Chapter 14
Epigenetic Regulations of mRNAs and miRNAs by Nutraceuticals

Yiwei Li, Dejuan Kong, Aamir Ahmad, Bin Bao, and Fazlul H. Sarkar

Abstract Both genetic alterations and epigenetic regulations of genes could lead to the development of human cancers. However, recent studies have shown that epigenetic alteration contributes significantly not only to the development of cancer but also responsible for the progression of cancer to metastatic disease. The epigenetic regulations of specific genes in human cancer cells include DNA methylation, acetylation, histone modification, nucleosome remodeling, and small non-coding RNA regulation including the regulation of microRNAs (miRNAs). Among many epigenetic regulations, DNA methylation is the most common event and has been well studied for understanding the mechanisms of epigenetic regulation of genes. The DNA hypermethylation occurs in the promoter sequences of tumor suppressor gene or tumor suppressive miRNAs leading to the down-regulation in the expression of tumor suppressor mRNAs or miRNAs, resulting in the development and progression of various cancers. Interestingly, recent studies have shown that several non-toxic natural agents known as nutraceuticals including isoflavone, curcumin, (−)-epigallocatechin-3-gallate, resveratrol, indole-3-carbinol, 3,3′-diindolylmethane, and lycopene could demethylate DNA at their hypermethylation sites or modulate histone, demonstrating their potential roles in the epigenetic regulation of mRNAs and miRNAs. These epigenetic regulations of mRNAs and miRNAs could be one of the molecular mechanisms by which nutraceuticals inhibit carcinogenesis and cancer progression, and thus either nutraceuticals or their synthetic analogs could serve as novel demethylating agents for the treatment of human malignancies.

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14.1 Introduction

Human cancer is the second leading cause of death after cardiovascular disease in the United States and in the world. It is known that both genetic alterations and epigenetic regulations of genes could cause human cancers. The genetic changes including DNA point mutations, gene amplification, gene translocation, etc. have been traditionally believed as major causes of cancer development. However, recent studies have demonstrated that epigenetic alterations contributes significantly to the development and progression of cancers [1]. Moreover, it has been found that genetic and epigenetic regulations are not separate biological events in cancer. Epigenetic regulations could cause genetic mutations while genetic mutations in epigenetic regulators could alter epigenome [1], suggesting the complex biological regulations of these genetic events in the development and progression of cancer.

The epigenetic regulation of specific genes in human cancer cells include DNA methylation, histone modification, nucleosome remodeling, and small non-coding RNA (ncRNA) regulation including microRNAs (miRNAs). These regulations lead to the alterations in the expression of genes without altering the DNA sequences. Among the different types of epigenetic regulations, DNA methylation is the most common event and has been well studied. DNA methylation is heritable and plays critical role in cell differentiation and embryogenesis. However, the hypermethylation occurs in the DNA sequences in the promoter of tumor suppressor genes which could cause gene silencing through the obstruction of transcriptional activators, leading to the development and progression of various cancers (Fig. 14.1).

In recent years, studies have focused on the investigations of the roles and the epigenetic regulation of miRNAs in cancer development and progression. The miRNAs could inhibit its target gene expression by binding to the 3′-untranslated region (3′-UTR) of target mRNA, causing either mRNA degradation or inhibition of translation. The miRNAs could be oncogenic or tumor suppressive depending on their specific functions during cancer development and progression. Interestingly, it has been found that some miRNAs are also epigenetically regulated in various cancers [2], resulting in altered expression of miRNAs and their target mRNAs. The DNA hypermethylation occurs in the promoter region of miRNA gene which could result in the low expression of miRNAs and, in turn, up-regulates the expression of specific target mRNAs and proteins. The epigenetically regulated tumor suppressive miRNAs could cause increased expression of oncogenes both at the mRNA and

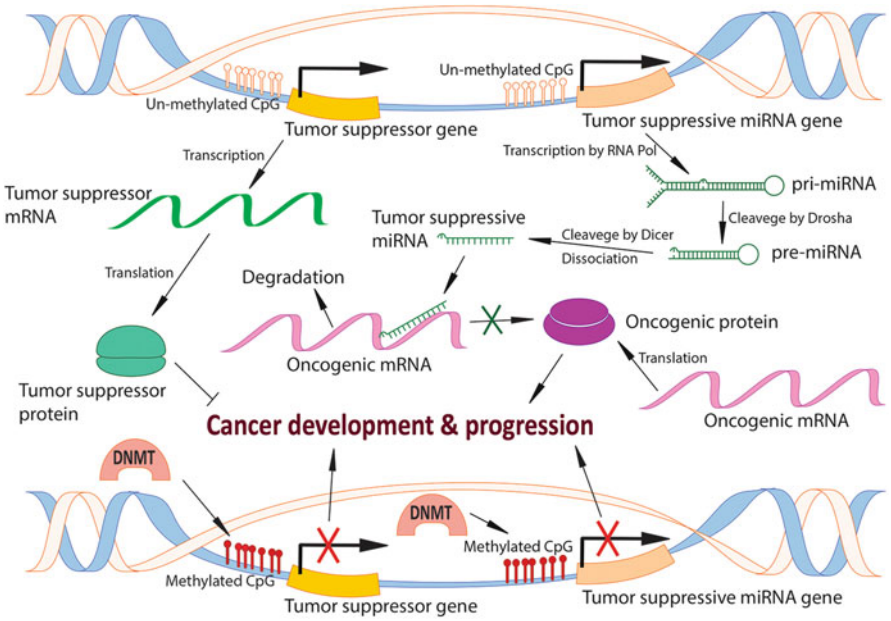

Fig. 14.1 DNA methylation regulated mRNA and miRNA expressions in cancer development and progression

protein levels, which in part could be responsible for the development and progression of various cancers (Fig. 14.1).

Since epigenetic regulations of mRNAs and miRNAs through DNA methylation and histone modification play important roles in cancer development and progression, targeting the epigenetic deregulations in cancers could become a novel and effective approach to fight the battle against cancers. Several epigenetic inhibitors have been synthesized and used in epigenetic therapy trials to re-express abnormally silenced tumor suppressor genes. However, the side-effects of the demethylating agents and histone deacetylase inhibitors (HDAC inhibitors) appear side-by-side with the beneficial effects [3]. Interestingly, recent studies have shown that several non-toxic natural agents known as nutraceuticals including isoflavone, curcumin, (−)-epigallocatechin-3-gallate (EGCG), resveratrol, indole-3-carbinol (I3C), 3,3′-diindolylmethane (DIM), and lycopene could demethylate DNA sequences or inhibit HDACs, demonstrating their roles in epigenetic regulation of mRNAs and miRNAs. These epigenetic regulations of mRNAs and miRNAs could be one of the molecular mechanisms by which nutraceuticals inhibit carcinogenesis and cancer progression, suggesting that either nutraceuticals or their synthetic analogs could serve as novel demethylating agents for the treatment of human malignancies.

14.2 Epigenetic Regulation of mRNAs in Cancers

Epigenetics refers to heritable as well as non-heritable changes in gene expression and cellular phenotype that are not due to alterations in DNA sequence. Epigenetic regulations could alter the expression of mRNA of specific genes. During cancer development and progression, the epigenome precedes multiple alterations including genome-wide loss of DNA methylation (known as hypomethylation), frequently increased methylation of CpG islands in the gene-specific promoter sequence, changes in histone modification and nucleosome, and alterations in ncRNA profile. These alterations are beginning to be appreciated as the molecular basis of carcinogenesis and cancer aggressiveness. Therefore there are significant efforts in the areas of drug development research focusing on epigenetic deregulation of genes for the treatment of human malignancies.

14.2.1 DNA Methylation

Among the epigenetic regulations, DNA methylation is the most widely investigated area in cancer research. During the process of DNA methylation, methyl group is added to cytosine base of CpG dinucleotides through enzymatic methyl transfer catalyzed by DNA methyltransferases (DNMTs). DNA methyltransferases consist of DNMT1, DNMT3A, DNMT3B, and DNMT3L, which are grouped into maintenance and *de novo* methyltransferases. DNMT1 is a maintenance methyltransferase, which recognizes hemimethylated DNA produced during cell division and methylates newly synthesized CpG dinucleotides, to maintain the status of methylation. DNMT3A and DNMT3B are *de novo* methyltransferases to produce DNA methylation during embryogenesis or tumorigenesis. DNMT3L does not possess enzymatic activity; however, it regulates the activity of other methyltransferases to alter the status of methylation.

DNA methylation is a fundamental event in epigenetic regulation, and plays critical roles in the control of gene expression. The methylation of CpG islands, which are the regions with a high density of CpG dinucleotide, in the promoters of genes obstructs transcriptional activators, leading to the down-regulation of mRNA expression. In addition, DNA methylation also influences the remodeling of nucleosome. Wrapped nucleosomal DNA is less accessible than linker DNA; therefore, compressed nucleosomes strongly prevent transcription activators binding to DNA sequences. The methylation of CpG islands allows compressed nucleosome formation and blocks transcription. Moreover, DNA methylation also provides an environment for several methyl-CpG binding proteins including MBD1, MBD2, MBD3, and MECP2, which recruit histone-modifying enzymes to modify histone and regulate gene expression. Therefore, DNA methylation together with other epigenetic regulations could lead to the aberrant expression of tumor suppressor genes, causing carcinogenesis and cancer progression.

14.2.2 DNA Hypermethylations in the Promoters of Tumor Suppressor Genes in Cancers

In normal cell, about 50 % of the CpG islands in the promoter region of genes are un-methylated and these genes are expressed for normal functions [4]. In cancer cells, more methylations occur within CpG islands of promoters, especially hypermethylations in the promoter region of tumor suppressor genes. It has been found that 5–10 % of normally unmethylated CpG islands in the promoter regions become highly methylated in various human cancer [5]. DNA hypermethylation has been commonly correlated with significant down-regulation of gene expression. The reported gene silencing due to hypermethylation in cancers include hMLH1, APC, E-cadherin, CHFR, CASP8, TGF-βRII, p73, HOX A11, COMT, SPRY2, RASSF1A, GPR54, CDH1, RSK4, etc. These DNA hypermethylations commonly do not appear in normal cells; however, it could be observed in hyperplasia, pre-cancerous cells, and in cancer cells.

It is now well known that APC is a tumor suppressor gene. The DNA hypermethylation in the APC gene promoter has been found in atypical hyperplasia, early pre-cancerous cells, and in cancer cells. The frequency of DNA hypermethylation in the APC promoter region has been shown to be negatively correlated with progression of some types of cancer, suggesting that APC hypermethylation could be an early event in tumorigenesis [6]. SPRY2 is another tumor suppressor gene involved in the control of cell proliferation, differentiation and angiogenesis through the inhibition of MAPK signaling. The expression of SPRY2 has been shown to be down-regulated in various cancers because of the DNA hypermethylation in SPRY2 promoter [7]. RASSF1A is also a tumor suppressor gene which inhibits RAS-MAPK signaling. It has been found that the RASSF1A promoter is hypermethylated in cancer cells, leading to reduced expression of RASSF1A consistent with malignant transformation of different types of cells [8]. In addition, more DNA hypermethylations have been observed in the promoters of other tumor suppressor genes in various cancers [9], demonstrating that the silencing of tumor suppressor genes is in part regulated through epigenetics in human cancers.

14.2.3 Histone Modifications in the Regulation of Gene Expression

It is well known that highly conserved histone proteins (such as H1, H2A, H2B, H3 and H4) and DNAs are the basic components of eukaryotic chromatin. The histones undergo a series of post-translational modifications including acetylation, methylation, phosphorylation, ADP-ribosylation, and ubiquitination. Among them, acetylation and methylation of histones are more relevant to the regulation of gene expression (Fig. 14.2). Histone acetylation has been widely investigated and believed to be one of the important modifications during cancer development. It has been found

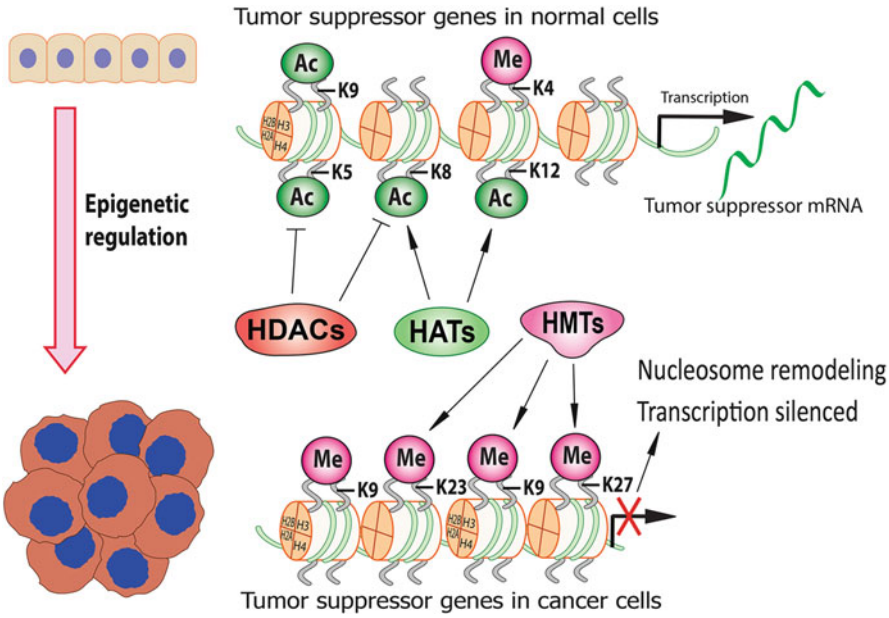

Fig. 14.2 Histone modifications in cancer development

that some selected lysines (such as lysines 9 and 12) are acetylated by histone acetyltransferases (HATs) or deacetylated by histone deacetylases (HDACs) to keep the balance of stable status in the DNA modification [10]. Methylation of lysine residues in histone is another important post-translational modification involved in cancer development. It has been known that methylation of H3 at different lysines could lead to deregulation in the expression of genes. The methylation of H3 at lysine 4 (H3K4) could activate gene expression while the methylation of H3 at lysine 9 (H3K9) and 27 (H3K27) could inhibit the expression of genes [10, 11].

Although both DNA methylation and histone modification have their own enzymes to catalyze different chemical reaction, DNA methylation and specific histone modifications could influence each other to regulate gene expression. Histone methylation could influence DNA methylation to form different methylation patterns whereas DNA methylation could serve as a template for some histone modifications [11]. The molecular interactions among histone, DNA methyltransferases, and other enzymes and proteins contribute to orchestrate interrelationship between DNA methylation and histone modifications. During DNA methylation, methylation-binding proteins (MBDs) and histone deacetylase (HDAC) are recruited to the chromosome. MBDs prevent transcription factors and cofactors binding to the promoter of genes, and thus, inhibit the expression of genes. HDAC in the region of methylated DNA also reduces the activity of the promoter and deacetylates the lysine of histone, resulting in the tightly packed chromosomes which block transcription factor access [1, 11]. Therefore, both DNA methylation and histone deacetylation

work together in regulating gene expression, which prompted the development of drugs that could function as demethylating agents of the inhibitors of HDACs. Advances have been made in the clinical arena for testing the anti-tumor activity of some agents; however, more clinical trials especially phase III clinical trials are warranted.

14.3 Epigenetic Regulations of miRNA Expression in Cancers

The miRNA is one type of short noncoding RNA that down-regulates the expression of its target genes though degradation of target mRNA or interruption of target translation. Emerging evidences have shown that DNA methylation in the promoter region of miRNA genes could also down-regulate the expression of specific tumor suppressive miRNAs, resulting in the up-regulation of oncogenic targets of these miRNAs (Fig. 14.1). The up-regulated oncogenic signaling caused by DNA methylation mediated through miRNA down-regulation could promote carcinogenesis, cancer invasion and metastasis. In the following section we will summarize the role of selected miRNAs whose expression has been found to the regulated through epigenetic events although we cannot catalog all miRNAs due to space limitation.

DNA hypermethylation in the region of miR-9 promoter has been found in renal, gastric, and lung cancers [12, 13]. The hypermethylation caused the silencing of miR-9 gene resulting in reduced expression of miR-9. Importantly, it has been found that the hypermethylation of the miR-9 promoter is associated with cancer development, metastasis, recurrence, and shorter overall survival [12, 13], suggesting the prognostic value of miR-9 methylation and further suggest that selective demethylating agents would be useful therapeutic approach for these malignancies.

The miR-34 belongs to a tumor-suppressor miRNA family. The expression of miR-34 family could be regulated by tumor suppressor p53 and DNA hypermethylation [14]. The down-regulation of miR-34 expression is commonly observed in various cancers. We and other investigators have found lower expression of miR-34 which was in part due to DNA methylation of the promoter region of miR-34 gene [15, 16]. It has been shown that androgen receptor (AR), Notch-1, and SIRT1 are the direct targets of miR-34. Therefore, the AR, Notch-1, and SIRT1 signaling is usually up-regulated in cancer cells due to the silencing of miR-34 expression, which could be causally linked with cancer development and progression.

The miR-29a is also a tumor suppressive miRNA. We and other investigators have found that miR-29a is down-regulated in lymphoma, prostate and pancreatic cancer cells and tissues due to DNA methylation of the promoter of miR-29a gene [17, 18]. Other studies have shown that miR-29 family directly targets both DNMT3A and DNMT3B and that the down-regulation in the expression of miR-29 family causes overexpression of DNA methyltransferases 3A and 3B [19]. These findings suggest a regulatory loop of miR-29/DNMT/methylation in the epigenetic regulation of cancer specific genes and their signaling.

The miR-124a is a known tumor suppressive miRNA. The DNA hypermethylation and the epigenetic silencing of miR-124a have been observed in different types of cancers [20]. The expression of miR-124a has been found to be significantly down-regulated which leads to the overexpression and activation of its target gene, CDK6. The epigenetic silencing of miR-124a expression also leads to the phosphorylation of tumor suppressor gene retinoblastoma [20], resulting in the promotion of cancer cell growth.

The miR-129-2 is another miRNA with tumor suppressor feature. The miR-129-2 directly targets the expression of SOX4 by 3′-UTR binding. It has been found that the level of miR-129-2 was significantly down-regulated while the expression of SOX4 was highly up-regulated in gastric and endometrial cancer cells [21]. Moreover, the DNA hypermethylation in the miR-129-2 CpG islands was observed in gastric and endometrial cancer cell lines and in 68 % of human endometrial cancer tissues. Histone acetylation and DNA demethylation has been shown to up-regulate the expression of miR-129-2, and consequently down-regulates the expression of SOX4, resulting in the inhibition of cancer cell proliferation [21], suggesting the epigenetic regulation of miR-129-2 in cancers.

The down-regulation of tumor suppressive miR-145 has been observed in various cancers. It has been found that the expression of miR-145 is silenced through DNA hypermethylation and p53 mutation. Moreover, the promoter region of miR-145 gene has been found to be highly methylated in both human prostate cancer tissues and cell lines [22]. Since miR-145 could down-regulate OCT, SOX2 and KLF4 which are markers of the embryonic stem cells, the epigenetic deregulation of miR-145 in cancers could contribute to the growth of cancer stem cells; however, further studies in this area is required.

The miR-152 is also a tumor suppressive miRNA which could be deregulated by DNA hypermethylation. The methylation of miR-152 promoter and low expression of miR-152 has been observed in acute lymphocytic leukemia, endometrial and other cancers [23]. The expression of miR-152 could be recovered by demethylating agent 5-aza-dC. It has been found that DNMT1, E2F3, and MET are targets of miR-152. The methylation of miR-152 promoter could increase the expression of DNMT1, E2F3, and MET [23], leading to high methylation status during cancer development.

The miR-200 family has been known to play important roles in the regulation of epithelial-to-mesenchymal transition (EMT) through the inhibition of ZEB1 and ZEB2. ZEB1 and ZEB1 are the transcriptional repressors of E-cadherin, which is a critical molecule for epithelial structure. The DNA hypermethylation in the region of miR-200 promoter has been found in lung and bladder cancers [24]. The methylation of miR-200 promoter caused lower expression of miR-200, leading to EMT and increased proliferation of cancer cells [24].

In addition, the epigenetic deregulation of other miRNAs including miR-92, miR-127, miR-137 miR-148a, miR-203, miR-26, etc. have also been observed in different types of cancers [2], which leads to the development and progression

of cancers. Therefore, targeting aberrant miRNA expression altered by epigenetic regulation could be an effective strategy for cancer prevention and treatment. Although there have been some progress in the areas of drug development such as demethylating agents or HDAC inhibitors, there remains many challenges especially the unwanted toxicity of these agents, which prompted many investigators to turn into agents that are abundantly found in the nature and are known to be non-toxic as discussed in the following paragraphs.

14.4 Epigenetic Regulations of mRNAs and miRNAs by Nutraceuticals

Emerging evidences have demonstrated that several nutraceuticals including isoflavone, curcumin, EGCG, resveratrol, and lycopene could serve as epigenetic regulators to reverse the deregulated expression of tumor suppressive mRNAs and miRNAs, leading to the inhibition of cancer development and progression (Fig. 14.3). The effects of these selected agents are discussed below although we cannot summarize all natural agents because of space limitation.

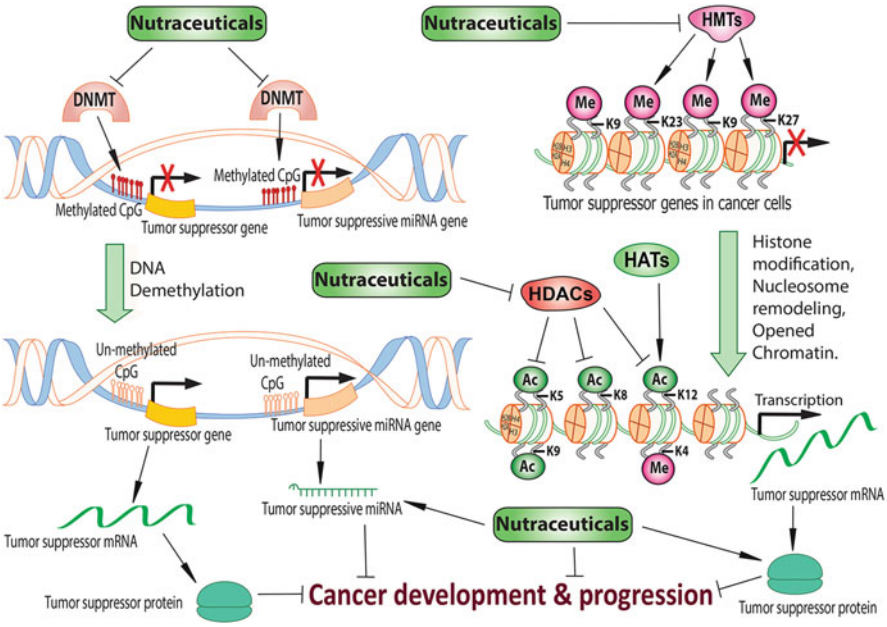

Fig. 14.3 The effects of nutraceuticals on epigenetic regulations in cancer development and progression

14.4.1 Epigenetic Regulations by Isoflavone

Isoflavones are mainly derived from soybean and could epigenetically up-regulate the expression of tumor suppressor mRNAs and miRNAs by modulating DNA methylation and chromatin configuration, leading to the suppression of cancer cell survival. To explore the effect of isoflavone genistein on epigenetic regulation of miRNAs, the miRNA expression profiles of PC-3, DU145, and LNCaP prostate cancer cells after genistein and Aza-dC treatment were compared. It has been found that genistein had similar effects on miRNA regulation compared to Aza-dC, suggesting that genistein and demethylating agent Aza-dC could have similar epigenetic regulatory effects on miRNAs [25] which is in part due to the role of genistein as a demethylating agents among many other effects of genistein. We have also found higher level of methylation in the promoter region of miR-29a and miR-1256 in prostate cancer cells compared to normal prostate epithelial cells [17]. Importantly, we found that isoflavone could demethylate the methylated promoter of miR-29a and miR-1256 and, in turn, up-regulate the expression of miR-29a and miR-1256. By up-regulation of miR-29a and miR-1256, isoflavone could reduce the expression of TRIM68 and PGK-1, which are targets of miR-29a and miR-1256. However, it is important to note that isoflavone was not a pan-demethylating agent like Aza-dC. We found that Aza-dC up-regulated oncogenic miR-155 and miR-421 expression by demethylation while isoflavone decreased the expression of miR-155 and miR-421, suggesting the specific targeting effect of isoflavone [17]. Other investigators also reported that isoflavone genistein could regulate the expression of miR-145, miR-221, and miR-222, leading to the inhibition of prostate cancer growth through epigenetic regulations [26, 27].

Studies have shown in LNCaP and DuPro prostate cancer cells that isoflavone genistein could up-regulate the expression of tumor suppressor genes $p21^{WAF1}$ and $p16^{INK4a}$. This effect of isoflavone genistein was mediated by epigenetic regulation. It has been found that genistein increased the expression of histone acetyltransferases and the level of acetylated histones 3, 4, and H3K4 at the transcription start sites of $p21^{WAF1}$ and $p16^{INK4a}$, leading to the up-regulation of tumor suppressor genes $p21^{WAF1}$ and $p16^{INK4a}$ [28]. In $ARCaP_E$ and $ARCaP_M$ prostate cancer model of EMT, isoflavone genistein affected histone H3K9 acetylation and increased the expression of histone acetyltransferase 1 (HAT1). Moreover, genistein combined with histone deacteylase inhibitor vorinostat could significantly enhance cell death in prostate cancer cells [29]. The effects of isoflavones, genistein and daidzein, on DNA methylations in the promoter regions of glutathione S-transferase P1 (GSTP1) and ephrin B2 (EPHB2) genes have also been tested in prostate cancer cells. After treatment with isoflavones, the authors have found significant demethylation of GSTP1 and EPHB2 promoters with corresponding increase in their protein expression [30]. All these findings demonstrate the potent effects of isoflavone on epigenetic regulations of genes in prostate cancer, and as such genistein may serve as a demethylating agent for the treatment of human malignancies although further in-depth investigations are required.

The effects of isoflavone on breast cancer in terms of epigenetic regulation have also been investigated. A study showed that the promoters of BRCA1 and BRCA2 tumor suppressor genes were highly methylated in MCF-7 and MDA-MB-231 breast cancer cells [31]. However, demethylation agent Aza-dC or isoflavones including genistein and daidzein could reduce DNA hypermethylation and consequently up-regulate the expression of BRCA1 and BRCA2, suggesting the demethylating effect of genistein and daidzein in breast cancer [31]. In MCF-7 breast cancer cells, isoflavone genistein also showed its ability to inhibit the expression of hTERT (human telomerase reverse transcriptase) and DNA methyltransferases (DNMT1, 3a and 3b). Moreover, isoflavone genistein could remodel chromatin structures of the hTERT promoter by induction of trimethyl-H3K9 and reduction of dimethyl-H3K4 in the hTERT promoter. The combination treatment with isoflavone genistein and demethylating agent Aza-dC led to a significant inhibition in the expression of hTERT, suggesting the epigenetic regulation of telomerase by isoflavone genistein [32]. In addition, lignans as isoflavones are one of the major classes of phytoestrogens. The nordihydroguaiaretic acid (NDGA) is a member of the lignan family and it was found that NDGA could reverse DNA hypermethylation in $p16^{INK4a}$ CpG islands and restore its expression in T47D breast cancer cells, leading to cell cycle arrest at G1 phase [33]. These findings suggest the effects of isoflavone on epigenetic regulations in breast cancer and similar effects may occur in other cancers.

BTG3 is a tumor suppressor gene and its expression has been found to be down-regulated in renal cancers due to DNA hypermethylation in the BTG promoter. However, isoflavone genistein and demethylating agent Aza-dC significantly inhibited the DNA hypermethylation in the BTG promoter [34]. Isoflavone genistein and Aza-dC also induced acetylated histones 3, 4, 2H3K4, 3H3K4 and RNA polymerase II at the BTG3 promoter. Moreover, genistein and Aza-dC decreased DNA methyltransferase and methyl-CpG-binding domain 2 activity, leading to increased BTG expression and cell cycle arrest. Similar effects of isoflavone genistein have also been observed in prostate cancer cells [35], suggesting the epigenetic effects of isoflavone on tumor suppressor BTG3 expression and cancer cell proliferation.

In myeloid and lymphoid leukemia, genistein exerted its anti-tumor activity through reactivation of tumor suppressor genes which are commonly silenced by DNA methylation [36]. In the clinical setting, Aza-dC has been used for the treatment of leukemia. It has been found that isoflavone genistein combined with Aza-dC could significantly enhance anti-leukemic activity against murine Aza-dC resistant cells [37], suggesting that genistein could increase the clinical efficacy of Aza-dC through epigenetic regulation. In esophageal squamous cell carcinoma cells, genistein has been shown to inhibit DNA methyltransferase activity and, in turn, up-regulate RARβ, $p16^{INK4a}$, and MGMT expression, causing the inhibition of cancer cell growth [38].

DKK1 is an antagonist of Wnt signaling and DNA methylation in DKK1 promoter has been found in colon cancer cells. The effects of isoflavone genistein on epigenetic regulation of DKK1 have been detected. DNA methylation at the DKK1 promoter was not altered by genistein treatment; however, genistein induced histone

H3 acetylation of the DKK1 promoter region in colon cancer cells, leading to increased expression of DKK1 [39]. These results suggest the epigenetic regulatory effects of isoflavone on Wnt signaling. In addition, *in vivo* animal studies showed that isoflavone exerted its inhibitory effects on DNA methylation. The overall methylation was found to be increased in liver and muscle tissues when monkeys switched from soy diets to no soy diets. The involved genes in epigenetic regulation by isoflavone *in vivo* are specifically homeobox genes (HOXA5, HOXA11, and HOXB1) and ABCG5 [40]. These reported results all support the epigenetic effects of isoflavone although further mechanistic and clinical studies are warranted.

14.4.2 Epigenetic Regulations by Curcumin

Curcumin is a natural compound present in turmeric and possesses anti-inflammatory, antioxidant, and anti-cancer activity. Experimental studies have demonstrated that curcumin could mediate epigenetic modulation of miRNA expression. The miR-203 is a tumor suppressive miRNA and it is frequently down-regulated in bladder cancer because of DNA hypermethylation in its promoter [41]. Curcumin could up-regulate the expression of tumor suppressive miR-203 in bladder cancer through demethylation of miR-203 promoter. Since Akt2 and Src are the targets of miR-203, the up-regulation of miR-203 by curcumin could down-regulate the expression of Akt2 and Src, leading to reduced proliferation and increased apoptosis of bladder cancer cells [41], suggesting the epigenetic regulatory effects of curcumin on miRNA expression.

In addition to the regulation of miRNA, curcumin could also epigenetically regulate mRNA expression, leading to cell growth inhibition. Curcumin has been found to inhibit the activities of DNMT, HAT, and HDAC. However, the molecular mechanism by which curcumin inhibits DNMT is unclear. By molecular docking analysis of curcumin and DNMT1 interaction, it was found that curcumin could block the catalytic thiolate of C1226 of DNMT1 to inhibit the activity of DNMT1 [42]. Another mechanism of DNMT inhibition by curcumin involves cyclic nucleotide phosphodiesterases (PDEs). The effects of curcumin on PDE-regulated DNMT1 have been investigated in B16F10 murine melanoma cells. It has been found that curcumin was able to down-regulate PDE1 and PDE4 activities and, in turn, inhibited the expression of DNMT1, leading to the inhibition of melanoma cell proliferation [43]. Histone deacetylase inhibitors have been used as epigenetic drugs but have shown low efficacy in cancer monotherapy. It was found that HDAC inhibitors could activate tumor-progressive genes to enhance cell migration and tumor metastasis. However, HDAC inhibitors combined with curcumin have been shown to suppress HDAC inhibitor-activated tumor progressive proteins and cell migration *in vitro* and significantly inhibited tumor growth and metastasis *in vivo* [44], suggesting the superior effects of HDAC inhibitor in combination treatment with curcumin.

In LNCaP prostate cancer cells, curcumin demethylated the first 14 CpG sites of CpG island in Neurog1 gene and, in turn, up-regulated the expression of Neurog1 [45].

Curcumin also significantly inhibited MeCP2 (one of the epigenetic modulators) binding to the promoter of Neurog1, leading to decreased expression of Neutog1. Moreover, curcumin inhibited the enrichment of H3K27me3 at the Neurog1 promoter region and the activity of HDAC [45], suggesting the strong effects of curcumin on epigenetic regulation in prostate cancer. In addition, Nrf2 has been found to be a regulator of cellular antioxidant defense system and it is epigenetically silenced during the development of prostate cancer in TRAMP mice. Curcumin could reverse the methylation of the first 5 CpGs in the promoter region of the Nrf2 gene. The demethylation of Nrf2 by curcumin has been found to be correlated with the re-expression of Nrf2 and its target gene NQO-1 [46], suggesting that curcumin could exert its chemopreventive effect through epigenetic modification of the Nrf2-mediated anti-oxidative stress pathway.

In cervical cancer cells, several tumor suppressor genes have been reported to be silenced by promoter methylation. It has been found that curcumin could demethylate the promoter methylation of RARβ2 gene in SiHa cervical cancer cells [47]. In HeLa cervical cancer cells, the hypermethylation of RARβ2 gene was also reversed after 6 days of treatment with curcumin. The reversal of RARβ2-methylation led to the induction of apoptosis. Curcumin could also reverse promoter hypermethylation and increase gene expression of FANCF in SiHa cervical cancer cells. Methylation specific PCR and bisulphite sequencing analysis showed that curcumin was able to demethylate 12 CpG sites in the region of FANCF promoter [48], suggesting the potent demethylating effects of curcumin on tumor suppressor genes.

Wnt inhibitory factor-1 (WIF-1) is another tumor suppressive gene and the hypermethylation of WIF-1 promoter has been found in lung cancer cells and tissues. To reactivate the expression of WIF-1, three major curcuminoids including curcumin, demethoxycurcumin and bisdemethoxycurcumin have been used [49]. It was found that bisdemethoxycurcumin had the strongest demethylation effect *in vitro*. The curcuminoids could restore WIF-1 expression through the demethylation effect [49], suggesting their therapeutic benefit for lung cancer. In acute lymphoblastic leukemia (ALL), it was found that the expression of several genes in the TP53 pathway was decreased due to DNA hypermethylation. The DNA methylation of genes in TP53 pathways was significantly associated with a higher relapse and mortality rate. Importantly, curcumin or Aza-dC treatment reversed the epigenetic abnormalities, resulting in the increased expression of genes in TP53 pathways, and also led to the induction of apoptosis of ALL cells [50], suggesting the epigenetic regulation of tumor suppressors by curcumin.

Histone methyltransferase EZH2 is a critical epigenetic regulator and plays important roles in the control of cell proliferation, apoptosis, and cancer stem cell function. We found that diflourinated-curcumin (CDF), a novel analogue of curcumin, down-regulated the expression of EZH2 and up-regulated the expression of several tumor-suppressive miRNAs including let-7a, b, c, d, miR-26a, miR-101, miR-146a, and miR-200, leading to the inhibition of cell survival, clonogenicity, formation of pancreatospheres, cell migration, and cancer stem cell function in human pancreatic cancer cells [51], suggesting the beneficial effects of CDF on epigenetic regulation.

14.4.3 Epigenetic Regulations by EGCG

EGCG is extracted from green tea and has been shown to have antioxidant and anti-cancer properties. It has been found that EGCG could decrease global DNA methylation in cancer cells. EGCG down-regulated 5-methylcytosine, DNMT1, DNMT3a, and DNMT3b. EGCG also inhibited the activity of histone deacetylase and promoted acetylation in lysine9 and 14 on histone H3 and lysine5, 12 and 16 on histone H4, leading to the up-regulation of silenced tumor suppressor genes, $p16^{INK4a}$ and $p21^{WAF1}$ in A431 cancer cells [52]. EGCG showed its inhibitory effect on the DNMT1-mediated DNA methylation. Computational modeling studies revealed that the gallic acid moiety of EGCG is critical for its inhibitory interaction with the catalytic site of DNMT1.

EGCG could also demethylate the DNA methylation in the promoter regions of several tumor suppressor genes including $p16^{INK4a}$, $p15^{INK4b}$, retinoic acid receptor β (RARβ), O(6)-methylguanine methyltransferase (MGMT), and human mutL homologue 1 (hMLH1) genes, resulting in the up-regulation of these genes in various cancer cells including HT-29 and Caco-2 colon cancer, KYSE 150 esophageal cancer, and PC-3 prostate cancer cells [53]. EGCG could also demethylate the DNA hypermethylation in the promoter region of tumor suppressor WIF-1 gene and restore the expression of WIF-1 in H460 and A549 lung cancer cells [54]. By epigenetic regulation of WIF-1, EGCG decreased the level of cytosolic β-catenin and suppressed the activity of Tcf/Lef reporter, suggesting the inhibitory effects of EGCG on Wnt signaling pathway through the epigenetic mechanism [54]. RECK is also a tumor suppressor gene which down-regulates matrix metalloproteinases (MMPs) and suppresses invasion, angiogenesis and metastasis of cancer. It has been found that EGCG could partially reverse the DNA hypermethylation in the region of RECK promoter and significantly up-regulate the expression of RECK, causing the down-regulation of MMP-2 and MMP-9, and the suppression of invasion in oral squamous cell carcinoma cells [55]. These findings demonstrate the up-regulation of tumor suppressors by EGCG through epigenetic regulation.

It is well known that the status of estrogen receptor-α (ERα) predicts the clinical prognosis and therapeutic outcome in breast cancer. ERα-negative breast cancer commonly has progressive disease and poor prognosis. The silence of ERα is believed to be due to epigenetic regulation in breast cancer cells. It has been found that EGCG could remodel the chromatin structure of the ERα promoter by the inhibition of transcription repressor complex binding to the regulatory region of the ERα promoter [56]. In this way, EGCG has been found to increase the expression of ERα in ERα-negative MDA-MB-231 breast cancer cells. Combination treatment with EGCG and HDAC inhibitor showed a synergistic effect by increasing ERα expression and sensitizing breast cancer cells to tamoxifen, suggesting the beneficial effects of EGCG in the treatment of breast cancer through epigenetic regulation.

It is known that polycomb group (PcG) proteins are epigenetic regulators of gene expression. Multiprotein PcG complexes such as PRC2 and Bmi-1 could up-regulate histone methylation and down-regulate acetylation, resulting in an altered chromatin

conformation and gene expression. In SCC-13 skin cancer cells, the expression and activity of PcG protein were up-regulated with increased cancer cell proliferation and survival. However, the treatment of SCC-13 cells with EGCG significantly inhibited the expression of Bmi-1 and EZH2, leading to reduced cell survival [57]. EGCG treatment could also reduce histone H3 lysine 27 trimethylation through inhibition of PRC2 complex deregulation. The decreased expression of PcG protein by EGCG caused reduced expression of cdk1, cdk2, cdk4, cyclin D1, cyclin E, cyclin A and cyclin B1, and increased expression of $p21^{WAF1}$ and $p27^{kip1}$. Further studies have shown that EGCG could reduce the expression of HDAC1 and the formation of H3K27me3 and H2AK119ub, leading to the up-regulation of tumor suppressors and the suppression of cell survival. The PcG-mediated epigenetic regulation could be one of the molecular mechanisms by which EGCG inhibits skin cancer cell survival.

EGCG could also regulate acetylation of NF-κB. It is known that p300/CBP-mediated hyperacetylation of RelA (p65) promotes the activation of NF-κB in cancer cells. EGCG could inhibit the acetylation of p65 and abrogate p300-induced p65 acetylation *in vitro* and *in vivo*, leading to the inhibition of NF-κB activation [58]. By the inhibition of p65 hyperacetylation, EGCG suppressed TNFα-induced p65 nuclear translocation. Furthermore, EGCG decreased the p300 binding to IL-6 promoter with an increased recruitment of HDAC3 [58]. These results demonstrate that EGCG could regulate NF-κB signaling by epigenetic regulation.

EGCG has also been found to down-regulate telomerase activity in breast cancer cells through the inhibition of hTERT by epigenetic mechanisms. EGCG decreased the level of acetyl-H3, acetyl-H3K9, and acetyl-H4 in the hTERT promoter and modulated chromatin structures of the hTERT promoter [59]. Moreover, EGCG promoted hTERT repressors including MAD1 and E2F-1 binding to the hTERT regulatory region. Furthermore, EGCG could demethylate DNA hypermethylation in the promoter of CTCF and increase the expression of CTCF which down-regulates hTERT expression by binding to hTERT promoter. These findings all suggest the effects of EGCG on epigenetic regulation in multiple cancers.

14.4.4 Epigenetic Regulations by Resveratrol

Resveratrol is a dietary compound from grapes and shows anti-carcinogenic activity. It has been found that resveratrol could epigenetically regulate the expression of several tumor suppressor genes. The BRCA1 protein is a tumor suppressor, especially in breast cancers. Aromatic hydrocarbon receptor (AhR) could down-regulate the expression of BRCA1. The activation and recruitment of AhR to BRCA1 promoter blocked the expression of BRCA1 with reduced acetylated histone 4 and AcH3K9, and increased DNMT1 and MBD2. However, this AhR-dependent repression of BRCA1 expression could be reversed by resveratrol treatment [60], suggesting that epigenetic silencing of BRCA1 gene could be prevented by resveratrol. Moreover, resveratrol could inhibit the function of tumor promoter, 2,3,7,8

tetrachlorodibenzo-p-dioxin (TCDD). It has been found that TCDD could inhibit 17β-estradiol-dependent stimulation of BRCA1, and could also induce hypermethylation of CpG sites that has been found in the start site of BRCA1 transcription, leading to the lower expression of BRCA1 in breast cancer cells. Therefore, resveratrol treatment could epigenetically reactivate BRCA1 by inhibition of AhR/TCDD/DNMT1 signaling [61]. In addition, it has been found that BRCA1 binds to the SIRT1 promoter and promotes the expression of SIRT1, which in turn suppresses survivin by epigenetic modification of histone H3. Resveratrol could increase the expression of Sirt1 and, in turn, could down-regulate the expression of survivin, suggesting that resveratrol treatment combined with conventional chemotherapeutics could be a strategy for the treatment of BRCA1-negative breast cancer [62]. Moreover, Resveratrol could inhibit RASSF-1α DNA methylation and, in turn, increase the expression of RASSF-1α, leading to the inhibition of prostaglandin PGE_2 in breast cancers [63], suggesting the beneficial effects of resveratrol in the epigenetic regulation of tumor suppressors in breast cancer.

Resveratrol could also inhibit the expression of some oncogenes which participate in epigenetic regulations. Metastasis-associated protein 1 (MTA1) is an oncogenic protein which promotes deacetylation of histones. It has been shown that MTA1 is overexpressed in prostate cancer and its overexpression is associated with tumor aggressiveness and metastasis. It has been found that resveratrol could decrease the expression of MTA1, leading to the acetylation and activation of p53 [64]. The acetylated p53 could recruit to $p21^{WAF1}$ and Bax promoters, resulting in the apoptosis of cancer cells. HDAC inhibitor SAHA shows similar effects as resveratrol, suggesting the epigenetic regulation of resveratrol in cancer cells [64]. It has also been found that lysine acetylation of the oncogenic transcription factor STAT3 is increased, leading to the high expression of STAT3 in cancers. Resveratrol could reduce acetylation of STAT3 at Lys685 and, in turn, increase the expression of several tumor-suppressor genes, leading to the inhibition of cancer growth. The reduction of acetylated STAT3 also caused demethylation and activation of ERα, which could sensitize triple-negative breast cancer cells to anti-estrogen therapy [65].

In addition, it has been shown that viruses, including HIV-1, could increase the expression of human DNA methyltransferases, leading to the development of cancers. Interestingly, the HIV-1 induced overexpression of DNA methyltransferase could be prevented with resveratrol treatment through the inhibition of transcription factor AP1 signaling [66], suggesting the chemopreventive effects of resveratrol through epigenetic regulation.

14.4.5 Epigenetic Regulations by I3C and DIM

I3C and its *in vivo* dimeric product DIM are phytochemicals derived from cruciferous vegetables and has been shown to have no known toxicity in humans. Both I3C and DIM could inhibit carcinogenesis in different types of cancers. In recent years,

HDAC inhibitors have been synthesized for cancer prevention and therapy; however, the side effects and toxicity limits the use of HDAC inhibitors in humans. Interestingly, it was found that both I3C and DIM could inhibit HDAC activity in prostate cancer cells [67]. I3C modestly inhibited HDAC activity in androgen sensitive LNCaP cells whereas DIM significantly inhibited the expression of HDAC2 and reduced the activity of HDAC with increased expression of $p21^{WAF1}$ in both LNCaP and PC-3 cells, suggesting that DIM is a better natural agent for the regulation of aberrant epigenetic patterns in prostate cancer prevention or treatment [67]. We have also found that DIM treatment could demethylate the DNA methylation in the promoter of miR-34a, leading to the up-regulation of miR-34a expression and the down-regulation of target genes, AR (downstream targets of AR, PSA) and Notch 1 in LNCaP and C4-2B cells [15]. Moreover, DIM intervention in prostate cancer patients prior to radical prostatectomy resulted in the re-expression of miR-34a and consequently led to decreased expression of AR, PSA and Notch-1 in prostate tumor tissues [15]. These results suggest that epigenetic silencing of tumor suppressive miR-34a in prostate cancer could be reversed by DIM treatment.

The overexpression of oncogenic cyclooxygenase-2 (COX-2) has been found in several types of cancers with activation of AhR signaling. It was found that AhR ligand could induce the rapid formation of complex with the AhR, the histone acetyl transferase p300, and acetylated histone H4 at the COX-2 promoter [68]. Importantly, DIM could inhibit the recruitment of AhR and acetylated histone H4 to the COX-2 promoter and, thereby, down-regulate the expression of COX-2 in MCF-7 breast cancer cells, suggesting that the use of DIM could be a novel strategy against epigenetic activation of COX-2 by AhR.

14.4.6 Epigenetic Regulations by Lycopene

Lycopene is the red pigment in tomatoes and has shown its chemopreventive potential in cancer research. The effects of lycopene on DNA methylation in the promoter of tumor suppressor genes have been tested in MDA-MB-468 breast cancer cells and MCF10A breast epithelial cells. It was found that lycopene partially demethylated the DNA hypermethylation in the promoter of glutathione S-transferase P1 (GSTP1) tumor suppressor gene in MDA-MB-468 cells. The expression of GSTP1 was significantly up-regulated after lycopene treatment. However, the demethylation of another tumor suppressor gene RARβ by lycopene was only observed in noncancerous MCF10A breast epithelial cells [69]. A controversial observation has been reported in prostate cancer cells. GSTP1 has been found to be hypermethylated in 90 % of prostate cancers; however, lycopene was unable to alter the methylation and expression of GSTP1 in LNCaP prostate cancer cells while a demethylating agent was able to significantly decrease the methylation of GSTP1 gene [70]. These results suggest that the effects of lycopene on epigenetic regulation could be cell type and context-dependent.

14.5 Conclusions and Perspectives

Mounting evidence suggests that epigenetic regulations of mRNAs and miRNAs by DNA methylation and histone modification play important roles in cancer development and progression; therefore, targeting the epigenetic deregulations in cancers is a key and effective approach to fight against cancers. Several epigenetic drugs or HDAC inhibitors have been synthesized and used in epigenetic therapy trials to re-express abnormally silenced tumor suppressor genes. However, the adverse side-effects of the demethylating agents and histone deacetylase inhibitors limit the broader application of these agents to appreciate their beneficial effects. Therefore, using non-toxic nutraceuticals including isoflavone, curcumin, EGCG, resveratrol, I3C, DIM, and lycopene to demethylate DNA sequences or inhibit HDACs could epigenetically deregulate the expression of tumor suppressive mRNAs and miRNAs.

Indeed, the *in vitro* experiments and *in vivo* animal studies have demonstrated that the epigenetic regulation of mRNAs and miRNAs could be one of the molecular mechanisms by which nutraceuticals could inhibit carcinogenesis and cancer progression. These natural agents exert their potent effects on the inhibition of cancer cell growth, invasion, and metastasis partly mediated through epigenetic regulation, suggesting that these non-toxic agents having anti-cancer effects could be useful in combination treatment with conventional chemotherapeutics for the treatment of cancers. It is important to note that recent development of technologies such as next-generation of sequencing coupled with chromatin immunoprecipitation (ChIP-seq) and DNA methylation profiling will lead to a deeper understanding of the epigenetic regulations in cancers and the effects of nutraceuticals on epigenome. However, more mechanistic experiments and clinical trials are needed to appreciate the value of nutraceuticals in cancer prevention and treatment which is mediated in part due to their roles in epigenetic deregulation of genes relevant to human cancers.

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