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Loss of 5-hydroxymethylcytosine correlates with increasing morphologic dysplasia in melanocytic tumors

Allison R. Larson[1,2], **Karen Dresser**[3], **Qian Zhan**[1], **Cecilia Lezcano**[1], **Bruce A. Woda**[3], **Benafsha Yosufi**[4], **John F. Thompson**[4], **Richard A. Scolyer**[4], **Martin C. Mihm Jr.**[2], **Yujiang G. Shi**[5], **George F. Murphy**[1,*], and **Christine Guo Lian**[1,*]

[1]Program in Dermatopathology, Department of Pathology, Brigham and Women's Hospital, Harvard Medical School

[2]Department of Dermatology, Brigham and Women's Hospital, Harvard Medical School

[3]Department of Pathology, University of Massachusetts Medical Center

[4]Royal Prince Alfred Hospital, Melanoma Institute Australia and Sydney Medical School, The University of Sydney

[5]Divison of Endocrinology, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School

Abstract

DNA methylation is the most well studied epigenetic modification in cancer biology. 5-hydroxymethylcytosine is an epigenetic mark that can be converted from 5-methylcytosine by the ten-eleven translocation gene family. We recently reported the loss of 5-hydroxymethylcytosine in melanoma compared to benign nevi and suggested that loss of this epigenetic marker is correlated with tumor virulence based on its association with a worse prognosis. In this study we further characterize the immunoreactivity patterns of 5-hydroxymethylcytosine in the full spectrum of melanocytic lesions to further validate the potential practical application of this epigenetic marker. 175 cases were evaluated: 18 benign nevi, 20 dysplastic nevi (10 low-grade and 10 high-grade lesions), 10 atypical Spitz nevi, 20 borderline tumors, 5 melanomas arising within nevi, and 102 primary melanomas. Progressive loss of 5-hydroxymethylcytosine from benign dermal nevi to high-grade dysplastic nevi to borderline melanocytic neoplasms to melanoma was observed. In addition, an analysis of the relationship of nuclear diameter to 5-hydroxymethylcytosine staining intensity within lesional cells revealed a significant correlation between larger nuclear diameter and decreased levels of 5-hydroxymethylcytosine. Furthermore, borderline lesions uniquely exhibited a diverse spectrum of staining of each individual case. This study further substantiates

*Co-corresponding authors, George F. Murphy, MD, Program in Dermatopathology, Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, 221 Longwood Avenue, EBRC Suite 401, Boston MA 02115, gmurphy@rics.bwh.harvard.edu, telephone 617-525-7484, fax 617-264-5149, Christine G. Lian, MD, Program in Dermatopathology, Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, 221 Longwood Avenue, EBRC Suite 401, Boston MA 02115, cglian@partners.org, telephone 617-525-7484, fax 617-264-5149.

Disclosure/Conflict of Interest

The authors have no other conflicts of interest to disclose.

the association of 5-hydroxymethylcytosine loss with dysplastic cytomorphologic features and tumor progression and supports the classification of borderline lesions as a biologically distinct category of melanocytic lesions.

Keywords

5-hydroxymethylcytosine; DNA methylation; melanocytic lesion; dysplastic nevus; melanoma

Introduction

The process of DNA methylation at the 5-carbon position of cytosine is a critical epigenetic mechanism in the regulation of gene expression. 5-methylcytosine constitutes 2–8% of all cytosines in human genomic DNA and impacts a broad range of biological functions and pathological processes including gene expression, maintenance of genome stability, genomic imprinting, X-chromosome inactivation, developmental regulation, aging-related processes, and cancer (1). DNA methylation is the most extensively studied epigenetic modification in cancer. Tumor cells of various cancer types have been found to exhibit global hypomethylation as well as selective hypermethylation at the promoters of tumor suppressor genes associated with silencing of these genes and tumorigenesis (2). In 2009, breakthrough studies show that 5-hydroxymethylcytosine can be converted from 5-methylcytosine by ten-eleven translocation family genes (3–5). We reported that loss of 5-hydroxymethylcytosine is associated with increased melanoma virulence and poor survival (6). Other studies have also shown 5-hydroxymethylcytosine loss in other cancers including breast, prostate, colon cancer, and hematologic myeloid malignancies (7–10).

In melanoma, we hypothesized that cytologically atypical cells (as characterized by enlarged nuclei and a coarse chromatin pattern) represent the subpopulations of melanoma cells with more aggressive biological behavior. In addition, studies have shown that nuclear size is helpful in differentiating benign from malignant melanocytic lesions (11–13). Accordingly, it would be expected that a greater loss of 5-hydroxymethylcytosine would be observed within cells with enlarged nuclear size as a consequence of the association with increased virulence.

Melanocytic lesions are extremely heterogeneous in their morphologies with many overlapping histological features between benign and malignant lesions. Among those, the most challenging category is the ‘gray zone’ or ‘borderline’ melanocytic lesions, not only in diagnosis and grading, but also in proper guidance of treatment due to the unknown and unpredictable biological potential of such lesions (14). In this study, we also assess the 5-hydroxymethylcytosine staining patterns and possible utilization of this epigenetic marker in these difficult melanocytic lesions. We analyzed groups of ‘borderline’ lesions including Spitz nevi with atypia and severely atypical melanocytic proliferations of uncertain malignant potential (also known as atypical Spitz tumors), for their 5-hydroxymethylcytosine staining pattern in comparison to clearly benign dermal nevi and clear-cut cases of melanoma. Furthermore, a challenging situation often encountered by dermatopathologists in practice is that of melanoma arising within a pre-existing nevus. In

these cases, particularly when the melanoma cells show “maturation” with depth, distinguishing the nevus and melanoma cells and thus precise determination of the Breslow depth can be difficult (15). We also examined this type of challenging case to determine if the 5-hydroxymethylcytosine stain could help to differentiate the benign nevus component with a high level of 5-hydroxymethylcytosine from the melanoma component with 5-hydroxymethylcytosine loss to facilitate accurate Breslow depth measurement.

Materials and Methods

Histopathologic samples

This study was conducted with approval of the Institutional Review Board of Brigham and Women’s Hospital, Harvard Medical School. In total, 175 cases were studied retrospectively: 18 benign nevi, 20 dysplastic nevi (10 low-grade, 10 high-grade), 30 borderline lesions (10 Spitz nevi with atypia, 20 severely atypical melanocytic proliferations of uncertain malignant potential also known as atypical Spitz tumors), 5 melanomas arising in the background of nevi, and 102 predominantly superficial spreading melanomas. The melanomas as well as 22 of the borderline lesions were obtained from the annotated Melanoma Institute of Australia cohort of specimens. The ages of the patients ranged from 19–92, they were 52% male, race was not recorded in the database, however, the population seen at this clinic is predominantly Caucasian. The remainder of the specimens were obtained from the pathology archives of Brigham and Women’s Hospital and the University of Massachusetts. The patient ages for the borderline lesions ranged from 3–66. For 21 of the borderline lesions follow-up information was available. These included 8 atypical Spitz nevi and 13 severely atypical melanocytic tumors of uncertain malignant potential or atypical Spitz tumors. Six patients underwent sentinel lymph node biopsies and all were negative for melanocytic lesions. 5 of these lesions fell into the category of severely atypical melanocytic proliferations of uncertain malignant potential or atypical Spitz tumors and one was classified as an atypical Spitz nevus. All 21 patients survived to their last-recorded follow-up (ranged from 16–137 months) with no evidence of disease recurrence. The 20 dysplastic nevi were grouped as low-grade (n=10) and high-grade lesions (n=10) according to Table 1 (16). Dysplasia was assessed in the junctional and/or superficial dermal components of the nevi (17). The borderline lesion group contained 10 Spitz nevi with atypia as well as 20 tumors carrying the diagnoses of atypical Spitzoid tumor (n=12), or severely atypical melanocytic proliferation of uncertain malignant potential (n=8).

Immunohistochemistry and nuclear measurements

Cases were included for analysis based on the diagnosis on the original pathology reports. Hematoxylin and eosin-stained slides were reviewed by two authors (CGL and ARL) to confirm diagnoses following immunohistochemical analysis to help blind the immunohistochemical analysis. Staining scores were not viewed upon hematoxylin and eosin slide review to eliminate the possibility of selection bias. An additional author (GFM) reviewed all atypical Spitz nevi and borderline tumors for appropriate categorization. Immunohistochemical studies were carried out in accordance with Lian et al. (6) using rabbit anti-5-hydroxymethylcytosine at 1:10,000 (Active Motif, Carlsbad, CA). Within all slides 2 random areas of the lesion were viewed and representative cells were evaluated in

terms of nuclear diameter and staining intensity relative to the staining of normal melanocytes at the basal layer of flanking normal epidermis as a positive internal control. The normal melanocytes were defined as an intensity of 2 in each case. There was some variation in the staining intensity of these basal melanocytes, thus the staining of each slide was calibrated separately based on this intensity. Some variability in tissue uptake of antibody is typical in immunohistochemistry so we used an internal control to scale staining intensity to correct for this. A total of 5 cells were evaluated per field (10 cells per tumor) on a 0–3 scale as shown in to Figure 1. The measurements from all cells were included in the analysis. For these same cells the nuclear diameter was measured at 400X with an optical micrometer to the nearest 2.5 micrometers (the smallest unit of measurement on the ocular micrometer). The long axis of the nucleus was chosen for measurement. There was some variability in nuclear size because within the tissue, each nucleus was sectioned in a random location. All measurements were taken by one author (ARL) and a random subset was reviewed by a second author (CGL) to ensure concordance. Reviewers were blinded to the diagnosis of each lesion (as far as possible). T-tests were used for all comparisons with p-values less than 0.05 considered significant.

Semi-quantitative analysis

To confirm our findings in a semi-quantitative manner, 5 cases were chosen at random from each of the six categories (benign nevi, low-grade dysplastic nevi, high-grade dysplastic nevi, atypical Spitz nevi, atypical borderline tumors, melanomas) and 3 photographs of 40× high power fields were taken of each case along with a photograph of normal melanocytes within the epidermis. Adobe Photoshop CS4 version 11.0 was used to eliminate all but the melanocytic component from each image as epithelium stains positively for this antibody. ImageJ software (NIH, Bethesda, MD) was used to analyze the nuclear perimeter in micrometers and percent of cells meeting or exceeding a threshold staining intensity with the threshold set at the intensity of normal epidermal melanocytes flanking the melanocytic lesion (termed strongly staining cells). This threshold was recalibrated for each slide. T-tests were used for all comparisons with p-values less than 0.05 considered significant.

Results

5-hydroxymethylcytosine levels were high in normal melanocytes that resided as solitary cells at the basal layer of the epidermis. The nuclear staining of 5-hydroxymethylcytosine in these normal melanocytes was used as the grading standard (Figure 1). Benign nevi were comprised of intermixed lightly and darkly staining cells with approximately 2/3 of the cells staining darkly (Figure 2A and Figure 3). Low-grade dysplastic nevi had a similar pattern to benign nevi (Figure 2B and Figure 3). The high-grade dysplastic nevi ranged from areas that were an admixture of about 90% slightly stained and 10% negatively stained cells (Figure 3) to areas with complete loss (Figure 2C). The predominant pattern seen in melanomas was complete loss of staining (Figure 2D), however, some melanomas had a mixture of negatively staining and very lightly staining cells (Figure 3). The 5-hydroxymethylcytosine staining pattern varied somewhat from melanoma to melanoma as well as within individual melanomas between subpopulations of cells with differing cytomorphology, highlighting the

heterogeneous nature of this cancer. Thus individual melanoma cells will not exhibit full loss of 5-hydroxymethylcytosine with 100% specificity.

In benign nevi, the majority of nevic cells retained strong nuclear staining for 5-hydroxymethylcytosine (Figure 2A). Interestingly, 5-hydroxymethylcytosine levels were similar in low-grade dysplastic nevus cases ($p = 0.462$ compared to dermal nevi), which are dysplastic nevi with mild atypia and dysplastic nevi with mild to focal moderate atypia (Figure 2B). We grouped dysplastic nevi with diffuse moderate atypical features and dysplastic nevi with severe atypia into high-grade dysplastic nevi (12). The high-grade dysplastic nevi group had significantly lower 5-hydroxymethylcytosine levels compared to benign and low-grade dysplastic nevi ($p = 0.0279$, 0.0120 respectively) (Figure 2C and Table 2). Consistent with our previous findings (6), 5-hydroxymethylcytosine positivity was significantly lower in melanoma cases compared to benign nevi, low-grade dysplastic nevi, and high-grade dysplastic nevi ($p < 2.2\times10^{-16}$ for all) (Table 2). There was a progression of loss of 5-hydroxymethylcytosine from high-grade dysplastic nevi to melanoma (Figure 3). Interestingly, in essentially all of the melanoma cases and select high-grade dysplastic nevi with diffuse 5-hydroxymethylcytosine loss, the keratinocytes directly overlying the melanocytic lesion also exhibited 5-hydroxymethylcytosine loss. The epidermis flanking the melanocytic lesion stained normally with 5-hydroxymethylcytosine and this was used as an internal control for staining quality in these lesions. A separate positive internal staining control for the dysplastic nevi was the deep (dermal) component of these lesions which exhibited darker 5-hydroxymethylcytosine staining than the superficial dysplastic portion.

The atypical Spitz nevi showed significant 5-hydroxymethylcytosine loss when compared with benign nevi, low-grade, and high-grade dysplastic nevi ($p = 7.141\times10^{-9}$, $p = 3.284\times10^{-8}$, $p = 0.000571$ respectively), and showed significantly more 5-hydroxymethylcytosine expression than melanoma ($p = 3.83\times10^{-13}$) (Table 2). The remainder of the borderline lesions (the superficial severely atypical melanocytic proliferations of uncertain malignant potential and atypical Spitzoid tumors) hereafter called the atypical borderline tumors, were the most variably staining both within the lesions themselves and between each lesion (Figure 4). This subgroup exhibited the most diverse spectrum of staining with nests comprised of darkly, lightly and negatively staining cells. The percent of cells with a total loss of 5-hydroxymethylcytosine varied from a few cells in some cases to roughly half of the cells in other cases. This group did show significant loss of 5-hydroxymethylcytosine compared to dermal nevi and low-grade dysplastic nevi ($p < 2.2\times10^{-16}$ for both), high-grade dysplastic nevi ($p = 5.859\times10^{-9}$), atypical Spitz nevi ($p = 0.0139$), and showed significantly more expression than melanomas ($p = 6.839\times10^{-9}$) (Table 2). In addition, to assess the possibility of applying 5-hydroxymethylcytosine as a marker to differentiate the benign nevus component versus malignant melanocytic component in the same lesion (to assist accurate measurement of Breslow depth), several cases of melanoma arising within associated dermal nevus components were studied (Figure 5). Strong staining of 5-hydroxymethylcytosine levels in the benign nevus component in the deep dermis are observed along with loss of 5-hydroxymethylcytosine in the melanoma component (Figure 5).

Linear regression illustrates a significant loss of 5-hydroxymethylcytosine staining with increased nuclear diameter in melanomas with a p-value of $< 2.2\times10^{-16}$ (Figure 6). The size differences between dermal nevi (ranged from 2.5–10, most were 5–7.5 micrometers) and low-grade dysplastic nevi, and between low and high-grade dysplastic nevi were significant ($p = 3.2\times10^{-8}$, 0.0017 respectively). Melanoma nuclei ranged from 2.5 to 27.5 micrometers in diameter, most fell in the 7.5–10 micrometer range. Both atypical Spitz nevi and atypical borderline tumors had significantly larger nuclear size than benign nevi ($p = 0.0007$, $p < 2.2\times10^{-16}$ respectively). Only atypical borderline tumors had larger nuclear size than low-grade dysplastic nevi ($p = 0.0133$). High-grade dysplastic nevi had significantly larger nuclear size than atypical Spitz nevi ($p = 3.978\times10^{-7}$) but not atypical borderline tumors. Atypical borderline tumors had significantly larger nuclear size than atypical Spitz nevi ($p = 1.149\times10^{-5}$). Melanomas showed a significantly larger nuclear diameter than every other lesion group ($p < 0.001$ for all comparisons) (Table 2).

The results of our semi-quantitative analysis on a small subset of cases from each category confirmed the majority of the above findings in a small subset of our cases. 15 high power fields were analyzed for each category except for the low-grade dysplastic nevi where 3 fields had to be eliminated due to difficulty of the software in determining nuclear size because of overlap. Similar observations in the proportion of strongly staining cells and nuclear perimeters were found (Table 3). Compared to melanoma, dermal nevi, low-grade dysplastic nevi, atypical Spitz nevi, and atypical borderline tumors had a significantly increased proportion of strongly staining cells. Compared to dermal nevi, high-grade dysplastic nevi, atypical Spitz nevi, atypical borderline tumors, and melanomas had a significantly lower proportion of strongly staining cells. With regard to nuclear perimeter, all groups had a significantly smaller perimeter than melanomas and atypical Spitz nevi, atypical borderline tumors, and melanomas had a significantly larger perimeter than dermal nevi.

Discussion

5-hydroxymethylcytosine loss is strongly associated with melanoma and specifically with a poor prognosis in superficial spreading and nodular melanomas (6). This study provides further support to the finding that 5-hydroxymethylcytosine loss is a putative epigenetic biomarker of tumor progression in melanocytic lesions. Progressive loss of 5-hydroxymethylcytosine is found between benign nevi, low-grade dysplastic nevi, high-grade dysplastic nevi, atypical Spitz nevi, atypical borderline tumors, and melanoma. On average, nuclear diameter likewise increases within the spectrum of benign to dysplastic to frankly malignant melanocytic neoplasms and the loss of 5-hydroxymethylcytosine staining pattern correlates with changes in nuclear size. Interestingly the correlation with 5-hydroxymethylcytosine staining intensity and nuclear diameter persists within the melanomas in this study indicating heterogeneity in nuclear atypia and 5-hydroxymethylcytosine staining within this group. Our measurement technique probably underestimates true nuclear diameter (and nuclear perimeter) since the sectioning technique catches each nucleus in a random orientation. The ‘nuclear diameters’ measured could be smaller than the maximal nuclear diameter because sectioning did not occur through the center of the nucleus. The impact of this measurement will likely underestimate true nuclear

diameter particularly for lesions with larger nuclei (melanomas). The possibility arises that the loss of 5-hydroxymethylcytosine staining intensity with increased nuclear size is purely a dilutional effect. This is highly unlikely given that previous studies have shown loss of 5-hydroxymethylcytosine in many low-grade malignancies with mild cytological atypia, including breast, prostate, and colon cancer implicating the loss of 5-hydroxymethylcytosine as an early event in carcinogenesis (7). Additionally, further studies reported by Lian et al. (6) strongly support a mechanistic role for 5-hydroxymethylcytosine in melanoma as overexpressing 5-hydroxymethylcytosine in human melanoma cells reduces tumor growth in mouse xenograft models and prolongs tumor-free survival in zebrafish models (6). Importantly, our prior work includes a deep sequencing analysis that shows a global decrease in 5-hydroxymethylcytosine levels in the entire melanoma genome compared to nevi indicating that this effect is not simply due to a similar number of binding sites spread over a larger nuclear area (6). Thus a reasonable conclusion is that 5-hydroxymethylcytosine is lost as cells undergo dysplastic or malignant transformation.

Interestingly, the loss of 5-hydroxymethylcytosine in keratinocytes overlying melanomas and occasional high-grade dysplastic nevi was also observed in our study. It indicates an epigenetic shift not just in the tumor itself but in the surrounding microenvironment, raising the possibility of an environment permissive for melanoma formation.

In summary, we have studied the expression pattern of 5-hydroxymethylcytosine in a full spectrum of melanocytic lesions from benign nevi to melanoma and demonstrate the progressive loss of this epigenetic marker with increasing dysplasia as well as nuclear diameter. In addition, the loss of 5-hydroxymethylcytosine was observed in severely dysplastic subpopulations of cells within individual lesions of dysplastic nevi, borderline melanocytic lesions and more diffusely in most melanomas. Future studies including PCR and genomic deep sequencing will be needed to confirm these findings within dysplastic nevi and borderline lesions. The mosaic pattern, seen most dramatically in the borderline lesions, supports classification of borderline lesions as biologically distinct as previously proposed (18). The current utility of this biomarker may revolve around distinguishing nevus cells from melanoma cells in cases where the two intersect to determine an accurate Breslow depth. Further studies utilizing larger cohorts of borderline lesions with long clinical follow-up are required to determine whether loss of 5-hydroxymethylcytosine can assist in predicting the clinical outcomes of patients with such lesions. It also raises the possibility that subpopulations of melanoma cells with complete loss of 5-hydroxymethylcytosine correlate with higher virulence in melanoma. In summary, these findings further implicate 5-hydroxymethylcytosine as a potential biomarker in melanocytic lesions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nuclear Staining Score	Description
0 	No nuclear stain
1 	Nuclear stain less intense than melanocyte nuclei in flanking normal epidermis
2 	Staining equal to intensity of flanking melanocyte nuclei
3 	Staining darker than flanking melanocyte nuclei

Figure 1. Nuclear staining intensity scoring system

Staining intensity of nuclei was graded according to the following criteria. Examples of each grade are shown on the left. Nuclei positive for 5-hydroxymethylcytosine stain brown, nuclei negative for 5-hydroxymethylcytosine stain gray/purple with the counterstain.

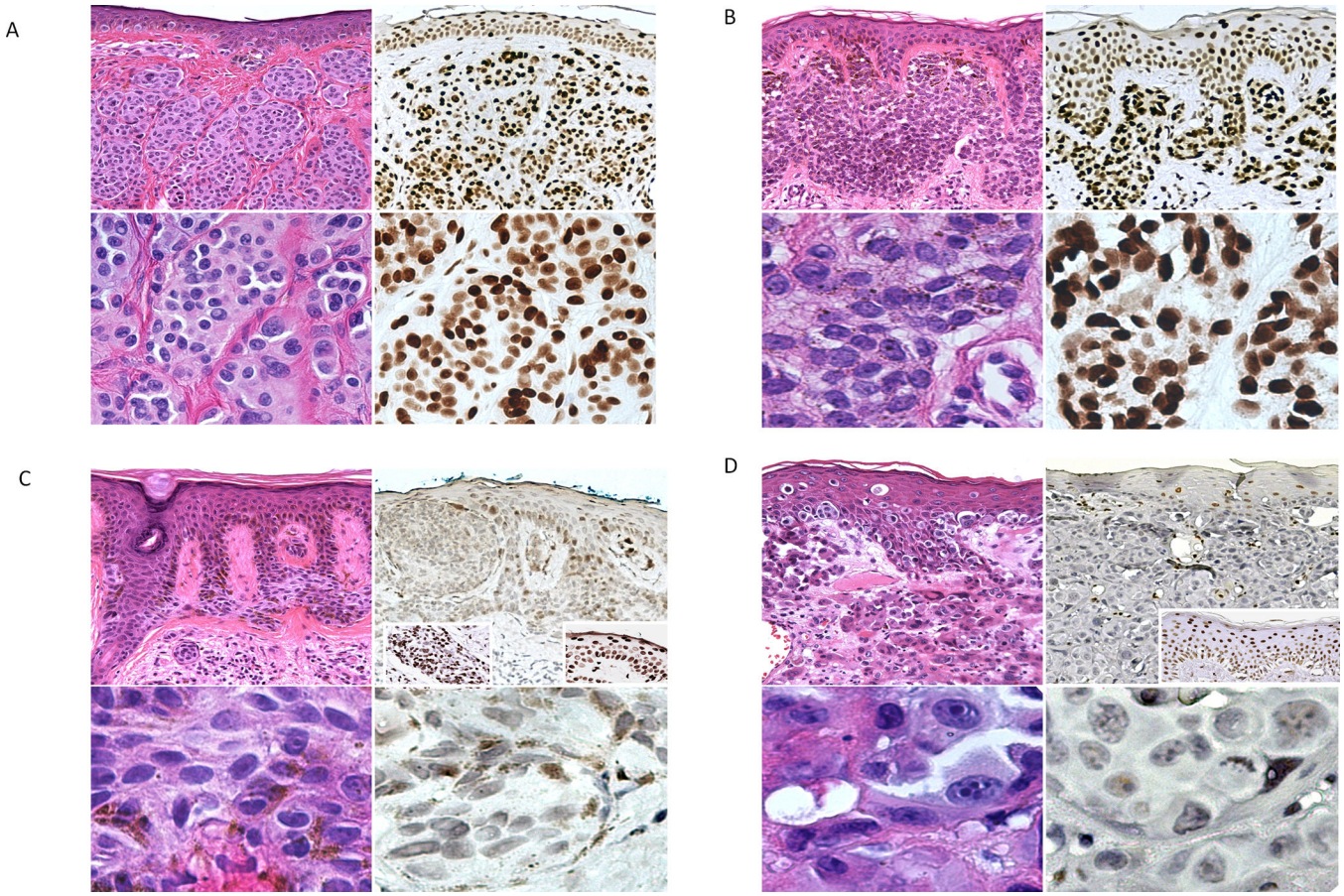

Figure 2. 5-hydroxymethylcytosine in benign or low-grade nevi compared to high-grade nevi and melanoma

Conventional histology (left, hematoxylin and eosin) and 5-hydroxymethylcytosine (right, immunohistochemistry) staining patterns (200× on top, representative area magnified below) of a dermal nevus (A), low-grade dysplastic nevus (B), high-grade dysplastic nevus (C), and melanoma (D). The 5-hydroxymethylcytosine staining pattern of low-grade dysplastic lesions (B) resembles that of benign nevi (A). Overall the high-grade lesions show some staining loss with a subset showing complete loss as shown here (C), resembling the staining pattern of melanoma (D). Within the high-grade dysplastic nevus there is cytologic atypia as well as prominent architectural atypia with extension down adnexal structures. The inserts within the 5-hydroxymethylcytosine staining pictures for C and D illustrate positive internal controls of normal staining of follicular epithelium (C) and the flanking epidermis (C, D).

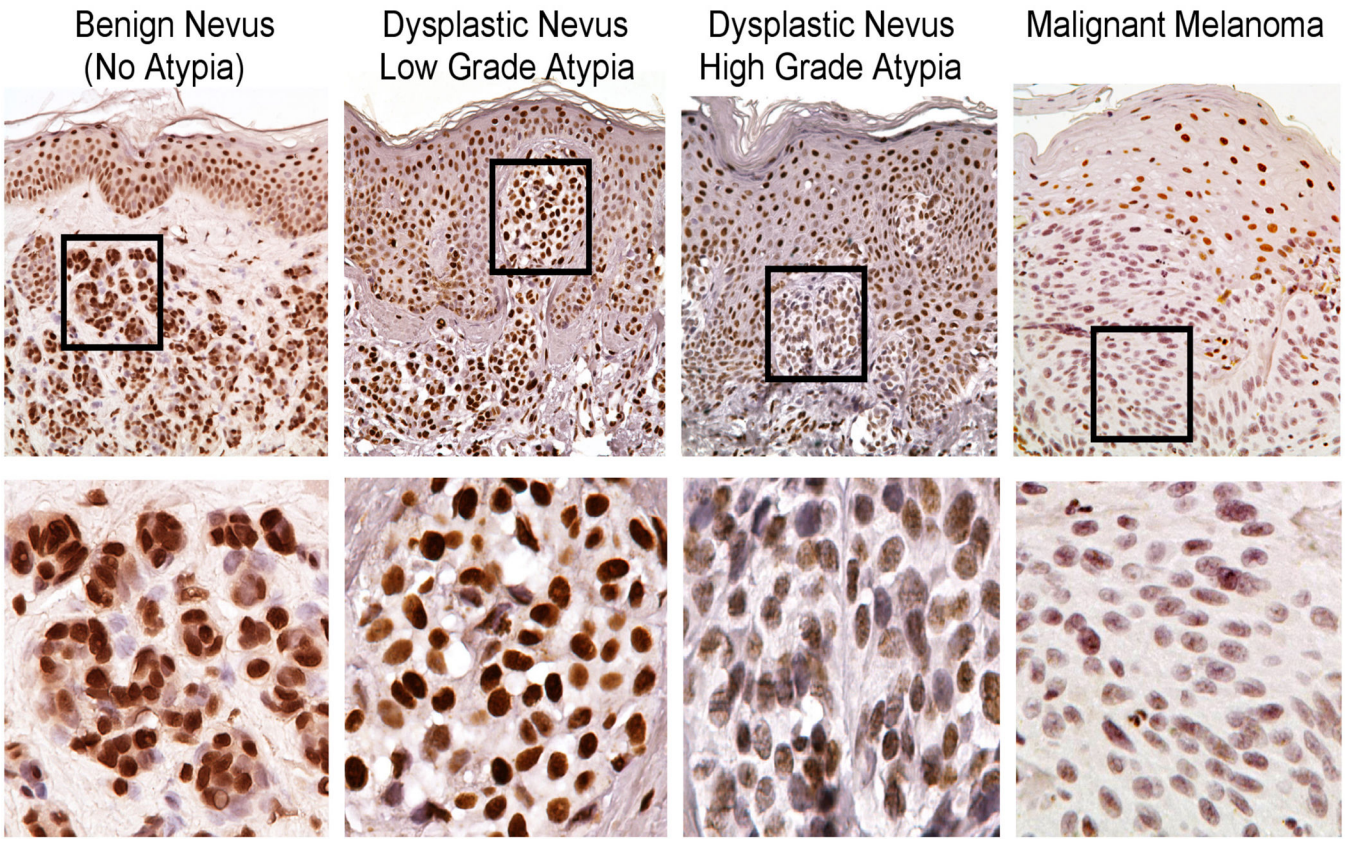

Figure 3. Progressive loss of 5-hydroxymethylcytosine with increasing dysplasia

5-hydroxymethylcytosine immunohistochemistry staining positivity is increasingly lost from benign and low-grade dysplastic nevi to high-grade dysplastic nevi to melanoma. 200× sections are shown above with selected areas magnified below.

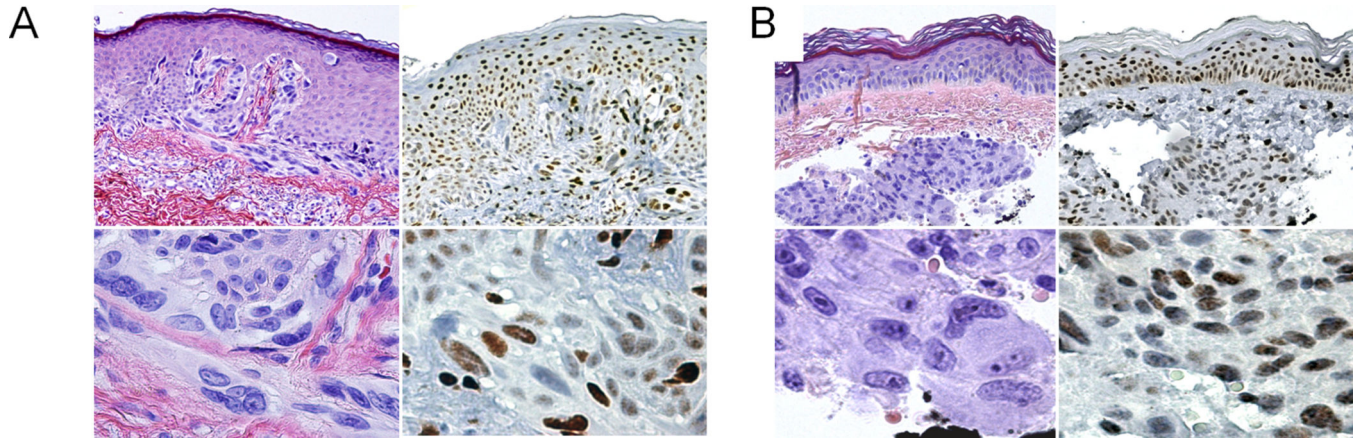

Figure 4. 5-hydroxymethylcytosine in borderline melanocytic lesions

Two borderline melanocytic lesions (left, hematoxylin and eosin and right, immunohistochemistry stains) show a mosaic pattern of 5-hydroxymethylcytosine staining with a mixture of positively and negatively staining cells.

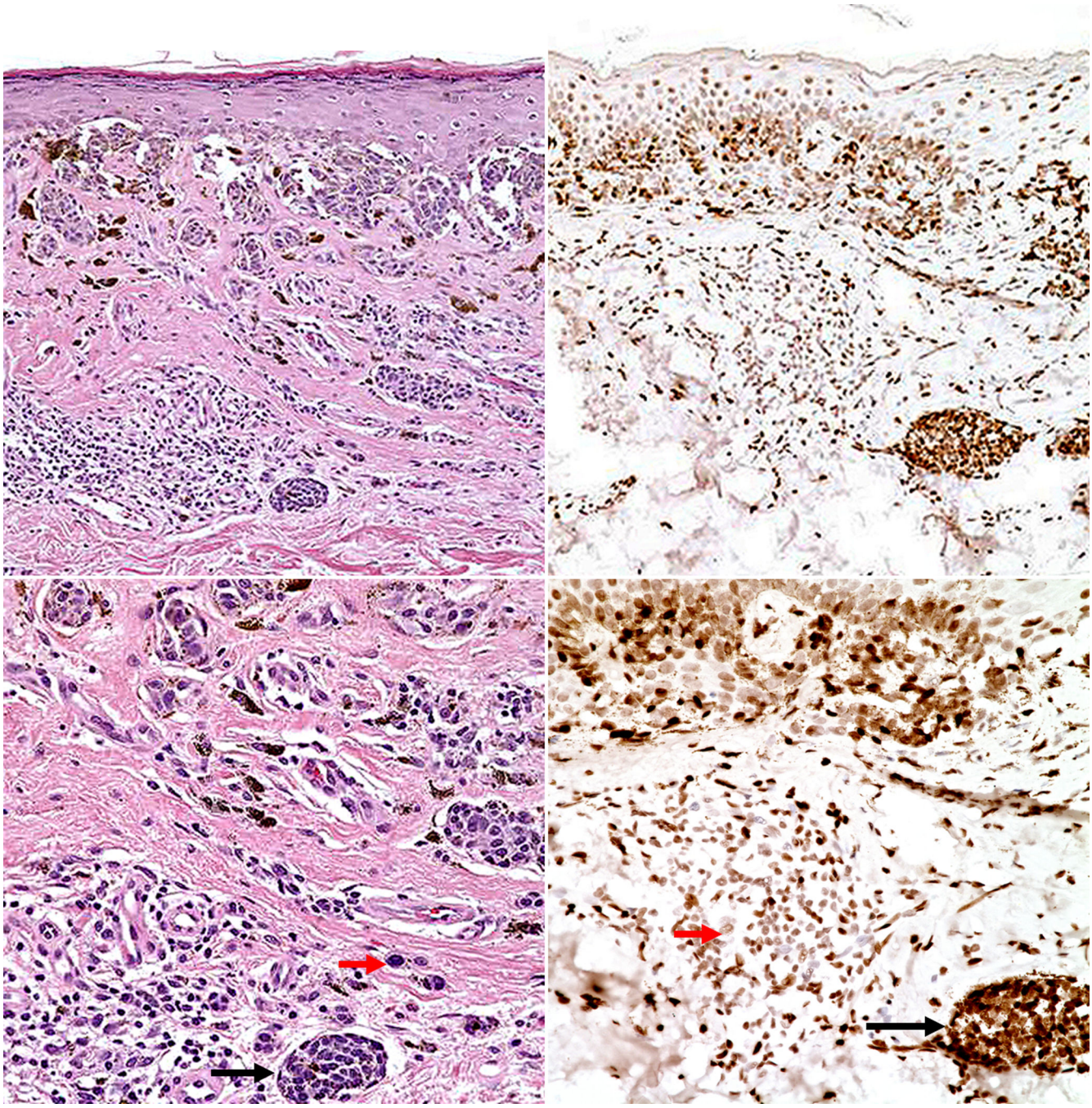

Figure 5. 5-hydroxymethylcytosine highlights nevic component within a melanoma

Conventional histology of a case of superficial spreading melanoma arising within a benign dermal nevus (left column, hematoxylin and eosin). 5-hydroxymethylcytosine immunohistochemistry stain (right column) show negativity in the superficial malignant melanoma components and positivity in dermal nested nevic components (right column). The black arrow highlights the nevic nests and red arrow indicates the larger melanoma cells surrounded by inflammation.

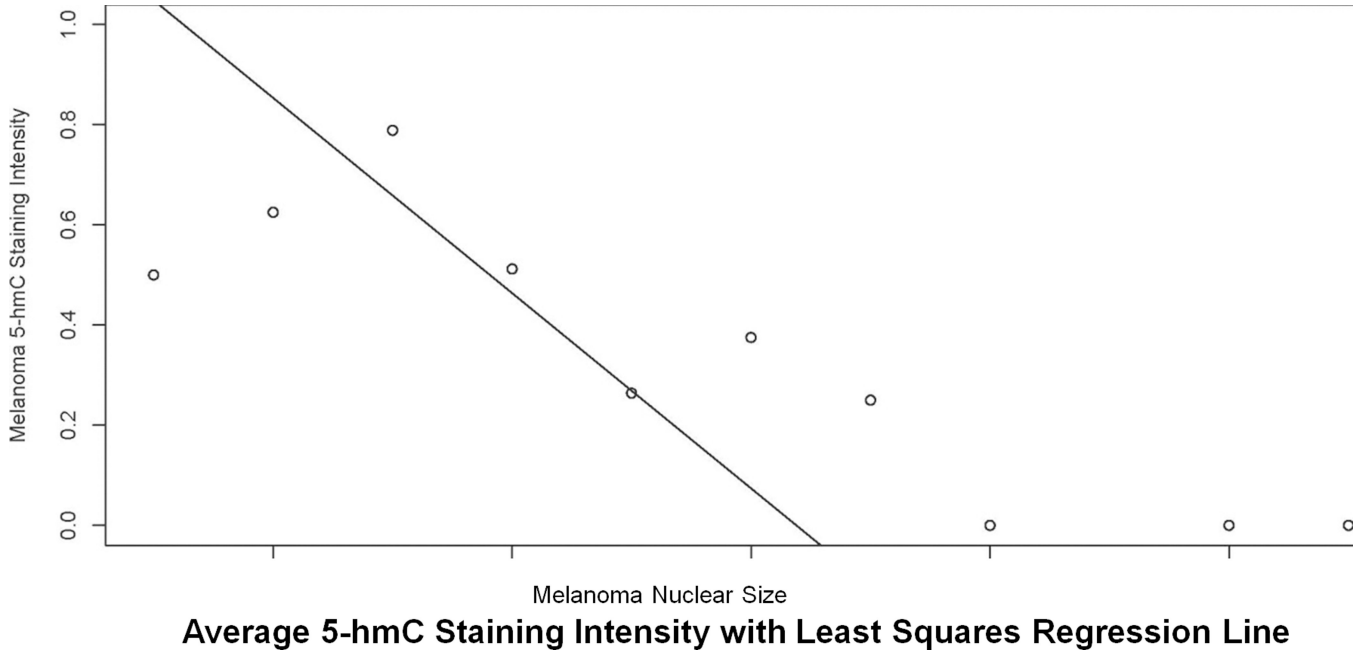

Figure 6. Loss of 5-hydroxymethylcytosine staining correlates with nuclear size

Linear regression of the staining intensity of melanomas as a function of nuclear size illustrates a significant loss of staining correlating with an increase in nuclear size ($p < 2.2\times10^{-16}$, t-test).

Table 1

Classification of Melanocytic Lesions

Architecture					Cytology			
	Location of cell/nest	**Bridging of nests**	**Adnexal extension**	**High-level pagetoid and/or continuous contiguous growth**	**Average nuclear size**	**Nuclear contour**	**Chromatin pattern**	**Nucleoli**
Normal flanking melanocyte	Basal layer of epidermis	Absent	Absent	Absent	< basal keratinocyte nucleus	Round	Condensed	Absent
Junctional/superficial nevus cell	Rete tip	Absent	Absent	Absent	= basal keratinocyte nucleus	Round	Delicate	Absent
Low-grade superficial dysplasia	Varies	Present	Absent	Absent	1–2× basal keratinocyte nucleus	Round or polyhedral	Delicate	Inconspicuous
High-grade superficial dysplasia	Varies	Present	Varies	Absent	>2× basal keratinocyte nucleus	Irregular, polyhedral, some marked angulation	Coarse	Usually prominent
Melanoma	Infiltration of epidermis and dermis	Present	Present	Present	Much larger than 2× basal keratinocyte nucleus	Irregular, polyhedral, some marked angulation	Coarse	Prominent

Table 2

Staining Intensity and Nuclear Size in a Spectrum of Melanocytic Lesions

Lesion	Mean nuclear intensity	Mean nuclear size (range)
Dermal nevi	1.7 *	6.2 (2.5–10) *
All dysplastic nevi	1.6 *	8.5 (2.5–12.5) *+
Low-grade dysplastic nevi	1.8 *	8 (2.5–12.5) *+
High-grade dysplastic nevi	1.3 *+	8.8 (5–12.5) *+
Atypical Spitz nevi	1.2 *+	7.4 (2.5–12.5) *+
Atypical borderline tumors	0.9 *+	8.7 (2.5–20) *+
Melanomas	0.5 +	9.5 (2.5–27.5) +

* $p < 0.001$ compared to melanoma,

+ $p < 0.05$ compared to dermal nevi (t-test)

Table 3

Semi-Quantitative Analysis of Strongly Staining Cells and Nuclear Perimeter

Lesion	Mean proportion of strongly staining cells	Mean nuclear perimeter (range)
Dermal nevi	0.63 *	17.3 (15.9–18.8) *
All dysplastic nevi	0.36 *+	18.0 (15.3–23.0) *
Low-grade dysplastic nevi	0.47 *	17.8 (15.3–21.2) *
High-grade dysplastic nevi	0.27 +	18.2 (16.0–23.0) *
Atypical Spitz nevi	0.37 *+	19.8 (17.4–22.3) *+
Atypical borderline tumors	0.14 *+	19.8 (16.7–24.5) *+
Melanomas	0.01 +	22.6 (18.4–25.7) +

* $p < 0.05$ compared to melanoma,

+ $p < 0.05$ compared to dermal nevi (t-test)