



DNA methylation and microRNAs in cancer

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Author contributions: De W conceived the review; Li XQ and Guo YY analyzed the data; and Li XQ wrote the review.

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Received: May 14, 2011 Revised: September 9, 2011

Accepted: January 18, 2012

Published online: March 7, 2012

Abstract

DNA methylation is a type of epigenetic modification in the human genome, which means that gene expression is regulated without altering the DNA sequence. Methylation and the relationship between methylation and cancer have been the focus of molecular biology researches. Methylation represses gene expression and can influence embryogenesis and tumorigenesis. In different tissues and at different stages of life, the level of methylation of DNA varies, implying a fundamental but distinct role for methylation. When genes are repressed by abnormal methylation, the resulting effects can include instability of that gene and inactivation of a tumor suppressor gene. MicroRNAs have some aspects in common with this regulation of gene expression. Here we reviewed the influence of gene methylation on cancer and analyzed the methods used to profile methylation. We also assessed the correlation between methylation and other epigenetic modifications and microRNAs. About 55 845 research papers have been published about methylation, and one-fifth of these are about the appearance of methylation in cancer. We conclude that methylation does play a role in some cancer types.

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Key words: Methylation gene expression; Transcriptional control; Cancer; MicroRNA; Gastric cancer

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Li XQ, Guo YY, De W. DNA methylation and microRNAs in cancer. *World J Gastroenterol* 2012; 18(9): 882-888 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i9/882.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i9.882>

INTRODUCTION

The occurrence of many diseases, such as cancer, diabetes mellitus and scleroderma, is related to the dysregulated gene expression resulting from epigenetic and genetic abnormalities. Genetic abnormalities can be caused by point mutations, gene amplification, or changes in the promoter (typically caused by chromosomal rearrangements)^[1], whereas epigenetic modifications include methylation, deacetylation of genes, and methylation of histone proteins, resulting in the accurate regulation of gene expression, without changing the DNA sequence, in response to changes in the environment and to meet the demands of differentiation^[2]. In fact, epigenetic modifications are as important as genetic modifications in regulating gene expression and controlling the onset of disease^[3]. DNA methylation, as a crucial component of epigenetics, plays an important role in cell differentiation and embryogenesis and is also heritable. Since it was first described by Feinberg and Vogelstein in 1983, our knowledge of methylation has grown at a dramatic rate^[4]. The recent development of methods to profile and study methylation in chromosomes has led to a deeper understanding of this process along with clinical applications to aid in the prognosis and treatment of disease^[5].

DNA METHYLATION

Definition of methylation

In 1999, the modern conception of epigenetics was put forward by Jones *et al*^[6]. Epigenetics is defined as the reg-

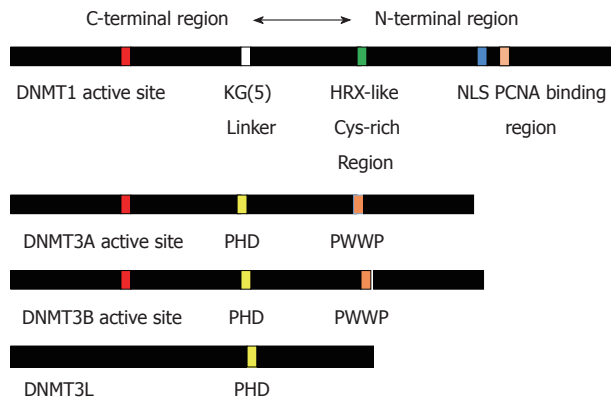


Figure 1 DNA methylation machinery. NLS: Nuclear localization signal; PCNA: Proliferating cell nuclear antigen.

ulation of gene expression without changing the genetic sequence. It is a flexible, heritable process that regulates some genes^[7]. DNA methylation and the modification of histones including acetylation and deacetylation are important components of epigenetics; however, the most common modification is DNA methylation.

DNA methylation is a type of covalent modification in which a methyl group is added to a cytosine in the genome *via* S-adenosylmethionine; this process occurs as an enzymatic reaction after DNA replication^[8]. In mammalian cells, methylation of DNA is typically restricted to the 5-position of the pyrimidine ring of cytosine residues that are located in CpG dinucleotides^[9]. CpG dinucleotides are frequently clustered into CpG islands, regions that are rich in CpG sites. These islands are generally about 0.5-3 kb, occur on average every 100 kb in the genome, and are found in approximately half of all genes in humans^[10]. Methylation of other CpGs seems to have no biological functions^[11].

There are four types of DNA methyltransferases (DNMTs), including DNMT1, DNMT3A, DNMT3B, and DNMT3L. DNMTs control the degree of methylation of the genome: DNMT1 is responsible for the maintenance of methylation, and DNMT3A and DNMT3B carry out *de novo* methylation. The DNMT3L does not have enzymatic activity, but it does regulate the activity of the other methyltransferases^[12,13]. DNMT1 is considered to be the maintenance methyltransferase because of its high activity and preference for hemimethylated DNA during DNA replication. All of the active DNA methyltransferases contain an active site motif in the C-terminal region (red box), whereas DNMT3L does not. DNMT1 contains other functional regions required for its interaction with proliferating cell nuclear antigen, which is adjacent to the nuclear localization signal. The N-terminal region of DNMT1 also contains a cysteine-rich HRX-like region and a lysine-glycine repeat [KG(5)] region. DNMT3A, DNMT3B, and DNMT3L contain a plant homeodomain; DNMT3A and DNMT3B contain a PWWP domain. These two domains are required for targeting DNMT3A and DNMT3B to pericentromeric heterochromatin and contribute to protein-protein interactions by recognizing histone modifications^[14] (Figure 1).

Normal and abnormal levels of methylation

The level of methylation changes during the growth of human beings and the development of diseases and, in different tissues, methylation varies substantially. Normally, about 50% of the CpG islands, which customarily are located in the promoter region of housekeeping genes, are unmethylated and thus are active. When those CpGs become methylated, the corresponding gene is silenced. There are, however, CpGs that are located elsewhere in genes and that do not influence transcription when they are methylated. DNA methylation is replicated with a high fidelity in mammalian cells and is almost at a stable state in a specified cell. It is regarded as having tissue and organ specificity^[15].

Once the rhythm of methylation is disturbed, many diseases develop^[16,17] (Table 1). Customarily, the abnormal condition includes two aspects: Hypermethylation and hypomethylation^[14].

DNA METHYLATION IN TRANSCRIPTIONAL CONTROL

The role of methylation in transcriptional control

When methylated, chromosomes become stabilized, and their activity is decreased. Gene expression is repressed by methylation in two separate mechanisms^[18,19]. In direct inhibition, the methylated chromosome prevents the approach of the transcriptase, holding back transcription. The second method is indirect inhibition, in which two types of protein, methylation-binding proteins (MBDs) and histone deacetylase (HDAC) are recruited to the chromosome (Figure 2). MBD proteins display homology within their MBD domains, whereas the transcription repression domains (TRDs) described for MeCP2, MBD1 and MBD2 are nonhomologous. In addition to its MBD domain, MBD1 is able to bind unmethylated DNA *via* its third CxxC zinc-finger motif. MBD2 features a characteristic stretch of glycine and arginine residues in the MBD domain, which when mutated prevent the binding of MBD to methylated CpGs in mammals^[20]. MBD3 is not able to bind methylated CpGs in mammals because of a mutation in the MBD domain. MBD4, a thymine glycosylase, contains a C-terminal glycosylase domain used for excision-based DNA repair. Three members of the Kaiso protein family which also influence the transcription, have been described so far. Kaiso, ZBTB4 and ZBTB38 share a triple zinc-finger domain and a BTB/POZ domain, which in the case of ZBTB4 contains a 60-amino-acid insertion. Furthermore, ZBTB4 and ZBTB38 contain, respectively, three and seven additional zinc-finger domains and have juxtaposed MBD and TRD domains^[21].

MBDs can also prevent the approach of transcription factor (TF) and cofactors, so that they cannot bind the promoter of the gene, thus stopping the transcription. The HDAC is also recruited to the region of methylated DNA, where it affects the activity of the promoter and deacetylates of the lysine of histone3/histone4 charged, and it then reacts with the negatively charged DNA. As a result, the chromosome becomes more tightly packed,

Table 1 Methylation and diseases

| AML | <i>hPer3</i> gene | Hypermethylation |
|---|--|--|
| Fragile X syndrome | Loss of FMR1/FMR2 function | Promoter methylation |
| ATR-X syndrome | Loss of ATRX function | Hypomethylation of certain repeat and satellite sequences |
| Immunodeficiency, centromeric region instability, and facial anomaly syndrome | DNMT3b mutation | Centromeric DNA hypomethylation |
| Beckwith-Wiedeman syndrome | Disruption of the imprinted IGF2/CDKN1C loci on 11p15.5 | Loss of genomic imprinting |
| Williams syndrome | Loss of WSTF function | Condensed chromatin structures |
| Rubinstein-Taybi syndrome | Mutations in the gene encoding CREB-binding protein | Reduced histone H3 acetylation |
| Prader-Willi syndrome | Disruption of the imprinted SNRF/SNRPN locus on 15q11-13 | Disruption of genomic imprinting |
| Coffin-Lowry syndrome | Mutation in RSK genes | Disrupted chromatin remodeling <i>via</i> activation of CREB-binding protein |

CREB: cAMP-response element-binding protein; WSTF: Williams syndrome transcription factor.

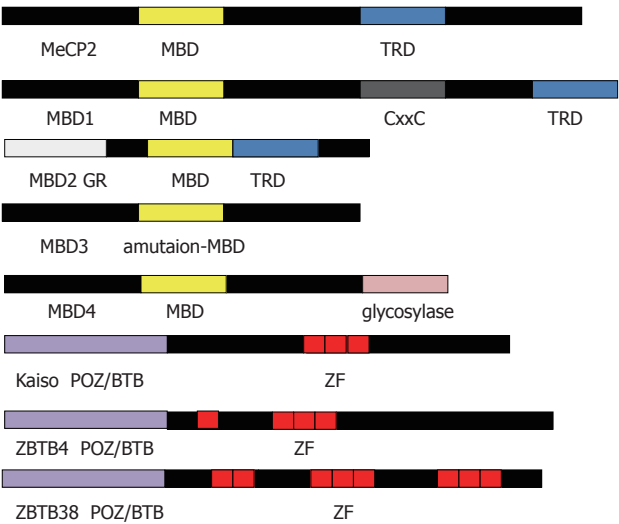


Figure 2 Proteins that bind the methylated DNA. MBD: Methylation-binding protein; TRD: Transcription repression domains.

blocking access to those proteins that are needed to start transcription. Genes with unmethylated (open lollipops), active CpG island promoters (Pro), have TFs (the radial pattern) at the transcription initiation site. Transcripts initiated here proceeding through the downstream elements even though they are methylated (closed lollipops) and presumably are coated with methyl CpG binding domain proteins (MBDs, the cylindricalcast) and HDACs (the trigon). The enhancer is functional because the silencer and insulator are methylated and, thus, not occupied by their respective cognate proteins. Methylation here is permissive for expression. For a permanently silenced gene such as an imprinted gene or a gene on the inactive X chromosome, the promoter is methylated, leading to binding of MBDs, HDACs, other transcriptional suppressors, and chromatin compaction. The TFs, which normally regulate gene expression, are not able to access the promoter. Figure 3 also shows how lack of methylation in a silencer or insulator can lead to binding of the cognate proteins, e.g., GCF2 (GC binding factor 2, the cube) or CTCF (CTC binding factor, the oblong), thus preventing the enhancer

from functioning^[22] (Figure 3).

From what has been described above, we know that methylation and deacetylation work in conjunction to regulate gene expression, but methylation is the triggering event. Under some conditions, however, deacetylation appears first, followed by methylation^[23]. The role of each process in the regulation of gene expression needs to be studied in more detail.

How methylation is maintained and induced

Although methylation is known to be important and has been the focus of research, we are still not clear why abnormal methylation occurs. Because methylation is catalyzed by DNMTs, changes in these enzymes may have some association. When DNMT1 is removed, the level of methylation of the whole genome is reduced by 3%, and when DNMT3 is removed, it is reduced by 4%. When they are both removed, the level is lowered by 98%^[14,24]. Thus, DNMTs are important for methylation. Their activity can, however, be influenced by many factors, such as ray, temperature. Because cells near the body surface are more easily influenced by the surrounding environment, the methylation of skin cells often becomes dysregulated. In addition, infection can lead to abnormal methylation. Infection with *Helicobacter pylori* (*H. pylori*) in the stomach can cause cancers, accompanied by changes in DNA methylation. Smoking is also a risk factor, as it can cause many genes to gain methylation^[25]. One study has also shown that eating foods lacking in folic acid, which is the carrier of the one carbon unit, leads to more-complex methylation patterns and increases the likelihood of cancer^[26].

DNA METHYLATION AND MICRORNAS IN CANCER

It is well accepted that cancer is a result of many events, including genetic and epigenetic and others. Since 1983, epigenetics has attracted the most attention of researchers, who have focused in particular on methylation. The appropriate DNA methylation within CpG dinucleotide islands plays a significant role in the regulation of gene

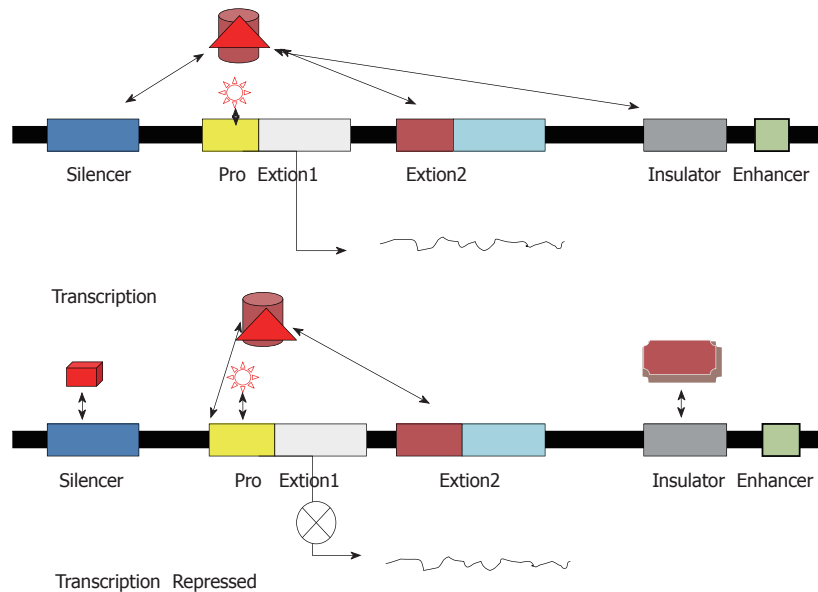


Figure 3 How methylation represses the gene expression..

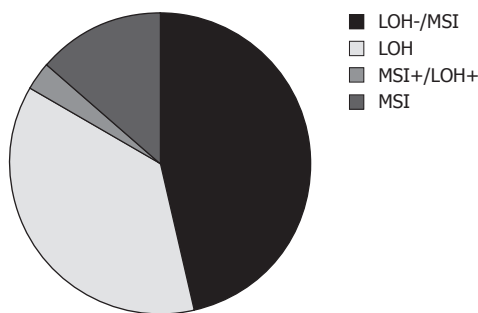


Figure 4 Factors for cancer development. LOH: Heterozygosity; MSI: Microsatellite instability.

expression. Abnormal patterns in DNA methylation often result in many diseases^[27]. Either as a result of DNMT overexpression or the occurrence of aberrant hypermethylation of tumor cell-specific promoters, the pattern of cell cycle, apoptosis and DNA repair changes following the aberrances of differentiation and adhesion of cells which is often a hallmark of diseases^[28]. It is reported that 48% of tumors had evidence of loss of heterozygosity (LOH), whereas 14% of tumors had microsatellite instability (MSI), including a minority of tumors (3%) that overlapped with LOH. Nearly one-third of tumors (38%) have neither MSI nor LOH^[1] (Figure 4).

Four abnormal aspects, inactivation of mismatch repair, instability of chromosomes, hypomethylation of oncogenes, and hypermethylation of tumor suppressor genes, can be observed when abnormal DNA methylation appears. The four aspects often cooperate to cause cancers to happen. The first genes found to be methylated were p16, TSP-1, and IGF2. In colorectal cancer, there is a significant degree of methylation at the previously identified CIMP-associated loci (MINT-1, -2, -31; p16; p14; MLH1), as well as in six new tumor suppressors or gene markers (PTEN, TIMP3, RUNX3, HIC1, APC, and RAR β 2), in combina-

tion with the chromosomal instability and microsatellites instability^[1].

In addition, it has been proposed that hypomethylation patterns of the genome exist and, more specifically, that hypomethylation and hypermethylation cooperate in cancer development^[29]. Transcriptional silencing *via* DNA hypermethylation can often be associated with poor clinical outcome in several malignancies, indicating that hypermethylation of tumor-suppressor genes or hypomethylation of tumor genes are related to cancer^[30,31].

In addition, methylation can be a marker to judge whether the cancer has been transferred to other tissues. HIN-1, CDH13, RIL, RASSF1A, and RAR β 2 were frequently methylated in both primary and metastatic tissues, whereas the methylation status of HIN-1, CDH13, RIL, and RAR β 2 isolated from lymph nodes was correlated with that in primary tumors in breast cancer^[32].

Recently, small interfering RNAs (siRNAs) have been found to participate in gene regulation together with methylation. siRNAs are RNAs that consist of 21-25 nucleotides. They make up nearly 2% of the genome and can be found either in the host gene or other genes. siRNAs can induce the methylation of the promoter, thus silencing the gene. This factor has been conserved during evolution and has complicated functions. There are at least three kinds of small RNAs, siRNA, miRNA, and others. It has been known that some small RNA can repress gene expression *via* a complex of proteins named the RNA-induced silencing complex, thus regulating gene expression after transcription. miRNA-223 is located in the X chromosome, and its expression is controlled by the upper sequence CCAAT box. Two kinds of proteins, NFI-A and C/EBP α , participate in the process. Abnormal expression of miRNA223 can lead to acute lymphocytic leukemia and acute myelocytic leukemia (AML). The fusion gene *AML1/ETO* will cause down-regulation of the miRNA223 *via* methylation or recruiting of related

enzymes. When miRNA223 is silenced, diseases appear. In the primary carcinoma of liver, miRNA223 is notably down-regulated, reflecting the coordination of methylation and miRNA. The influence of methylation on the expression of miRNAs should be further studied and will be a hotspot of research in the next few years^[33-37].

DNA METHYLATION AND MICRORNAS IN GASTRIC CANER

The roles of DNA methylation and miRNA in gastric cancer have been extensively studied recently. Many genes are methylated specifically in gastric cancer, as are miRNAs and siRNAs. Because oncogenes and tumor suppressor genes can have important roles in cancer and because methylation can repress gene expression, the level of methylation of a specific gene in gastric cancer may reflect whether that gene is an oncogene or tumor suppressor gene. Weak gene expression and loss of gene expression because of promoter hypermethylation may be a cancer-specific event^[38].

Ulcer-healing genes (*TFF1*, *TFF2*, *CDH1* and *PPARG*) are methylated in earlier gastric carcinoma, and methylation of hsa-miR-124 is involved in cervical cancer^[39-41]. The gene encoding BMP3 has been found to cause gastric carcinoma in Chinese population^[42]. If combined with other aspects, the situation may be more severe. For example, epigenetic inactivation of GATA-4 and GATA-5 by methylation of CpG islands is an early frequent event during gastric carcinogenesis and is significantly correlated with *H. pylori* infection^[43]. If expression of DNMT is abnormal, methylation will be abnormal, leading to a disease state. It has also been reported that methylation can have an additive effect with other chromosomal abnormalities. This can result in a positive feedback loop that progresses to a disease state^[11]. Infection with *H. pylori* induces IRX1 promoter methylation and downregulation of the promoter activity as well as a significant reduction in gene expression. Gene silencing of the IRX1 tumor suppressor by promoter CpG methylation, combined with LOH, has been identified in human gastric cancer^[44]. SLC19A3 was epigenetically down-regulated in gastric cancer, and *via* the technique of quantitative real time polymerase chain reaction (RT-qPCR), it has been shown that aberrant SLC19A3 promoter hypermethylation in plasma may be a novel biomarker for breast and gastric cancer diagnosis^[45]. Promoter hypermethylation of p16, Runx3, DAPK and CHFR is frequent in gastric cancer. DAPK and CHFR promoter hypermethylation may be important for evaluating the differentiation grade and lymph node status in patients with gastric cancer. Silencing of HIC1 and TOB1 expression is a common occurrence in gastric cancer and may contribute to the development and progression of the disease^[46].

Methylation silencing of *miRNA* genes, in addition to that of protein-coding genes, may contribute to the formation of a field defect for gastric cancers^[47]. Down-regulation of miR-212 may be related to gastric carcinogenesis through its target genes, such as *MECP2*^[48].

Hypermethylation of hsa-miR-124a is present in gastric cancer^[49]. miRNA-34b and miRNA-34c are novel tumor suppressors that are frequently silenced by DNA methylation in gastric cancer, and methylation of miR-34b/c is involved in an epigenetic field defect and that the methylation might be a predictive marker of gastric cancer risk^[50]. The transformation from gastritis to lymphoma of mucosa-associated lymphoid tissue is epigenetically regulated by miR-203 promoter methylation, and ABL1 is a novel target for the treatment of this malignancy^[51]. miR-10b methylation may be a useful molecular biomarker for assessing the risk of gastric cancer development, and modulation of miR-10b may represent a therapeutic approach for treating gastric cancer^[52].

CLINICAL APPLICATIONS

Based on how methylation works to repress gene expression, several methods can be used to treat the related diseases. Current research has focused on methods to demethylate the gene of interest. Both HDAC inhibition and DNA demethylating agents have shown clinical efficacy respectively; yet a combination of the two agents has a strong synergistic effect on the reactivation of silenced genes and antiproliferative and cytotoxic effects on cancer cells^[30,53]. The two compounds do not, however, reverse the methylation entirely^[54]. Both nucleoside analogs and non-nucleoside analogs can be used to demethylate the gene of interest, but with severe side effects^[55,56]. The first kind of agents includes 5-azacytidine, 5-aza-2-deoxycytidine (5-aza-CdR), 5,6-dihydro-5-azacytidine, and zebularine, among which 5-azacytidine has been clinically shown to reduce the degree of methylation and prolong the survival of the patients. The second includes procaine, mitoxantrone, N-acetyl-procainamide, procainamide, hydralazine, and the main polyphenol compound of green tea, (-)-epigallocatechin-3-gallate. Although the two kinds of agents are effective, their severe side effects cannot be ignored, for they both will make the whole genome hypomethylated which can cause many problems including the development of new diseases^[57].

For these reasons, these agents just act as some assistance ones. To reduce the side effects, small molecules targeting DNMT are being developed. It has been reported that they are exquisitely S-phase specific, which makes them less toxic^[58]. RG108 and MG98 are among them. They can apparently inhibit methylation with fewer side effects and activate the repressed genes, but clinical trials have not yet been carried out^[59].

Because we now have accurate ways to profile the methylation in a genome or in an individual gene, demethylation can be monitored frequently, which will allow the prompt correction of therapeutic agents, giving greater promise to this approach. It is, however, more important to prevent the occurrence of abnormal methylation.

METHODS IN METHYLATION PROFILING

Methylation profiling can be approached in two ways:

analysis of genome-wide methylation and analysis of single CpGs. Both approaches can be carried out by the same methods: colorimetry, fluorescence, methylation-sensitive restriction endonuclease treatment and PCR.

The first step in the colorimetric method is to hydrolyze the target DNAs into nucleotides using hydrochloric acid and then test the resulting absorbance. Differences in absorbance suggest that there are differences in the level of methylation between the two chromosomes. This is used to test the whole-genome methylation of DNAs. The fluorescence method has something in common with the colorimetric approach. It uses chloroacetaldehyde to treat the chromosomes, so that the chromosomes will increase in fluorescence, and the fluorescence intensity reflects the level of methylation of the whole genome. The methylation-sensitive restriction endonuclease method is used to analyze single CpGs, and the enzyme pairs used include Hpa II-Msp I and Sma I-XmaI. These enzymes specifically degrade the unmethylated chromosomes into small pieces, whereas the methylated DNA will escape the shearing. The pieces then can be tested *via* PCR or Southern blotting^[60].

When treated with bisulfite, methylated cytosines are stable, whereas unmethylated cytosines are modified to uracils. The amplification results thus can indicate whether the CpG is methylated or not^[61].

CONCLUSION

With every new discovery in the epigenetic landscape of tumors, there comes a new opportunity for producing targeted agents to cure cancer. As the understanding of the intricate machinery of tumor growth continues, there is greater hope that methylation-based therapies will prove successful. It is, however, essential to determine the safety of these treatments in the long run as we administer the agents to healthier populations of patients. It is also important to consider the context of all epigenetic processes, as the known treatments for blocking methylation are either non-specific or have severe side effects.

Our understanding of the relationship between DNA methylation and transcriptional control is being deepened but is still far from complete. It may be unrealistic to expect that any unified theory will encompass all the biological consequences of DNA methylation. It must be linked with deacetylation, siRNAs and phosphorylation. The mechanisms by which methylation patterns are generated are still not fully understood. After the human genome project comes into the post-genome period, methylation is of great importance. It is feasible to use the technique of methylation in the future to induce cell differentiation, guide clinical treatment and explore the early stages of cancer^[62].

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