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TOPIC HIGHLIGHT

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Current trends in the development and application of molecular technologies for cancer epigenetics

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Abstract

Current progress in epigenetic research supports the view that diet and dietary components are important in cancer etiology by enhancing or inhibiting carcinogenesis. Since diet and dietary factors may significantly contribute to the causation and progression of many cancers, it is important to find the molecular mechanisms of action of such dietary factors for cancer prevention and treatment. Recently, the role of epigenetic mechanisms in the cancer development and progression has attracted more attention as additional evidence along with traditional DNA sequence based mechanisms such as mutations and structural re-arrangements. Such an increasing interest in cancer epigenetics has also accelerated the development and application of molecular assays and tools for DNA methylation detection and histone modification enrichment analysis. In this paper, key assays and methods for epigenetic research are reviewed and discussed in terms of their utility and usability. In addition, more advanced methods for genome-wide analysis are introduced as part of upcoming research trends and directions.

Key words: Cancer; Epigenetic; Technology; Butyrate; Histone modifications; Diet; Methyl sources

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INTRODUCTION

Diet and dietary factors play an important role in many biological processes and are also involved in the regulation of pathological progressions including cancers. Several epidemiological and preclinical studies suggested that increased intake of bioactive dietary components may modulate cancer risk. Many studies provide compelling evidence that part of the anti-cancer properties contributed to several bioactive dietary components may relate to modulation of epigenetic process including DNA methylation and histone protein modifications. Here, we provide a brief overview of dietary modulation of DNA methylation and histone modifications and its potential role in cancer prevention. Also, we will discuss several new epigenetic methods to help understand the effect of dietary factors on epigenetic modifications.

EPIGENETICS AND CANCER

The growing interest in the role of epigenetics in cancer came from the demonstration that epigenetic modifications are involved in tumor development and progression. Epigenetics can be defined as phenomena that alter the expression of the information in the genome at the transcriptional, translational, or posttranslational

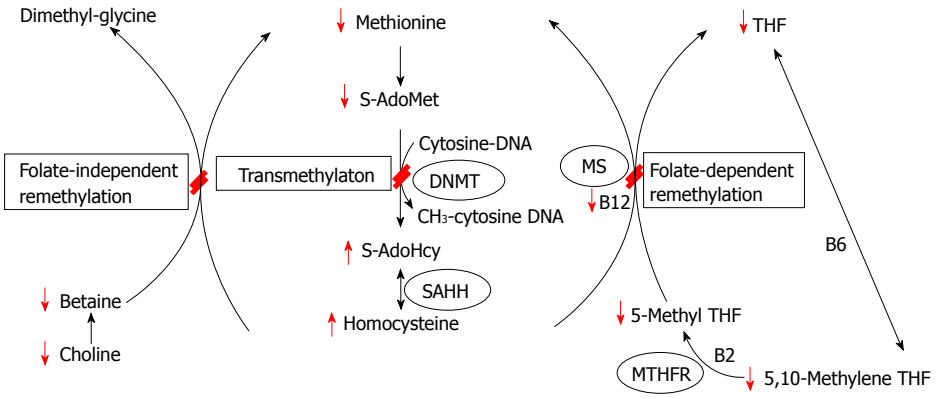

Figure 1 Effect of methyl-deficiency on biological methylation pathway. S-AdoMet: S-adenosylmethionine; S-AdoHcy: S-adensylhomocysteine; DNMT: DNA methyltransferase; SAHH: S-adenosylhomocysteine hydrolase; MS: Methionine synthase; THF: Tetrahydrofolate; MTHFR: Methylenetetrahydrofolate reductase.

level without change in DNA sequence[1,2]. Epigenetic information is maintained to preserve cellular identity in normal cells, while cancer cells are characterized by profound alteration of epigenetic regulation[3-7]. The overall disruption of epigenetic phenomena is a common feature of all human tumors and includes alteration of DNA methylation and histone modification patterns[8]. DNA methylation patterns of neoplastic cells have been recognized as being substantially altered compared with normal cells[3,4]. Two types of changes in the DNA methylation pattern can occur in cancer: global DNA hypomethylation and hypermethylation of CpG islands which are associated with gene silencing[3,4,7]. DNA in eukaryotic cells is intimately associated with a family of small, basic histone proteins forming a highly ordered and condensed DNA-protein complex termed chromatin. Because of this chromatin structure, changes in DNA methylation in cancer cells are not isolated events; they occur in the context of more complex epigenetic deregulation[9]. Chromatin is the physiological template of the genetic information and is composed of DNA, histones, and other chromosomal proteins. The fundamental repeating unit of chromatin is the nucleosome octamer, which consists of 147 base pairs of DNA wrapped around 2 copies each of histones H2A, H2B, H3 and H4[10]. The amino-terminal tails of histones are subject to posttranslational modifications, including acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, and ADP-ribosylation[11], and multiple histone modifications may occur on a given histone tail[12]. Histone modifications patterns distinguish the structure of chromatin status, in particular, acetylation of histone H3 and H4 is associated with active gene expression with open chromatin structure. Histone acetylation is regulated by several enzymes such as histone acetyltransferase and histone deacetylases activity. Aberrations in post-translational modifications of histones have been shown to occur in cancer cells. Although alterations in global histone modification patterns in cancer cells have remained unknown, recent studies on global histone modifications at specific amino acids have been suggested as predictive clinical outcomes for various cancers[13-15]. Additionally, a number of studies have been focused only on changes of a particular histone modification at individual gene promoters in cancer cells.

DNA METHYLATION

Methyl-deficient diet induced hepatocarcinogenesis

In last 4 decades, researchers have developed various tools for exploring DNA methylation, and started to apply those new technologies to the field of nutrition science. The methyl-deficient model of endogenous hepatocarcinogenesis is one of which in DNA methylation has been extensively studied. This animal model is unique in that dietary omission rather than chemical carcinogens addition can lead to tumor formation[16]. Specifically, deficiency of the major dietary sources of methyl groups - methionine, choline, folic acid and vitamin B12 - leads to the development of liver cancers in rats and certain mouse strains[17-19]. From early 1990, these animal models have shown that the methyl-deficiency is associated with several defects, including genome-wide DNA hypomethylation and gene-specific hypermethylation[20-22]. Importantly, the aberrant epigenetic alterations imposed by this diet have been hypothesized to be the primary mechanism responsible for malignant transformation of rat liver cells[15,20,22,23]. Figure 1 displays a simplified version of biological methylation pathway from one-carbon metabolism, emphasizing that various dietary methyl sources (methionine, choline, various coenzymatic forms of folate and vitamin B2, B6 and B12) play an important roles in DNA methylation. Methyl source deficiency has marked effects on the flow of one-carbon units through this web of reactions as the effect of methyl source deficiency are highlighted in red. The major effect observed in methyl deficiency models is a rapid decrease in hepatic S-adenosylmethionine (SAdoMet) levels and genomic DNA hypomethylation. In other recent studies examining the early stages of hepatocarcinogenesis induced by methyl deficiency in rats, substantial alterations in other aspects of the epigenetic machinery have been observed, including aberrant expression of DNA methyltransferases and methyl-CpG

Table 1 DNA methylation analyses used in methyl-deficient model of hepatocarcinogenesis in rodents

Dietary component	Model	Observations	Methylation assay	Ref.
Amino acid-defined diet lacking choline, methionine, folic acid and vitamin B12	Rat	Depletion of SAdoMet and DNA hypomethylation	Liver DNA methyltransferase activity assay with labeled SAdoMet	[20]
Amino acid-defined diet lacking choline, methionine, folic acid and vitamin B12	Rat	Hypomethylation of CCGG site of c-myc, c-fos and c-Ha-ras	Enzyme digestion by *Hpa* Ⅱ/*Msp* Ⅰ	[21]
Diet low in methionine lacking in choline and folic acid	Rat	Hypermethylation of $p16^{INK4A}$	MS-PCR	[94]
Diet low in methionine lacking in choline and folic acid	Rat	Decrease in the total percent of methylated CCGG sites in DNA	*Hpa* Ⅱ/*Msp* Ⅰ -based cytosine extension assay	[22]
Diet low in methionine lacking in choline and folic acid	Rat	Depletion of S-AdoMet, decrease in S-AdoMet/S-AdoHcy and global DNA hypomethylation	*Hpa* Ⅱ/*Msp* Ⅰ -based cytosine extension assay	[23]
Diet low in methionine lacking in choline and folic acid	Rat	Hypomethylation of ID element and LINE-1 in preneoplastic livers and liver tumors; Decrease in histone H4-Lys20 trimethylation and increase in histone H3-Lys9 trimethylation; Decrease in histone H4-Lys20 trimethylation at the LINE-1 regulatory region	ID methylation by methylation-sensitive McrBC-PCR array; LINE-1 methylation by COBRA-assay; global histone methylation by Western blotting; LINE-1-associated histone methylation by ChIP	[15]
Diet deficient in methionine lacking in choline and folic acid	Rat	Changes in the DNA methylation machinery	Indirect methods by DNA methyltransferases and Methyl CpG binding proteins	[24]
Amino acid-defined diet lacking choline	Rat	Hypermethylation of upstream of E-cadherin and Cx26	Bisulfite sequencing	[95]
Diet deficient in methionine lacking in choline and folic acid	Rat	global loss of DNA methylation; hypermethylation of CpG islands	Global DNA methylation by cytosine extension assay and [^{3}H-methyl] incorporation; CpG island methylation by [^{32}P]dGTP incorporation	[30]
Diet deficient in methionine lacking in choline and folic acid	Mouse	Global DNA hypomethylation; substantial loss of repetitive sequences (LINE-1, SINES, IAP elements) cytosine methylation. Increase in histone H3-Lys9 trimethylation and decrease in histone H4-Lys20 trimethylation	Global DNA methylation by cytosine extension assay; methylation-sensitive McrBC-qPCR assay; global histone modifications by Western blot	[96]
Diet deficient in methionine lacking in choline and folic acid	Mouse	Detection of CpG island methylation profiles	MeDIP	[97]

S-AdoMet: S-adenosylmethionine; ID: Identifier; LINE-1: Long interspersed nucleotide elements; SINES: Short interspersed nuclear elements; IAP: Intracisternal A-particle; MS-PCR: Methylation-specific polymerase chain reaction; ChIP: Chromatin immunoprecipitation; MeDIP: Methylated DNA immunoprecipitation.

binding proteins[24], defects in histone methyltransferase protein expression and histone posttranslational modifications[15]. In Table 1, various DNA methylation assays were summarized in methyl-deficient model of hepatocarcinogenesis in rodents.

Genomic DNA methylation assays

One of widely used methods for global DNA methylation assay is a radioassay that utilizes the enzyme *Sss* I DNA methyltransferase to catalyze the *de novo* methylation of the CpG sites with radiolabeled [^{3}H]-SAdoMet, a universal methyl donor *in vitro*[20,25-28]. And another method was developed thanks to the discovery of methylation-sensitive restriction endonucleases. In 1999, Pogribny *et al*[29] developed a new method based on methylation-sensitive endonucleases followed by single nucleotide extension with radiolabeled [^{3}H]-dCTP. This cytosine extension assay was used in various studies of methyl-deficient model of hepatocarcinogenesis for genomic DNA methylation[22,23,29,30]. These enzyme based methods have wide variations in precision as a result of inconsistency in the activity of methyl-sensitive endonucleases and the instability of methyltransferase activity[31]. In 2002, Friso *et al*[32] developed a method for quantitative determination of 5-methyl-2'deoxycytidine using liquid chromatography/electrospray ionization/mass spectrometry (LC/ESI/MS). This method allows accurate measurement of the absolute amount of 5-methyl-2'deoxycytidine relative to the total amount of cytosine residues, furthermore, it requires relatively lower amount of DNA and has a shorter run time for each sample than other high-performance liquid chromatography-based methods[33-35]. DNA methylation assay by LC/ESI/MS has been widely used for quantitative DNA methylation in animal studies and population-based studies in the light of its greater reproducibility and precision in large number of samples[32,36-40].

Gene-specific DNA methylation measurements

DNA methylation has long been recognized as an important factor on the silencing of genes, therefore it has become important to know the methylation status of individual CpG site. The first generation of DNA methylation detection assay is Southern blot or polymerase chain reaction (PCR) amplification that follows the enzyme digestion with methylation-sensitive restriction endonucleases[41-46]. Currently, the most commonly used methods for gene-specific DNA methylation can be categorized into three major methods.

Bisulfite DNA sequencing and methylation-specific

PCR: Treatment of DNA with bisulfite converts cytosine residue to uracil, but leaves 5-methylcytosine residue unaffected. Bisulfite sequencing involves chemical conversion of cytosine to uracil, followed by PCR, and DNA sequencing[47]. While providing single-base resolution, the high cost and labor-intensive steps limit the use of this method for high-throughput analyses[48]. Methylation-specific-PCR also employs bisulfite conversion, but avoids the need to sequence the area of interest. Instead, methyl-specific and unmethyl-specific primer sets are designed, to distinguish methylated from unmethylated DNA in bisulfate-converted DNA[49]. This method is powerful to explore CpG islands with high methylation density, as increased numbers of CpG in the primer increase the specificity of the assay. However, these two methods using bisulfite conversion are not currently suitable for whole-genome analysis on multiple samples but commonly used for data validation from array-based methods.

Methods that focus specific single-CpG: These include Combined Bisulfite Restriction Analysis (COBRA)[50], MethyLight[51], and bisulfite pyrosequencing[52]. In COBRA, the combination of bisulfite conversion and PCR amplification is used, therefore it results in sequence conversion (unmethylated cytosine residue to thymidine and methylated cytosine to cytosine) which can lead to new methylation-dependent restriction enzyme sites. The following digestion of the PCR product with at least one CpG site in the recognition sequence only proceeds if the CpG site is protected from bisulfite conversion by methylation. For this reason, the signal ratio of restriction products indicating methylation to undigested PCR product representing unmethylated sequences can be used as a measure for the methylation level of this specific CpG. MethyLight is a bisulfite-dependent, fluorescence-based, quantitative real-time PCR method for DNA methylation. MethyLight relies on methylation-specific priming combined with methylation-specific fluorescent probing. This combination of methylation-specific detection principles results in a highly methylation-specific detection technology, with an accompanying ability to sensitively detect very low frequencies of hypermethylated alleles. Bisulfite pyrosequencing has been used to analyze bisulfite-converted DNA without using methylation-specific PCR. Following PCR amplification of the region of interest, pyrosequencing is used to determine the bisulfite-converted sequence of specific CpG sites in the region. The ratio of cytosine to thymidine at individual sites can be determined quantitatively based on the amount of cytosine and thymidine incorporation during the sequence extension. While the methods mentioned above are sensitive, specific, and relatively inexpensive, none of these methods is suitable for analysis of the whole genome, which includes about 28 million CpGs.

Microarray-based methods: These enable to interrogate larger numbers of CpG, there are three major types of microarray-based methylation analysis. Direct hybridization to CpG island arrays is the first high-throughput approach capable of detecting DNA methylaion in genes across several CpG sites. Based on the bisulfite modification of DNA, this method utilizes methylation-specific oligonucleotides arrayed on glass slides for detection of all possible methylation in target DNA[53]. Methylated DNA immunoprecipitation (MeDIP) is also a large-scale, genome-wide purification method that is used to enrich for methylated DNA sequence using antibody raised against 5-methylcytosine[54]. DNA from MeDIP can be used for either array-based hybridization (MeDIP-chip) or high-throughput sequencing (MeDIP-seq). Although MeDIP helps generate comprehensive DNA methylation profiles, both applications have their typical limitation of array-based technology, restricted resolution. The HELP assay (*Hpa*Ⅱ tiny fragment enrichment by ligation-mediated PCR) is comparative isoschizomer profiling of DNA methylation[55]. DNA is digested by *Hpa*Ⅱ in parallel with *Msp* Ⅰ (resistant to DNA methylation), and then the *Hpa*Ⅱ and *Msp* Ⅰ products are either amplified by ligation-mediated PCR and hybridized using separate fluorochromes to a customized array, or directly sequenced[56]. These high-throughput array-based approaches for DNA methylation are relatively inexpensive tool suitable for genome-wide analysis, therefore, help to target aberrant methylation patterns in various cancer models. Furthermore, methylation profiling achieved from high throughput methods will offer differentially methylated regions to understand the effect of dietary factors on epigenetic modifications in cancer, subsequently, provide insight in prevention strategies to reduce the burden of cancer.

HISTONE MODIFICATION

Histone deacetylase inhibition by butyrate

In addition to the effects on DNA methylation, dietary components can affect posttranslational modifications of histones. The dietary agent best studied in histone modifications is the short chain fatty acid butyrate which is generated in the colon as a result of bacterial fermentation of dietary fiber. Higher intake of dietary fiber is associated with reduced risk of colorectal cancer[57,58]. The molecular mechanisms underlying this anti-cancer effect of dietary fiber are poorly understood, however, the strongest evidence is based on the anti-carcinogenic actions of butyrate. Butyrate can be found at millimolar concentrations in the lumen of the colon[59], and has inhibitory effects on types Ⅰ and Ⅱ histone deacetylase enzymes. Butyrate-induced alterations in histone marks, especially acetylation at histone H3 and/or H4, have been associated with several processes, including cellular differentiation[60,61], cell cycle arrest[62-64], apoptosis[65-67], and inhibition of invasion[68] in a number of cancer cell studies. Table 2 summarized some of evidence of the effects of butyrate on histone acetylation. Although butyrate has strong marks

Table 2 A summary of selected evidence for effects of butyrate in histone modification and histone modification assays in cancer cell culture models

Dietary component	Cell culture model	Observations	Histone modification assay	Ref.
Sodium butyrate	SW620 human colon carcinoma cells	Increased global histone H4 acetylation	Western blot	[70]
Sodium butyrate	A375 human melanoma and S91 mouse melanoma	Increased global histone H4 acetylation	Western blot	[98]
Sodium butyrate	Colo-320 human colon cancer cells	Increased acetylation of histone H3 and H4 within CDKN1A promoter site	ChIP	[64]
Sodium butyrate	EBC-1 human lung epithelial cells	Increased histone H3 and H4 acetylation associated with promoter of cathelicidin	ChIP	[99]
Sodium butyrate	HepG2 human hepatocarcinoma	Increased global histone H3 and H4 acetylation; Genome-wide changes in acetylation of DNA-bound histones	Western blot; ChIP-chip (ChIP and microarray hybridization)	[100]

CDKN1A: Cyclin-dependent kinase inhibitor 1A; ChIP: Chromatin immunoprecipitation.

on histone acetylation, a small fraction of cellular genes is regulated in response to butyrate[69-71]. Therefore, it should be noted that site-specific approach by chromatin immunoprecipitation (ChIP) based experimental tools will provide a better understanding on the chemopreventive effects of butyrate, showing gene-specific histone acetylation and its associated gene expression.

Global histone acetylation assays

The first estimates for the rate of acetylation turnover were measured by pulse, pulse-chase, and steady-state acetylation labeling in hepatoma tissue culture cells in 1975[72]. Boffa *et al*[73] showed that sodium butyrate suppressed histone deacetylation *in vivo* and *in vitro* by measuring the kinetics of [^{3}H] acetate release from histone proteins. Since specific antibodies to modified histones were developed, Western blot has been used to detect histone modifications. As shown in Table 2, butyrate-induced histone acetylation was confirmed by Western blot in many studies.

Gene-specific histone acetylation measurements

ChIP: The antibodies to acetylated histone H3 and H4 have been used for ChIP to determine histone acetylation in specific regions of gene promoter and other regulatory regions. ChIP is a specialized immunoprecipitation used to detect the covalent interaction between the DNA sequence and DNA-binding proteins such as transcription factors or histone proteins. ChIP using histone antibodies is able to determine the specific location in the genome that various histone modifications are associated with, indicating the target of the histone modifiers[74]. For example, ChIP experiment unveiled that butyrate induced an increase in histone H3 and H4 acetylation within the CDKN1A promoter, which regulates the p21 protein, in Colo-320 human colon cancer cells[64]. Due to its ability to precisely detect the DNA binding of modified histones, transcription factors, and non-histone chromosomal proteins, ChIP has been widely used to generate and test numerous hypotheses regarding transcriptional and epigenetic regulations. However, it remains to be still challenging to conduct ChIP on an "epigenome" level, since ensuring an antibody of high specificity is often laborious and time-consuming. Another important concern with ChIP scalability is the maximum range of target regions that can be investigated by a single assay. For instance, a typical experiment of ChIP coupled with qPCR is designed to measure the enrichment levels of a DNA binding protein at a handful of sites (*e.g.*, gene promoters). However, in general, even a single epigenetic event in the cell pervasively occurs over a wide range of genomic regions, often involving thousands of genes and their associated regulatory elements. Thus, it becomes more important to have an ability to run the assay on a genome-wide scale for having a more balanced and unbiased perspective on the underlying mechanisms. Coupled with genomic profiling technologies such as tiling arrays or next generation sequencing (NGS), ChIP can be extended over the whole genome. In the following sections, we will introduce two major methods coupled with ChIP that enable epigenome-scale research of histone marks and transcription factors.

ChIP-chip: ChIP-chip is based on the combination of ChIP and a genomic tiling array technology (*i.e.*, chip), in which DNA sequences extracted after ChIP hybridize with probes that are designed to cover the whole genome or specific regions of interest such as promoter[75,76]. Due to bias in microarray hybridization, a control experiment using chromatin input or DNA from non-specific immunoprecipitation (IP) (*e.g.*, IP against immunoglobulin G) is often recommended. Most algorithms for ChIP-chip are designed to compute the normalized ratio between the hybridizations of ChIP and control after removing random and/or systemic noise. Then, they call binding sites as those significantly enriched in ChIP over control[77-79]. Since its emergence in the early and mid 2000s, ChIP-chip has been widely adopted in many transcriptional and epigenetic regulation studies, assisting scientists to more understand the role of each histone mark in physiological and pathological processes[80-83]. However, the utility of ChIP-chip is heavily restricted by a tiling array probe design, which determines the resolution of

the measurement (*i.e.*, intervals between adjacent probes) and the regions that can be explored (*e.g.*, omission of repetitive sequence areas). These weaknesses of ChIP-chip have accelerated the major platform shift to NGS.

ChIP-Seq: In ChIP-Seq, the extracted DNA sequences are directly sequenced using a NGS technology instead of being hybridized onto tiling arrays. NGS refers to sequencing technologies that newly emerged since the mid 2000s as an alternative to the traditional automated Sanger sequencing. NGS is characterized as massive parallel sequencing of template DNA or RNA (cDNA) molecules by a relatively short length ranging over 50-400 bp[84]. One advantage of ChIP-Seq over ChIP-chip is that ChIP-Seq does not require any predefined array design, which allows a more unbiased assay at a much higher resolution (100-1000 bp in ChIP-chip *vs* 10-100 bp in ChIP-Seq). Since NGS generally produces a notoriously large amount of data than array-based methods, more powerful bioinformatics support is essential for data processing and analysis[85,86]. Bioinformatics analysis for ChIP-Seq in epigenetic research includes the pre-processing for sequence data such as quality control and read mapping, the identification of candidate sites enriched by the target histone mark, and further down-stream analysis for revealing biological implications of the observations from the precedent steps[85,86].

In cancer studies, the down-stream analysis is focused on finding the most associated genes or regulatory elements (*e.g.*, promoters or enhancers) with the histone mark of interest and investigating how these genes and regulatory elements can be understood in the context of biological pathways. Since Barski *et al*[87] and Wang *et al*[88] studies on 19 histone methylations and 18 histone acetylations using the human CD4+ T cell, many studies have been done to understand the biological implications of histone marks in normal conditions[80-82]. However, due to the plasticity of epigenome and heterogeneity of cancer, cancer epigenetics of examining histone modifications on a genome scale still remains in its beginning stage. For this reason, most of currently on-going efforts in cancer epigenetics still largely target DNA methylation (*e.g.*, The Cancer Genome Atlas, http://cancergenome.nih.gov/)[89]. Therefore, it will be a long-term goal to accumulate the knowledge on cancer epigenetics from histone modifications and use it for cancer studies, which will require a great amount of public and private investments. Another interesting research direction is an attempt to comprehend how genetic variations lead to epigenetic changes in cancer. In 2011, several studies have been published about the possibility of multiple chromatin remodelers and histone enzymes as potential oncogenes or tumor suppressor genes[90-93]. These studies suggest that the disruption of chromatin remodelers and histone enzymes due to driving somatic mutations in their coding regions may cause aberrant epigenetic changes, which eventually lead to cancer development or evolution in at least several cancer indications. Such approach is particularly interesting because it may be able to provide a genuine perspective on the target histone mark by observing somatic mutations in several key chromatin remodelers and histone enzymes.

CONCLUSION

In conclusion, a number of aberrant epigenetic modifications have been found in cancer cells, and diet and dietary factors play an important role to prevent cancer as well as to stimulate carcinogenesis. The use of epigenetic technology offers significant advantages to study the epigenetic mechanisms of cancer development and progression. Also, the newly developed technologies for epigenetic study expand the scope of nutrition study in the field of cancer research by helping monitor and pin down specific epigenetic pathways in diet-related cancers.

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