Chapter 5
Epigenetics of Colorectal Cancer

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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

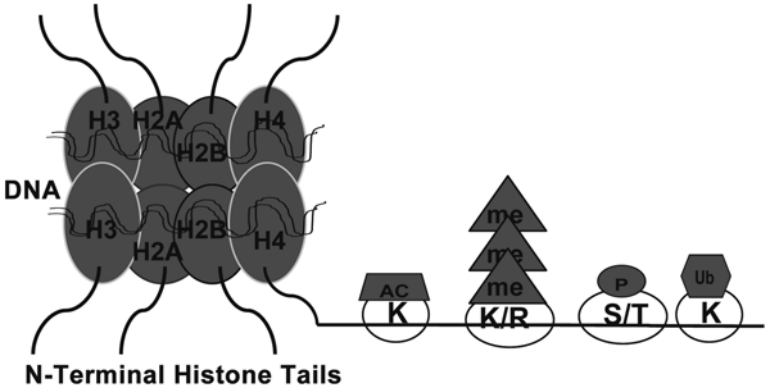

Fig. 5.1 N-terminal histone tails protruding from the nucleosomal units of DNA can be post-translationally modified by acetyl (Ac), methyl (me), phosphate (P), ubiquitin (Ub) and other groups at the basic amino acids including lysine (K), arginine (R), serine (S) and threonine (T). These post-translation 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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