the most commonly known

genes, such as BRCA1, RASSF1, CCND2, APC, ESR1, TWIST1, and PTEN, which are reported to be frequently hypermethylated in breast tumors (Reviewed in [14, 15]).

Studies that focused on specific genes established the basis of our understanding on the role of DNA methylation in breast cancer. However, they were limited with the few number of genes or genomic regions due to technical inadequacies. Recent advances in high throughput technologies allowed the analysis of complete epigenome in numerous tumor samples. Similar to whole genome expression profiling, whole genome DNA methylation analysis brought insights into molecular basis of breast tumors, their classification, and prediction of prognosis (Table 9.1).

One of the first studies that analyzed high number of CpGs simultaneously in breast tumors made use of methylation sensitive restriction enzymes and hybridization on immobilized CpG sequences derived from human CpG island library [16]. Using this approach, 1104 CpG islands in 28 paired breast tumor and normal samples were analyzed. The results showed that 9 % of the CpG islands were hypermethylated in tumors compared to normal samples while there were little or no changes in the rest of the CpG islands. Furthermore, hypermethylation was found to be associated with poor differentiation. In this study, other clinical parameters did not show any correlation with the methylation status of the analyzed CpGs [16]. This initial screening comparing breast tumor and normal samples was expanded with extensive analysis of CpGs in several studies (Table 9.1). The most commonly used methods was applying DNA, which is bisulfite treated or immunoprecipitated with antibodies against methylated cytosines, on arrays that covers CpGs across the genome. Analysis of 27,578 CpGs in 19 inflammatory breast tumors, 43 non-inflammatory breast tumors and 10 normal samples revealed 1353 CpGs differentially methylated between normal and tumor samples. 77 % of these CpGs were hypermethylated, while the remaining 23 % were hypomethylated in tumors. Differentially methylated genes were related to the focal adhesion, ECM receptor interaction and cytokine-cytokine receptor interaction [17]. Using the same methodology, analysis of a larger set (119 tumor and 8 normal samples) identified larger number of CpGs (6309) that were differentially methylated between tumor and normal samples. The nature of the hyper- and hypo-methylated regions were consistent with the previous findings that hypermethylation was mostly observed within CpG islands, while hypomethylation was detected outside CpG islands [18]. In another study, 108 breast tumors and 11 adjacent normal breast tissue were analyzed and 100 gene associated CpGs were found to be differentially methylated between tumor and normal samples. The list contained both previously identified genes that are hypermethylated in breast tumors such as RUNX3 and PITX2, and novel genes such as GPR10, DRD5 and CDKN1C. Increased methylation of GPR10, DRD5 and CDKN1C in tumors were validated in an independent sample set indicating that those genes could be novel candidates as tumor markers in breast [19]. In addition to genes, CpGs within 5 kb of several microRNAs were also differentially methylated between normal and tumor samples. Among those microRNAs, miR335 has previously been shown to be lost in tumors, which was associated with increased risk of metastasis [19, 20]. In summary, high throughput analyses further confirmed

Table 9.1 Whole genome DNA methylation analyses

Main conclusions	Coverage	Samples	Reference
• Methylation profile reflects T-lymphocyte infiltration in breast tumors	27,578 CpGs 14,495 genes	DS: 119 IDC, 4 NB VS: 117 IDC, 8 NB	[18]
• Luminal progenitor signature is associated with methylation pattern of the cluster that is enriched in basal-like tumors			
• Methylation profile clusters tumors into five groups	27,578 CpGs-14,495 genes 482,421 CpGs-99 % of RefSeq genes	802 BT	[7]
• The cluster with hypermethylation is enriched in luminal B tumors			
• The cluster with lowest levels of DNA methylation is enriched in basal-like tumors			
• ERBB2(+) tumors are not associated with a specific cluster			
• Luminal B or basal-like specific methylation patterns, which are characterized by hyper- or hypomethylation, respectively, are generated	482,421 CpGs 99 % of RefSeq genes	DS: 40 IBT, 17 NB VS: 121 BT(TCGA), 310 BT	[29]
• Methylation based subtypes, Epi-LumB and Epi-Basal, are associated with unfavorable clinical parameters			
• 196 CpGs associated with molecular subtypes are more methylated in luminal B subtype than in basal-like subtype	1505 CpGs 807 cancer related genes	189 BT	[28]
• Distinct methylation patterns between normal and tumor tissues are observed	1505 CpGs 807 cancer related genes	215 IDC and aNB	[27]
• Differential methylation of 5 genes can discriminate between basal-like and ERBB2 overexpressing (ERBB2(+) and luminal B subtypes) tumors			
• Methylation profile could distinguish tumor tissue from normal tissue	151 cancer specific differentially methylated regions	27 BT, 9 NB	[21]
• Methylation profile predicts BRCA1, BRCA2 and non-BRCA1/2 mutation status more accurate than gene expression profile	25,500 promoters	DS: 33 FBT VS: 47 FBT	[33]
• EPCAM(−) SRAM genes can identify claudin-low tumors, which has mesenchymal characteristics	27,578 CpGs 14,495 genes	47 BT, 19 BCCL	[35]

Main conclusions	Coverage	Samples	Reference
• Breast CpG island methylator phenotype (B-CIMP), associated with HR(+) tumors, is described	27,578 CpGs	DS: 39 BT	[23]
• HR(+) B-CIMP(−) tumors have worse prognosis than HR(+) B-CIMP(+)tumors	14,495 genes	VS: 132 BT	
• Methylation profile clusters cell lines into two groups (i: ER(−), ii: ER(+))	21,570 CpG islands	7 BCCL	[24]
• 148 differentially methylated CpGs are identified in HR(+) and HR(−) tumors	27,578 CpGs 14,495 genes	12 (HR)(+) tumors, 12 (HR)(-) tumors	[25]
• Methylation profile is associated with tumor size, race, alcohol intake and total dietary folate	1505 CpGs 807 cancer related genes	162 IBT	[26]
• Luminal A tumors, which are classified into two different methylation groups, have different overall survival	1505 CpGs	80 BT	[37]
• p53 status, ER status and tumor grade influences methylation clusters	807 cancer related genes		
• 100 CpGs can distinguish tumor tissue from normal tissue	27,325 CpGs	108 BT, 11 aNB	[19]
• 921 CpGs can classify patients into poor or good prognosis groups			
• Methylation profile classifies tumors into two groups (i: high methylation phenotype, enriched in patients with distant metastasis, ii: low methylation phenotype)	27,578 CpGs 14,495 genes	19 InBT, 43 non-InBC, 10 NB	[17]
• Methylation profile clusters each metastasis with its own primary tumor	482,421 CpGs 99 % of RefSeq genes	44 paired BT and LNM	[39]
• Metastases, except luminal A subtype, have global hypermethylation compared to primary tumors			
• Luminal A metastases mainly confer hypomethylation			
• 90 differentially methylated CpGs can discriminate between subtypes of BBM	27,578 CpGs 14,495 genes	32 BBM, 12 NB, 15 NBr, 48 BT	[38]
• Basal-like metastases have the lowest methylation levels			

aNB Adjacent Normal Appearing Breast Tissue, *BBM* Breast Brain Metastasis, *BCCL* Breast Cancer Cell Lines, *BT* Breast Tumors, *DS* Discovery Set, *ER* Estrogen Receptor, *FBT* Familial breast tumors with BRCA1, BRCA2 or non-BRCA1/2 mutations, *HR* Hormone Receptor, *IBT* Invasive Breast Tumor, *IDC* Infiltrating/Invasive Ductal Carcinoma, *InBT* Inflammatory Breast Tumor, *LNM* Lymph Node Metastasis, *NB* Normal breast tissue, *NBr* Non-neoplastic Brain Tissue, *SRAM* Significantly Repressed in Association with Methylation, *TCGA* The Cancer Genome Atlas Network, *VS* Validation Set

the previous findings that there are significant differences in DNA methylation between tumor and normal breast tissues and that differences are not limited to a few gene associated CpGs or repetitive sequences but affects thousands of CpGs across the genome. Furthermore, a list of differentially methylated CpGs can distinguish breast tumors from normal breast tissues. Interestingly, the selected CpGs did not need to be derived from breast cancer, but 151 differentially methylated regions identified in colon cancer could successfully distinguish tumors from normal tissue in breast as well [21]. All these data indicate that methylation changes are involved in a global mechanism that differentiates tumor tissue from normal tissue.

Encouraged by success of mRNA profiling, an intriguing question whether DNA methylation profiling could improve our understanding of heterogeneity of breast cancer came up with the development of high throughput assays.

Analysis of genome wide DNA methylation of numerous samples in many studies derived clusters that are mainly enriched in a specific histological characteristic such as estrogen receptor (ER) expression, or a specific molecular subtype. Methylation profile of over 200 invasive ductal carcinomas successfully clustered the tumors into two: Cluster I: enriched in ER(−) and Cluster II: enriched in ER(+) tumors [18]. To understand whether this DNA methylation pattern is functionally important for tumor phenotype, the authors looked into the genes that were represented in differentially methylated CpGs. The genes that were shown to be positively correlated with ER expression were highly methylated in Cluster I, while the genes negatively correlated with ER expression were hypermethylated in Cluster II [18, 22]. These data indicate that expression of the gene sets that can distinguish ER(−) from ER(+) tumors are at least partially regulated by DNA methylation. In an independent study, methylation profile clustered more than 160 breast tumors into two as one of them being enriched in hormone receptor (HR) (+) tumors, and the other was a mixture of HR(+) and HR(−) tumors [23]. Cell line analysis provided similar results with the tumor studies. A panel of 7 breast cancer cell lines, 4 ER(+) and 3 ER(−), were analyzed for gene expression, gene copy number and DNA methylation. Gene expression analysis were able to cluster all the cell lines as ER(+) or ER(−), while methylation status of all 21,570 CpGs did the same except that one ER(−) and one ER(+) cell line failed to cluster with their respective groups. Among 21,570 CpGs, 444 that mapped within the 5 kb of the 5′ end of the gene were differentially methylated between ER(−) and ER(+) cell lines. Top 100 of this list illustrated a very robust methylation signature for ER(+) and ER(−) cell lines. The results obtained from cell lines were complemented with tumor data showing that 67 genes that are hypermethylated in ER(−) cell lines were overexpressed in ER(+) tumors and 17 genes that were hypermethylated in ER(+) cell lines were overexpressed in ER(−) tumors [24]. Another study identified only 148 CpGs out of 27,578 to be differentially methylated between 12 ER/PR(+) and 12 ER/PR(−) tumors [25]. Only one of the four genes that was found to be differentially methylated between this group of ER/PR(+) and ER/PR(−) tumors was found to be differentially methylated between ER(+) and ER(−) cell lines [24, 25]. Discrepancies between the studies could be due to low number of samples, different techniques that are applied or differences between the nature of cell line models and primary tumors. An analysis

of 162 invasive breast tumors, which were clustered into eight groups, initially showed no association between methylation clusters and ER status. However, when samples were restricted to post-menopausal patients, methylation clusters increased to 11, which were significantly associated with ER status showing the importance of sample diversity [26]. Overall, the data indicates that ER status not only affects the gene expression pattern of tumors but also represents a specific DNA methylation profile, which at least partially contributes to the regulation of gene expression.

Molecular subtypes are based on gene expression. Since DNA methylation is one of the mechanisms that regulate gene expression, many recent studies focused on whether it could represent distinct profiles in different molecular subtypes. Furthermore, DNA methylation profiling is considered as a promising strategy to improve classification of breast tumors and predictions on clinical outcomes such as survival and metastasis. Using a cancer specific panel screening 1505 CpGs from 807 cancer related genes in more than 200 tumors, 15 CpGs were found to be differentially methylated in different molecular subtypes. Basal-like tumors were shown to have the lowest methylation levels. Interestingly, methylation status of five genes (NPY, FGF2, HS3ST2, RASSF1, Let-7a) was able to discriminate basal-like and HER2-overexpressing tumors. The marker genes were hypermethylated mostly in luminal B and ERBB2+ tumors, were not methylated mostly in basal-like tumors and were variably methylated in a group of tumors composed of mainly luminal A with a few luminal B and ERBB2+ tumors [27]. Another study, using the same panel, clustered 189 tumors into three. Each cluster was associated with either luminal A and luminal B or basal-like subtypes, while ERBB2+ and normal-like tumors were not enriched in any of the clusters. 196 CpGs associated with molecular subtypes were identified. These CpGs were more frequently methylated in luminal B tumors and less methylated in basal-like tumors consistent with the finding that the cluster dominated by basal-like tumors had lower overall methylation levels compared to luminal B dominated ones [28]. Screening 27,578 CpGs in a different set of 119 tumors, revealed 6 distinct methylation clusters. Three of these clusters were enriched in ERBB2+, basal-like or luminal A tumors, pointing to a significant difference between the methylation profiles of these three subtypes [18]. 100 loci that were initially found to distinguish tumor from normal samples were not informative for molecular subtypes. However, 600 loci that were found in the same study clustered tumors into three classes, one of them being enriched in luminal A and other in basal-like [19]. The Cancer Genome Atlas (TCGA) Network had the largest data set with 802 breast tumors that were analyzed for whole genome DNA methylation [7]. In this study, 574 differentially methylated CpGs were found to cluster the breast tumors into five groups. Consistent with the previous findings, one of the clusters had hypermethylated phenotype and enriched in Luminal B subtype, while another one had the lowest methylation and was enriched in basal-like subtype. ERBB2+ group was not associated with any of the clusters [7]. A recent study initially analyzed 482,421 CpGs in 40 tumors and showed that DNA methylation pattern clustered the tumors into three, each of which were enriched in luminal B, basal-like or luminal A tumors [29]. Then, the authors compared their data with the

one released by TCGA Network and looked for the CpGs that were commonly changed in both groups. Interestingly, 254 CpGs were determined for luminal B, 202 for basal-like, while there were no common CpG changes detected for luminal A and ERBB2+. Luminal B specific CpG pattern, which was also observed in a number of luminal A and ERBB2+ tumors, predominantly consist of methylated CpGs in promoter region. On the other hand, basal-like specific CpG pattern was exclusively observed in basal-like tumors and dominated by hypomethylated CpGs in gene bodies, CpG shores and CpG poor promoter regions [29]. Differences between luminal B and basal-like subtypes were also observed in promoter specific analysis. Promoters of ten genes (APC1, BRCA1, BRCA2, CDH1, Cyclin D2, ESR1, HIN-1, RAR-β, RASSF1A and TWIST) that are involved in breast cancer were analyzed by quantitative multiplex methylation specific PCR in 114 primary breast tumor sample group, which is composed of basal-like, luminal and HER2+ tumors. Average methylation ratio of basal-like tumors were lower than the luminal and HER2+ tumors. Only BRCA1 methylation level were higher in basal-like subtype [30]. In another study, promoters of 15 genes (APC, DLEC1, GRIN2B, GSTP1, HOXA1, HOXA10, IGF2, MT1G, RARB, RASSF1A, RUNX3, SCGB3A1, SFRP1, SFRP4, and TMEFF20) were analyzed by PCR based assay in 179 primary breast tumors. 12 genes were differentially methylated between the subtypes and had the lowest values in basal-like group [31]. In conclusion, strong data accumulated by independent research groups showed that molecular subtypes have distinct methylation patterns. The most striking difference was repeatedly found between luminal B and basal-like subtypes, of which the former had hypermethylated phenotype while the latter had low methylation profile. High throughput data were further confirmed by promoter specific analysis of selected genes. ER status, which was shown to have a dominant effect on DNA methylation profile, is an important component of molecular subtypes. Thus, it is plausible to expect DNA methylation differences between luminal and basal-like subtypes, one of the main differences of which is being HR(+) or HR(−). However, ER status alone is unlikely to explain all the methylation changes.

Familial breast tumors could be assigned into one of the molecular subtypes based on their expression profile. A study analyzing 75 familial breast tumors showed that 74 % of BRCA1 tumors were classified as basal-like, 73 % of BRCA2 tumors were luminal A or luminal B, and 52 % of non-BRCA1/2 tumors were luminal A [32]. Analysis of 1505 CpGs from 807 cancer related genes in 71 familial breast tumors showed that BRCA2 tumors had higher methylation levels compared to BRCA1 or other familial tumors. In this tumor set, 50 % of the BRCA2 tumors were classified as luminal B, and 60 % of the BRCA1 tumors were basal-like. Since it was shown that luminal B subtype has higher methylation levels than basal-like, further analysis was required to eliminate the possibility that the methylation profile was an outcome of subtype differences in the particular sample set [7, 27–29]. The complementing data came from analysis of 25,500 transcription start sites in familial breast tumors including BRCA1, BRCA2 and non-BRCA1/2 groups [33]. In contrast to somatic tumors, molecular subtypes of familial tumors did not show any significant changes in methylation profile. However, 822 genes were found to be

differentially methylated between the mutation groups and methylation profile predicted BRCA1, BRCA2 or non-BRCA1/2 status more accurate than the gene expression profile. Furthermore, methylation profile clustered non-BRCA1/2 tumors into two groups, which were heterogeneous in molecular subtype composition [33]. Overall, the data showed that familial breast tumors represent unique DNA methylation profiles that are associated with the mutation type not the molecular subtype. Thus, DNA methylation profiling could expand our knowledge to understand the clinical phenotype of the familial breast tumors.

DNA methylation profile not only improved our understanding on the heterogeneity of breast tumors, but it also provided valuable information of their biology and clinical phenotypes. DNA methylation profile was shown to be able to reveal immune cell infiltration in breast tumors [18]. Gene ontology analysis showed that immunity related genes were differentially methylated between six different methylation clusters of breast tumors. These genes were hypomethylated in immune cells but hypermethylated in normal and cancer cell lines. Tumor clusters that have hypomethylation of the immunity related genes were found to be infiltrated by lymphocytes [18]. Furthermore the same group found out that DNA methylation pattern could reflect the cellular origin of the tumor. They showed that luminal progenitor signature was associated with one of the methylation clusters. This methylation cluster was enriched in basal-like tumors, for which candidate population for transformation was identified as luminal progenitor cells [18, 34]. Another study showed that, methylation profile reflects cell lineage origins of the breast tumors [35]. The authors analyzed 27,578 CpG islands in 19 breast cancer cell lines and found out that 120 genes that were significantly repressed in association with methylation (SRAM) clustered the cell lines into two; EPCAM(+) epithelial cells and EPCAM(−) mesenchymal cells. 71 SRAM genes that were methylated in EPCAM(+) breast cancer cell lines were also repressed in normal human luminal and luminal progenitor cells, which are EPCAM(+). Similarly SRAM genes that were methylated in EPCAM(−) mesenchymal cell lines were repressed in normal human basal/myoepithelial and stromal cells, which are EPCAM(−). Thus, methylation pattern of breast cancer cell lines matched expression patterns of specific lineages of normal breast cells. Furthermore, EPCAM(−) SRAM were able to identify claudin-low tumors, which carry mesenchymal characteristics, while EPCAM(+) SRAM were frequently methylated in primary tumors reflecting their epithelial origin [35]. Stem cell component of the breast tumors were also linked to methylation profile [31]. Stem cell rich tumors, determined by presence of CD44(+)/CD24(−) or ALDH1(+) cell content, had lower methylation levels. However, CD44(+)/CD24(−) tumors were enriched in basal-like subtype, which was shown to have lower methylation levels [7, 27–29]. To make sure that low methylation levels were a result of stem cell component not molecular subtype, the authors analyzed stem cell rich or poor basal-like tumors. They showed that basal-like tumors enriched in stem cells had lower methylation levels compared to basal-like tumors that do not have CD44(+)/CD24(−) cells indicating that stem cell phenotype contributes to DNA methylation profile independently of molecular subtype [31]. Breast cancer risk related exposures were also found to be associated with methylation profiles. Analysis of 1413

autosomal CpGs clustered 162 invasive breast tumors into 8 classes, which were significantly associated with alcohol intake and total dietary folate indicating the effect of environmental factors on DNA methylation pattern of breast tumors [26].

One of the practical benefits of DNA methylation profiling is its contribution to prediction of prognosis. Screening of 27,578 CpGs clustered breast tumors into two, one of which was defined as having breast CpG methylator phenotype (B-CIMP) [23]. B-CIMP was composed of 3297 differentially methylated CpGs of 2543 genes, more than two third of which was hypermethylated. B-CIMP(+) tumors had improved metastasis-free survival. However, B-CIMP(+) cluster was composed of HR(+) tumors, while the other cluster was a mix of HR(+) and HR(−) tumors. To validate that the association of B-CIMP(+) phenotype with better metastasis free survival was independent of HR status, the authors limited the analysis with HR(+) tumors. They showed that B-CIMP(−) HR(+) tumors had worse prognosis than B-CIMP(+) HR(+) tumors. B-CIMP status was a strong predictor of prognosis independent of tumor stage, age, nodal status and HR status. B-CIMP genes that has decreased mRNA expression, were enriched in genes listed in breast cancer prognostic expression signatures [23]. Thus, B-CIMP phenotype could partly explain the differential gene expression in poor vs. good prognosis tumors and could be used as a prognostic marker. Another study showed that DNA methylation profile was associated with the molecular signature that is an indicator of poor prognosis [17]. 500 differentially methylated CpGs clustered a tumor set that consists of 19 inflammatory and 43 non-inflammatory breast tumors into two. The cluster that has high methylation levels was enriched in tumors from patients with distant metastasis and poor prognosis as determined by 70 gene prognostic signature [17, 36]. There were no association between the methylation clusters and age, tumor stage, histological grade or HR and ERBB2 status of the tumors, but one of the clusters were enriched in inflammatory breast tumors. However, it is unlikely that the distribution of the inflammatory breast tumors would affect prognosis because only four CpGs corresponding to four genes were differentially methylated between inflammatory and non-inflammatory tumors [17]. It was also shown that methylation status of individual genes could cluster patients into poor or good prognosis groups [19]. Analysis of more than 100 tumors showed that 921 CpGs, including 490 genic and 431 non-genic loci, could derive two groups with different prognosis. 25 of the genes that were associated with these CpGs were able to cluster patients into poor and good prognosis groups individually. Interestingly, all of these genes were in a different methylation state in normal tissue compared to tumor tissue. Furthermore, these genes belong to gene ontology groups of vasculature development, cell death, proliferation, and cell cycle processes indicating that they could be functionally important in metastatic cascade [19]. DNA methylation profile could also reveal heterogeneity within the molecular subtypes. Analysis of 1505 CpGs clustered 80 breast tumors into three, none of which were strongly dominated by a specific group [37]. Interestingly, it was found that luminal A tumors, which were distributed between two different clusters, differ significantly in survival [37]. However, it should be noted that TP53 status and size of the tumors were also found to affect survival in different methylation clusters and status of luminal A tumors in these

parameters might be a factor in differential survival as well [37]. A recent study identified two new subtypes based on DNA methylation profile: Epi-LumB and Epi-Basal, which are luminal B and basal-like associated CpGs, respectively [29]. Both signatures were associated with reduced survival and observed in most but not all of the relevant molecular subtypes, reflecting the heterogeneity between them. Three genes for Epi-LumB and two genes for Epi-Basal phenotype were selected as proxy to analyze by locus specific assays. Analysis of 301 tumors showed that survival of patients with luminal B or basal-like tumors were not changed by having Epi-LumB or Epi-Basal phenotype. However, within ER(+) tumors, having luminal B or Epi-LumB phenotype significantly increased the death risk by approximately fivefold compared to having only luminal B phenotype. Similarly, within ER(−) tumors, having basal-like or Epi-Basal phenotype increased the death risk by tenfold compared to basal-like only phenotype although it did not reach to statistical significance [29]. In summary, DNA methylation profiling was shown to provide additional information to estimate prognosis and stands out as a prominent approach for clinical use.

Metastasis is another important mechanism that is tightly associated with the survival of the patient. Thus, its molecular characterization has long been a focus of interest. To understand the landscape of breast brain metastasis, DNA methylation profile was analyzed in 32 metastases, 12 non-neoplastic breast tissue and 15 non-neoplastic brain tissue [38]. 425 CpGs were found to be differentially methylated in metastases, majority being hypermethylated. Similar to primary tumors, subtypes of metastases were discriminated by 90 differentially methylated CpGs. Basal-like metastases were found to have the lowest methylation levels consistent with the primary tumor data [38]. The differences between metastases and matched primary tumors were not that striking. A recent study analyzed methylation pattern of more than 400,000 CpGs in 44 paired primary tumors and lymph node metastases [39]. Metastases did not form an independent group but each metastasis was grouped with the matching primary tumor instead, indicating that metastasis specific changes do not dominate the methylation profile. Metastases have global DNA hypermethylation compared to primary tumors mostly outside the core promoter regions, with the most significant increase being observed in basal-like subtype. Only luminal A metastases mainly confer hypomethylation leading to the question whether different subtypes acquire metastatic capacity via different mechanisms. Only 155 genes were differentially methylated between primary tumor and its matched metastasis in at least one of the molecular subtypes. Among these 155 genes, only 8 were differentially expressed indicating that majority of the methylation changes that are associated with gene expression must have occurred early in tumorigenesis [39].

Overall, high throughput DNA methylation analysis improved our understanding in breast tumor heterogeneity and clinical outcomes. However, how DNA methylation contributes to these processes is still an open question. Many of the studies combined DNA methylation profiling with mRNA profiling. Although there was an association, DNA methylation did not always correlate with gene expression. B-CIMP phenotype that is associated with better prognosis was composed of 1764

hypermethylated genes, only 102 of which were downregulated at the mRNA level [23]. 444 CpGs were found to be differentially methylated between ER(−) and ER(+) cell lines, but only 151 of them were inversely correlated with the expression of 149 genes [24]. Among 79 genes that were differentially methylated between three clusters of tumors, only 33 showed a significant anti-correlation between methylation and gene expression [19]. The tumor group with hypermethylation profile has 4283 genes that are differentially methylated, while only 1899 of them were differentially expressed [7]. All these data point to alternative mechanisms that regulate gene expression in coordination with or independent of DNA methylation to reach to a certain context in the cell. Loss of copy number could be one of the candidate mechanisms. In poorly metastatic MDA MB 468-GFP and highly metastatic MDA MB 468-LN cell lines, copy number alterations, gene expression and DNA methylation profiles were analyzed. It was shown that loss in copy number was correlated with hypermethylation, while copy number increase was associated with hypomethylation [40]. Gene expression and DNA methylation analysis in ERBB2(+) cell line HCC1954 showed that hypomethylated genes had greater tendency to be repressed [41]. In 50 ERBB2(+) breast tumors, the genes hypomethylated in HCC1954 were enriched in repressed genes compared to 23 normal breast samples. The repression mechanism of hypomethylated genes were revealed by the analysis of histone modifications. Regions showing hypomethylated DNA were enriched in H3K9me3 and H3K27me3. Allele specific analysis showed that in hypomethylated genes, one allele was methylated and the unmethylated allele was occupied by H3K9me3 and H3K27me3, resulting in repression of gene expression [41]. Thus, DNA hypomethylation was tightly linked to formation of closed chromatin structures and gene silencing.

In conclusion, DNA methylation profiling proved to be useful as a novel approach to understand the heterogeneity of breast cancer and classify breast tumors into better defined subtypes with predictable clinical characteristics. Since DNA could be well preserved in different kinds of clinical specimen and represent a more stable profile than mRNA, methylation pattern as a prognostic or diagnostic marker would benefit clinic.

9.1.2 Global Histone Modifications

Eukaryotes maintain their DNA content in a highly compact and ordered structure by the help of small, basic histone proteins. This condensed structure is formed by wrapping 147 bp of DNA around the octamer of four core histone proteins, composed of H2A, H2B, H3 and H4 dimers [6, 42–44]. As a result of this configuration, two types of chromatin regions are generated in the genome; transcriptionally inactive heterochromatin and transcriptionally active euchromatin regions. This chromatin structure is strictly regulated by post-translational covalent modifications of histone tails and at least 16 different types of histone modifications including the addition/removal of methyl, acetyl, phospho, ubiquitin, SUMO and

poly-ADP-ribose groups, have been identified that regulate DNA replication, transcription and repair mechanisms [45–50]. The specific function of each modification and their modifiers in DNA-based mechanisms have been extensively studied to determine their relevance to cellular functions and their contribution to the generation and progression of several human disorders including different types of cancer [6].

Identification of various types of histone modifications suggested the importance of combinations of histone marks in the genome that are referred to as the "histone code" for the regulation of gene function [50–52]. This also implicated the crosstalk among different histone modifier enzymes and their effector proteins. Examination of individual histone marks enabled the determination of the function of each mark and recent efforts now focus on the interplay between different modifications and their involvement in cellular functions [47, 50].

Among all modifications, acetylation and methylation (mono, double and triple) of basic lysine and arginine residues are the most widely investigated histone marks. The levels of acetylation and methylation are governed by the activities of histone acetyltransferases (HATs) and deacetylases (HDACs and Sirtuins); and by histone methyltransferases (HDMTs) and demethylases (HDMs), respectively [47, 53, 54]. Studies on the identification of functions of single histone modifications on gene activation and repression have shown that differential acetylation (ac), mono- (me), di- (me2) and tri- (me3) methylation of histones produce diverse functional outcomes in cells. For example, while H3K4me3, H3K36me3, H3K79me3, H3K9ac, H3K14ac, H3K20me and H2BK4me modifications were demonstrated to be involved in gene activation, H2BK5me3, H3K9me3, H3K27me3 and H4K20me3 were associated with gene repression [50, 52, 55–57]. Changes in the levels of histone modifications can affect their function in gene transcription and therefore can alter cell homeostasis.

Histone modifications are highly dynamic processes that can change in a cell-context dependent manner by altering the activities of modifier proteins that consist of modification inducers, erasers and readers that interact and bind to histone complexes. Changes in histone mark status will eventually alter histone-DNA, histone-histone, histone-non-histone protein interactions that control many DNA-template processes important for regulation of cellular events [49, 58, 59]. This type of crosstalk among DNA, histones, modifiers and effector proteins provide an enormous amount of complexity to the regulation of the gene function [6, 48]. That is why regulation and maintenance of histone modifications are crucial for normal cellular functions and abnormalities in histone modifications, alteration in modifier activities and alteration in the interaction with reader proteins might contribute to the generation of various human disorders including cancer [10, 15, 58, 60].

Cancer is a multistep process that involves both genetic and epigenetic changes of whom cooperation play essential part in the development and progression of cancer [6, 60, 61]. The contribution of epigenetic changes to cancer generation and progression was initially demonstrated by the observation of alterations in methylation status of CpG islands in promoters of cancer related genes [62–64]. This was followed by the identification of the involvement of histone modifiers that were

shown to be mutated to silence and/or activate cancer related-genes during cancer formation [6]. Since then, roles of modifiers and their specific histone modifications in various cancer types have been extensively studied. Although, initial studies examined functions of individual modifier and/or modification in cancer formation and progression, recent developments in global proteomic and genomic technologies enabled the study of global assessment of histone modifications and modifier enzymes in a genome-wide scale. Global changes in histone modifications in different cancer types including breast cancer have been focused on the potential usage of these modifications for correlation with prognostic and histopathological outcomes [65, 66].

Epigenetic factors are thought to be one of the contributors of the breast cancer heterogeneity. For this, effects of epigenetic modifications to the development of breast cancer and their association with breast cancer prognosis, recurrence risk and subtype classification have been extensively studied in recent years [66–68]. Similar to other cancers, breast cancer tissues and cells have been observed to have altered epigenetic modifications compared to normal tissues and cells. Although initial studies have focused on the analysis of modifications on a gene-by-gene basis, recent trends switched to investigation of global epigenetic changes and their relevance to breast cancer-specific subtypes, prognosis and patient outcomes [66]. In the following parts, the global changes in histone acetylation and histone methylations and their association with breast cancer will be mainly explained with a brief summary of involvement of other histone marks in breast cancer development and progression.

Analysis of normal tissues, cancer cell lines and primary tumors have shown that global loss of monoacetylation (H4K16ac) and trimethylation (H4K20me3) of histone 4 have been commonly observed in initial steps of different cancers and considered to be one of the hallmarks of cancer generation [69]. This suggested the involvement and usage of global histone modification changes for the prediction of risk assessment, prognosis, recurrence and overall survival in breast cancer [66, 70, 71]. The global changes in H3K4me2, H3K9ac, H3K18ac, H4R3me2, H4K12ac, H4K16ac and H4K20me3 histone modifications were compared using immunohistochemistry staining on tissue microarrays of 880 normal and primary invasive breast carcinoma cases [66]. Here, authors observed a correlation between histone modification status, breast cancer subtypes and clinical outcome in analyzed tissues. Although a heterogeneous staining of different histone marks were detected in tumor samples compared to normal tissue, majority of tumor cases (78.9 %) was shown to have a very low or undetectable acetylation of K16 on H4 (H4K16ac) levels. As a general observation, high levels of histone acetylation and methylation were associated with a better prognosis, ER(+) and PR(+) tumors and luminal-like breast tumors while low-to-moderate levels of all histone marks were generally implicated in poorer prognostic tumors such as basal-like and HER2-positive subtypes. Moreover, low levels of H3K18ac, H3K9ac, H3K4me2, H4R3me2, and H4K16ac were associated with unfavorable patient outcome while high levels were linked to more favorable breast cancer specific survival and metastatic specific survivals. Finally, high levels of H3K9ac, H3K18ac and H4R3me2 were associated

with longer disease free survival rate [66]. In summary, global hypermodified or hypomodified histones were differentially correlated with breast cancer subtypes, prognosis and patient outcomes.

Genome-wide changes in acetylation of K12 of H4 (H4K12ac) were also correlated with differential patient outcomes, where high levels of H4K12ac were implicated in better breast cancer specific and metastatic specific survivals and low levels were associated with unfavorable patient outcome [66]. In a similar way, immunostaining of global histone H4 acetylation (H4ac) and H4K12ac modification in 58 breast tissues containing concurrent non-cancerous breast epithelium, ductal carcinoma in situ (DCIS), and invasive ductal carcinoma (IDC) in the same block and 22 normal breast samples from reduction mammoplasties indicated that normal epithelium had a higher level of H4ac and H4K12ac expression compared to tumor samples. In tumors, on the other hand, a progressive hypoacetylation of H4ac and H4K12ac was observed as the tumor progresses from normal to DCIS and to IDC stages [72]. This suggested involvement of global histone H4 hypoacetylation, specifically H4K12ac, in the progression of cancer to more invasive stages.

Repressive histone modifications were among the widely studied histone marks that have been shown to be associated with different breast cancer subtypes [67, 73, 74]. The expression of trimethylation of histone 3 on K27 (H3K27me3) was observed to be higher in normal breast tissues (88 %) compared to breast tumor samples as shown by immunohistochemical staining of 142 primary breast tumor and 43 normal breast tissues [67]. Authors suggested a correlation between the reduction of H3K27me3 mark and ER negative tumors and also large tumor sizes which was supported by Holm et al. (2012) who observed a correlation between high expression of H3K27me3 and small tumor sizes [67, 75]. Moreover, low H3K27me3 levels were shown to have adverse prognostic values as low H3K27me3 levels were significantly correlated with poor prognosis in breast cancer patients with shorter overall survival time compared to patients with high H3K27me3. H3K27me3 was regulated by histone methyltransferase EZH2 (enhancer of zeste homolog 2) that was observed to be upregulated in breast cancers and this overexpression was shown to be mostly associated with aggressive breast cancer phenotypes [76–79]. Holm et al compared the global expression of H3K27me3 and EZH2 in more than 400 tumor samples and also in breast cancer cell lines to relate their expression pattern with different breast cancer subtypes [75]. There was an inverse correlation between H3K27me3 and EZH2 expression in different tumor types and tumor grades as high expression of EZH2 was associated with high grades and ER(−)/PR(−) tumors and these tumors were observed to have low H3K27me3 levels [75]. Consistent with this study, examination of H3K27me3 on tissue micro arrays in the Nurses' Health Study demonstrated a significant association of H3K27me3 mark with lower tumor grade and a positive association with ER(+) and PR(+) tumors [68]. Finally, H3K27me3 was also observed to be positively associated with luminal subtype A breast cancers [68, 75].

The levels of other repressor marks, H3K9me3 and H4K20me3, were also evaluated in a small group of primary tissues (15 breast cancer patients and 28 healthy individual) and both of them were observed to be increased in breast cancer patients

compared to the control group [46]. Assessment of global changes of H3K9me3 modification in a larger cohort suggested no correlation between H3K9me3 levels and clinical data as tumor samples had a diverse staining of H3K9me3 while non-cancerous cells of epithelium and myoepithelium had positive H3K9me3 staining [80]. Similarly, Healey et al. (2014) also observed no association between H3K9me3 and clinical outcomes even though there was a significant overlap between H3K9me3 and H3K27me3 positive tumors [68]. Studies in cell lines further supported this, where the global levels of H3K9Me3 were not observed to be significantly changed among non-cancerous (H16N2), atypical ductal hyperplasia (21PT), ductal in situ carcinoma (21NT) and metastatic carcinoma (21MT1) cell lines [81].

Loss of H4K20me3 is considered to be a common hallmark for different cancers [69, 82]. The studies in breast cancer tissues also suggested a global loss of H4K20me3 modification and its association with clinical outcomes [66, 80]. Low levels of H4K20me3 in breast tumor samples were correlated with poor prognostic features and higher tumor grades [66]. In the same way, Yokoyama et al. (2014) observed a high expression of H4K20me3 in noncancerous regions adjacent to tumor sites and benign cases and a low H4K20me3 levels in 63.9 % of cancer tissues in a study comprised of benign and tumor samples from 112 breast cancer patients. The heterogeneous H4K20me3 staining in cancer tissues was also correlated with different histopathological characteristics. For example, the loss of H4K20me3 mark was shown to be associated with poor prognosis and shorter disease-free survival while patients with high H4K20me3 expression had higher overall and disease-free survival rates [80]. Furthermore, a positive association between H4K20me3 levels and luminal subtypes was suggested in this study as the expression of H4K20me3 was correlated with ER and PR expression but not with HER2 expression. As a result, the loss of H4K20me3 might be a candidate for detection of poor prognostic cases in breast cancer patients. The loss of H4K20me3 in breast cancer was further verified by detection of a decrease in the expression of H4K20me3-specific methyltransferases, SUV420H1 and SUV420H2, as observed in Methylation and Expression of Normal and Tumor tissues, MENT, database as well as in invasive breast cancer cell lines, MDA MB 231 and BT474, compared to other cell lines [80]. Ectopic expression of SUV420H1 and SUV420H2 in these cell lines resulted in elevated H4K20me3 levels and limited invasive activities of cells while knockdown of SUV420H2 in MCF10A, immortalized non-tumorigenic mammary epithelial cell line, increased invasion potential of these cells, suggesting a possible role for SUV420H1 and SUV420H2 and their histone modifications, H4K20me3, in cancer invasion.

In addition to acetylation and methylation, histones are phosphorylated on various sites and histone phosphorylation has been shown to play role in mitosis, DNA repair and transcriptional regulation [83–86]. Phosphorylation of histone H3 (Phosphohistone H3, PPH3) has been used as a marker for proliferating cells and was suggested to have a prognostic value for rapidly proliferating tumor cases such as for early breast cancer cases [87, 88]. Skaland et al. (2007, 2009), observed a significant correlation between PPH3 levels and tumor size, estrogen receptor, histological grade, and mitotic activity index in lymph node-negative invasive breast

cancers in patients less than 55 years old and patients less than 71 years old, respectively [88, 89]. Further, PPH3 was suggested to have a strong prognostic value in all patients especially in ER-positive and histological grade 1 and 2 patients in lymph node-negative breast cancers [89, 90]. Another study investigated the global phosphorylation of linker histone H1 in breast cancer samples, where a correlation between the presence of pT146 of histone H1 staining and tumor grades and subtypes was observed by the immunohistochemical analysis of 242 primary breast tumors and 97 nonbreast cancer tissue [91]. An increase in pT146 staining of histone H1 was also observed in the nuclei of tumor cells as the tumor progressed from grade I to grade III. Further, an association between pT146 staining and tumor subtypes was suggested as higher pT146 intensity was correlated with triple negative breast tumors and lower staining was strongly linked to the luminal A subtype. A similar trend was also observed in cell lines where pT146 level was higher in MDA MB 231, a metastatic breast cancer cell line compared to the MCF-10A, immortalized non-tumorigenic mammary epithelial cell line and MCF7, non-invasive breast cancer cell line. This study suggested that pT146 staining of H1 might be used to differentiate tumor grades in breast cancer [91].

Aside from global changes in histone acetylation, methylation and phosphorylation levels, genome-wide alterations in other histone modifications such as ubiquitination and deimination/citrullination have been also investigated in breast cancer [92–94]. For example, a reversible histone ubiquitination process, governed by the activities of ubiquitin ligases, E1, E2, and E3 enzymes that adds ubiquitin and deubiquitinating enzymes (DUBs) that removes the ubiquitin moiety, regulates the activities of histones H2A and H2B [95, 96]. Ubiquitination of histones have been implicated in the transcriptional regulation and DNA repair mechanisms and their function in disease development including cancer have been recently started to be investigated [96, 97]. Monoubiquitination of histone H2B (H2Bub) levels were analyzed in 109 samples containing normal breast epithelial tissue samples, benign, malignant, and metastatic samples and it was shown that its level did not change in benign breast tumors compared to normal breast epithelium as observed in other cancers [93]. On the other hand, global loss of H2Bub expression was observed in malignant and metastatic samples, suggesting its role in breast cancer progression and metastasis [93, 98]. Further studies in a large group of breast cancer tissues can help to establish the association of H2Bub and other histone ubiquitination marks with different breast subtypes and clinical outcomes.

Citrullination is another histone modification that involves the conversion of positively charged arginine and methylated arginine residues to neutral citrulline by peptidylarginine deiminase (PAD) family of enzymes in a process called deamination or citrullination [94, 99]. Methylation of arginine at different sites of histones H3/H4 can have either repressive or activator effect on gene transcription and therefore, citrullination of these residues could alter chromatin structure and lead to transcriptional activation or repression, respectively [100–102]. The contribution of PADs and citrullinated histones (HCit) to the development of different tumors including breast cancer have been recently started to be investigated. For example, PAD4 was initially shown to be extensively expressed in various tumor types com-

pared to the normal or benign tissues that basically lacked the PAD4 expression [103, 104]. Guertin et al. (2014) further investigated the association of ER, PAD2 and H3R26Cit in tumor sections of 21 breast cancer patients [94]. They observed a correlation between the degree of ER staining, PAD2 and H3R26Cit staining. Additionally, a significant link between PAD2 expression and relapse free survival time of patients was detected in luminal A subtypes. These results might suggest the importance of PADs and citrullination in tumor development/progression but further studies will elicit their prognostic and histopathological values.

Genome also contains other rare modifications that include O-GlcNacetylation, sumoylation, ADP-ribosylation, proline isomerization, crotonylation, propionylation, butyrylation, formylation and biotinylation [6]. Involvement of some of these modifications (O-GlcNacetylation, sumoylation) in breast cancer development and progression have started to be documented recently [105, 106]. Development of new tools to detect these minor histone marks in the genome will enable the investigation of their global changes in different cancers including breast cancer and their relevance to prognosis and clinical outcomes.

In conclusion, breast cancer tissues contained altered global histone modification profiles compared to normal breast tissue, implicating the importance of chromatin regulation for tumorigenesis. Elucidation of genes affected by these changes would provide clearer picture about how histone modifications contribute to cellular functions as well as carcinogenesis.

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Chapter 10
Enhancer of Zeste Homology 2 (Ezh2), an Epigenetic Regulator: A Possibility for Prostate Cancer Treatment

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Abbreviations

ATRA	All Trans retinoic form
CRPC	Castration-resistance prostate cancer
DAB2IP	Disabled homology 2-interaction protein
DNMT	DNA methyltransferase
DZNep	3Deanzeaneplanocin-A
EED	Embryonic ectoderm development
HDAC	Histone deacetylase
miRNA	microRNA
MMP	Matrix metalloproteinase
PCAT	Prostate cancer-associated noncoding RNA transcript
PcG	Polycomb group
PI3K	Phosphoinositide-3-kinase
PRC	Polycomb repressive complex
RKIP	Raf-1 kinase inhibitors protein
SAHA	Suberoylanilide hydroxamic acid
SUZ12	Suppressor of Zeta 12

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10.1 Introduction

Enhancer of zeste homology 2 (Ezh2) is a histone-lysine *N*-methyltransferase enzyme. It is regulated by *Ezh2* gene that is involved in DNA methylation, which ultimately leads to the suppression of transcription. Ezh2 catalyzes the addition of methyl (–CH3) groups to histone H3 at lysine 27 with the help of a cofactor *S*-adenosyl-L-methionine (SAM). The methylation in Ezh2 induces heterochromatization, which is responsible for the remodeling of chromatin thereby silencing gene function(s). Further, Ezh2 is the functional catalytic core protein of Polycomb Repressor Complex 2 (PRC2), which is essential for normal embryonic development through the epigenetic modifications. Ezh2 is also responsible for PRC2 methylation and catalyzes the trimethylation of histone3 lysine27 (H3K27). Ezh2 induces silencing of target genes, which are involved in suppressing tumor growth and cellular homeostasis [1–7]. These target genes are associated with cellular proliferation, invasiveness, senescence, angiogenesis and metastasis of cancer development [8]. Studies suggest that over expression/dysregulation of Ezh2 could be an important factor for tumor development and progression [2–7]. Therefore, the prevention of Ezh2 over expression is a promising strategy for effective therapeutic interventions in many aggressive cancers including prostate cancer [2–7].

Studies have established the location of the *Ezh2* gene on chromosome 21q22.2 in almost all mammals [9]. However, later findings presented by Cardoso and his colleagues found the location of *Ezh2* on chromosome number 7q35 and the sequence isolated from chromosome 21 corresponded to a pseudo gene [10]. Structurally, the human *Ezh2* gene contains 20 exons, which encode 746 amino acid residues respectively. Additionally, human *Ezh2* gene has evolutionarily conserved sequences such as domain-1, domain-2 and a cysteine-rich amino acid stretch that leads to the carboxy-terminal SET domain. The SET domain is directly associated with the activation of histone methyltransferases (HMTase). The removal of a single amino acid (Tyr641) in SET domain significantly reduces histone methyltransferase (HMTase) activity *in vitro* [11–13]. However, studies on human PRC2 demonstrated that optimal HMTase activity requires Ezh2, Embryonic Ectoderm Development (EED), and SUZ12 [11]. Biochemically, EED is essential to the enzymatic activity of Ezh2 which organizes the EED-Ezh2 complex. The formation of this important protein complex typically leads to increased activity and functionality of PRC2's HMTase [12, 13]. The WD40 (Trp-Asp) domain of EED is crucial to the proper funcition of the EED-Ezh2 complex; however, point mutations in WD40 domain showed interrupted interaction between EED and Ezh2 [11, 14]. Studies show that PRC2-induced activation of H3K27me3 plays an important role in cell proliferation, senescence and carcinogenesis [8, 15, 16]. Therefore, targeting over expression of EZh2 in cancer cells will certainly prove successful in paving the way to novel epigenetic drug discoveries and presenting as a viable therapeutic regimen in the treatment of cancer.

Ezh2 resides in both the nucleus and the cytoplasm of the cell. It produces nuclear localization signals (NLS) that activate downstream signaling of F-actin polymerization, which may eliminate the possibility of Ezh2-mediated prostate cancer progression and invasion [17]. A past study showed that increased expression of Ezh2 was observed in malignant prostate cancer tissues as compared to normal prostate tissues, which suggest that over expression of Ezh2 is associated with prostate cancer development and progression [18]. Therefore, inhibition of Ezh2 can be achieved using pharmacological inhibitors such as 16-hydroxycleroda-3, 13-dien-15, 16-olide (PL3) and small moles DZNep. Besides these pharmacological strategies, silencing of gene function using microRNA has gained great attention for further research in this direction. The use of microRNAs, specifically microRNA-101 is crucial to this avenue of research [16]. Several studies suggest that the expression of microRNA-101 decreases during cancer progression; however, in some cases there exists an inverse relationship between microRNA-101 and Ezh2 expression [19]. Antiparallel expression profiles have been observed between microRNA-101 and Ezh2 further cementing such studies [16]. Furthermore, knockdown of microRNA-101 in cancer may lead to over expression of Ezh2 and deregulation of epigenetic pathways, thus resulting in cancer progression [3, 20–22]. Interestingly, it was found that AKT phosphorylates Ezh2, which also increases the likelihood of carcinogenesis [23, 24]. In addition, Akt-dependent ser-21 phosphorylation was found in breast cancer cells after treatment with IGF-1 or estrogen. Therefore, it is believed that phosphorylation results in the weak interaction between Ezh2 and other PRC2 subunits, which has shown decrease methylation of H3K27. Furthermore, phosphorylation results in activation of JNK-STAT3-AKT signaling that leads to trimethylation of histone H3 lysine 27 (H3K27me3) [25, 26].

10.2 Role of Ezh2 in Prostate Cancer

Previous studies suggest that Ezh2 is highly expressed in a wide range of malignancies, including: cancers of prostate, colon, bladder, lung, breast, pancreatic cancer as well as lymphomas and sarcomas as compared to normal tissue/cells [1, 8, 19, 20, 27–51]. Increased expression of Ezh2 is often correlated with an advanced state of cancer progression and poor survivability [8]. Cells that express more Ezh2 demonstrate a high rate of cellular proliferation and oncogenic properties [4, 12, 18, 28, 52–56]. Li et al. showed over expression of Ezh2 in mammary epithelial cells of the tumorigenic mouse model results in the development of an epithelial hyperplasia phenotype [57]. Furthermore, mutations in Ezh2 result in B cell lymphoma, follicular lymphoma, myelodysplasic and myelo-proliferative disorders [1, 2, 21, 49–51, 58–62].

It has been shown that prostate cancer patients showed enhanced expression of Ezh2 with increased cellular proliferation, invasiveness, and metastasis of cancer cells [63]. In cases of human prostate cancer, increased expression of Ezh2 results in extra prostatic extension, positive surgical margins and a recurrence of prostatic

specific antigens [63]. Opposite to this, knockdown of endogenous Ezh2 using siRNA showed reduce cellular proliferation, differentiation and invasion in prostate cancer patients [18]. Additionally, Ezh2 induced prostate cancer cell invasion and metastasis by repressing RKIP (Raf-1 kinase inhibitor protein), which is a metastasis suppressor gene [15]. Therefore, it may be plausible to assume that metastasis is the outcome of Ezh2 over expression. In addition, studies indicate that Ezh2 plays a vital role in the regulation and suppression of the expression of metalloproteinase and the inhibitors of metalopeptidases-2 and -3 in prostate cancer cells [64]. However, Ezh2 showed 11 genetic variations in prostate cancer, which are not accountable for the linkage of 7q to prostate cancer [65]. The individual variation did not show significant differences in the allele frequencies between the experimental and controls. Although, one haplotype may be higher in frequency than those of another haplotype which showed significantly higher levels in low grade tumors and vice versa in high grade tumors [65]. Therefore, the mechanism of Ezh2 over expression in prostate cancer is not well understood and requires further investigation. In castration-resistance prostate cancer cells, Ezh2 may be a transcriptional co-activator of androgen receptor instead of a transcriptional repressor of PCR2 [66]. Moreover, the phosphatidylinositol 3-kinase-Akt pathway mediated by Ezh2 phosphorylation at ser-21 could act as transcriptional activator [36].

PCR2 maintains cellular homeostasis during chromatin remodeling [67]. In mammals, there are two types of polycomb group complexes, PCR1 and PCR2. The PCR2 complex consists of four core components: Ezh2, Suppressor of Zeta 12 (SUZ12), EED, and retinoblastoma associated protein 46/48 [13]. Ezh2 with SET domain forms a complex, which catalyzes H3K27me3 and is involved in the silencing of tumor suppressor genes such as Disabled homology2-interaction protein (DAB2IP) (Fig. 10.1) [75].

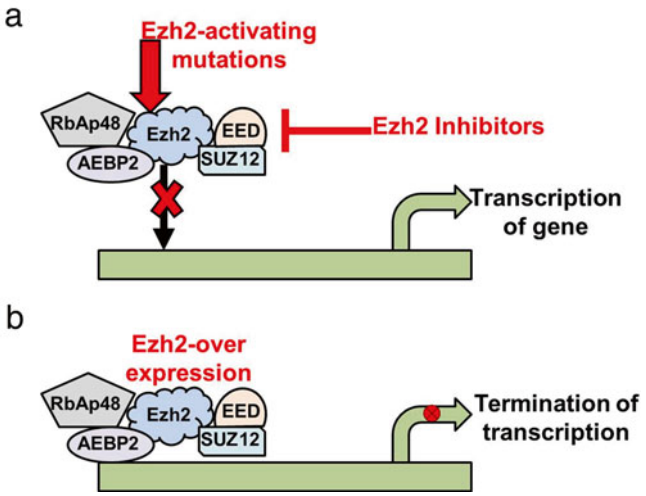

Fig. 10.1 Role of Ezh2 in tumor progression. (**a**) The mutation in Ezh2 activates gene transcription. (**b**) Over expression of Ezh2 results in trimethylation of H3K27 to terminate gene transcription, especially the inhibition of tumor suppressors [53, 58, 68–74]

It has been shown that Ezh2 and STAT3 (signal transducer and activator of transcription 3) affect self-renewal, tumorigenicity, chemo-resistance, pluropotency, and proliferation in cancer cells [22, 76, 77]. Akt-dependent Ezh2 phosphorylation at ser21 was also observed in breast cancer cells treated with insulin-like growth factors 1 or estrogen [23]. Furthermore, JNK-STAT3 and JNK-STAT3-Akt signaling induces phosphorylation of Ezh2 [24]. Transcriptional repression of c-Myc by Ezh2 may be a novel mechanism for the treatment of gliomablastoma and stem cells maintenance [78]. In addition, phosphorylation of Ezh2 induces H3K29 trimethylation and target gene silencing. Cyclin-dependent kinase (CDK) is a family of protein kinases, which are involved in cell cycle regulation. CDKs have also been found to be involved in gene transcription, mRNA processing, and differentiation. Mutations alter the functions of CDKs, which may result in uncontrolled cell division and progression of cancer. In addition, CDKs also induced Ezh2 over expression by phosphorylating Thr350 [79]. CDK1/2 harbors and phosphorylates Thr350 of Ezh2; however, Ezh2 has a mutation at a site of an amino acid located within Thr350 (Thr350A) that silences CDK1/2 thus decreasing the expression of Ezh2. Therefore, CDKs regulates the expression of Ezh2 and cancer cell proliferation. Further, chromatin immune-precipitation shows that inhibition of Ezh2 decreased H3K27me3 levels in the promoter of HOXA9 and DAB2IP, which are downstream targets of Ezh2 (Table 10.1) [79]. In the mammalian cell system, phosphorylation of Ezh2 results in altered biological functions by suppressing the transcription of other genes [1, 13, 51, 91]. Furthermore, a large number of genes that were transcriptionally repressed by CDK1/2 restored wild type Ezh2 expression [16]. It is suggested that the phosphorylation of Ezh2 is critical to ensuring the proper regulation of targeted genes [1, 13, 56, 91–93]. In addition, Ezh2 is frequently over expressed in several types of cancers, such as advanced human prostate [18, 65, 76, 94, 95]. It has been shown that thr350 phosphorylation is essential for the tumorigenic function of Ezh2 in prostate cancer cells. However, dephosphorylation of Ezh2 at the site of Thr350 increases its tumor suppressor gene DAB2IP expression in LNCaP cells, while abnormal activation of CDK1/2 contributes to the aggressive phenotypes

Table 10.1 List of Ezh2 targets in prostate cancer

Ezh2 targets in prostate cancer		
Gene	Roles in pathways inhibition	Role in carcinogenic
DAB2IP	NF-kB/Ras pathways	Invasion, proliferation and transformation [75, 80, 81]
PCAT-1	Transcription	Proliferation [82–84]
TIMP2/3	ECM deletion	Invasion [85]
RKIP	Raf and NF-kB pathways	Invasion [15]
PSP94	MPM secretion	Invasion [86]
CDH1	Cell adhesion	Invasion [87]
SLIT2	Chemorepellent protein	Proliferation and invasion [88, 89]
ADRB2	Anderenegic signaling	Invasion and transformation [90]

typically found in tumors. This is accomplished via phosphorylation and the subsequent tumorigenic/gene silencing mechanisms of Ezh2 (Table 10.1) [79, 96, 97]. Therefore, dephosphorylation of Ezh2 at Thr350 may serve as a viable therapeutic target to prevent the tumor inducing functions of Ezh2 in human prostate cancer [79, 98] (Table 10.1).

10.3 Ezh2: Prostate Cancer Therapy

The main difference between genetic and epigenetic mechanisms is the degree of reversibility of each respective process [7, 91, 93, 94, 99, 100]. Genetic changes that alter nucleotide sequences are difficult to restore and affect gene product(s). Conversely, epigenetic modifications have shown the ability to be reversed without disrupting the DNA sequence. Therefore, it is possible that Ezh2 can be targeted using several inhibitors against the enzymes, which are directly involved in the epigenetic modification of Ezh2 [91, 93, 100–103]. This may prove to be a novel therapeutic strategy for prostate cancer treatment and tumor elimination. A number of lead treatment studies are in the process to develop an effective pharmacological agent.

Keeping these facts in view, we analyzed effective molecular targets currently being used in prostate cancer treatment therapies such as 16-hydroxycleroda-3, 13-dien-15, 16-olide (PL3) and DZNep. It was demonstrated that small molecules like 3-deazaneplanocin-A (DZNep) can be used in inhibiting *S-adenosyl-l* -homocysteine (SAH) hydrolase, a cofactor, essential for Ezh2-dependent methylation and synergistically enhanced the anti-proliferative activity [104]. Furthermore, DZNep deplete PRC2 complex proteins (Ezh2) and inhibits H3K27me3; therefore, DZNep may be the first targeting compound in this area [105]. Furthermore, *in vivo* studies suggest that DZNep induces apoptosis in cancer cells without affecting normal cells [95, 105]. Therefore, treatment with DZNep not only showed anti-proliferative and anti-cancer activities but also blocked migration and invasion of prostate cancer cells [95, 106, 107]. As a result, DZNep has gained attention from cancer researchers and is being used as a chemotherapeutic agent against several types of human cancers. DZNep has a short half-life (1.10 h); therefore, the liposome method was used to improve the pharmacokinetics of DZNep [40, 108–110]. DZNep acts on enzymes H3K27me3 and H3K4me3, which stimulate gene transcription and inhibits histone methylation resulting in poor histone demarcation [106, 107]. DZNep can be implemented as a potential therapeutic agent due to its ability to suppress many cancers; however, it also has some limitations that need to be investigated in further detail.

The emerging approach to target Ezh2 over expression is to block HDAC and DNMTs activity. Inhibition of HDAC and DNMTs, which results in Ezh2-mediated epigenetic gene silencing, is depicted in Fig. 10.2. Inhibition of the enzymes can be achieved by suberoylamilide hydroxamic acid (SAHA) and desi-peptide (Romidespin) which are FDA approved inhibitors of enzymes HDAC and DNMTs

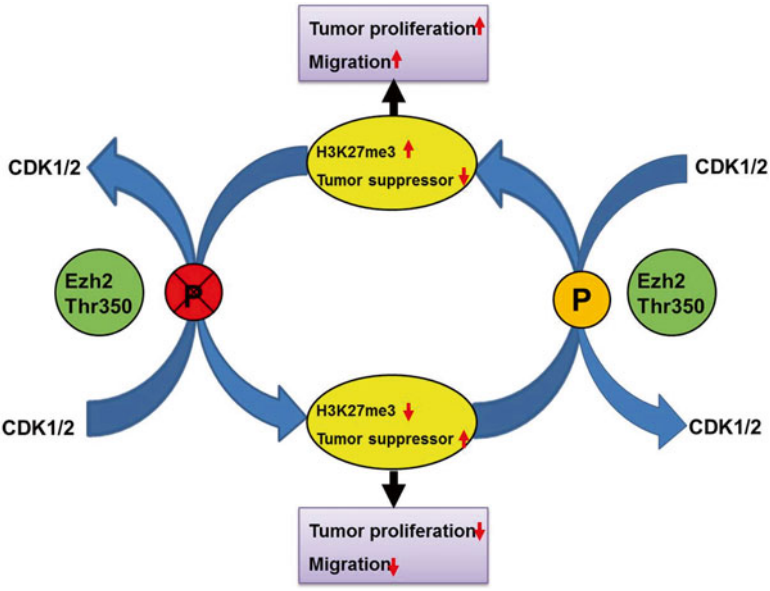

Fig. 10.2 Role of CDK1/2 in Ezh2 over expression and carcinogenesis. CDK1/2 induces Ezh2 phosphorylation at Thr350, which results in decreased expression of tumor suppressor genes by enhancing H3K27me3 at the promoters of Ezh2 targeted genes. However, inhibition of Ezh2 phosphorylation by of CDK1/2 or Thr350 of Ezh2 inhibitors suppressed cancer cell proliferation and migration [53, 58, 68–74]

[111]. SAHA directly targets the catalytic site of the enzymes HDAC and inhibits its activation while Romidespin, a pro-drug binds to zinc (Zn) present in HDAC's active site resulting in diminished catalytic activity [99, 112]. Therefore, SAHA and Romidepsin have been considered as potential inhibitors of prostate cancer growth and proliferation [98, 113, 114]. These enzymes are considered to be among the most effective emerging therapy strategies against prostate cancer [19, 20, 51, 106, 115, 116]. 5aza-2′-deoxycytodine (5aza), a nucleotide analogue to be a potent inducer of apoptosis in prostate cancer cells. Although, the specific mechanism remains to be elucidated [117, 118]. DZNep, HDAC, and DNMT inhibitors have number of limitations and serious concerns in their clinical applications (side effects).

Small molecules can inhibit the enzymatic activity of Ezh2 by blocking its phosphorylation [55, 72, 110]. Previous studies shows that the formulated drug 3,3′-diindolylmethan is able to inhibit Ezh2 over expression [21, 52, 55, 119–121]. In fact, prostate cancer patients treated with BR-DIM in phase-II clinical trials showed increase expression of let-7; however, Ezh2 expression was diminished significantly [21, 52, 55, 119–121]. Other molecules such as, 16-hydroxycleroda-3, 13dien-15, 16-olide (PL3), which is a naturally isolated compound from the bark of polyathialongifolia has shown great promise in therapeutic applications against prostate cancer [18, 22, 54, 63, 65, 84, 98, 122]. PL3 inhibited histone modifying enzymes

including two PRC2 components, Ezh2, and SUZ12 [54, 79, 113, 114, 121–124]. PL3 induced the reactivation of genes, which were repressed by PRC2 and induced apoptosis in K562 cells. Further studies show that PL3 also induced apoptosis in human leukemia cells by suppressing the expression of Ezh2 and SUZ12 which further leads to the reactivation of the PRC2 tumor suppressor gene [56, 92, 115, 116, 123–126]. These findings reveal the link between the anti-inflammatory and cytotoxic effects of PL3 against breast and hepatocellular carcinomas, and provide new insight into the modulation of Ezh2 over expression in prostate cancer [127]. Studies indicate that all trans-retinoic acid (ATRA), a potent anticancer agent, induces apoptosis in leukemia, gastric cancer, and prostate cancer by inhibiting Ezh2 and DNMT3B-induced hypermethylation of HOXB13 [54, 128–131]. However, Ezh2 recruits DNMT3B to the promoter regions of specific gene loci and induces DNA methylation [132]. In addition, ATRA treatment showed androgen-independent cell growth arrest in prostate cancer DU145 cells by blocking Ezh2 and DNMT3B methylation resulting in the subsequent reactivation of HOXB13 [54].

In order to analyze more therapeutic targets against Ezh2 over expression, we moved towards the agents that can act as competitive inhibitors against methyl transferase enzymes. For example, GSK-A acts as a competitive inhibitor against both Ezh2 and methyltransferase. GSK-A displaces the endogenous substrate for the enzymes, which results in the marked reduction of H3K27 trimethylation [5, 7, 58, 59, 133, 134]. Furthermore, *in vivo* studies suggest that a slightly different compound, GSK126, inhibits Ezh2 in a highly specific manner as compared to GSK-A [21, 59, 135, 136]. However, in lymphoma models, mutations in Ezh2 lead to enhance activity of GSK126, which reduces the activity of H3K27me3 and PRC2 target genes resulting in impeded growth and proliferation of cancer cells [137, 138]. Keeping these facts in view, it may be possible to assume that GSK compounds hold great therapeutic potential by targeting Ezh2 in prostate cancer but further investigations are needed to explore the underlying mechanisms.

Taken together, Ezh2 inhibitors and agents that block HDAC and DNMT may work as potential anti-cancer agents and could be used in combination therapy to inhibit prostate cancer growth. Therefore, these therapeutic strategies may effectively reduce tumorigenesis/carcinogenesis without affecting the normal cells and reduce disease recurrence (Fig. 10.3).

10.4 Conclusion and Future Perspective

Previous studies have shown that expression of Ezh2 increase many times over during cancer progression and development [18, 68, 77]. In this chapter, we reviewed mechanism of Ezh2 regulation including over expression of Ezh2 can be regulated by various mechanisms such as the inhibition of , histone modification and chromosomal remodeling [40, 111, 134]. Therefore, it may be safe to assume that the modulation of Ezh2 regulatory mechanisms could highly impact Ezh2 activity and subsequently be therapeutically effective in many cancers. However, several inhibi-

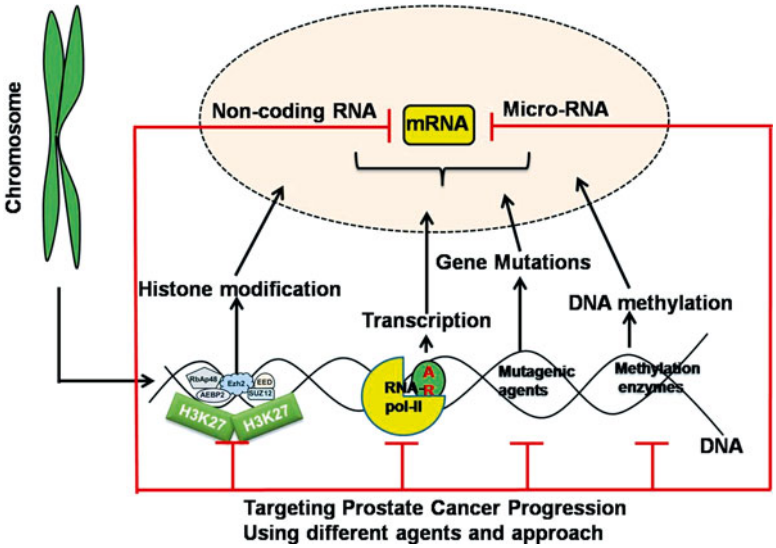

Fig. 10.3 A schematic representation of the regulation of prostate cancer progression using different regulatory mechanism(s) [15, 21, 40, 52, 55, 59, 64, 66, 77, 110, 115, 120, 121, 126, 135, 136, 139]

tors of enzymes like HDAC, DNMTs and Ezh2 are being used in clinical trials and have shown great potential in inhibiting cancer growth and metastasis [15, 41, 93, 140]. Evidence regarding these inhibitors suggests that the molecules could potentially act in suppressing Ezh2 over expression and prevents the recurrence of prostate cancer [57, 58, 68, 69, 73]. In addition, the use of DZNep, HDAC and DNMT inhibitors result in decreased expression of Ezh2, which further leads to inhibition of cancer cell proliferation [106, 107]. Therefore, use of these inhibitors may disable Ezh2-mediated tumorigenesis. More interestingly, inhibition of CDK1/2 also results in down regulation of the tumor suppressor gene DAB2IP, which plays an important role in inhibiting cancer growth by phosphorylating Ezh2 at Thr350 [79, 125, 141]. miRNA inhibits invasiveness and proliferation of cancer cells *in vitro* in a similar fashion like knocking down Ezh2 over expression [96, 116, 126]. Therefore, it will be interesting to test whether miR-101 treatment can be therapeutically effective *in vivo* as microRNA inhibition of overexpression has been exploited in preclinical and clinical trials as a potential cancer treatment regimen [7, 96, 116, 126]. Interestingly, other, studies show that the prevention of Ezh2 over expression in mouse adult stem cells could produce small imperfections in normal organ development or function [60–62, 134, 142–144]. In brief, the administration of Ezh2 inhibitors using specific delivery systems may be necessary to avoid adverse side effects in normal cells [92, 96, 111, 114, 118, 136]. Therefore, better characterization of blocking Ezh2-induced tumorigenesis targets/signaling pathways can be more practical and effective as compared to previously described techniques. Understanding the regulatory mechanisms and the function of *Ezh2* gene targets will help to expedite the development of novel cancer therapeutic regimens.

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