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Attenuation of genome-wide 5-methylcytosine level is an epigenetic feature of cutaneous malignant melanomas

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Abstract

Epigenetic modification of DNA, namely covalent changes of cytosine residues, plays a key role in the maintenance of inactive chromatin regions, both in health and in disease. In the vast majority of malignant melanomas, the most notable known epigenetic abnormality is the attenuation of 5-hydroxymethylcytosine (5-hmC) residues. However, it remains unknown whether a decrease in 5-hmC represents a primary defect of melanoma cancer epigenome or whether it is secondary to the loss of 5-methylcytosine (5-mC), a chemical substrate for 5-hmC. Here, we evaluated 5-mC levels in a spectrum of melanocytic proliferations. To study the epigenetic features of melanocytic nuclei, we began by measuring 5-mC levels in histologic specimens semiquantitatively by immunohistochemistry. We next treated established melanoma cell lines with S-adenosyl methionine (SAM), a universal methyl group donor, in an effort to cause changes in 5-mC levels. We detected a marked reduction in 5-mC levels in both primary and metastatic melanomas compared with 5-mC levels in benign melanocytic nevi. We also empirically induced changes in 5-mC in melanoma cell lines by incubation with SAM. To our surprise, we observed a significant cytoreductive effect of SAM on all melanoma cell lines examined. At subcytotoxic levels, SAM treatment is accompanied by a genome-wide increase in 5-mC. Moreover, we recorded a dose-dependent increase in genome-wide 5-mC levels in melanoma cell lines following SAM treatment. Taken together, we report that genome-wide attenuation of 5-mC is a hallmark of malignant melanomas. We propose that genome-wide attenuation of 5-mC is not merely an epiphenomenon

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Conflicts of interest

There are no conflicts of interest.

as it is required for melanoma cell growth, albeit by an as of yet undetermined mechanism. Given its potential benefit in slowing down the growth of melanoma cells, SAM should be studied further to determine its role in epigenome modulation.

Keywords

cancer epigenetics; 5-methylcytosine; S-adenosyl methionine

Introduction

Chromatin, or the organization of primary nuclear genome, is a heterogeneous and complex aggregate composed of RNA, protein, and DNA. Although a lot is known about the helical structure of DNA, comparably less is known about higher-level DNA packaging [1]. We know that some DNA encodes active genes, whereas other DNA encoding inactive genes are tightly packaged into what is termed heterochromatin. In normal cells, the organization of heterochromatin is maintained through cell division, by covalent modification of histones, as well as DNA [2]. These heritable modifications to the state of the chromatin are called epigenetic modifications because they are not born out of the change in the DNA sequence of the primary nuclear genome [2].

The most well-studied DNA modification is DNA methylation. It signifies covalent addition of a methyl group to the C5 position of the cytosine residue. In humans, this modification is catalyzed by three DNA methyltransferase enzymes (DNMT1, DNMT3A, or DNMT3B) and requires S-adenosyl methionine (SAM) as the methyl group donor in methyltransferase reactions. Together with a range of covalent modifications of histones, DNA methylation occurs through the genome and is believed to play a central role in the maintenance of constitutively inactivated, heterochromatic genomic regions. Traditionally, the most well-studied epigenetic modification, 5-methylcytosine (5-mC), is likely central to the chromatin organization as loss of 5-mC in murine models leads to arrest in embryonic development [3–5]. Conversely, conversion of 5-mC back into unmodified cytosine entails a serial oxidation reaction from 5-mC to 5-hydroxymethylcytosine (5-hmC) to 5-formylcytosine (5-fC) to 5-carboxylcytosine (5-caC). 5-caC is then converted into unmodified cytosine by thymine DNA glycosylase or terminal deoxynucleotidyl transferase [6]. The exact function of 5-hmC/5-fC/5-caC is not entirely elucidated at present because although some mice with genome-wide 5-mC loss are embryonically lethal, mice lacking 5-hmC are viable with abnormal imprinting and reduced fertility as the only observable phenotypes [7].

Since its discovery in 1983 by Feinberg and Vogelstein [8], global loss of DNA methylation is known to be a feature of many carcinomas, including carcinomas of the gastrointestinal tract, liver, and gynecologic origin [9–11]. However, loss of 5-mC has only been considered to be slightly decreased in malignant melanomas [12,13]. Thus far, the research focus has been on loss of 5-hmC as a major epigenetic abnormality in the majority of malignant melanomas [13]. In a seminal study by Lian *et al.* [13], loss of 5-hmC was linked to both less favorable pathologic staging and adverse survival in a collection of malignant melanomas. Because the reported loss of 5-hmC was not accompanied by a comparable

increase in 5-mC [13], we reasoned that 5-mC may be the primary epigenetic defect in melanomas. Here, we report that attenuation of 5-mC is indeed readily detectable in the vast majority of malignant melanomas and that partial reconstitution of 5-mC levels by SAM inhibits the growth of established melanoma cell lines.

Methods

Case selection

The histopathologic subtypes and pathologic stages from the cohort have been described previously [14]. Following application and review of the present study, we obtained the NCI melanoma cohort from the NCI Cancer Diagnosis Program (<http://chtn.sites.virginia.edu/tissue-microarrays#CDPMelprog>).

Immunolabeling and 5-methylcytosine assessment

Immunohistochemical and immunofluorescence protocols were performed as reported previously [15,16]. Briefly, paraffin tissue sections were dewaxed and rehydrated following standard tissue processing. Heat-induced antigen retrieval was performed by steaming slides in citrate buffer (pH 6.0) for 30 min and subsequent incubation in 3.5 N HCl for 15 min at room temperature. Slides were washed and equilibrated in Tris-buffered saline with 0.05% Tween 20 (Sigma-Aldrich, St Louis, Missouri, USA) for 10 min. For 5-mC detection, slides were incubated with mouse monoclonal-specific antibody (Calbiochem; EMD Chemicals Inc., San Diego, California, USA) at a 1: 2000 working dilution for 1 h at room temperature. Immunocomplexes were detected with 3,3'-diaminobenzidine tetrahydrochloride as the chromogen using the PowerVision+ immunohistochemistry detection system (ImmunoVision Technologies Co., Norwell, Massachusetts, USA). After immunohistochemical staining, tissue sections were counterstained with hematoxylin. For immunofluorescence analysis, slides were pretreated as outlined above and incubated with mouse 5-mC specific antibody (Calbiochem; EMD Chemicals Inc.) at a 1: 500 working dilution. Immunocomplexes were further labeled with secondary antibodies conjugated with Alexa 568 dye (Invitrogen, Grand Island, New York, USA) and DNA was counterstained with 4',6-diamidino-2-phenylindole (DAPI). Slides were then visualized using a Nikon E400 fluorescence microscope (Nikon Instruments, Melville, New York, USA). To quantify 5-mC levels, representative photomicrographs of 5-mC and DAPI colabeled slides were analyzed using the Telometer software application (Johns Hopkins University, Baltimore, Maryland, USA) [17]. The signal intensities of individual lesional cell nuclei were determined. To account for differences in the overall DNA content, 5-mC signal intensities were normalized to DAPI intensities. For every evaluable tissue microarray (TMA) core, 40 lesional nuclei were counted to ensure uniform results. Each case was scored by two trained pathologists (N.R. and J.M.T.). The 5-mC intensity was scored as none (0), weak (1), moderate (2), or marked (3). Specifically, 0=no immunolabeling; 1=less intense than immunolabeling of in adjacent benign cells (e.g. normal keratinocyte or lymphocyte nuclei in cutaneous or metastatic cores, respectively); 2=comparable with normal nuclei; and 3=more intense than normal nuclei. An immunohistochemical 5-mC staining score was developed, ranging from 0 to 3. This score represents the percentage of cells expressing 5-

mC×intensity of staining/maximum possible value. The maximum possible value was 3, that is, 100% of melanocyte nuclei showing marked (grade 3) staining intensity.

Cell culture

Melanoma cell lines were obtained from the Yale SPORE (gift from Dr Halaban), are part of the Yale University melanoma cell line collection, and have been described previously [18]. They were originally isolated from melanoma specimens collected with participants' informed consent according to Health Insurance Portability and Accountability Act regulations with the Human Investigative Committee protocol at Yale University. Human melanoma cell lines were grown at 37°C and 5% CO₂, in Opti-MEM (GIBCO, Grand Island, New York, USA) media supplemented with heat-inactivated fetal bovine serum (Sigma-Aldrich) and penicillin–streptomycin (GIBCO), as described previously [19]. For 5-aza-2-deoxycytidine (5-aza-dC) treatment, growth medium was supplemented with 10 µmol/l 5-aza-dC (Sigma-Aldrich) every 24 h over a 72 h period. For SAM treatment, growth medium was supplemented with 10, 25, 50, or 100 µmol/l SAM (New England Biolabs, Ipswich, Massachusetts, USA) for up to 72 h.

DNA extraction and methylation-sensitive digestion

Genomic DNA was isolated from treated and untreated established human melanoma cell lines using the Blood and Tissue kit (Qiagen, Redwood City, California, USA) according to the manufacturer's specifications. Extracted genomic DNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Grand Island, New York, USA). Two aliquots of 1 µg each of DNA were separately digested with HpaII or MspI restriction enzymes (New England Biolabs) overnight at 37°C. Reactions were stopped by incubation at 65°C for 15 min. Reactions were separated on a 2% Agarose (Invitrogen) gel. Gels were imaged using a ChemiDoc MP (Biorad, Hercules, California, USA) and semiquantified using ImageJ Software (National Institutes of Health, Bethesda, Maryland, USA).

Cell viability assays

Reconstituted CellTiter-Glo (Promega, Madison, Wisconsin, USA) reagent was added to 96-well optical bottom black plates (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) containing 3×10^3 cells in 100 µl of Opti-Mem (GIBCO) supplemented with 10% fetal bovine serum (Sigma-Aldrich) according to Promega's instructions. Assay plates were sealed and shaken on a plate shaker for 2 min at 400 rpm and incubated for 10 min. Luminescence was then recorded on either a SpectraMaxL (Molecular Devices, Sunnyvale, California, USA) or a SynergyMX (Biotek, Winooski, Vermont, USA) luminometer. Cell growth was determined by comparing assay signals of treated cells with the control conditions of untreated cells (defining 0% growth inhibition and 100% of normalized value). Three replicates were performed for each condition and the mean of the signal was used to determine proliferation at each time point.

Flow cytometry-based apoptosis assay

For flow-based apoptosis measurement, supernatant media were first carefully aspirated after 72 h following either 5-aza-dC or SAM treatment. Then, adherent cells were

trypsinized and added to the supernatant. Pellets were stained using the BD Pharmingen Apoptosis Detection Kit II according to the manufacturer's specifications (BD Biosciences, Franklin Lakes, New Jersey, USA). Each specimen was analyzed separately using the BD LSRII flow cytometer to at least 10 000 events per specimen. Each melanoma cell line was treated independently and gates were fixed on the basis of negative control signals. Plots were generated using FlowJo 9.6.2 (FlowJo LLC, Ashland, Oregon, USA).

Global DNA methylation 5-methylcytosine quantification

Global DNA methylation 5-mC levels of cultured melanoma cell lines were quantified colorimetrically using commercially available kits. Briefly, DNA isolation was performed using the FitAmp General Tissue Section DNA Isolation Kit (EpiGentek Group, Farmingdale, New York, USA) according to the user guide protocol. Next, input DNA samples were applied onto a 96-well microplate and processed according to the user guide protocol of the MethylFlash Methylated DNA Quantification Kit (EpiGentek Group). Microplates were then loaded onto a SpectraMax M3 Multi-Mode Microplate Reader (VWR International, Radnor, Pennsylvania, USA) to measure absorbance at 450 nm. The average OD450 of samples were determined and used for 5-mC calculation.

Focus formation assay

Melanoma cell lines were trypsinized, counted using a Countess II FL Automated Cell Counter (Thermo Fisher Scientific Inc.), and diluted into 0.5% UltraPure Agarose (Thermo Fisher Scientific Inc.) in cell culture media. Cells were plated at 500 cells per well and allowed to grow for 24 h before SAM treatment. Cell culture media were changed every 72 h. Following cell growth for 14 days, cells were fixed in ice-cold methanol (Thermo Fisher Scientific Inc.) for 10 min and stained with a 0.5% crystal violet (Sigma-Aldrich) solution prepared in 25% methanol for 5 min at room temperature. An estimated value for transformation efficiency was determined by counting the number of foci obtained.

Statistical analysis

5-mC by immunolabeling was treated both as a qualitative variable during pathological evaluation by a trained pathologist (N.R.) and as a semiquantitative variable on an ordinal scale (N.R.). The immunofluorescence signal was evaluated as a quantitative variable on an interval scale. All experiments, apart from the MethylFlash-based experiment, were conducted in triplicate. The MethylFlash-based experiment was performed in quadruplicate. The statistical significance of all variables examined was reached when the two-sided Student's *t*-test reached *P*-value less than 0.05.

Results

Attenuated global nuclear 5-methylcytosine levels in cutaneous malignant melanomas

To investigate global 5-mC levels in melanocytic proliferations, we carried out a 5-mC immunohistochemical analysis on clinical samples from an independent patient cohort. The cohort included 31 benign acquired melanocytic nevi, 35 primary malignant melanomas, and 62 metastatic malignant melanomas.

Careful microscopic evaluation of the cohort showed that lesional nuclei within benign melanocytic nevi immuno-labeled strongly and uniformly with 5-mC (Fig. 1a–c). In comparison, lesional nuclei from both primary malignant melanomas and metastatic malignant melanomas were largely devoid of 5-mC immunolabeling (Fig. 1d–i). We observed decreased 5-mC immunolabeling in two available malignant melanomas *in situ* (Fig. 1d–f), suggesting that 5-mC attenuation is an epigenetic abnormality that occurs early during melanomagenesis. On average, the 5-mC signal detected in benign melanocytic nevi was 10-fold higher than that in malignant melanoma samples (Fig. 2, $P < 0.001$). The mean 5-mC score in benign melanocytic nevi was 1.19 ± 0.53 , whereas the mean 5-mC score in malignant melanomas was 0.09 ± 0.13 . Taken together, the results support the interpretation that the epigenome of malignant melanoma is comparably DNA hypomethylated relative to that of benign melanocytic nevi.

S-adenosyl methionine induces DNA methylation in cultured melanoma cells

A genome-wide decrease in 5-mC has been detected in previous studies using human carcinoma cultures. Two of the studies suggest that culture of carcinoma cells in the presence of SAM, a common cosubstrate involved in methyl group transfers, leads to a rapid and global increase in global 5-mC levels [20,21]. Conversely, culture of murine somatic cells with 5-aza-dC, a DNA hypomethylating agent, is known to induce a decrease in global 5-mC levels [22]. To test these two hypotheses on melanoma cells, we incubated a cultured established melanoma cell line, YUSIT1, in the presence of either a solvent carrier (water), 10 $\mu\text{mol/l}$ 5-aza-dC, or 100 $\mu\text{mol/l}$ SAM. Following 48 h of treatment, cells were collected and procured for a quantitative immunofluorescence analysis of global 5-mC levels. Relative to solvent-treated melanoma cells, we detected a slight, but statistically significant, decremented decrease in genome-wide 5-mC levels following 5-aza-dC and an increase in global 5-mC levels following SAM treatment (Fig. 3). Specifically, when the results were normalized to 1 in solvent only control, the mean nuclear fluorescence and SD of the 5-aza-dC treated group were 0.86 ± 0.17 , and 1.94 ± 0.23 in the SAM-treated group. This experiment suggests that the global decrease in 5-mC is not a static epigenetic abnormality, but a dynamic feature of the melanoma cell epigenome, one that can be affected by incubation with readily available DNA methylation drugs.

To confirm our observation that SAM induces DNA methylation in cultured melanoma cells, we quantified global 5-mC levels by adapting a traditional method that utilizes a combination of methylation-sensitive (HpaII) and methylation-insensitive (MspI) restriction enzymes [23]. Following overnight incubation of YUSIT1 melanoma cell line-derived DNA with restriction enzymes, we again detected an increase in global 5-mC levels following 48 h of SAM treatment (Fig. 4a and b). To establish a dose-dependent relationship, we repeated the experiment in the presence of varying final concentrations of SAM. We detected a decremented increase in global 5-mC levels by differing levels of exposure to a 72 h SAM treatment (Fig. 4c and d). We detected a strong positive correlation between global 5-mC levels and SAM exposure ($r = 0.949$, $P = 0.0079$) (Fig. 4d). Using another technique, a spectrophotometry-based quantitative method termed the MethylFlash assay, we also detected an increase in global 5-mC levels following 72 h of SAM treatment (Fig. 4e). Thus,

the attenuation of 5-mC in malignant melanoma-derived cultured cells can be partially restored following treatment with SAM.

S-adenosyl methionine treatment induces cell death in some cultured melanoma cells

Serendipitously, we observed a marked cytotoxic effect of SAM on YUSIT1 melanoma cells. To establish that this observation is not an idiosyncratic phenomenon potentially unique to a single established melanoma cell line, we tested all readily available melanoma cell lines in our laboratory for sensitivity to SAM. All five melanoma cell lines examined showed comparable sensitivity to SAM. Following a 72 h treatment with 100 $\mu\text{mol/l}$ SAM, we detected readily discernable cytotoxicity, measured as decreased number of viable cells, in YUSIT1, YULAC, YUGEN8, 501mel, and WM1366 melanoma cell lines (Fig. 5a). However, increased cell apoptosis was only detected in YUSIT1, YULAC, YUGEN8, and mel501 melanoma cell lines (Fig. 5b). Although there was a tendency toward increased cell death of the WM1366 melanoma cell line following of SAM treatment, the finding did not reach statistical significance (Fig. 5b, last panel). To further investigate these findings, we performed colony formation assays on the YUSIT1 melanoma cell line treated with different concentrations of SAM ranging from 0 to 100 $\mu\text{mol/l}$. We observed a significant decrease in the transforming potential of the YUSIT melanoma cell line following SAM treatment (Fig. 5c). Because cytostatic effects changed when the concentration of SAM was changed, we conclude that SAM effects are dose dependent (Fig. 5d). Therefore, SAM shows a broad-spectrum cytostatic activity in a panel of human melanoma cell lines.

Discussion

In this report, we make two novel observations on the epigenetic features of cutaneous malignant melanomas. First, we note a global DNA hypomethylation in melanoma clinical specimens, observed as a significant decrease in nuclear 5-mC staining of malignant melanoma compared with benign nevi. Second, we observed that 5-mC levels could be increased in cultured melanoma cells by treatment with SAM. Interestingly, treatment with SAM resulted in decreased proliferation and viability of all five melanoma cell lines studied. Therefore, our findings suggest that epigenomes of malignant melanomas are DNA hypomethylated relative to benign nevi and that DNA hypomethylation may play important roles in melanoma cell proliferations.

In mammals, DNA methylation almost exclusively occurs in the CpG dinucleotide context [24]. In turn, most CpG dinucleotides are either scattered through the genome in so-called, intergenic regions, or situated near one another in regions known as CpG islands. Importantly, most methylated CpG dinucleotides are located in intergenic regions in the genome, which are devoid of protein-coding genes. Although the functional role of these CpG dinucleotides is relatively poorly understood, their putative role may be inactivation of endogenous repetitive DNA, mostly retrotransposons [25]. In contrast, CpG islands are usually not methylated and are associated with promoters of genes. DNA methylation in promoter-associated CpG islands plays well-appreciated roles in silencing protein-coding gene transcription by inhibiting transcriptional initiation [26].

Back in 1983, Feinberg and Vogelstein [8] were the first to empirically observe that global DNA methylation levels are reduced in cultured colorectal carcinoma cells. Subsequent studies firmly corroborated the initial observation that the global 5-mC level is decreased in carcinoma cells relative to benign counterparts [27,28]. However, the research focus changed, in part because of the availability of newly developed DNA sequencing techniques, to studies of methylated moieties within the context of CpG dinucleotides within individual promoter regions, that is, CpG islands. What emerged has been termed an ‘epigenetic paradox’, whereby global cancer 5-mC levels are decreased, but individual CpG dinucleotides, particularly those situated within the CpG islands of tumor-suppressor genes, are hypermethylated. Silencing of tumor-suppressor genes by focal hypermethylation has been observed in many cancers, including silencing of *CDKN2A*, *VHL*, *RBI*, and *BRCA1* genes [29–32]. Therefore, although methylations in a CpG island of a tumor-suppressor genes are well-described epigenetic abnormalities to date, global 5-mC decrease remained a relatively understudied epigenetic feature of human cancer.

Change came as an antibody to 5-mC became available, allowing for a global 5-mC evaluation of clinical specimens. Global DNA hypomethylation has been recorded to be a characteristic of human carcinomas, including those of gastrointestinal, breast, liver, lung, prostatic, keratinocytic, and astrocytic origin [33–38]. One notable feature is that this epigenetic abnormality is observed in the vast majority of neoplasms examined. For instance, the epigenomes of roughly 96% of prostate cancers are hypomethylated, as well as 100% of germ cell carcinomas *in situ*, and 100% of gastric carcinomas [34,39,40]. Therefore, although the level of 5-mC decrease in carcinomas varies, the vast majority of carcinomas are devoid, in large part, of global 5-mC moieties. In the present study, we make a similar observation following the evaluation of a melanoma cohort.

The cause for global DNA methylation decrease in human cancers has not been firmly established to date. Broadly, both hypoxia and vitamin C excess have been independently evoked as putative physiologic causes of global DNA hypomethylation. The proposed molecular link between hypoxia and DNA hypomethylation is activation of HIF- α , which has been observed in hepatocellular carcinomas [41]. Both culture of hepatocellular carcinoma cells under hypoxic conditions and increasing HIF- α levels correlated with global DNA hypomethylation [41]. Similarly, a recent study of murine embryonic stem cells showed that vitamin C treatment induces global DNA hypomethylation and a blastocyst-like state [42]. The molecular link for global DNA hypomethylation following vitamin C treatment is enhanced activity of TET1 and TET2 enzymes, both of which catalyze the conversion of 5-mC into another heritable epigenetic modification, 5-hmC [42]. It is noteworthy that loss of *TET1* and *TET2*, in double knockout embryonic stem cells, completely blocked the effect of vitamin C on DNA methylation. To date, two mechanistic studies provide evidence for a role for DNMT 3B loss and UHRF1 overexpression in the establishment of global DNA methylation decrease in human cancers. First, it has been observed that loss of DNMT3A activity leads to global DNA methylation decrease in murine models of lung carcinomas [43,44]. Finally, increased expression of UHRF1, a protein that regulates DNA methyltransferases activity, leads to global DNA methylation in murine models of hepatocellular carcinomas [45].

In turn, ramifications of global DNA hypomethylation in cancer are also only partially understood. Major breakthroughs came with a series of elegant empirical observations by the Jaenisch group, who uncovered that global DNA hypomethylation causes both cancer and aneuploidy [46,47]. Specifically, mice with low levels of Dnmt1 enzyme developed sarcomas and lymphomas depending on the presence or absence of accompanying mutations of the *tp53* gene, respectively [46,47]. Finally, global DNA hypomethylation might also contribute toward increased mutational rates by an as of yet unelucidated mechanism [48]. It has also been suggested that global DNA hypomethylation may play a key role in genome instability [48].

Although global DNA hypomethylation in melanoma has not been studied in depth previously, hypomethylation of CpG islands within the gene promoters has been described in a group of melanoma specific antigens, such as *NY-ESO-1*, *MAGE*, *BAGE*, *GAGE*, and cancer testis genes [49]. It is noteworthy that *MAGE* genes have been shown to directly contribute toward malignant growth and there have been attempts to exploit the antigenicity for immunotherapies [50].

Unlike changes in 5-mC, the attenuation of 5-hmC has been well documented in human cutaneous malignant melanomas. Our findings are in partial agreement with the seminal study by Lian *et al.* [13] that described loss of 5-hmC as a common feature of malignant melanomas. These investigators also noted that partial restoration of 5-hmC by TET overexpression suppresses tumor growth in xenografts [13]. It is noteworthy that the authors reported a slight, but nonreproducible, decrease in global 5-mC levels in malignant melanomas [13]. This latter feature is suggestive that 5-hmC attenuation is not a primary epigenetic abnormality because it is not accompanied by an expected increase in 5-mC levels. A subsequent study by Gamblicher *et al.* [12] also recorded that the loss of TET2 protein is a common occurrence in malignant melanomas. Therefore, although previous studies suggest that loss of 5-hmC is a primary epigenetic abnormality in malignant melanomas, we strongly favor that loss of 5-mC, a biochemical substrate for 5-hmC formation, is the cause of 5-hmC loss.

It is also of interest to understand whether more globally hypomethylated malignant melanomas behave more aggressively, or are more indolent, from their less hypomethylated counterparts. Because of previous work by Lien and colleagues, which shows that less hydroxymethylated malignant melanomas are more aggressive, we favor that more globally hypomethylated malignant melanomas are also more aggressive. Such putative future studies are best suited for analysis using well-annotated TMAs and quantitative immunofluorescence assays, such as the automated quantitative analysis assay [51].

We reason that not only are 5-mC and 5-hmC decreased in malignant melanomas but also the other two iterative oxidation derivatives: 5-fC, and 5-caC. As alluded to in the introduction, 5-hmC arises by TET-mediated oxidation of 5-mC residues. TET enzymes also mediate further oxidation of 5-hmC to 5-fC and eventually 5-caC. Conversion in the opposite direction has not been described, that is, reduction of 5-fmC to 5-hmC and 5-hmC to 5-mC is not believed to occur. We hypothesize that in the future, the combined use of

antibodies against, 5-mC/5-hmC/5-fmC/5-caC, may be of use in the diagnosis and/or the prognosis of malignant melanoma.

Our second major finding is that SAM, a global methyl group donor, causes an increase in 5-mC and cytotoxicity in cultured melanoma cells. Upon donating the methyl group, SAM becomes S-adenosyl-homocysteine, which competes with SAM for the cofactor binding pocket in DNMTs, and is thus a competitive inhibitor of DNA methylation. Excess SAM has been shown to inhibit 5-aza-dC-mediated demethylation [52]. A current model accounting for this observation is that exogenous administration of SAM increases the intracellular ratio of SAM to S-adenosyl-homocysteine, thus stimulating DNMT activity, resulting in increased DNA methylation. In melanoma cell lines, SAM treatment has been shown to inhibit 5-aza-mediated demethylation and cause methylation of all cytosines in the promoter region of MAP2, a highly methylated promoter in metastatic melanoma [53].

We treated five low-passage established human melanoma cell lines with SAM and measured the amount of global DNA methylation. Consistent with reports in the literature [21,54], we observed a dose-dependent increase in DNA methylation by immunofluorescence, methylation-specific endonuclease digestion, and colorimetric ELISA assay. Furthermore, decreased proliferation of melanoma cells was associated with a significant reduction in proliferation in all five cell lines examined at 72 h. These results support our novel hypothesis that DNA hypomethylation is intrinsically required for melanoma survival. Our findings are consistent with studies in osteosarcoma cell lines, where SAM treatment blocked cell proliferation and invasion *in vitro* and tumor metastasis *in vivo* [54]. Additional previous studies show that SAM is cytotoxic to carcinoma cells because in-vitro culture with SAM inhibited the growth of human gastric and colon cancer cells [21]. It is interesting that culture with SAM did not inhibit the growth of normal liver nor neural cells, which supports the notion that SAM targets an epigenetic abnormality that selectively incurred in transformed cells [21,55]. To our knowledge, this is the first report to suggest that induction of DNA methylation decreases the viability of melanoma cells.

At first glance, our findings may seem in contrast to the well-described toxicity of acutely depleting DNA methylation in cancer. Acute depletion of de-novo methyltransferases, by knockdown or 5-aza-dC treatment, has also been shown to decrease cancer cell viability [56]. However, combined treatment with the methyl donor SAM and the DNA methylation inhibitor 5-aza-dC has been shown to have synergistic anti-breast cancer effects [52]. The mechanism proposed for this effect is that SAM inhibits global hypomethylation induced by 5-aza-dC, preventing activation of prometastatic genes *uPA* and *MMP2*, while augmenting the growth-inhibitory effects of 5-aza-dC and its effects on tumor-suppressor genes [52]. This combinatorial approach against both hallmark changes in DNA methylation (global DNA hypomethylation and focal CpG island hypermethylation) may be a fruitful strategy for epigenetic therapy. To evaluate the therapeutic potential in melanoma, we are currently expanding our studies to assess the in-vivo effect of SAM, 5-aza-dC, and combined treatment in melanoma grafts *in vivo*.

Importantly, an alternative hypothesis is that SAM melanoma growth-inhibitory effect is not related to an increase in 5-mC. We accordingly note that SAM contributes methyl groups to

other macromolecules, such as proteins and lipids [57]. Specifically, methyl transfer from SAM has been documented in more than 40 metabolic reactions [57]. We, therefore, cannot formally exclude the possibility that the cytotoxic effect of SAM on melanoma cells is not directly related to global 5-mC increase.

Yet another alternative hypothesis is that the cytotoxic effect of SAM is not even related to methyl group transfer, but rather associated with polyamine biosynthesis. SAM can be decarboxylated by adenosylmethionine decarboxylase to form S-adenosylmethioninamine. Subsequently, S-adenosylmethioninamine donates the n-propylamine group to the production of polyamines, such as spermidine and spermine, from putrescine. Because reduced putrescine and increased spermine are important for neoplastic cellular growth, the treatment of cancer cells with SAM is expected to increase tumor cell growth. To our knowledge, SAM treatment has not been reported to be associated with increased neoplastic cell growth; rather, SAM treatment slows down the growth of colorectal and hepatocellular carcinoma cells [21]. In addition, in transgenic mice with SAM decarboxylase overexpression, there is a concomitant increase in SAM and an accompanying inhibition of mouse skin keratinocytic tumor growth [57]. We therefore reason that the inhibitory effect of SAM in melanoma cells may not be related to polyamine biosynthesis.

Although we report that SAM causes global 5-mC increase in culture melanoma cells, we provide no additional molecular evidence for a more precise mechanism of this epigenetic change. Previous mouse-based studies of colon and hepatocellular carcinomagenesis provide a putative link for the tumor-inhibitory effect of SAM. In the study by Li *et al.* [20], SAM inhibited colon cancer proliferation by inhibiting CTNNB1, NF- κ B activation, and IL-6 mediated signaling. In a similar study by Li *et al.* [58], SAM inhibited the growth of hepatocellular carcinoma by attenuation of WNT/CTNNB1 signaling. Therefore, we hypothesize that the inhibitory effect of SAM in melanoma may be mediated in a similar manner and that inflammation-induced signaling plays a critical role in the establishment of global 5-mC hypomethylation in melanoma.

Conclusion

We find that global DNA hypomethylation occurs nearly universally in cutaneous melanomas. Previous work has focused on identifying differentially methylated genes between nevi and melanoma [59], but a direct comparison of global levels of methylation has been inconclusive thus far. A global decrease of 5-hmC has also been reported more recently in melanoma with important functional consequences on melanoma growth and progression [13]. Our results indicate a notable decrease in 5-mC in melanoma relative to nevi and suggest a potential functional role of DNA hypomethylation in melanoma cell survival, meriting further investigation to increase our understanding of the mechanism, kinetics, and functional consequences of DNA hypomethylation in melanoma. In the future, we plan to evaluate 5-mC levels on additional TMAs that are well annotated for clinicopathologic variables such as survival and pathological staging. As another step in the preclinical evaluation of SAM, we also plan to evaluate the tumor growth-inhibitory effect of SAM in murine xenograft melanoma models.

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Fig. 1.

Notable qualitative decrease of 5-mC in cutaneous malignant melanoma nuclei as evaluated by immunohistochemistry. (a) Representative H&E photomicrograph showing benign acquired intradermal melanocytic nevus. (b) Nevus immunolabeled with the 5-mC biomarker. (c) High-power photomicrograph showing strong nuclear expression in the vast majority of lesional nevi nuclei. (d) Representative H&E photomicrograph showing malignant melanoma *in situ*. (e) Malignant melanoma *in situ* immunolabeled with the 5-mC biomarker. (f) High-power photomicrograph showing focal attenuation of 5-mC expression in most lesional malignant melanoma *in situ* nuclei. (g) Representative H&E photomicrograph showing metastatic malignant melanoma. (h) Metastatic malignant melanoma immunolabeled with the 5-mC biomarker. (i) High-power photomicrograph showing attenuation of 5-mC expression in most lesional metastatic malignant melanoma nuclei. a, b, d, e, g, and h, $\times 100$; c, f, and i, $\times 400$. a, b, d, e, g, and h, the black bar denotes 250 μm ; c, f, and i, the black bar denotes 60 μm . H&E, hematoxylin and eosin; 5-mC, 5-methylcytosine.

Fig. 2.

Semiquantitative decrease of 5-mC in a cohort of malignant melanomas as measured by immunohistochemistry. The mean 5-mC scores in 128 melanocytic lesions (31 benign acquired melanocytic nevi and 97 malignant melanomas). Error bar, SD; * $P < 0.05$, statistical significance by two-sided Student's t -test. 5-mC, 5-methylcytosine.

Fig. 3.

5-mC level in the YUSIT1 cultured melanoma cell line can be modulated using 5-aza-dC and SAM. (a) Left panel showing YUSIT1 treated for 48 h either with solvent (water) only; middle panel showing 5-aza-dC treated cells (10 $\mu\text{mol/l}$); right panel showing SAM-treated cells (100 $\mu\text{mol/l}$). (b) Quantification of 5-mC immunofluorescence signal. Error bar, SD; * $P < 0.05$, statistical significance by two-sided Student's t -test. (a) $\times 400$ final magnification. (a) All three images, the white bar denotes 60 μm . 5-aza-dC, 5-aza-2-deoxycytidine; 5-mC, 5-methylcytosine; SAM, S-adenosyl methionine.

Fig. 4.

SAM causes a global increase in DNA methylation of YUSIT1, YUMAC, and 501mel cultured melanoma cell line. (a) Representative agarose gel photograph showing 1 µg of genomic DNA from either untreated YUSIT1 cells (lanes 2 and 3) or treated for 72 h with either 5-aza-dC (lanes 4 and 5) or SAM (lanes 6 and 7). (b) Semiquantification of dominant band intensities (lanes 3, 5, and 7). (c) Representative agarose gel photograph showing 1 µg of genomic DNA from either untreated YUSIT1 cells (lanes 2 and 3) and cells treated for 72 h with increasing SAM (lanes 4–9). (d) Semi-quantification of dominant band intensities in lanes (3, 5, 7, and 9). (e) Quantification of DNA methylation in melanoma cell lines YUSIT1, 501mel, and YUMAC treated for 72 h with SAM (0, 25, or 50 µmol/l) by a calorimetric ELISA assay. The y-axis represents the relative absorbance as measured by spectrophotometry. (a, c) The first lane indicates the DNA size marker. Error bar, SD; * $P < 0.05$ and *** $P < 0.001$, statistical significance by two-sided Student's *t*-test, respectively. 5-aza-dC, 5-aza-2-deoxycytidine; h, HpaII; m, MspI; SAM, S-adenosyl methionine.

Fig. 5.

Decreased viability and increased apoptosis in four of five cultured melanoma cell lines following SAM treatment. (a) Quantification of 72 h SAM cytoreductive effect on YUSIT1, YULAC, YUGEN8, mel501, and WM1366 cell lines. (b) Quantification of 72 h SAM apoptotic effect on YUSIT1, YULAC, YUGEN8, mel501, and WM1366 cell lines. (c) Representative images of YUSIT1 colony formation following 14 days of growth in media supplemented with SAM (concentrations ranging from 0 to 100 μmol/l). (d) Quantification of colony formation assay shown in (c). Error bar, SD; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, statistical significance by two-sided Student's t -test, respectively. SAM, S-adenosyl methionine.