For reprint orders, please contact: reprints@futuremedicine.com

Epigenetic analysis in the search for tumor suppressor genes

"Although the epigenetic silencing by promoter hypermethylation and histone modifications is a well-known hallmark of many tumor suppressor genes, the majority of genes silenced by these epigenetic events in cancer cells could be passengers, not drivers, during tumor initiation and progression. Therefore, these silenced genes should be further investigated by analyzing their genetic lesions in tumor cells and functions in normal cells."

KEYWORDS: bisulfite ■ DNA methylation ■ DNA treatment ■ epigenetics ■ epigenome ■ histone modification ■ tumor suppressor gene

Ze-Guang Han

Shanghai-MOST Key Laboratory for Disease and Health Genomics, Chinese National Human Genome Center at Shanghai, 351 Guo Shou-Jing Road, Shanghai 201203, China
Tel.: +86 215 080 1325
Fax: +86 215 080 0402
hanzg@chgc.sh.cn

Increasing evidence has shown that the epigenetic silencing of tumor suppressor genes (TSGs) as a result of aberrant hypermethylation of CpG islands in promoters and chromatin modification is a crucial event during carcinogenesis and metastasis [1,2], although the genetic inactivation of TSGs is considered to be essential to the development and progression of tumors.

DNA methylation provides a stable gene silencing mechanism that plays an important role in regulating gene expression and chromatin architecture, in combination with histone modifications and other chromatin associated proteins [2]. In mammalian cells, DNA methylation is regulated by a family of DNA methyltransferases (DNMTs), including DNMT1, DNMT3A and DNMT3B, through a covalent modification with a methyl group transferred from S-adenosylmethionine to the C-5 position of cytosine of CpG dinucleotides. Although CpG dinucleotides are underrepresented in human genome, there exist short regions rich in CpG content, known as CpG islands, most of which are found in proximal promoter regions of approximately half the human genes, where these CpG islands of these genes are generally unmethylated [3]. Methylated CpG regions affect the transcription of downstream genes by recruiting methyl-CpG-binding domain proteins (MBDs) that function as adaptors between methylated DNA and chromatin-modifying proteins or block transcription factors from accessing target-binding sites of some genes. The DNA methylation patterns are generated by the cooperative activity of the *de novo* methyltransferases, DNMT3A and DNMT3B, which act independently of replication and show equal

preference for both unmethylated and hemimethylated DNA, whereas the DNA methylation is heritably maintained by DNMT1, which acts by preferentially methylating hemimethylated DNA during replication. The global reduction of 5-methyl cytosine content as a common feature was observed in various tumor types as compared with levels in normal tissues, however, promoter hypermethylation that leads to transcriptional silencing of TSGs is well-characterized in human tumors [4]. The promoter regions of genes can contain 100–400 CpG dinucleotides. Aberrant hypermethylation of the CpG islands in the promoter regions of many genes involved in the cell cycle, DNA repair, metabolism of carcinogens, cell–cell interactions, and apoptosis has been shown to be an important mechanism by which tumor suppressors are inactivated in tumorigenesis and tumor development [1,5]. The loss of function of numerous TSGs owing to the hypermethylation of CpG islands in promoters is considered as a more general mechanism than classical genetic mutations of these genes. More than 100 genes with tumor-specific promoter hypermethylation are currently recognized [6]. Many known TSGs, including the cyclin-dependent kinase inhibitor *CDKN2A* (p16), mismatch repair enzyme *MLH1*, *CDH1* and *BRCA1* were found to be silenced by promoter hypermethylation.

In addition to the aberrant hypermethylation of CpG islands in promoters of TSGs, the abnormal chromatin modification, including histone modification, is also commonly recognized as the cause of inactive TSGs. Nucleosomes which are the smallest unit of chromatin contain 146 bp of DNA wrapped around the core comprised of

future
medicine part of fsg

eight histone proteins, where the octamer has two copies each of the H2A, H2B, H3 and H4 proteins. Each of all these four histones has an amino-terminal lysine-rich tail that protrudes out of the nucleosome and is subject to post-translational modifications. These posttranslational modifications of the amino-terminal tails are critical to maintaining chromatin structure and controlling gene expression without changes in the DNA sequence. These posttranslational modifications include acetylation, methylation, phosphorylation, ubiquitylation, sumoylation and ADP-ribosylation [1,7,8]. The major forms of histone modifications are acetylation/deacetylation, methylation/demethylation, in combination with histone variants, and ATP-dependent chromatin remodeling. These modifications regulate gene expression through modulating how tight or loose the chromatin is compacted to change the accessibility of chromatin, which leads to either activation or repression depending upon the type of modifications and residues modified. The acetylated lysine residues on histones H3 and H4 are correlated with active or open chromatin that allows various transcription factors access to the promoters of target genes, and thereby enhances their transcription. Both histone acetyltransferases (HATs) and histone deacetylases (HDACs) can affect the acetylation of histone proteins [9,10]. HATs can acetylate the lysine residues in the N-terminal tails of histones, and by contrast, HDACs can remove the charge-neutralizing acetyl groups from histone lysine tails, which leads to the condensation of chromatin and to gene inactivation. So far, histone modifications with lysine acetylation have been increasingly recognized as important epigenetic features in tumorigenesis [1]. The genome-wide mapping of chromatin changes revealed a global loss of acetylated H4-lysine 16 (H4K16ac) as a common hallmark during tumorigenesis [11]. Such loss of histone acetylation could be mediated by HDACs that are often found overexpressed in various types of cancer [2]. A common mechanism by which HDACs as transcriptional corepressors are recruited to TSGs with or without the hypermethylated CpG islands is via other transcription factors, including methyl-binding proteins and oncogenic DNA-binding factors.

Unlike histone acetylation, histone methylation is regulated by histone methyltransferases (HMTs) and demethylases (HDMs) and is widely regarded as a mark of long-standing cellular memory, which results in either activation or repression of gene expression, depending on the residue methylated [12]. For example, the

methylation of histone H3-lysine 4 (H3-K4), H3-K36 or H3-K79 is considered as a marker of active chromatin and gene expression, whereas the methylation of H3-K9, H3-K27 or H4-K20 corresponds to inactive chromatin and gene silencing [2,8]. Cancer cells display widespread changes in histone methylation patterns, where the changing H3K9 and H3K27 methylation patterns are found in various forms of cancer. Dysregulation of HMTs responsible for repressive marks leads to aberrant silencing of TSGs in cancer, whereas overexpression of key histone methyltransferases that catalyze the methylation of either H3-K9 or H3-K27 residues is a frequent event in cancer cells [13]. For example, EZH2, a H3-K27 methyltransferase as a member of the polycomb group complex PRC2, was found to be upregulated in many cancers [2], which is associated with the retinoblastoma (Rb) family of proteins to inactivate the tumor suppressor p16/INK4 [14]. Increased levels of G9a, the H3-K9 HMT, is implicated in perpetuating malignant phenotype possibly through modulation of chromatin structure [15]. Several histone lysine demethylases with Jumonji C domain have been discovered. However, these HDMs can perform both activating and repressive functions in a precise context-dependent fashion. For example, LSD1, as the first identified lysine demethylase, can effectively remove histone marks (H3-K4 and H3-K9 methylation, respectively) depending on its specific binding partners [16].

.....
“...the bisulfite DNA treatment followed by genomic sequencing might provide a comprehensive view of the heterogeneous methylation patterns that exist in cancer cells.”

Both DNA methylation and histone modifications together determine gene expression through their interaction at multiple levels [17]. This indicates a convergence of DNA and histone methylation pathways, which may cooperate to silence TSGs in cancer cells [18]. DNMTs recruit HDACs that lead to histone deacetylation and thereby transcriptional repression. MBPs that bind the methylated DNA also recruit HDACs and ATP-dependent chromatin-remodeling proteins, which results in chromatin condensation and gene inactivation [18]. Histone methylation affects gene expression in part through its association with DNA methylation. Histone methylating enzymes, including HMTs and HDMs, directly recruit many DNA

methylation-related proteins, such as DNMTs and methyl-binding proteins, to specific genomic targets to stably silence genes by DNA methylation [19–21]. Moreover, they also influence DNA methylation levels by regulating the stability of DNMT proteins [22,23].

Screening and identification of candidate TSGs silenced by CpG-island-promoter hypermethylation and histone modifications are a compelling challenge to oncologists. To address these TSGs, the powerful high-resolution epigenomic approaches should be employed to explore TSGs undergoing epigenetic disruption in a given tumor, although previous studies on a gene-by-gene basis have provided important insights into cancer biology.

■■■■■■■■■■
“...ChIP-based technology followed by DNA sequencing using new generation of DNA sequencer is a more powerful tool for the high-throughput genome-wide mapping of epigenetic modifications...”
 ■■■■■■■■■■

With regard to TSGs with promoter hypermethylation, there are two rationales for readily approaching these candidate TSGs, although several other strategies were introduced [5,24]. One is bisulfite DNA treatment-based approaches, because the bisulfite treatment reproducibly changes unmethylated cytosines to uracil but fails to change methylated cytosines, and the other approach is chromatin immunoprecipitation (ChIP)-based approaches using the antibodies against 5-methyl-cytosine (methyl-DIP) or MBDs. The bisulfite DNA treatment, in combination with amplification by methylation-specific PCR has allowed us to easily study DNA methylation of particular sequences. But PCR-based assays address the methylation status only at CpG sites that are complementary to the designed primers, while the predominant methylation pattern in a tumor is not necessarily reflected in the results of such experiments. Therefore, the bisulfite DNA treatment followed by genomic sequencing might provide a comprehensive view of the heterogeneous methylation patterns that exist in cancer cells. More recently, combining bisulfite treatment of genomic DNA with ultra-high-throughput DNA sequencing technology, allows one to sensitively measure cytosine methylation on a genome-wide scale [25]. The genome-wide state of DNA methylation, termed the ‘methylome’, will not be limited to specific predefined genes. Moreover, bisulfite treatment of genomic DNA in combination with array-based analysis that is similar

to a SNP array also displays a comprehensive genome-wide view by measuring the amount of DNA in a methylation state [26,27]. However, the major limitation of bisulfite treatment approach is the incompleteness of changing unmethylated cytosines to uracil, which misleads to estimation of DNA methylation in a given tumor.

Chromatin immunoprecipitation-based approach employs the antibodies against 5-methyl-cytosine (methyl-DIP) or MBDs to purify methylated DNA, which is subjected to hybridization with high-density genome arrays (ChIP-on-chip) or DNA sequencing (ChIP-seq). The ChIP-on-chip or ChIP-seq approaches have provided another important recent advance in the epigenomic profiling of cancer cells. Immunoprecipitation using antibodies against MBDs that have a great affinity for binding to methylated cytosines has been used to identify hypermethylated genes in cancer cells [28]. The DNA that is immunoprecipitated with an antibody against 5-methyl-cytosine (methyl-DIP) can be used as a probe for hybridization to genomic microarray platforms containing CpG island and promoter or genomic tiling microarrays [29]. This approach promises to simplify and universalize the analysis of the DNA methylome as the first step to screen for the potential TSGs silenced by promoter hypermethylation. However, the entire human genomic analysis is not yet represented in these microarrays. Moreover, the whole-genome amplification procedure after a ChIP assay could produce biases from PCR [5]. Thus, ChIP-based technology followed by DNA sequencing using new generation of DNA sequencer is a more powerful tool for the high-throughput genome-wide mapping of epigenetic modifications that enable researchers to define the epigenome in cancer cells [30].

An alternative genome-wide approach for screening TSG silenced by promoter hypermethylation and histone modifications is the pharmacologic unmasking strategy with gene-expression profiling, which is now widely used and may prove to be particularly useful for searching for TSGs [5]. This strategy involves comparing mRNA levels from cancer cell lines before and after treatment with demethylating agent and HDAC inhibitor alone or in combination. The approach has proven successful at identifying TSGs silenced by promoter hypermethylation and histone modification [31,32]. However, it is important to note that not all of the epigenetically silenced genes became re-expressed after the use of the demethylating agent and/or a HDAC inhibitor. Moreover, the approach does

not provide direct proof of the presence of DNA methylation, bisulfite genomic sequencing is always required for confirmation [5].

“...it is important to note that not all of the epigenetically silenced genes became re-expressed after the use of the demethylating agent and/or HDAC inhibitor.”

In addition to the HDAC inhibitors employed by the pharmacologic unmasking strategy for silenced TSGs due to deacetylation of histones as a common mechanism in cancer cells, theoretically, H3–K9 or H3–K27 HMTs inhibitors are also used for TSGs silenced by H3–K9 or H3–K27 trimethylation. Similarly, ChIP-based approaches with antibodies against either H3–K9 trimethylation or H3–K27 trimethylation, followed by PCR, hybridization of microarray and genomic sequencing, would also be applied for screening TSGs silenced by these type of histone modifications.

It should be pointed out that new generation sequencing technology will facilitate the epigenomic approaches, including bisulfite-treated genomic sequencing, ChIP-seq and methylated DNA fractionation based on restriction endonuclease digestion, for screening the candidate TSGs. Sequencing-based techniques can define the genomic loci of the affected regions by hypermethylated CpG islands and histone modifications, and even quantitatively assess the methylation status of CpG dinucleotides. Moreover, a particular advantage of this global DNA sequencing analysis is the ability to ascertain allele-specific methylation by virtue of DNA polymorphisms [24].

Although the epigenetic silencing by promoter hypermethylation and histone modifications is a well-known hallmark of many tumor-suppressor genes, the majority of genes silenced by epigenetic events in cancer cells could be passengers, not drivers, during tumor initiation and progression. Therefore, these silenced genes should be further investigated by analyzing their genetic lesions in tumor cells and functions in normal cells. The extensive mutations and allelic loss of these genes will strongly support the epigenetic inactivation as biologically relevant to carcinogenesis. The functional significance of epigenetic silencing of certain genes can be appreciated in the context of the ‘two-hit’ hypothesis of TSG inactivation, where one allele of the gene stably maintains mutations with loss of functions, while the other allele is hypermethylated or adhered by the repressed histone modifications, which thereby leads to functional inactivation. However, many genes silenced by epigenetic mechanisms do not undergo genetic mutations in human cancer, and their tumor-suppressor function is based on their function in normal cells, which could represent a nonclassical pathway that leads to tumorigenesis.

Financial & competing interests disclosure

The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Bibliography

- Iacobuzio-Donahue CA: Epigenetic changes in cancer. *Annu. Rev. Pathol.* 4, 229–249 (2009).
- Sharma S, Kelly TK, Jones PA: Epigenetics in cancer. *Carcinogenesis* 31(1), 27–36 (2010).
- Klose RJ, Bird AP: Genomic DNA methylation: the mark and its mediators. *Trends Biochem. Sci.* 31, 89–97 (2006).
- Esteller M: Epigenetic gene silencing in cancer: the DNA hypermethylome. *Hum. Mol. Genet.* 16, R50–59 (2007).
- Esteller M: Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat. Rev. Genet.* 8, 286–298 (2007).
- Shames DS, Minna JD, Gazdar AF: DNA methylation in health, disease, and cancer. *Curr. Mol. Med.* 7, 85–102 (2007).
- Jenuwein T: The epigenetic magic of histone lysine methylation. *FEBS J.* 273, 3121–3135 (2006).
- Kouzarides T: Chromatin modifications and their function. *Cell* 128, 693–705 (2007).
- Roth SY, Denu JM, Allis CD: Histone acetyltransferases. *Annu. Rev. Biochem.* 70, 81–120 (2001).
- Dokmanovic M, Clarke C, Marks PA: Histone deacetylase inhibitors: overview and perspectives. *Mol. Cancer Res.* 5, 981–989 (2007).
- Fraga MF, Ballestar E, Villar-Garea A *et al.*: Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. *Nat. Genet.* 37, 391–400 (2005).
- Shi Y: Histone lysine demethylases: emerging roles in development, physiology and disease. *Nat. Rev. Genet.* 8, 829–833 (2007).
- Feinberg AP, Tycko B: The history of cancer epigenetics. *Nat. Rev. Cancer* 4, 143–153 (2004).
- Kotake Y, Cao R, Viatour P *et al.*: pRB family proteins are required for H3 K27 trimethylation and polycomb repression complexes binding to and silencing *p16INK4a* tumor suppressor gene. *Genes Dev.* 21, 49–54 (2007).
- Kondo Y, Shen L, Ahmed S *et al.*: Downregulation of histone H3 lysine 9 methyltransferase G9a induces centrosome disruption and chromosome instability in cancer cells. *PLoS One* 3, e2037 (2008).
- Shi Y, Lan F, Matson C *et al.*: Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* 119, 941–953 (2004).

- 17 Cedar H, Bergman Y: Linking DNA methylation and histone modification: patterns and paradigms. *Nat. Rev. Genet.* 10, 295–304 (2009).
- 18 D'Alessio AC, Szyf M: Epigenetic *tete-a-tete*: the bilateral relationship between chromatin modifications and DNA methylation. *Biochem. Cell Biol.* 84, 463–476 (2006).
- 19 Klose RJ, Zhang Y: Regulation of histone methylation by demethylation and demethylation. *Nat. Rev. Mol. Cell Biol.* 8, 307–318 (2007).
- 20 Tachibana M, Matsumura Y, Fukuda M, Kimura H, Shinkai Y: G9a/GLP complexes independently mediate H3K9 and DNA methylation to silence transcription. *EMBO J.* 27, 2681–2690 (2008).
- 21 Zhao Q, Rank G, Tan YT *et al.*: PRMT5-mediated methylation of histone H4R3 recruits DNMT3A, coupling histone and DNA methylation in gene silencing. *Nat. Struct. Mol. Biol.* 16, 304–311 (2009).
- 22 Estève PO, Chin HG, Benner J *et al.*: Regulation of DNMT1 stability through SET7-mediated lysine methylation in mammalian cells. *Proc. Natl Acad. Sci. USA* 106, 5076–5081 (2009).
- 23 Wang J, Hevi S, Kurash JK *et al.*: The lysine demethylase LSD1 (KDM1) is required for maintenance of global DNA methylation. *Nat. Genet.* 41, 125–129 (2009).
- 24 Feinberg AP: Genome-scale approaches to the epigenetics of common human disease. *Virchows Arch.* 456, 13–21 (2010).
- 25 Cokus SJ, Feng S, Zhang X *et al.*: Shotgun bisulphite sequencing of the Arabidopsis genome reveals DNA methylation patterning. *Nature* 452(7184), 215–219 (2008).
- 26 Bibikova M, Fan JB: GoldenGate assay for DNA methylation profiling. *Methods Mol. Biol.* 507, 149–163 (2009).
- 27 Bibikova M, Lin Z, Zhou L *et al.*: High-throughput DNA methylation profiling using universal bead arrays. *Genome Res.* 16, 383–393 (2006).
- 28 Lopez-Serra L, Ballestar E, Fraga MF, Alaminos M, Setien F, Esteller M: A profile of MBD protein occupancy of hypermethylated promoter CpG islands of tumor suppressor genes in human cancer. *Cancer Res.* 66, 8342–8346 (2006).
- 29 Weber M, Davies JJ, Wittig D *et al.*: Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nat. Genet.* 37, 853–862 (2005).
- 30 Falk J: Using ChIP-based technologies to identify epigenetic modifications in disease-relevant cells. *IDrugs* 13(3), 169–174 (2010).
- 31 Suzuki H, Gabrielson E, Chen W *et al.*: A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. *Nat. Genet.* 31, 141–149 (2002).
- 32 Yamashita K, Upadhyay S, Osada M *et al.*: Pharmacologic unmasking of epigenetically silenced tumor suppressor genes in esophageal squamous cell carcinoma. *Cancer Cell* 2, 485–495 (2002).