Biochemical and Biophysical Research Communications 455 (2014) 16–23

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Review

The role of mutation of metabolism-related genes in genomic hypermethylation

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ARTICLE INFO

Article history:
Received 2 June 2014
Available online 8 August 2014

Keywords:
DNA methylation
Metabolism
Cancer
IDH
SDH
FH

ABSTRACT

Genetic mutations, metabolic dysfunction, and epigenetic misregulation are commonly considered to play distinct roles in tumor development and maintenance. However, intimate relationships between these mechanisms are now emerging. In particular, mutations in genes for the core metabolic enzymes IDH, SDH, and FH are significant drivers of diverse tumor types. In each case, the resultant accumulation of particular metabolites inhibits TET enzymes responsible for oxidizing 5-methylcytosine, leading to pervasive DNA hypermethylation.

Contents

1. Introduction

Pioneering work in the first half of the twentieth century established many of the core steps in human metabolism including the tricarboxcylic (TCA) or Krebs cycle through which sugars, amino acids, and fatty acids are broken down to produce chemical energy and molecular building blocks [1]. In the 1950s Otto Warburg discovered that cancer cells have a characteristic type of metabolism, with a high rate of glycolysis despite elevated oxygen levels, and argued that this metabolic shift (the "Warburg effect") is in fact the central cause of cancer [2]. While the relative contributions to tumorigenesis of metabolism, genetic mutation, and epigenetic misregulation have sometimes been debated, new findings in multiple tumor types have shown the three to be intimately related. Certain malignancies arising in hematopoietic, mesenchymal, neural, as well as epithelial tissues share the common quality that mutations in genes coding for core metabolic proteins drive the

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http://dx.doi.org/10.1016/j.bbrc.2014.08.003
0006-291X/

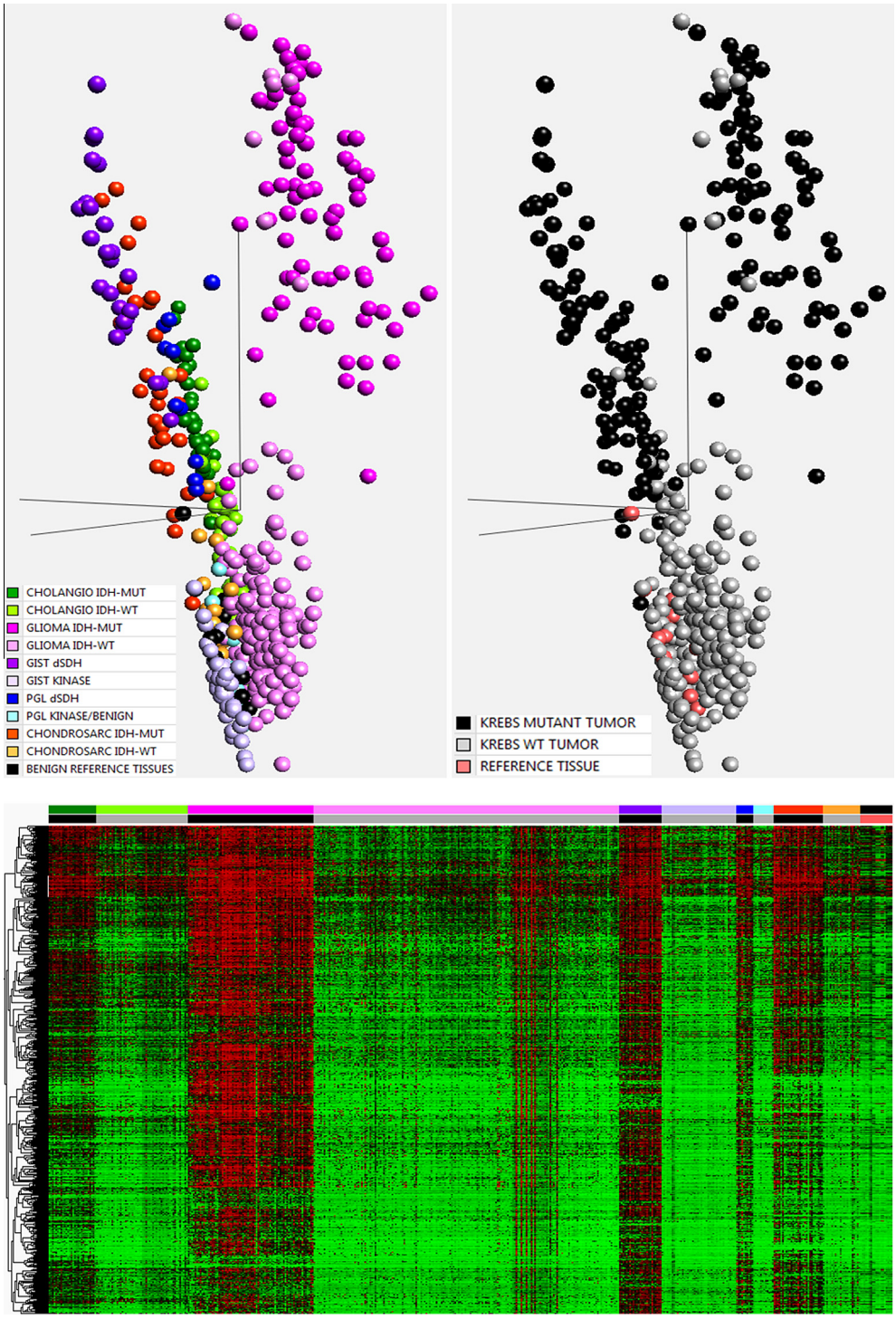

Fig. 1. Epigenomic relatedness of *IDH*/*SDH*/*FH* mutant- versus non-mutant- tumors, as shown by PCA plots and heatmap of DNA methylation profiles. Samples included here are: (1) *IDH*-mutant versus -wildtype cholangiocarcinoma (GSE49656, $n = 32$; GSE32286, $n = 50$), *IDH*-mutant versus -wildtype glioma (GSE36278, $n = 136$; GSE48461, $n = 56$; GSE32286, $n = 62$) and *IDH*-mutant versus -wildtype chondrosarcoma (GSE40853, $n = 51$); (2) *SDHx*- versus kinase-mutant GIST (GSE34387, $n = 69$) and *SDHx*- versus kinase-mutant paraganglioma/pheochromocytoma (GSE43293, $n = 22$); and (3) multiple normal associated tissue lineages ($n = 19$). (Sample color legends shown in PCA plots also correspond to heatmap colorbars.) Variables included here are the CpG methylation β-values, as measured by Infinium 450 K array, of the top 10 K differentially-methylated CpG targets between *IDH*/*SDH*/*FH* mutant- and non-mutant tumor groups (statistical calculations and graphics performed with Qlucore Omics Explorer software). In general, *IDH*/*SDH*/*FH* mutant tumors of diverse histological types and embryonic lineages show significantly greater global DNA hypermethylation than non-mutant counterparts.

accumulation of DNA hypermethylation (Fig. 1). While much remains to be learned about these phenomena, the recurrent reconciliation of these distinct pathways across such diverse tumor types provides new insights into oncogenic mechanisms and tumor biology.

2. Discovery of IDH mutations

One of the most unanticipated discoveries of the early genome wide cancer mutation profiling efforts was the report of recurrent somatic mutations in the *IDH1* gene. In 2008 and 2009 recurrent

hotspot mutations at codon 132 of *IDH1* were identified in glioblastoma multiforme (GBM) [3] and acute myeloid leukemia (AML) [4], respectively. *IDH1* encodes isocitrate dehydrogenase 1, an enzyme that functions as a dimer, localizes to the cytoplasm and peroxisomes, and that couples the oxidative decarboxylation of isocitrate to alpha-ketoglutarate (αKG) with the reduction of NADP+ to NADPH. Additional studies quickly identified somatic mutations in the analagous position (R172) of *IDH2* [5], a mitochondrially localized homologue of *IDH1*. Based on the crystal structure and biochemistry of the enzymes it was known that these highly conserved arginines contact substrate within the active site and that the mutated proteins had drastically lower enzymatic activity. The conversion of isocitrate to αKG is a core component in cellular metabolism of glucose, fatty acids, and glutamine (in the mitochondria it is an essential step in the Krebs cycle) and this led initial hypotheses to focus on a possible tumor suppressor mechanism for *IDH1/2* in tumor metabolism [3].

3. IDH mutants produce 2-hydroxyglutarate

The genetic evidence did not fit a simple tumor suppressor model however because the tumors retained one wild type copy of the gene and other loss of function mutations such as deletions, frameshifts, and nonsense mutations were not observed. The apparent discrepancy between the pattern of genetic mutations and the enzymatic activity was resolved with the demonstration that these mutations in fact confer neomorphic enzymatic activity [6]. In this study metabolic profiling could not identify any mutant *IDH1*-dependent changes in the abundance of standard Krebs cycle intermediates and instead revealed the rapid accumulation of a novel metabolite, 2-hydroxyglutarate (2-HG). Detailed biochemical assays showed that in fact the preferred reaction for multiple *IDH1* R132 mutants is the NADPH-dependent conversion of αKG to the D enantiomer of 2-hydroxyglutarate (D2-HG). The model this suggests is that the tumor must retain a wild type copy of *IDH1* to catalyze the standard reaction generating NADPH and αKG. The mutated enzyme, possibly in the context of a WT/mutant heterodimer, then carries out the pseudo-reverse reaction to generate D2-HG. The initial cell culture results extended to human glioma samples comparing *IDH1* wild type gliomas to those with a variety of *IDH1* R132 mutations, demonstrating both the lack of any consistent difference in abundance of standard Krebs cycle intermediates and an increase of at least two orders of magnitude in the abundance of D2-HG.

Accumulation of D2-HG also occurs in human AML samples where it was shown to be produced by both the *IDH1* R132 mutants as well as the analogous *IDH2* R172 mutants [7]. By sequencing *IDH* mutations in AML samples with high 2-HG accumulation Ward et al. were also able to discover *IDH2* R140 as a third hotpot that shares the neomorphic activity [7]. Mutations at the analogous arginine in *IDH1*, R100, are also found in some gliomas, albeit at extremely low frequency [8–10]. These two pairs of analogous arginines, which reside in the active site of the enzymes and participate in isocitrate binding [11], account for the vast majority of all IDH mutations identified to date. These mutations reduce the enzyme's affinity for isocitrate while stabilizing the interaction with NADPH and αKG [6]. The majority of *IDH1* R100, R132 and *IDH2* R140 mutations are C > T transitions in CpG dinucleotides resulting in IDH1 R100Q, R132C, R132H, or IDH2 R140Q but other substitutions also occur. The most common *IDH2* R172 mutation is also a C > T transition encoding IDH2 R172K but this is not in the context of a CpG site. While IDH1 and IDH2 both use NADP+ as an electron acceptor, a third isocitrate dehydrogenase, IDH3, uses NAD+ instead and is structurally, functionally, and mechanistically distinct from IDH1 and IDH2. While wild-type IDH1 and IDH2 are capable of catalyzing the "reverse" reaction, converting αKG to isocitrate under certain conditions [12,13], IDH3 appears to be only capable of catalyzing the "forward" reaction [14]. Somatic mutations in *IDH3* have not been reported to date. Interestingly, patients with germline IDH3 deficiencies manifest symptoms only in the retina [15]. In all other tissues studied the predominant isocitrate dehydrogenase activity is associated with the NADP+ dependent IDH1 and IDH2 enzymes [15].

The accumulation of 2-HG established a link to a group of rare neurometabolic disorders characterized by high levels of 2-HG in the body. A number of cellular enzymes produce the L and D enantiomers of 2-HG that are then converted to αKG by L-2-hydroxyglutarate dehydrogenase (L2HGDH) and D-2-hydroxyglutarate dehydrogenase (D2HGDH), respectively. The recessive disorder L-2-hydroxyglutaric aciduria (L2HGA) is caused by loss of both copies of *L2HGDH* and leads to accumulation of specifically the L chirality of 2-HG [16,17] while D-2-hydroxyglutaric aciduria (D2HGA) is characterized by the accumulation of the D chirality and in roughly 50% of cases is due to loss of both copies of *D2HGDH* [18,19]. Germline deficiency in the mitochondrial citrate transporter SLC25A1 causes accumulation of both enantiomers and combined L2- and D2-HGA [20]. Amongst other phenotypes L2HGA patients have a clear predisposition to tumors of the central nervous system [21]. The fact that germline deficiency of D2HGDH does not exhibit such a tumor predisposition is not completely understood at this point but is likely related to quantitative differences in compound accumulation [22] as well as enantiomer specific effects on enzyme targets [23–25]. Following the demonstration that *IDH* mutations from glioma and AML generate D2-HG, it was shown that patients with D2HGA type II, the form of the disease without D2HGDH deficiency, harbored germline *IDH2* R140 mutations [26].

4. IDH mutant tumors have genomic hypermethylation

The first connection of *IDH* mutations with DNA methylation patterns also came from the glioma field [27]. By integrating multiple molecular characterizations of a large set of glioma samples the authors were able to identify a set of samples that shared a characteristic DNA methylation profile dominated by hypermethylation of certain loci that they termed glioma CpG island methylator phenotype, G-CIMP. This G-CIMP set of tumors was enriched for the pro-neural subtype of glioma, tended to occur in younger patients, was associated with a better prognosis, had a characteristic profile of copy number changes, and was tightly associated with *IDH* mutations (Fig. 1). While the association between G-CIMP and *IDH* mutations was identified, and the global nature of the hypermethylation was presciently taken to suggest deficiency of a trans-acting factor normally required to protect CpG islands from invading DNA methylation, the mechanism connecting *IDH* mutation and G-CIMP remained unknown [27].

Extending the connection between DNA hypermethylation and *IDH* mutations, Figueroa et al. performed a similar integrative analysis of AML patient samples [28]. They demonstrated that a subset of AML tumors were also characterized by a global hypermethylation phenotype and that this was strongly associated with *IDH1* and *IDH2* mutations (Fig. 1). *IDH1* and *IDH2* mutations were mutually exclusive and no differences in the associated methylation patterns could be observed between them. The strikingly higher degree of DNA methylation associated with *IDH* mutations was true in comparison not only to WT *IDH* AML samples but also to normal bone marrow. Furthermore, introduction of either mutated gene into 293T cells was sufficient to confer a hypermethylation phenotype, suggesting a dominant role for the *IDH* mutations in setting the hypermethylation.

5. Inhibition of αKG-dependent dioxygenases

The human genome encodes more than sixty αKG-dependent dioxygenases, a superfamily of enzymes that use Fe(II) to couple the oxidation of a substrate with the conversion of αKG to succinate and carbon dioxide, involved in such processes as collagen maturation, oxygen sensing, epigenetic regulation, and fatty acid metabolism [29,30]. The structural similarity between αKG and D2-HG raised the possibility that this "oncometabolite" [6] could inhibit this vast array of enzymes [29] and thus affect numerous cellular processes. It has subsequently been shown biochemically that both D2-HG and L2-HG inhibit members of this enzyme family with a range of potencies [23,30,31]. In fact it had already been suggested that *IDH*-mutated gliomas showed activation of the HIF-1 pathway and higher levels of HIF-1α [32], a transcription factor regulated both at the level of protein stability and co-activator recruitment by αKG dependent prolyl hydroxylases [33]. Whether *IDH* mutations are sufficient for this effect however has been questioned [34].

By further mining the pattern of somatic mutations Figueroa et al. discovered that *IDH1/2* mutations were mutually exclusive with mutations in the gene *TET2* [28]. *TET2* and its closely related family member *TET1* were already known to be mutated in AML: *TET2* in roughly 15% of patients by diverse loss of function mutations and *TET1* by rare t(10;11)(q22;q23) translocations generating fusions with *MLL*. The gene family, which also includes *TET3*, had been discovered by mapping the MLL fusion partner, giving rise to the nomenclature TET for Ten-Eleven Translocation [35]. Excitingly, the TET family members had recently been demonstrated to be αKG-dependent dioxygenases that convert 5-methyl cytosine (5mC) to 5-hydroxymethylcytosine (5hmC), leading to DNA demethylation [36,37]. This led to the hypothesis that the TET enzymes would be inhibited by 2HG, which the authors confirmed genetically [28]. The mutant *IDH* associated hypermethylation phenotype is thus understood to be the result of the inhibition of a DNA demethylation pathway involving the conversion of 5mC to 5hmC (Fig. 2). The biochemical evidence thus shows that one consequence of the *IDH* hotspot mutations and consequent 2HG accumulation is inhibition of TET enzymes and the genetic evidence is that, at least in AML, this axis is selected upon and contributes to the malignancy.

6. IDH mutations in cancer

As larger patient series with detailed clinical data have been profiled it has become clear that amongst gliomas *IDH* mutations occur frequently in grade II/III astrocytomas, oligodendrogliomas, and oligoastrocytomas as well as the secondary glioblastomas to which these may progress (70–90%) but are rare in primary glioblastomas (7%) or pediatric tumors (11%) [38]. Furthermore the *IDH* mutations are associated with younger age as well as improved survival [39]. The proneural phenotype of *IDH*-mutated glioma is thought to reflect an epigenetic block to differentiation established by inhibition of not only TET enzymes but also αKG-dependent histone lysine demethylases containing the Jumonji-C enzymatic domain [23,31,40–43]. In addition to direct inhibition of these various epigenetic modifying enzymes, the complex network of interactions between DNA methylation and various histone methylation marks undoubtedly contributes to the epigenetic profile of these tumors.

IDH mutations have now been shown to occur in approximately 15% of unselected AML with higher frequencies in certain subclasses [44] as well as in roughly 5% of myelodysplastic syndromes (MDS) and myeloproliferative neoplasms (MPN), both of which can progress to AML [45–48]. Similarly, loss of function *TET2* mutations are found not only in AML but in these precursor conditions [49,50], suggesting that they are amongst the earliest steps in transformation and at least partial overlap in the oncogenic function of these mutations through establishment of a DNA hypermethylation phenotype. As in glioma, *IDH* mutations are rare in pediatric AML compared to the adult form of the disease [51–53]. In hematologic malignancies mutations in the *IDH* and *TET* genes are not restricted to the myeloid lineage [54]. In particular, they are commonly mutated in angioimmunoblastic T cell lymphoma (AITL) [55,56]. While DNA methylation profiling has

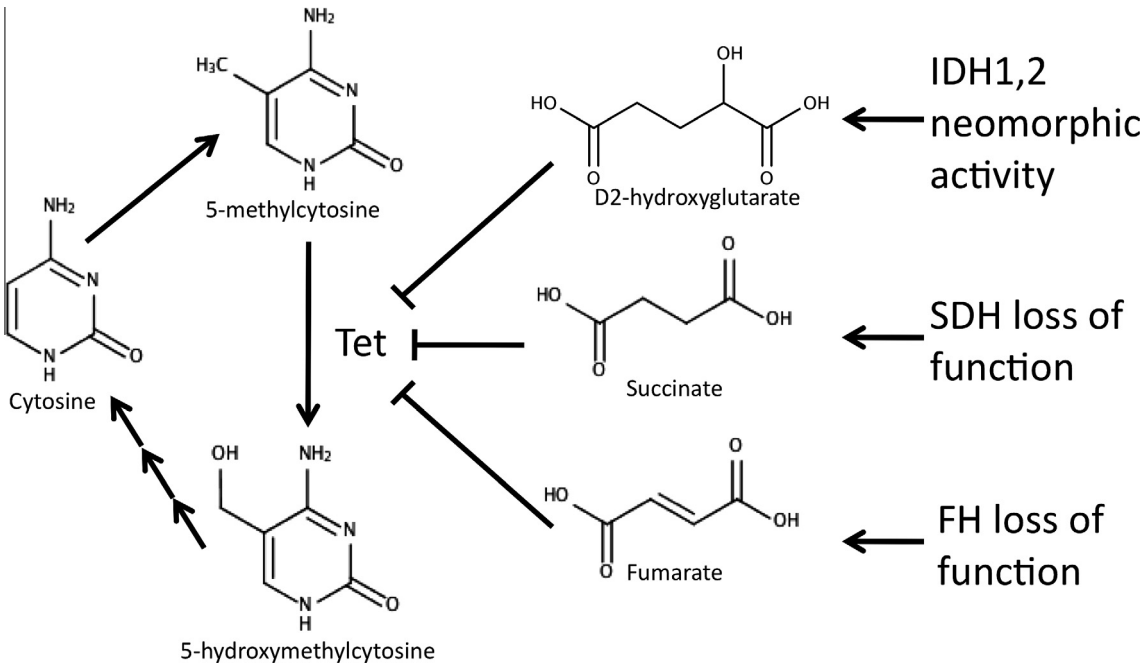

Fig. 2. Model linking metabolism-related genes and DNA hypermethylation. Mutations in metabolism-related genes *IDH1,2*, *SDHx*, or *FH* lead to accumulation of metabolites D2-hydroxyglutarate, succinate, or fumarate, respectively, which in turn inhibit hydroxylation of 5-methylcytosine by TET enzymes.

not yet been reported in AITL, there is no reason to assume these mutations are not associated with a hypermethylated genome.

Ollier disease and Maffucci syndrome are rare, typically non-familial, conditions characterized by the development of multiple cartilage producing tumors called enchondromas (Maffucci syndrome also entails the development of multiple spindle cell hemangiomas) which also carry a risk for glioma and AML [57–59]. This constellation of tumor predispositions was explained by the discovery that these patients are mosaic for *IDH* mutations [60,61]. In a large survey it was further shown that *IDH* mutations occur not only in the chondromas from these syndromes but are also very common in sporadic chondromas and chondrosarcoma, (56%) but were not observed in other mesenchymal tumor types [62]. Molecular profiling confirmed that, as in glioma and AML, chondrosarcomas with mutant *IDH* specifically accumulate 2-HG and display DNA hypermethylation (Fig. 1) [63,64]. Retroviral transduction of mutant *IDH2*, but not WT *IDH2* or vector control, into 10T1/2 murine embryonic cells was sufficient to induce DNA hypermethylation, block differentiation into multiple mesenchymal lineages, and generate undifferentiated sarcomas in a xenograft setting [63].

Cholangiocarcinomas are a rare tumor of the epithelial lining of the bile duct. While *IDH* mutations are very uncommon in most types of gastrointestinal carcinomas, they occur in roughly 15% of cholangiocarcinomas located in the intra-hepatic region [65,66]. IDH mutations in cholangiocarcinomas are associated with accumulation of 2-HG as well as increases in DNA and histone methylation (presumably through inhibition of TET and JumonjiC enzyme families, respectively) and the hypermethylated regions of the genome show extensive overlap with those that occur in *IDH*-mutated gliomas (Fig. 1) [65,67].

7. SDH

The succinate dehydrogenase (SDH) complex resides in the inner mitochondrial membrane and serves as a core component of both the Krebs cycle and the electron transport chain coupling the conversion of succinate to fumarate with the reduction of ubiquinone. SDH is composed of four core subunits, SDHA, SDHB, SDHC, and SDHD and is served by two assembly factors, SDHAF1 and SDHAF2, all of which are encoded by the nuclear genome. The active enzyme complex also contains several other chemical groups including flavin adenine dinucleotide (FAD), Fe–S clusters, ubiquinone, and haem b [68].

8. SDH mutations in cancer

Paragangliomas are neuro-ectodermal chromaffin cell tumors that are typically benign and highly vascularized. Linkage analysis on several families with hereditary paraganglioma syndrome showed *SDHD* to be a causative gene, the first tumor predisposition syndrome associated with a nuclear encoded mitochondrial enzyme [69]. Tumors of the chromaffin cells in the adrenal medulla are known as pheochromocytomas and it was quickly established that *SDHD* mutations contribute to sporadic pheochromocytoma development [70]. Consistent with their function as a complex, mutations in *SDHA*, *SDHB*, *SDHC*, as well as the assembly factor *SDHAF2* can also drive these types of tumors [71–76] by destabilizing the complex and causing loss of enzymatic activity [77–80]. Germline mutations in *SDH* genes are also associated with predisposition to gastrointestinal stromal tumors (GIST) [81,82], a mesenchymal malignancy thought to originate from the interstitial cells of Cajal (ICC) that effect gut peristalsis [83]. Patients with deleterious germline *SDH* variants may also present with both paraganglioma and GIST [84–86]. Germline *SDH* mutations can lead to aggressive renal cell carcinomas, either in combination with hereditary paraganglioma [87–89] or without [90]. Finally, mitochondrial dysfunction due to germline deleterious variants in SDH components are also a cause of nonmalignant neonatal and childhood conditions such as the severe neurological disorder Leigh syndrome, certain childhood leukodystrophies, and cardiomyopathy [91–95].

9. SDH mutant tumors have genomic hypermethylation

It was recognized early on that oxygen sensing and the hypoxic response played a major role driving tumor development downstream of SDH deficiency [69,96,97]. The mechanistic basis for this came with the demonstration that the loss of SDH activity leads to accumulation of its substrate, succinate, which in turn can exit the mitochondria. The first targets identified for the accumulated cytoplasmic succinate were the prolyl hydroxylase (PHD) enzymes that regulate stability of the HIF1-α transcription factor [98]. PHDs are αKG dependent enzymes that produce succinate through their catalytic reaction and are therefore sensitive to product inhibition by succinate. All of the αKG dependent dioxygenases produce succinate and thus are susceptible to this mechanism of inhibition, including the TET enzymes that convert DNA 5mC to 5hmC [30] (Fig. 2). Inhibition of TET activity by succinate has been verified in cell culture as well as in mouse models, where inhibition of SDH either through knockdown or expression of mutations from human patients resulted in DNA hypermethylation [99]. Extending the *in vitro* and model system work which showed that it is possible for SDH deficiency to lead to DNA hypermethylation, it has also been shown to occur in patient tumors, in SDH deficient paraganglioma, pheochromocytoma and GIST (Fig. 1) [100,101].

10. FH mutations in cancer

A leiomyoma is a benign smooth muscle tumor and predisposition to multiple leiomyomata, primarily of the skin and uterus, can be inherited in an autosomal dominant manner. They are also associated with predisposition to type II papillary renal cell carcinoma in a condition known as hereditary leiomyomatosis and renal cell carcinoma (HLRCC) [102,103]. Fumarate hydratase, *FH*, was first identified as the HLRCC gene through linkage analysis in large kindreds [104] and quickly verified in other large collections [105]. Recently germline loss of function mutations in *FH* have also been shown to predispose to pheochromocytomas and paragangliomas [100,106]. In all of these conditions *FH* functions as a tumor suppressor: one allele has a germ line loss of function mutation and a somatic second hit removes the WT copy [104]. The autosomal recessive metabolic disorder fumarate hydratase deficiency is caused by loss of function mutations in both alleles of FH and is so severe that most individuals do not survive long enough to develop neoplasia. Fumarate hydratase follows succinate dehydrogenase in the Krebs cycle, converting fumarate to L-malate. Complete FH deficiency leads to fumarate accumulation and, similar to succinate and 2-HG, high levels of fumarate are capable of inhibiting numerous αKG dependent dioxygenases [107] (Fig. 2). This includes the TET enzymes and both knockdown of FH as well as expression of *FH* mutations from HLRCC patients in the mouse has been shown to drive DNA hypermethylation [99]. While DNA methylation profiling in FH-deficient renal cell carcinomas and leiomyomata has not yet been published to date, one patient with an FH-deficient paraganglioma shows hypermethylation by array profiling [100], and immunohistochemistry in multiple FH deficient paragangliomas and pheochromocytomas show low 5hmC levels [106].

11. Conclusion

The gain-of-function nature of the *IDH* mutations [6,7], along with evidence that they continue to be necessary for tumor maintenance [25,108], creates a natural target for therapeutic development that has already begun to be exploited [109–111]. Although not directly targetable, loss of SDH and FH do afford clinical opportunities such as synthetic lethal interactions that are also being developed. Of particular relevance to this review, the DNA methyltransferase inhibitors 5-azacytidine and decitabine are under preclinical investigation [100,112]. These inhibitors are already clinically approved for MDS, however the link between degree of genomic hypermethylation and clinical drug response is not straightforward [113].

The direct connections between genetic mutations, core cellular metabolism, and misregulation of epigenetic states outlined above were completely unexpected just a few years ago. Mutations in the *IDH*, *SDH*, and *FH* genes are undoubtedly pleiotropic but the pervasive, common effect on DNA methylation across such diverse tumor types is striking (Fig. 1). While some efforts have been made to identify particularly significant target genes misregulated by DNA methylation, the broader model of "locking in" a progenitor state and instituting a block to terminal differentiation has been applied to at least some of the tumor types [43,63]. In addition to providing insights into the mechanism of tumor development, knowledge of mutations of *IDH*, *SDH*, and *FH* in particular patient's tumors can have diagnostic and prognostic relevance for the patient as well as genetic counseling implications for relatives. While DNA hypermethylation is emerging as a cohesive feature of *IDH*, *SDH*, and *FH* mutated tumors, the converse is not true. In particular, CIMP was first reported in colorectal cancer [114] and while much has been learned about this phenomenon the underlying cause is still unclear [115]. Similarly many other tumor types have genomic hypermethylation-positive subsets, and while recurrent *IDH*, *SDH*, *FH* or *TET* mutations do not appear to be the cause [116], it will be interesting to see if mitochondrial or metabolic pathways still play a role.

Acknowledgments

This work was supported by the Intramural Research Program of the US NIH, NCI, CCR. Marvin 14.7.14.0, 2014, ChemAxon (http://www.chemaxon.com) was used for drawing the chemical structures in Fig. 2.

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