Cell

Loss of 5-Hydroxymethylcytosine Is an Epigenetic Hallmark of Melanoma

Christine Guo Lian,[1,2,13] Yufei Xu,[1,13] Craig Ceol,[3,6] Feizhen Wu,[9] Allison Larson,[5] Karen Dresser,[7] Wenqi Xu,[9] Li Tan,[9] Yeguang Hu,[1] Qian Zhan,[2] Chung-wei Lee,[2] Di Hu,[1] Bill Q. Lian,[1,8] Sonja Kleffel,[5] Yijun Yang,[10] James Neiswender,[6] Abraham J. Khorasani,[1] Rui Fang,[1] Cecilia Lezcano,[2] Lyn M. Duncan,[4] Richard A. Scolyer,[11] John F. Thompson,[11] Hojabr Kakavand,[11] Yariv Houvras,[3,12] Leonard I. Zon,[3] Martin C. Mihm Jr.,[5] Ursula B. Kaiser,[1] Tobias Schatton,[5] Bruce A. Woda,[7] George F. Murphy,[2,*] and Yujiang G. Shi[1,9,*]

[1]Division of Endocrinology, Diabetes and Hypertension, Department of Medicine, Brigham and Women's Hospital
[2]Division of Dermatopathology, Department of Pathology, Brigham and Women's Hospital
[3]Stem Cell Program and Hematology/Oncology, Children's Hospital Boston, Howard Hughes Medical Institute, Harvard Stem Cell Institute
[4]Division of Dermatopathology, Department of Pathology, Massachusetts General Hospital
[5]Department of Dermatology, Brigham and Women's Hospital
Harvard Medical School, Boston, MA 02115, USA
[6]Department of Cancer Biology
[7]Department of Pathology
[8]Department of Medicine
University of Massachusetts, Worcester, MA 01605, USA
[9]Laboratory of Epigenetics, Institutes of Biomedical Sciences, Fudan University, Shanghai 200032, P.R. China
[10]ZIOPHARM Oncology, Inc., Charlestown, MA 02129, USA
[11]Royal Prince Alfred Hospital, Melanoma Institute Australia, and Sydney Medical School, The University of Sydney, NSW 2006, Australia
[12]Departments of Surgery and Medicine, Weill Cornell Medical College and New York Presbyterian Hospital, New York, NY 10065, USA
[13]These authors contributed equally to this work
*Correspondence: gmurphy@rics.bwh.harvard.edu (G.F.M.), yujiang_shi@hms.harvard.edu (Y.G.S.)
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SUMMARY

DNA methylation at the 5 position of cytosine (5-mC) is a key epigenetic mark that is critical for various biological and pathological processes. 5-mC can be converted to 5-hydroxymethylcytosine (5-hmC) by the ten-eleven translocation (TET) family of DNA hydroxylases. Here, we report that "loss of 5-hmC" is an epigenetic hallmark of melanoma, with diagnostic and prognostic implications. Genome-wide mapping of 5-hmC reveals loss of the 5-hmC landscape in the melanoma epigenome. We show that downregulation of isocitrate dehydrogenase 2 (IDH2) and TET family enzymes is likely one of the mechanisms underlying 5-hmC loss in melanoma. Rebuilding the 5-hmC landscape in melanoma cells by reintroducing active *TET2* or *IDH2* suppresses melanoma growth and increases tumor-free survival in animal models. Thus, our study reveals a critical function of 5-hmC in melanoma development and directly links the IDH and TET activity-dependent epigenetic pathway to 5-hmC-mediated suppression of melanoma progression, suggesting a new strategy for epigenetic cancer therapy.

INTRODUCTION

Melanoma is a unique and highly aggressive type of cancer that occurs more frequently with increasing age and often with a significant contribution of environmental factors to its etiology (Jemal et al., 2001, 2006; Marks, 2000). As one of the most virulent human cancers, melanoma is capable of distant and lethal metastases when the primary tumor volume is as little as 1 mm^3. Studies of biomarkers predictive of clinical outcome are impeded by latent periods for detection of metastases that may range from several years to more than a decade, and thus clinically annotated bio-specimen archives serve as valuable surrogates for the otherwise impractical prospective approaches. Such studies are further compounded by the difficulties inherent in the diagnosis of melanoma, as certain benign nevi and melanomas show significant histological overlap. Presently, there is a dearth of molecular markers that facilitate detecting the differences between benign and malignant melanocytic lesions and assist in predicting their biological behaviors. Thus, there is a pressing need for novel biomarkers that define the malignant potential of primary lesions, predict clinical outcome, and forecast therapeutic responses.

Abnormal DNA methylation at the 5 position of cytosine (5-mC) is a well-known epigenetic feature of cancer. Melanoma exhibits global hypomethylation within the bulk genome and local hypermethylation at specific tumor suppressor genes (Hoon et al., 2004; Liu et al., 2008; Shen et al., 2007). Nonetheless, the degree of global hypomethylation in melanoma is not sufficient to distinguish benign nevus from melanoma (Paz et al., 2003). Gene-specific hypermethylation may be a better discriminator, as recent studies indicate that multilocus DNA methylation signature genes may differentiate melanomas from nevi (Conway et al., 2011; Tellez et al., 2009). However, this requires sophisticated molecular biological tools that are not easily applicable

in routine clinical practice, and the small biopsy size of melanocytic lesions presents another technical limitation. Thus, despite the increasing recognition that abnormal DNA methylation (and/or histone modification) is a crucial participant in melanoma progression, no characteristic epigenetic modifications have been discovered that can be readily used as molecular markers for diagnosis and evaluation of melanoma virulence.

The recent discovery of the ten-eleven translocation (TET) family of 5-mC hydroxylases, including TET1, 2, and 3, which convert 5-mC to 5-hydroxymethylcytosine (5-hmC), also known as the ''sixth base,'' has added an additional layer of complexity to the epigenetic regulation of DNA methylation (Ito et al., 2010; Tahiliani et al., 2009; Zhang et al., 2010). 5-hmC exists at a high level in self-renewing and pluripotent stem cells (Szwagierczak et al., 2010; Tahiliani et al., 2009). However, 5-hmC levels are greatly reduced in most cultured, immortalized tumor cells (Haffner et al., 2011; Song et al., 2011; Yang et al., 2012). Frequent *TET2* mutational inactivation has been reported to associate with decreased 5-hmC levels in various myeloid leukemias (Delhommeau et al., 2009; Langemeijer et al., 2009). In addition, the cofactor α-ketoglutarate (α-KG) is absolutely required and plays a positive and critical role in the conversion of 5-mC to 5-hmC (Xu et al., 2011a). Isocitrate dehydrogenases (IDHs) catalyze oxidative decarboxylation of isocitrate, producing α-KG and CO_2 (Reitman et al., 2011; Xu et al., 2011a). There are two major IDH enzymes in mammalian cells—IDH1 in cytoplasm and its homolog, IDH2, in mitochondria—which catalyze the same reaction. It has been reported that gain-of-function mutations in *IDH1* and *IDH2* in cancer cells produce the oncometabolite 2-hydroxyglutarate (2-HG), an antagonist of α-KG (Chowdhury et al., 2011; Xu et al., 2011a), which inhibits the TET-mediated conversion of 5-mC to 5-hmC. Moreover, similar to the frequent mutation rate of *IDH1* or *IDH2* in glioma and myeloid leukemia (Dang et al., 2010; Krell et al., 2011), 10% of melanomas harbor a neomorphic mutation in *IDH1* or *IDH2* (Shibata et al., 2011). These studies suggest a role of 5-hmC, TET, and IDH in malignancy. However, it remains elusive as to how 5-hmC is lost and what roles TET and IDH proteins play during tumor progression. In particular, it remains unknown as to how this epigenetic mark and these related enzymes partake in melanoma progression.

Using melanoma as a paradigm of aggressive cancer, here we report that ''loss-of-5-hmC'' is a new epigenetic hallmark of melanoma. We functionally characterize the significant impact of 5-hmC, IDH2, and TET2 in melanoma progression. Importantly, we show that the activity of IDH2 and TET2 enzymes required for the production of 5-hmC and the re-establishment of the 5-hmC landscape in melanoma cells is essential to regulation of melanoma virulence, contributing to our current understanding of cancer epigenetics.

RESULTS

5-hmC Level Is High in Mature Melanocytes and Nevi and Is Lost in Human Melanomas

High levels of 5-hmC were detected by immunofluorescent (IF) staining in the nuclei of isolated melanocytes that coexpressed MART-1, a melanocyte-specific marker, within the epidermal basal cell layer (Figures 1A and 1B). A more sensitive method for IF or immunohistochemical (IHC) staining of 5-hmC using HCl-treated formalin-fixed, paraffin-embedded tissue sections resulted in loss of the MART-1 epitope but significantly improved the detection of 5-hmC as demonstrated by staining in normal human tissues (Figure S1A available online). By this method, strong IF staining of 5-hmC was detected in melanocytes within the otherwise negative basal layer, as seen in Figures 1A and 1B, as well as variably within more differentiated suprabasal keratinocytes (Figure 1C). The IF staining pattern was confirmed by IHC staining (Figure 1D), and this more sensitive method for 5-hmC detection was utilized for all subsequent studies of melanocytic nevi and melanomas.

More than 50 individual cases of representative melanocytic lesions, including benign nevi, primary melanomas, and metastatic melanomas, were initially evaluated. Benign nevus cases ($n = 30$) showed strong nuclear 5-hmC staining, whereas virtually all tumor cells in primary ($n = 15$) and metastatic ($n = 10$) melanomas showed partial or complete loss of 5-hmC (Figures 1E–1H). Significant differences in other epimarks were not observed between benign nevi and melanomas (Figure S1B and Table S1), suggesting the unique discriminatory nature of 5-hmC staining as it relates to cells of melanocytic lineage. We next purified genomic DNA from nevi and melanomas and confirmed higher 5-hmC levels in nevi than in melanomas by two independent methods, the anti-5-hmC antibody-based dot blot (Figure 1I) and T4 phage β-glucosyltransferase-mediated 5-hmC glucosylation assay (Figure 1J). Taken together, these data demonstrate that, while a high level of 5-hmC is a distinctive epigenetic signature for melanocytes and benign nevi, significantly diminished or complete loss of 5-hmC is a feature of melanomas.

5-hmC Is a Putative Molecular Marker of Melanoma Progression

We next examined 5-hmC levels by IHC using a melanoma progression tissue microarray (TMA) representing four major diagnostic tumor types: benign melanocytic nevus, primary cutaneous melanoma, melanoma metastases to lymph nodes, and metastases to viscera (Kabbarah et al., 2010; Schatton et al., 2008). Consistent with the individual cases examined above (Figure 1), the TMA confirmed significant 5-hmC loss in primary melanomas and metastatic melanomas compared with nevi ($p < 0.001$; Figures 2A and S2 and Tables S2 and S3). In two additional commercially available melanoma TMAs, there was significant loss of 5-hmC in melanomas compared to benign nevi ($p = 1.1 \times 10^{-7}$) and loss in nodal compared to visceral metastases ($p = 0.016$) (Figure 2B). Taken together, these data further support loss of 5-hmC as a distinctive epigenetic event in melanoma and suggest that 5-hmC may represent a new epigenetic mark for melanoma recognition and progression.

We next correlated the 5-hmC level with critical melanoma staging parameters (tumor depth and mitotic rate) using the melanoma specimens from a clinically annotated cohort including 70 superficial spreading and nodular melanomas (Table S4). There was a negative correlation between 5-hmC staining score and primary melanoma Breslow depth, a standard predictor of prognosis (Figure 2C, $r = -0.4$, $p = 0.0005$), as well as between 5-hmC level and mitotic rate (Figure 2D, $r = -0.23$, $p = 0.054$). Furthermore, 5-hmC levels were significantly reduced