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Review

The role of TET family proteins and 5-hydroxymethylcytosine in human tumors

Yi-Chen Wu and Zhi-Qiang Ling
Zhejiang Cancer Research Institute, Zhejiang Province Cancer Hospital, Zhejiang Cancer Center, Hangzhou, P.R.China

Summary. Tumorigenesis correlates with hypermethylation of tumor suppressors and hypomethylation of oncogenes. DNA methyltransferases (DNMTs) catalyze DNA methylation, and mutations and aberrant expression in *DNMT* genes are found in multiple human tumors. The discovery of the DNA demethylation function of TET proteins has opened up new avenues for the study of DNA methylation regulation. TET proteins regulate the DNA demethylation pathway through oxidizing 5-mC into 5-hmC, 5-fC, and 5-aC. *TET* genes have been reported to be frequently mutated in hematopoietic malignancies and are associated with the malignant transformation of cells. Loss-of-function mutations in *TET* genes have not been reported in human solid tumors. However, 5-hmC has been found to be reduced in various solid tumors, indicating that *TET* genes may contribute to cellular transformation via regulation of DNA demethylation. As a new epigenetic modification, 5-hmC may be a useful biomarker for the diagnosis of cancers. To better understand the roles of TET and 5-hmC in tumors, the biological functions of TET and 5-hmC should be studied further.

Key words: TET family proteins, 5-hmC, Tumor, α-KG, IDH

Offprint requests to: Zhi-Qiang Ling, M.D., Ph.D, Zhejiang Cancer Research Institute, Zhejiang Province Cancer Hospital, Zhejiang Cancer Center, No.38 Guangji Rd., Banshanqiao District, Hangzhou 310022, China. e-mail: lingzq@hotmail.com

Introduction

5-hmC was first identified in T-even bacteriophages in 1953. However, because its physiological functions were not known, and due to limited detection technologies, it did not become a major focus of studies until recently (Wyatt and Cohen, 1953), when the demethylation function of TET was discovered. Like 5-mC, 5-hmC is also found universally and dynamically throughout the genome and participates in the regulation of various cellular functions. Currently, studies of 5-hmC mainly involve cell development and differentiation, including investigation of germ cells, zygotes, and embryonic cells. Compared with other research areas, studies of human cancer are quite limited. Several studies have confirmed that *TET* genes are frequently mutated in hematopoietic malignancies and influence 5-hmC levels in the genome. Additionally, while 5-hmC levels in human solid tumors are significantly correlated with tumorigenesis, tumor progression, and prognosis, the regulatory mechanisms mediating the *TET* gene and 5-hmC are still elusive. Therefore, clarification of these mechanisms will contribute to our further understanding of the role of DNA methylation dysregulation in tumorigenesis and tumor progression.

Domain structure of the TET family of DNA hydroxylases

TET family proteins are DNA dioxygenases whose catalytic activity is dependent on α-ketoglutarate (α-KG) and Fe^{2+}. α-KG acts as a cosubstrate, and Fe^{2+} acts

as a cofactor; TET proteins facilitate the binding of one oxygen atom of O_2 to the substrate, generating a hydroxy group, while the other oxygen atom is taken up by α-KG for decarboxylation, leading to the release of CO_2 and succinate (Xiao et al., 2012). The mammalian TET family has 3 members, TET1, TET2, and TET3, and the genes encoding these proteins are located on chromosomes 10q21, 4q24, and 2p13, respectively. These proteins share a high degree of homology in the carboxy-terminal catalytic domain (Mohr et al., 2011). All TET proteins contain a catalytic carboxy-terminal CD domain (including Cys-rich and DSBH regions), which are generally associated with the Cupin-like dioxygenase superfamily and confer α-KG/Fe^{2+}-dependent dioxygenase activity. TET proteins oxidize 5-mC into 5-hmC via these CD domains, and α-KG is treated as a cosubstrate (Tahiliani et al., 2009). In addition, TET proteins contain the CXXC zinc finger domain in the amino terminus; this domain exists in TET1 and TET3, but not TET2 (Fig. 1) (Tan and Shi, 2012). During evolution, the *TET2* gene has undergone a chromosomal gene inversion and is separated into two parts, the main gene and the CXXC domain. The main gene region encoding the catalytic region gradually evolved into the *TET2* gene, while the CXXC domain evolved into a distinct gene, *IDAX*. *IDAX* localizes to promoters and CpG islands through binding unmethylated CpG dinucleotides and regulates TET2 directly by interacting with its catalytic domain. However, the expression of *IDAX* results in downregulation of TET2 protein (Ko et al., 2013). Other proteins likely have functions that replace the CXXC domain and assist TET2 to recognize specific binding sites in the genome during DNA demethylation. To date, the function of the CXXC domain in TET proteins has not yet been fully elucidated. The CXXC domain of TET1 has been shown to recognize unmodified cytosine, 5-mC, and 5-hmC and can bind to regions of high CpG content (Xu et al., 2011). In addition to the above functional domains, TET proteins also have a spacer region that splits the DSBH domain. The DSBH domain can bind Fe^{2+} and exhibits DNA dioxygenase activity.

The spacer region is common among TET family proteins and differs in length in the various TET isoforms. Although the function of the spacer region remains unknown, Upadhyay et al (Upadhyay et al., 2011) found that the sequence of the spacer region of TET1 was similar to the C-terminal domain (CTD) of RNA polymerase II of *Saccharomyces cerevisiae*. Among cases of myelodysplastic syndrome (MDS) associated with *TET2* mutations, about 25% of these mutations are located in the spacer region, emphasizing the importance of this region (Ko et al., 2010). Dawlaty et al. (2013) knocked out both the *TET1* and *TET2* genes in embryonic stem cells in mice and found that loss of TET1 and TET2 proteins resulted in reduced 5-hmC and genomic hypermethylation. Moreover, TET3 has been shown to compensate for TET1 and TET2 deficiency, contributing to DNA demethylation. TET3 is unique in that it contains a PRK12323 region, which is not found in any other superfamily and does not have any known function.

Catalytic mechanism of TET family proteins

As a stable epigenetic modification, 5-mC has been studied for several decades and is known to play a vital role in gene expression, genomic imprinting, and suppression of transposable elements (Ito et al., 2011). According to a large body of evidence, DNA oxidation results in DNA damage, and the defect is subsequently excised by the DNA repair pathway. However, recent studies have indicated that DNA oxidizing reactions, which catalyze the conversion of 5-mC to 5-hmC, represent a type of DNA modification (Wu and Zhang, 2010). The DNA modification catalyzed by TET proteins plays a critical role in DNA demethylation pathways (Fig. 2). 5-mC and 5-hmC can be deaminated by activation-induced cytidine deaminase (AID), and this deamination reaction generates T and 5-hmU. Compared with 5-mC, 5-hmC is more sensitive to AID, and these deamination products activate the base-excision repair (BER) pathway for DNA demethylation (Guo et al., 2011). TET proteins not only oxidize 5-mC to generate 5-hmC, but also further oxidize 5-hmC to generate 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-aC), which are subsequently recognized and excised by thymine DNA glycosylase (TDG) (He et al., 2011). This is another mechanism through which DNA is actively demethylated. In addition to activating DNA demethylation pathways, some researchers have found that 5-hmC, 5-fC, and 5-aC, which are associated with the paternal genome in zygotes, are gradually lost during pre-implantation development, resulting in a DNA replication-dependent passive demethylation pathway (Inoue and Zhang, 2011). Thus, multiple DNA demethylation pathways, involving both active and passive DNA demethylation mechanisms, are associated with various proteins and enzymes. However, the conversion of 5-mC to 5-hmC is the initial step for the complete cycle of DNA demethylation, regardless of the

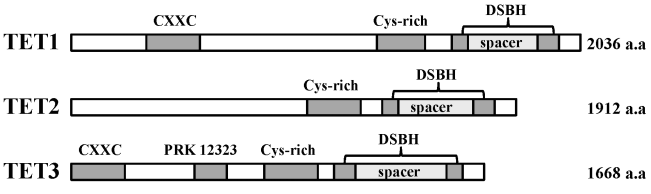

Fig. 1. The domain structure of mouse TET family proteins. (Modified with permission from reference (Tan and Shi, 2012). All TET proteins contain a C-terminal CD domain (Cys-rich and DSBH regions) that exhibits 2-oxoglutarate (2-OG)- and iron(II)-dependent dioxygenase activity and a spacer region that bridges the 2 parts of the disconnected DSBH region. TET1 and TET3, but not TET2, contain a CXXC domain, which can bind CpG islands. TET3 also has a unique PRK12323 domain. a.a. amino acids.

subsequent steps mediated by the AID, BER, or TDG pathways. Thus, TET-mediated conversion of 5-mC to 5-hmC is a prerequisite for DNA demethylation. 5-hmC acts as an intermediate product during the DNA demethylation process, and TET proteins function as rate-limiting factors in the regulation of DNA demethylation. Additionally, 5-hmC can inhibit the methyl-CpG binding domain (MBD) to bind to the oligonucleotide duplex (Valinluck et al., 2004). Since 5-hmC cannot be recognized by DNMT1, the methylation modification is gradually lost during DNA replication, and the daughter strand generates new unmethylated cytosine derivates (Valinluck and Sowers, 2007). However, there are also many questions about the mechanisms of DNA methylation that still need to be solved. For example, what are the functions of various domains within the TET proteins? Does a 5-hmC-specific binding protein exist? Do DNA decarboxylases for 5-aC or DNA deformylases for 5-fC exist to mediate DNA methylation? How do TET family proteins and DNMTs regulate the DNA methylation balance? How do TET family proteins and 5-hmC function in cancer?

Mutations in TET family genes in hematopoietic malignancies

TET1 and hematopoietic malignancies

The discovery of TET1 protein was dependent on an *MLL-TET1* translocation at t(10;11)(q22;q23), which generates the MLL-TET1 fusion protein in acute myeloid leukemia (AML). The *TET1* gene is expressed in various tissues, including hematopoietic cells. Burmeister et al. (2009) found that the *MLL-TET1* translocation occurs not only in AML, but also in acute lymphoblastic leukemia (ALL). The MLL-TET1 fusion protein may gain a new function and lose the normal functions of MLL and/or TET1. However, whether these new characteristics promote oncogenesis remains to be determined. To date, this translocation event is the only mutation identified in *TET1* in the context of hematopoietic malignancies.

TET2 and hematopoietic malignancies

TET2 mutations have been found in multiple hematopoietic malignancies, including AML, chronic myelomonocytic leukemia, myelodysplastic syndromes, polycythemia vera, primary myelofibrosis, essential thrombocythemia, mastocytosis, and more. Langemeijer et al. (2009) detected the expression of *TET2* mRNA in multiple tissues and found that *TET2* expression was highest in hematopoietic cells, especially granulocytes. Gaidzik et al. (2012) analyzed the presence of *TET2* mutations in 783 younger adult patients with AML. Of these patients, 60 (7.6%) exhibited 66 *TET2* mutations, including 37 missense, 16 frameshift, and 13 nonsense mutations. All *TET2* mutations were heterozygous except one. These results indicate that most *TET2* mutations in AML are heterozygous and that expression of the functional wild-type allele may act as a tumor suppressor. Itzykson et al. (2011) sequenced the *TET2* gene in 86 patients with MDS and AML following treatment with AZA and analyzed the effects of *TET2* mutations on patient response to AZA. In this study, 13 patients (15%) carried *TET2* mutations. The response rates in patients with mutant and wild-type alleles were 82% and 45%, respectively. Thus, *TET2* mutations and favorable cytogenetic risk could predict a higher response rate independently. Additionally, *TET2* status may act as a genetic predictor of response to AZA in higher-risk MDS and AML patients with low blast

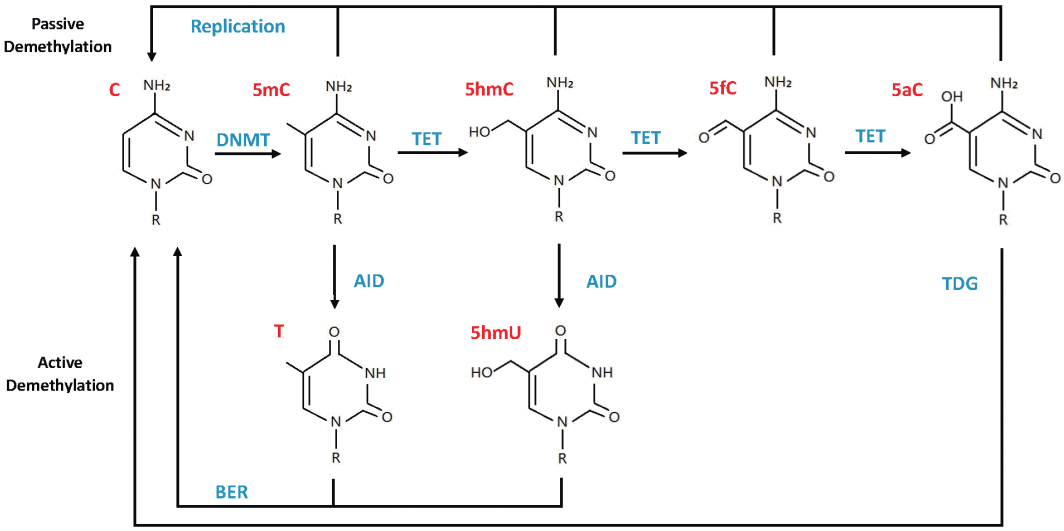

Fig. 2. Potential mechanisms of the DNA demethylation pathway. TET proteins not only oxidize 5-mC to 5-hmC, but also further oxidize 5-hmC to 5-fC and 5-aC, which can be recognized and excised to produce an unmethylated cytosine by TDG. 5-mC and 5-hmC are deaminated to T and 5-hmU by AID. These deamination products activate BER pathway-mediated demethylation. 5-hmC is more sensitive than 5-mC to AID-dependent deamination. 5-mC and its oxidative derivatives may undergo replication-dependent passive demethylation.

counts. *TET2* mutation was an early event in MPN (Abdel-Wahab et al., 2010), but a late event in the transformation from MPN to AML (Schaub et al., 2010). Abdel-Wahab et al. (2010) investigated several genes involved in leukemic transformation in 63 patients with AML secondary to a pre-existing MPN. They found that *TET2, ASXL1,* and *IDH1* were frequently mutated in sAML derived from a pre-existing MPN. *TET2*/*ASXL1* mutations might occur earlier than *JAK2* mutations, as demonstrated by MPN clones. When leukemia transforms, *TET2* mutations (not *ASXL1*) are always present. However, the mutational order of events in MPN and sAML varies in different patients, and *TET2* and *ASXL* mutations play different roles in MPN pathogenesis and leukemic transformation. Although loss of the catalytic activity of TET family proteins may theoretically elevate the levels of 5-mC and lower the levels of 5-hmC, the effects of *TET2* mutations on the levels of DNA methylation and 5-hmC are opposite in some patients. Ko et al. (2010) used both HELP assays and LC-MS to detect global levels of DNA methylation in AML patients with *TET2* mutations and showed hypermethylation with low global 5-hmC levels. However, it is unclear whether these differences are the result of differences in diseases or in the ability to distinguish between 5-mC and 5-hmC in varying experimental platforms. Chou et al. (2011) analyzed *TET2* mutations in 486 adult patients with primary AML. Sequential analysis indicated that patients whose *TET2* gene had been mutated at diagnosis frequently lost *TET2* mutations at relapse, but patients without *TET2* mutations at diagnosis rarely acquired *TET2* mutations at relapse. Thus, *TET2* mutations were unstable during disease evolution. Patel et al. (2012) analyzed the correlation between mutations in 18 genes and prognoses in 398 patients with AML. Their results revealed that *TET2* mutations were mutually exclusive with *IDH1* and *IDH2* mutations and were associated with reduced overall survival among patients with intermediate-risk AML. Combined with mutation information for *FLT3-ITD* (internal tandem duplication), *CEBPA, trisomy8, DNMT3A,* and *MLL-PTD* (partial tandem duplication), intermediate-risk AML could be further classified, and the prognosis of patients with AML could be predicted more accurately. Accumulating genetic evidence has shown that TET proteins are the pathological target of *IDH1/2* mutations. 2-hydroxyglutarate (2-HG), the product of mutated IDH1/2, inhibits TET-mediated hydroxylation. Thus, either *TET2* or *IDH1/2* mutations may result in genomic hypermethylation. *TET2* and *IDH1/2* mutations are mutually exclusive, suggesting that *TET2* mutations and *IDH1/2* mutations may share the same mechanism in hematopoietic transformation. Although *TET2* and *IDH1/2* mutations in AML elicit the same biological effects, patients with *TET2* or *IDH1/2* mutations show different clinical characteristics. In addition, *TET2* and *IDH1/2* mutations have contradictory effects on the overall survival of patients with AML (Patel et al., 2012). Thus, elucidation of the roles of *TET2* and *IDH1/2* mutations in AML will require further studies.

TET3 and hematopoietic malignancies

To date, only one report describing genetic aberrations of *TET3* has been published. One patient with refractory anemia with ringed sideroblasts (RARS) and idiopathic myelofibrosis carried a deletion in 2p23, where the *TET3* gene is located (Gelsi-Boyer et al., 2009). However, whether the *TET3* gene plays a role in myeloproliferative diseases, similar to *TET1* and *TET2*, remains to be studied.

5-hmC in human solid tumors

Currently, *TET* mutations influencing DNA methylation have only been discovered in hematopoietic malignancies, but have not been found in solid tumors. However, many studies have revealed that 5-hmC levels are reduced in solid tumor tissues compared with normal tissues (Haffner et al., 2011).

5-hmC in digestive system tumors

Kudo et al. (2012) measured 5-hmC levels in human tumor tissues (colon, liver, brain, kidney, skeletal muscle, and lung) and normal tissues by immunostaining and found that fluorescence signals representing 5-hmC were significantly decreased in tumor tissues compared with normal tissues. Dot blotting results revealed that 5-hmC levels were reduced in most colorectal cancer samples (16/22, 72.7%) and gastric cancer sampled (9/12, 75.0%), indicating the presence of a pathway that can downregulate 5-hmC levels in solid tumor. 5-hmC levels may be reduced due to decreased 5-mC levels, as suggested by experiments using anti-5mC antibodies to detect 5-mC levels in the above samples. These results indicated that the reduction in 5-hmC levels was not always due to decreased 5-mC levels. Thus, 5-hmC levels may be reduced via various pathways. Additionally, TET and DNMT family proteins can catalyze DNA demethylation and methylation reactions, respectively; thus, the expression of *TET* and *DNMT* genes plays a critical role in regulating 5-hmC levels. According to an analysis of the expression of *TET* and *DNMT* family genes, *TET1* expression was decreased in 50% (11/22) of tumor tissues compared with its expression in adjacent normal tissues. Among the samples with decreased *TET1* expression, 73% (8/11) also exhibited reduced 5-hmC levels. In contrast, samples without decreased expression of *TET1* showed increased expression of *DNMT* genes (88%, 7/8). The expression of *TET2* was low in both tumors and normal tissues, and low expression of *TET1* was associated with reduced *TET3* mRNA. The reason that upregulation of *DNMT* genes results in the reduction of 5-hmC level is still unknown. However, it is speculated that upregulation of *DNMT* may suppress *TET1* expression,

thereby affecting 5-hmC levels.

5-hmC in brain tumors

TET family proteins depend on α-KG and Fe^{2+} for their catalytic activity. α-KG participates in various metabolic pathways, including the Krebs cycle, fatty acid synthesis, protein and nucleic acid hydroxylation, and anaplerosis. Four enzymes can produce α-KG in human cells: IDH1, IDH2, IDH3, and glutamate dehydrogenase (GDH). Levels of α-KG have been shown to influence the catalytic activity of TET proteins. *IDH1* and *IDH2* genes are frequently mutated in human cancers. Mutated IDH1/2 protein exhibits lack of normal catalytic activity, which is responsible for α-KG production; however, the resulting mutated proteins also gain the ability to produce 2-HG. 2-HG is structurally similar to α-KG and can antagonize α-KG to competitively inhibit the enzymatic activity of α-KG-dependent dioxygenases, including both lysine histone demethylases and the TET family of DNA hydroxylases (Yang et al., 2012).

In 2008, the cancer genome project systematically sequenced 20,661 genes in 22 samples of human glioblastoma multiforme, and the *IDH* mutation (R132H) was first discovered (Parsons et al., 2008). Subsequently, mutations in *IDH1/2* have been detected in a variety of tumors, including glioma, astrocytoma, AML, cartilagonous tumor, thyroid carcinoma, cholangiocarcinoma, prostate cancer, B-cell ALL, colorectal carcinoma, and more.

Jin et al. (2011) used liquid chromatography/tandem mass spectrometry (LC/MS- MS) to assess the levels of 5-hmC and 5-mC in normal brain tissue and stage II and III astrocytoma and glioblastoma samples. The results showed that the levels of 5-hmC in normal human brain prefrontal cortex ranged from 0.82% to 1.18% that of dG. However, the levels of 5-hmC in brain tumors were strongly reduced compared with those in normal brain tissues; 5-hmC levels in astrocytomas were only 0.03%–0.04% that of dG. Additionally, according to these data, 5-mC levels were slightly reduced in some brain tumors. Therefore, the loss of 5-hmC is not simply due to reductions in 5-mC levels. They also analyzed levels of 5-hmC in tumors with the IDH^{R132H} mutation and in tumors without any *IDH* mutation. Surprisingly, 5-hmC levels in *IDH1*-mutant tumors were not lower than that in tumors with wild-type *IDH1*. These results were contradictory to those of a previous report demonstrating that 2-HG produced by mutated IDH inhibited the enzymatic activity of TET toward 5-hmC reduction. Moreover, tumors with *IDH1* mutations and no *IDH1* mutations exhibited no significant differences in 5-hmC levels. These data indicated that loss of 5-hmC may be due to enhanced cell proliferation in tumors, leading to passive demethylation.

Kraus et al. (2012) used immunohistochemistry and isotope-based liquid chromatography-mass spectrometry (LC-MS) to detect the presence and distribution of 5-hmC in normal brain tissue and brain tumors. The results revealed that both the number of 5-hmC positive cells and the amount of 5-hmC/dG in brain tumors were distinctly reduced compared with those in normal brain tissues. In addition, levels of 5-hmC correlated with tumor differentiation. Orr et al. (2012) also used immunohistochemistry to assess the distribution of 5-hmC in gliomas. The results showed that 5-hmC levels were high in low-grade tumors and decreased in malignant gliomas. In addition, 5-hmC levels were significantly correlated with reduced survival in malignant glioma. In silico analysis further supported these results, revealing the differential expression of genes involved in 5-hmC homeostasis in glioblastoma. Several genes involved in regulating 5-hmC levels are prognostic indicators in malignant glioma, and 5-hmC levels may play an important role in tumor differentiation and survival and may represent a potential novel therapeutic target.

5-hmC in other solid tumors

Lian et al. (2012) used genome-wide mapping of 5-hmC and found that loss of 5-hmC is an epigenetic hallmark of melanoma. Re-introducing active TET2 or IDH2 to rebuild the 5-hmC landscape in melanoma cells could inhibit melanoma growth and increase tumor-free survival in animal models. These results indicate that 5-hmC participates in melanoma development and that downregulation of TET and IDH2 is one of the mechanisms resulting in reduced 5-hmC levels.

In a separate analysis, Chen et al. (2013) developed an online trapping/capillary hydrophilic-interaction liquid chromatography (cHILIC)/in-source fragmentation/tandem mass spectrometry system to quantify 5-mC and 5-hmC in hepatocellular carcinoma tissues and adjacent normal tissues. The results showed that the 5-hmC content of hepatocellular carcinoma tissues was significantly reduced compared to that in adjacent normal tissues and that 5-hmC content was highly correlated with tumor stage. Thus, we can speculate that 5-hmC levels could be a potential biomarker for the early diagnosis and prognosis of hepatocellular carcinoma.

Conclusion

5-hmC acts as an intermediate product of DNA demethylation catalyzed by TET proteins. Reductions in 5-hmC are significantly correlated with tumorigenesis and tumor progression. According to the above argument, 5-hmC levels could be reduced through several mechanisms as follows. The structural abnormalities of *TET* family genes, including *TET2* mutations and the *MLL-TET1* translocation, influence the expression of *TET* genes and result in reduced enzymatic activity and expression of TET proteins. *IDH1/2* mutations not only reduce the amount of α-KG, but also produce 2-HG, which binds to and inhibits the

catalytic activity of TET proteins. Therefore, even if the expression of *TET* genes is normal, 5-hmC levels may be reduced. DNMT proteins functionally compete with TET proteins on DNA strands; thus, upregulation of *DNMT* may suppress the function of TET proteins. BER proteins and the AID/APOBEC family also mediate DNA demethylation, and increased expression of APEX1 may result in the reduction of 5-hmC in tumor cells. Enhanced proliferation leads to a passive 5-hmC reduction in tumor cells.

TET genes are frequently mutated in hematopoietic malignancies and significantly correlate with tumorigenesis and tumor development. Moreover, TET mutations may become genetic hallmarks for the early diagnosis and prognosis of tumors. As a new epigenetic modification, reduced 5-hmC is closely related to the occurrence and development of hematopoietic malignancies and solid tumors, suggesting that 5-hmC could be a valuable tumor marker for the early diagnosis of cancers. However, the mechanisms through which TET proteins and 5-hmC mediate their cancer-associated activities are still unknown, and the biological significance of 5-hmC loss in human tumors remains to be studied.

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