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Succinate dehydrogenase deficiency is associated with decreased 5-hydroxymethylcytosine production in gastrointestinal stromal tumors: implications for mechanisms of tumorigenesis

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Gastrointestinal stromal tumors (GISTs) usually harbor activating mutations in *KIT* or *PDGFRA*, which promote tumorigenesis through activation of growth factor receptor signaling pathways. Around 15% of GISTs in adults and >90% in children lack such mutations ('wild-type' GISTs). Most gastric wild-type GISTs show loss of function of the Krebs cycle enzyme complex succinate dehydrogenase (SDH). However, the mechanism by which SDH deficiency drives tumorigenesis is unclear. Loss of SDH leads to succinate accumulation, which is thought to inhibit α-ketoglutarate-dependent dioxygenase enzymes, such as the TET family of DNA hydroxylases. TET proteins catalyze the conversion of 5-methylcytosine to 5-hydroxymethylcytosine (5-hmC), which is required for subsequent DNA demethylation. Thus, TET-mediated 5-hmC production alters global DNA methylation patterns and may thereby influence gene expression. We investigated 5-hmC levels in a cohort of genotyped GISTs to determine whether loss of SDH was associated with inhibition of TET activity. 5-hmC levels were examined via immunohistochemistry in a cohort of 30 genotyped GISTs, including 10 SDH-deficient tumors (5 *SDHA* mutant; 1 *SDHB* mutant; 1 *SDHC* mutant; 3 unknown), 14 tumors with *KIT* mutations (10 in exon 11; 3 in exon 9; 1 in exon 17), and 6 tumors with *PDGFRA* mutations (all in exon 18). Staining for 5-hmC was negative in 9 of 10 (90%) SDH-deficient GISTs, 3 of 14 (21%) *KIT*-mutant GISTs, and 1 of 6 (17%) *PDGFRA*-mutant GISTs. The other SDH-deficient GIST showed weak staining for 5-hmC. Thus, 5-hmC was absent in nearly all SDH-deficient GISTs. These findings suggest that SDH deficiency may promote tumorigenesis through accumulation of succinate and inhibition of dioxygenase enzymes. Inhibition of TET activity may, in turn, alter global DNA methylation and gene expression in SDH-deficient tumors.
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Gastrointestinal stromal tumors (GISTs), the most common mesenchymal neoplasms of the gastrointestinal tract, are derived from interstitial cells of Cajal or their precursors. The majority of GISTs harbor activating mutations in the *KIT* (75–80%) or *PDGFRA* (10%) genes,[1,2] which drive tumorigenesis through activation of growth factor receptor signaling pathways. As such, these tumors are often effectively treated with the small molecule tyrosine kinase inhibitors imatinib and sunitinib.[3,4] In contrast, approximately 15% of GISTs in adults and >90% of GISTs in children are categorized as 'wild-type' GISTs because they lack *KIT* and *PDGFRA* mutations.[5–7] In recent years, a defined set of genetic changes in these so-called wild-type GISTs have begun to be characterized, including activating mutations in *BRAF*,[8–10] loss of function mutations in *NF1*,[11] and mutations leading to loss of function of the succinate dehydrogenase (SDH) enzyme complex.[12]

The SDH enzyme complex is localized to the inner mitochondrial membrane, where it functions both as complex II of the electron transport chain

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and as a member of the Krebs cycle, converting succinate to fumarate. The SDH complex is composed of four protein subunits (SDHA, SDHB, SDHC, and SDHD); mutations in any one subunit lead to destabilization of and subsequent loss of function of the complex. Mutations in all four SDH genes have been demonstrated in gastric 'wild-type' GISTs.[13–17] Recent studies have identified *SDHA* as the most commonly mutated subunit, with *SDHA* mutations found in approximately 30% of SDH-deficient GISTs.[18–21] However, around 40% of SDH-deficient GISTs lack demonstrable mutations in an SDH subunit gene.

SDH-deficient GISTs demonstrate distinct histological and clinical characteristics that differentiate them from *KIT*- or *PDGFRA*-mutant GISTs.[22] SDH-deficient GISTs arise exclusively in the stomach, demonstrate a multinodular architecture, and have a predominantly epithelioid morphology. In addition, SDH-deficient GISTs are often multifocal and frequently demonstrate lymph node metastases. Although many patients with such tumors develop peritoneal and liver metastases, these tumors tend to pursue a protracted, indolent clinical course. Finally, although SDH-deficient tumors express high levels of KIT by immunohistochemistry, they respond poorly to treatment with imatinib,[23] suggesting that alternative signaling pathways are driving tumor formation. Although the distinct clinicopathologic features of SDH-deficient GISTs have been well documented, the mechanism by which SDH-deficiency promotes oncogenesis remains unclear.

Several potential roles for SDH in tumorigenesis have been proposed. Loss of SDH activity leads to the accumulation of succinate within cells.[24,25] Because of structural similarities between succinate and α-ketoglutarate, succinate is thought to inhibit the activity of α-ketoglutarate-dependent dioxygenase enzymes.[26] In particular, recent work has demonstrated that the TET family of DNA hydroxylases may be inhibited by elevated levels of succinate.[26] TET proteins have been shown to have a role in the regulation of DNA methylation. Methylation of cytosine at the 5-position generates 5-methylcytosine, an important modification in the epigenetic regulation of gene expression. TET proteins convert 5-methylcytosine to 5-hydroxymethylcytosine (5-hmC), which is required for subsequent DNA demethylation.[27] TET proteins are thought to have a role in tumor development, as inhibition of TET activity, as well as alteration of global DNA methylation, has been demonstrated in multiple tumor types.[28–32] Thus, succinate accumulation in the context of SDH deficiency could potentially drive tumorigenesis via the inhibition of TET family proteins and subsequent changes in DNA methylation and gene expression patterns. Indeed, recent work has shown that siRNA-mediated knockdown of SDHA in cultured cells and in mice leads to an inhibition of TET activity and decreased levels of 5-hmC.[26] However, it remains unknown whether SDH deficiency leads to alterations in TET activity in human tumors. Therefore, in this study, we evaluated 5-hmC levels in a cohort of genotyped GISTs to determine whether loss of SDH is associated with inhibition of TET activity.

Materials and methods

The study group included 30 GISTs with known *KIT* and *PDGFRA* genotypes and known SDH status. Genotyping was performed as previously reported.[22] Immunohistochemistry was performed on 5-μm-thick formalin-fixed paraffin-embedded whole-tissue sections following incubation in 2 N HCl for 30 min, using a rabbit anti-5-hmC polyclonal antibody (1:10 000 dilution; 60 min incubation; Active motif, Carlsbad, CA, USA; catalog # 39769). The Envision Plus detection system (Dako, Carpinteria, CA, USA) was used as a secondary antibody. Nuclear staining for 5-hmC in tumor cells was compared with endothelial cells and tumor-infiltrating lymphocytes, which served as internal positive controls, and was scored as positive, weak (<50% intensity of controls), or negative.

Results

Of the 30 GISTs examined, 10 (33%) were SDH-deficient (5 *SDHA* mutant; 1 *SDHB* mutant; 1 *SDHC* mutant; 3 unknown mutation status), 14 (47%) carried *KIT* mutations (9 in exon 11; 3 in exon 9; 1 in exon 17; and 1 with mutations in both exon 11 and exon 17), and 6 (20%) carried *PDGFRA* mutations (all in exon 18). The results of the immunohistochemical analysis are summarized in Table 1. Nine of ten (90%) SDH-deficient GISTs demonstrated negative 5-hmC staining, whereas one (10%) SDH-deficient GIST showed weak staining for 5-hmC (Figure 1). Of the 14 *KIT*-mutant tumors, eight (57%) cases were positive for 5-hmC (Figure 2), whereas three (21%) demonstrated weak 5-hmC staining (all exon 9 mutants). Three (21%) *KIT*-mutant tumors were negative for 5-hmC,

Table 1 Comparison between classes of gastrointestinal stromal tumor and staining for 5-hydroxymethylcytosine by immunohistochemistry

Tumor class	*5-Hydroxymethylcytosine staining*		
	Positive (%)	*Weak (%)*	*Negative (%)*
SDH deficient ($n=10$)	0	1 (10)	9 (90)
KIT mutant ($n=14$)	8 (57)	3 (21)	3 (21)
PDGFRA mutant ($n=6$)	3 (50)	2 (33)	1 (17)

Abbreviation: SDH, succinate dehydrogenase.

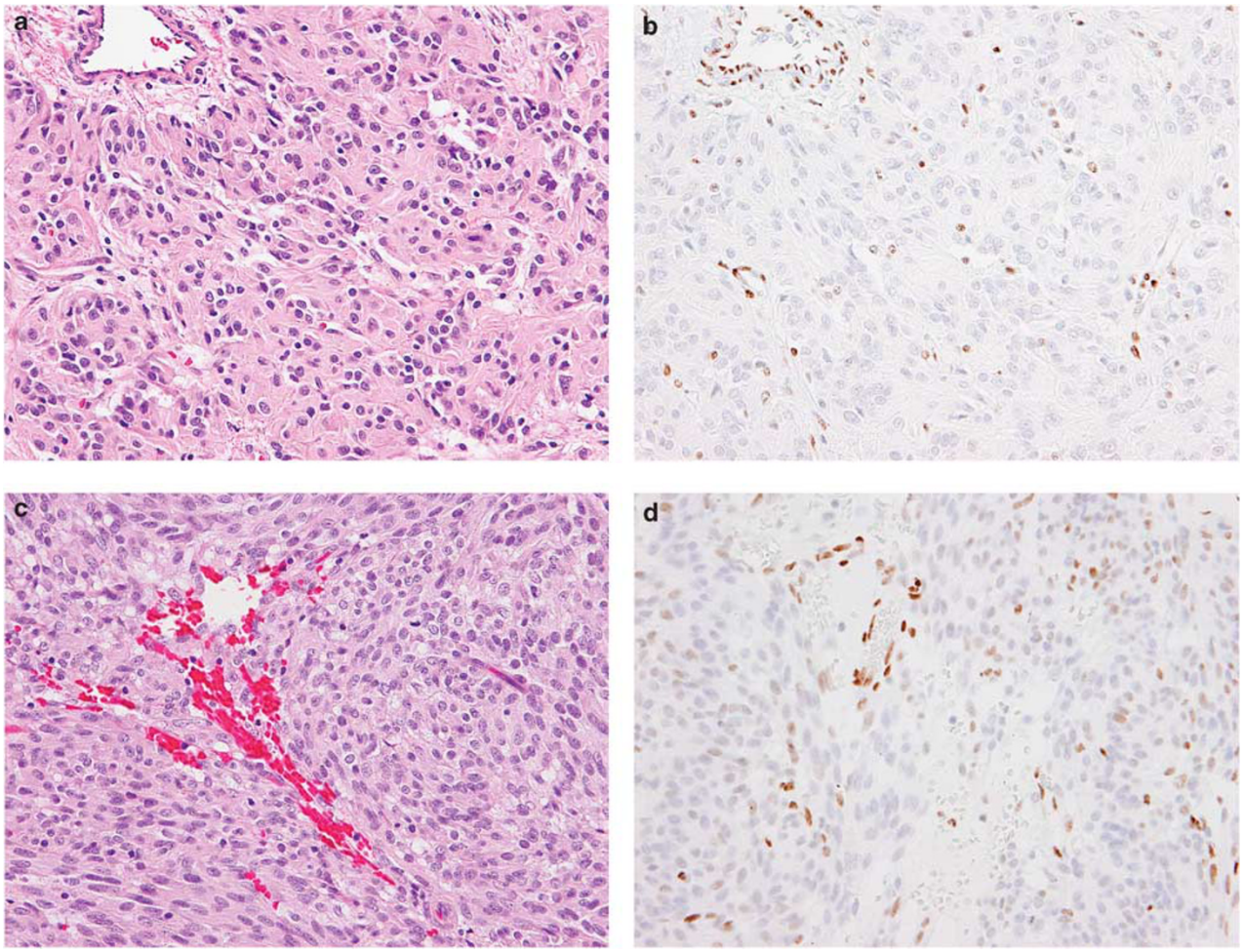

Figure 1 Immunohistochemistry for 5-hydroxymethylcytosine in succinate dehydrogenase (SDH)-deficient gastrointestinal stromal tumors (GISTs). (**a**) SDH-deficient GIST with *SDHA* mutation (**a**, H&E) showing loss of 5-hydroxymethylcytosine staining (**b**). SDH-deficient GIST (**c**, H&E) showing weak staining for 5-hydroxymethylcytosine (**d**). In both cases, note intact, strong nuclear 5-hydroxymethylcytosine staining in intratumoral endothelial cells and lymphocytes.

including two tumors with exon 11 mutations and one tumor with an exon 17 mutation. Three (50%) *PDGFRA*-mutant tumors demonstrated positive staining for 5-hmC (Figure 2), two (33%) demonstrated weak 5-hmC staining and one (17%) was negative for 5-hmC.

Discussion

Although the clinicopathologic features of SDH-deficient GISTs have been well characterized, it remains unclear how mutations in SDH subunit genes drive tumorigenesis. Recent *in vitro* work has demonstrated that the accumulation of succinate in SDH-deficient tumors may lead to the inhibition of α-ketoglutarate-dependent enzymes, including TET family proteins, which convert 5-methylcytosine to 5-hmC.[26] In the current study, we examined 5-hmC levels by immunohistochemistry in a cohort of 30 genotyped GISTs and demonstrate that 5-hmC staining is absent in 90% of SDH-deficient GISTs, whereas loss of 5-hmC staining is seen in approximately 20% of *KIT*- or *PDGFRA*-mutant GISTs. The alteration of 5-hmC content through inhibition of TET activity seen in the SDH-deficient GISTs demonstrates the dysregulation of epigenetic modifications in these tumors. Recent studies have highlighted the role of epigenetic changes in tumor formation. Mutations in a number of different epigenetic modifier proteins have been described in myeloid malignancies, including mutations in *TET2*, which are seen in 10–20% of cases of acute myeloid leukemia.[33] In addition, TET activity is thought to be inhibited in the context of mutations in isocitrate dehydrogenase 1 or 2 (IDH1/2), which are present in 15–30% of acute myeloid leukemias,[33] as well as in >70% of low-grade gliomas,[34] >50% of enchondromas and chondrosarcomas,[32,35] and approximately 25% of cholangiocarcinomas.[36] Our findings suggest that alterations in SDH should be added to the growing list of mutations that lead to altered epigenetic regulation within tumor cells.

SDH deficiency has been proposed to promote tumorigenesis in multiple ways. Mutations in

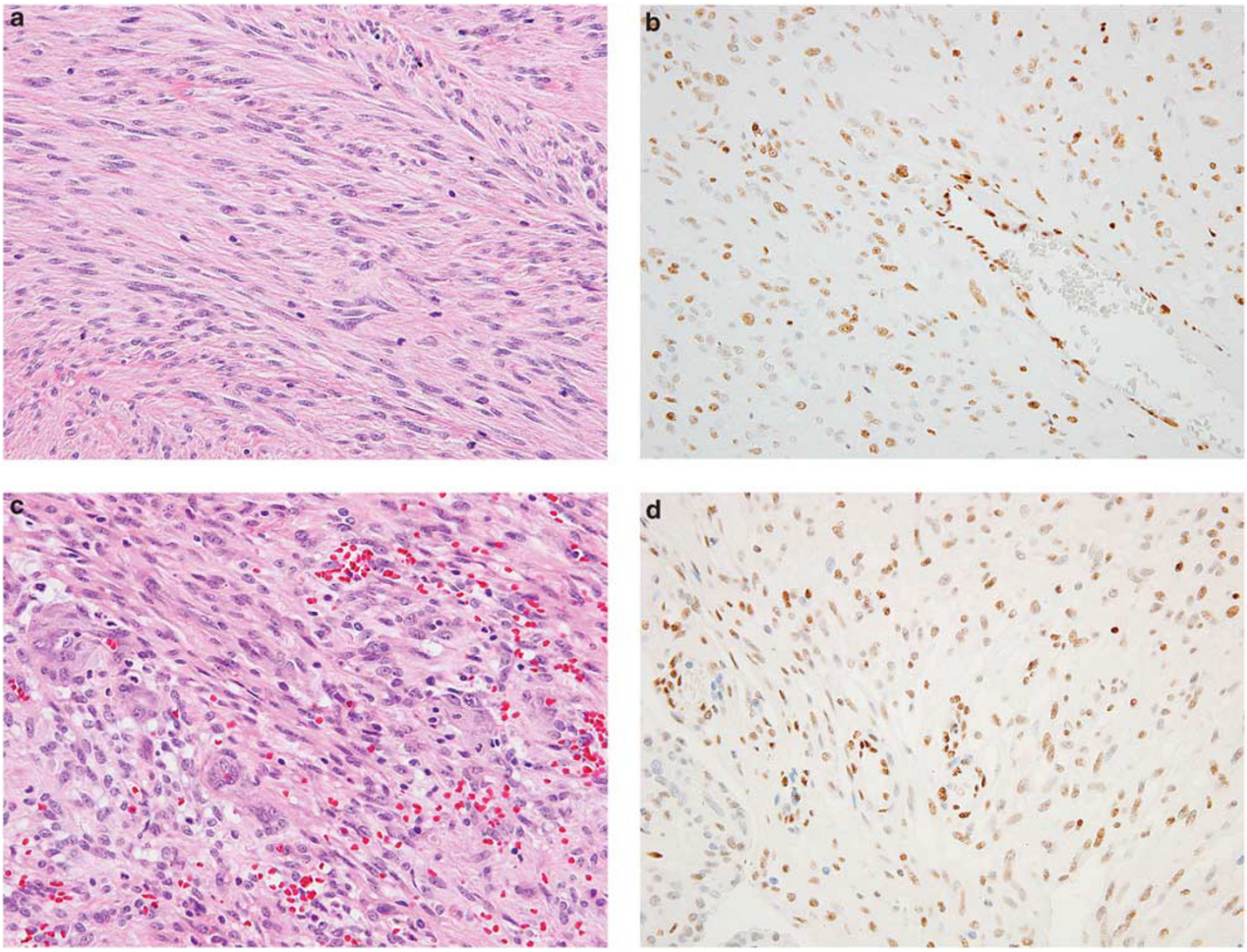

Figure 2 Immunohistochemistry for 5-hydroxymethylcytosine in conventional gastrointestinal stromal tumors (GISTs). *KIT* exon 11-mutant GIST (**a**, H&E) showing strong nuclear staining for 5-hydroxymethylcytosine (**b**). *PDGFRA* exon 18-mutant GIST (**c**, H&E) showing intact nuclear staining for 5-hydroxymethylcytosine (**d**).

SDH subunit genes may alter the mitochondrial (or intrinsic) apoptotic pathway[37] and are also thought to promote the production of reactive oxygen species, leading to DNA damage, genomic instability, and tumor formation.[38] Alternatively, elevated levels of succinate may inhibit the activity of other α-ketoglutarate-dependent dioxygenase enzymes, in addition to TET family proteins.[26] For example, the EgIN family of prolyl hydroxylase enzymes are α-ketoglutarate-dependent dioxygenase enzymes, which, in the presence of oxygen, hydroxylate the transcription factor HIF1α, leading to HIF1α degradation.[39] Thus, succinate accumulation may contribute to tumorigenesis through inhibition of prolyl hydroxylase and stabilization of HIF1α, which can, in turn, promote the expression of genes involved in glycolysis and angiogenesis.[25] α-Ketoglutarate is also used as a substrate by JmjC-domain containing histone demethylases.[40] Therefore, SDH deficiency may promote tumor formation by altering the methylation of both cytosine residues within DNA and histone proteins.

Because TET-mediated production of 5-hmC is required for DNA demethylation, loss of TET activity is expected to promote increased levels of DNA methylation. In support of this idea, studies have predominantly demonstrated hypermethylation signatures in both *TET2*- and *IDH*-mutant acute myeloid leukemias,[28] as well as in *IDH*-mutant gliomas[29] and enchondromas,[32] although other authors have reported conflicting results.[41] However, although inhibition of TET function appears to be associated with DNA hypermethylation, it remains unclear how these epigenetic changes regulate gene expression, as studies have, as of yet, failed to demonstrate direct links between changes in the promoter methylation status of specific genes and alterations in the expression levels of those genes. This may reflect the fact that although epigenetic changes can alter the accessibility of loci to transcriptional machinery, additional factors, including the availability of transcription factors, coactivators, and corepressors, also have a role in dictating changes in gene expression. Thus, further work is needed to

clarify precisely how epigenetic dysregulation is related to tumor formation, be it through changes in gene expression that regulate specific signaling pathways to drive oncogenesis or through alternative mechanisms.

The inhibition of TET activity in the context of SDH deficiency is thought to depend on the accumulation of succinate in SDH-deficient cells. Given the structural similarities between succinate and α-ketoglutarate, succinate is hypothesized to directly inhibit the binding of α-ketoglutarate to TET proteins and other α-ketoglutarate-dependent enzymes.[26] A similar mechanism is thought to underlie the tumor suppressor functions of fumarate hydratase (FH) and IDH1/2. FH is a Krebs cycle enzyme that converts fumarate to malate. Germline mutations in *FH* lead to hereditary leiomyomatosis and renal cell cancer and cause loss of function of FH and an accumulation of fumarate within cells.[24] IDH enzymes normally convert isocitrate to α-ketoglutarate. In contrast, tumor-associated IDH1/2 mutants lose the ability to produce α-ketoglutarate from isocitrate and also gain the neomorphic function of converting α-ketoglutarate to 2-hydroxyglutarate, which accumulates to high levels within IDH1/2 mutant cells.[42] Similar to succinate, both fumarate and 2-hydroxyglutarate share structural similarities with α-ketoglutarate, and both metabolites are thought to competitively inhibit α-ketoglutarate-dependent enzymes.[43] Hence, a common theme emerging from work examining cancer-associated mutations in genes encoding metabolic enzymes seems to be the generation of so-called 'oncometabolites' that may drive tumor formation through effects on post-translational and epigenetic modification. Given the role that these metabolites appear to have in oncogenesis, the targeting of metabolites and metabolic enzymes may represent a novel strategy for cancer therapy. In the context of SDH-deficient GISTs, which often fail to respond to treatment with tyrosine kinase inhibitors, a greater understanding of the molecular mechanisms underlying tumorigenesis could provide additional therapeutic targets, including metabolites themselves, such as succinate and α-ketoglutarate, or the proteins or cellular processes affected by these metabolic intermediates.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

Authors' note added in proof

Following acceptance of this manuscript, Killian *et al* reported similar findings (Killian *et al*. Succinate dehydrogenase mutation underlies global epigenomic divergence in gastrointestinal stromal tumor. *Cancer Discov* 2 April 2013 [e-pub ahead of print] PMID: 23550148). In that study, 16 of 24 SDH-deficient GISTs showed loss of 5-hydroxymethylcytosine by immunohistochemistry, compared to only one of 12 GISTs with *KIT* mutations. Moreover, the methylome signature of *KIT*-mutant GISTs closely resembled normal tissues, whereas SDH-deficient GISTs showed marked genomic hypermethylation. A similar phenomenon was observed for *SDH*-mutant paragangliomas. This study further implicates succinate accumulation and TET inhibition in failure of DNA demethylation as a mechanism for tumorigenesis of SDH-deficient GIST.

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