Epigenetic Dysregulation in Cutaneous Malignant Melanoma: Advancing Approaches to Diagnosis, Prognosis, and Therapeutic Targeting

ABSTRACT

Evidence is rapidly accumulating that epigenetic mechanisms, including DNA methylation and demethylation, histone modifications, and non-coding RNAs, may play a central role in the pathobiology of melanoma. Previous studies have demonstrated the utility of the novel epigenetic mark, 5-hydroxymethylcytosine, to distinguish between benign cutaneous nevi and primary cutaneous melanoma. Further applications of this epigenetic biomarker to enhance diagnostic accuracy and prognostic precision in the evaluation of a unique set of diagnostically-challenging primary cutaneous melanomas and sentinel lymph node biopsies will be explored. In addition, clinically-applicable next-generation sequencing platforms enable us to identify novel gene mutations present in patient melanoma samples. Accordingly, the prevalence and nature of mutations in genes encoding epigenetic regulators (as well as non-epigenetic regulators) in patient melanoma samples will also be explored in this study.

Fifty-two histologic sections of primary cutaneous melanoma (N_P=52) were obtained from the pathology archives of two academic institutions. These cases were intentionally selected based on the presence of pseudomaturation (n_{MPM}=24) or associated pre-existing nevus (n_{MPEN}=28). Immunohistochemistry for 5-hydroxymethylcytosine was performed on all primary melanoma cases (N_P=52). In addition, a collection of twenty eight histologic sections of sentinel lymph node biopsies (N_S=28) containing either metastatic melanoma (n_{MM}=18) or nodal nevus (n_{NN}=10) was also retrieved from the pathology archive of one academic institution. Dual-labeling direct immunofluorescence and immunohistochemistry for MART-1/5-hydroxymethylcytosine was performed on all sentinel lymph node biopsy cases. Finally, targeted next generation sequencing was performed on thirty-eight patient melanoma specimens (N_M=38) to detect exonic mutations in 275 cancer genes, 41 of which encode known epigenetic regulators.

Collectively, regions containing pseudomaturing cells within primary melanomas with pseudomaturation demonstrated intermediate immunopositivity for 5-hydroxymethylcytosine, in stark contrast to the overlying melanoma, which showed complete, diffuse loss. The staining intensity in pseudomaturing regions was quantifiably distinct and intermediate to that of pre-existing nevi (strong, homogeneous positivity) and bona fide melanoma (complete, diffuse loss), providing further support to the hypothesis that pseudomaturing melanoma cells may reflect a more indolent subpopulation. 5-hydroxymethylcytosine immunoreactivity was strongly retained in 10 of 10 (100%) cases of nodal nevus but 'lost' in 18 of 18 (100%) of cases of metastatic melanoma, thus representing a useful adjunctive strategy to definitively diagnose histologically subtle micrometastases. Targeted next generation sequencing demonstrated that 20.2% of all somatic mutations (107 of 530) affected an epigenetic regulator, with 35 of 38 samples (92.1%) harboring at least one mutation in an epigenetic gene. Genes with the highest percentage of UVB-signature mutations encoded epigenetic regulators. In addition, MECOM, a novel, central epigenetic regulator, as well as TET2/IDH1, critical enzymatic and metabolic regulators of DNA 5-hydroxymethylation, were found to be more frequently mutated than previously described.

The present study provides direct genomic evidence that epigenetic regulators may be involved in the pathobiology of melanoma and that novel, personalized therapeutic targets may be revealed with next generation sequencing. In addition, our immunohistochemical investigations demonstrate that the epigenetic biomarker, 5-hydroxymethylcytosine, can enhance diagnostic and microstaging accuracy in the histopathologic evaluation of pseudomaturing primary cutaneous melanoma and refine prognostic evaluations by enabling the distinction of metastatic melanoma from its diagnostic mimic, nodal nevus, in sentinel lymph node biopsies.

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α-KG: alpha-ketoglutarate; **5-mC**: 5-methylcytosine; **5-hmC**: 5-hydroxymethylcytosine; **5-caC**: 5-carboxylcytosine; **A**: adenine; **AJCC**: American Joint Commission on Cancer; **ANOVA**: analysis of variance; **C**: cytosine; **CpG**: cytosine-(phosphodiester bond)-guanine; **DAPI**: 4',6-diamidino-2-phenylindole; **DIF**: direct immunofluorescence; **DNA**: deoxyribonucleic acid; **DNMT**: DNA methyltransferase; **G**: guanine **H&E**: hematoxylin and eosin; **HDAC**: histone deacetylase; **IDH**: gene encoding isocitrate dehydrogenase; **IHC**: immunohistochemistry; **FDA**: United States Food and Drug Administration; **FISH**: fluorescence in situ hybridization; **MECOM**: MDS1 and EV1 complex locus; **MPM**: melanoma with pseudomaturation; **MPEN**: melanoma arising in association with pre-existing nevus; **NGS**: next-generation sequencing; **RNA**: ribonucleic acid; **SLN**: sentinel lymph node; **T**: thymine; **TET**: ten-eleven translocase; **UVR**: ultraviolet radiation;

The 'black cancer': A historian's lens

The term 'melanoma' is derived from the Greek term *'melas'*, meaning 'dark', and *'oma'*, referring to 'tumor'. The first descriptions of melanoma appear in the writings of the Greek physician, Hippocrates of Kos (5th century B.C.), and again in 18th century European medical literature, wherein it was described, post-mortem, as "fatal black [tumors] with metastases and black fluid in the body" (1). Scottish surgeon John Hunter is accredited with being the first to perform surgery on a patient with melanoma, having then described it as a 'cancerous fungous excrescence' and, descriptively, as the 'black cancer'. He also noted that the tumor, whose identity would remain a mystery for many years, appeared grossly heterogeneous with at least "two distinct parts: one white in colour and firm to feel, and the other spongy and dark-black" (**Figure 1**) (2).

By the 19th century, fundamental features of melanomagenesis had begun to become understood through close clinical and pathologic observation. In 1820, general practitioner William Norris published one of the first, complete clinical descriptions documenting the natural history of melanoma, including the post-mortem findings of his patient who ultimately succumbed to metastatic disease. In his original publication, he described a case of a 59-year-old male, who had noticed a malignant change in an abdominal mole, and ultimately suffered from local recurrence and a violent course of widespread metastasis. Unbeknownst to this general practitioner and the medical community, this index case would provide one of the first documentations of the virulent potential of malignant melanoma (3). Also remarkable was that Dr. Norris, approximately one-half century prior to the publication of Mendel's seminal work on genetics in 1866, had raised suspicion that this malignancy may be, at least in part, hereditary (**Figure 2**).

By the 20th century, clues regarding the mechanisms of melanoma pathogenesis began to surface. In 1968, British plastic surgeon D. C. Bodenham published an empirical, observational study of 650 melanoma cases seen at one institution over a period of twenty years (2). Of interest, his publication proposed that the rising incidence of melanoma on the lower legs of females at that time may be related to the adornment of post-war era stockings, which "permit 75 per cent

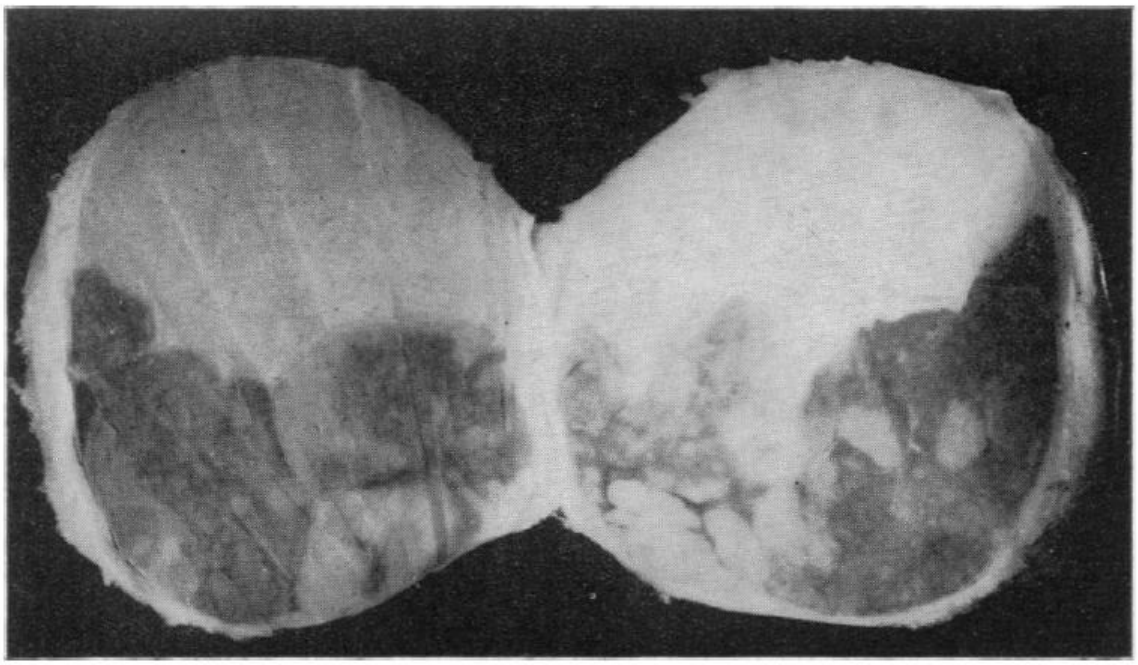


Figure 1. Original photograph of surgical resection specimen removed surgically by Dr. John Hunter. The official diagnosis of 'melanoma' was established centuries later by microscopy of the original, preserved specimen. This specimen is remains on display at the Hunterian Museum at the Royal College of Surgeons of England. Reprinted with permission (Royal College of Surgeons of England) from Bodenham (1968) Ann R Coll Surg Engl (2).

Case of Fungoid Disease. By WILLIAM NORRIS, M. D. Stourbridge.

death took place. This tumour, I have remarked, originated in a mole, and it will be worth mentioning, that not only my patient and his children had many moles on various parts of their bodies, but also his own father and brothers had many of them. The youngest son has one of these marks exactly in the same place where the disease in his father first manifested itself. These facts, together with a case that has come under my notice, rather similar, would incline me to believe that this disease is hereditary.

Figure 2. Photographic excerpt taken directly from Dr. Norris' original publication (1820, Edin J Med Surg) (3). In this original publication, Dr. Norris raised suspicion that this fatal malignancy, whose true biological identity was unknown at this time ('Case of Fungoid Disease'), might be, at least in part, hereditary.

[sic] ultra-violet light to reach skin, whereas pre-war stockings offered a high level of protection" (2). Moreover, he also prophetically expressed hope for therapeutic efficacy via 'immuno-defence' mechanisms against melanoma[i] (2). Perhaps most poignantly, however, melanoma had by this time achieved a notorious reputation for having "varied," "mysterious," and "unpredictable" behavior, gaining infamy for "the **speed** with which it [could] sometimes kill" (2). Most certainly, melanoma's historical notoriety still echoes, centuries later, into the present day.

Within the gates of Harvard University, great tributes must be paid to more recent insights into human malignant melanoma, including the landmark contributions of Thomas B. Fitzpatrick, for his detailed clinical descriptions of melanoma and for revealing the biological impact of ultraviolet radiation (UVR) on the melanocyte and its physiologic response (4, 5); Wallace H. Clark, Jr., for his classification of distinct stages of microscopic depth and invasion for use during the histopathologic evaluation of melanoma and their correlation with prognosis (6); Martin C. Mihm for advancing the concepts of the radial and vertical growth phase as well as for unveiling the many clinicopathologically-distinct subtypes of melanoma (7); Arthur J. Sober for advancing clinical management and prognostication strategies for late-stage melanomas (8); George F. Murphy for furthering the concept of pathobiologically-relevant melanoma stem cells (9) and refining our understanding of 'borderline' melanocytic tumors and the ever-challenging practice of evaluating such lesions histopathologically; and many others. Above all, the training, mentorship, and guidance provided by these individuals have and will continue to provide the foundations for future discoveries and advancements made by current and future generations to come.

The 'modern black plague': Mechanistic insight from the 21st century

Since this seminal body of observations and academic achievements, significant current advancements in our understanding of melanoma pathogenesis are being made. Today, it is well understood that melanoma arises from the malignant transformation of the melanocyte, the neural crest-derived, pigment-producing cell present in the skin, eye, squamous mucosal epithelia, and meninges. Known risk factors for developing melanoma are genetic and environmental in nature, including a personal or family history of melanoma, bearing multiple atypical nevi, fair skin, immunosuppression, and exposure to UVR. The strongest risk factor

[i] See discussion on 'spontaneous regression' and 'some observations on factors concerning prognosis' (2).

implicated by epidemiologic studies is intense, intermittent UVR exposure, such as that accrued through tanning bed use, which has been shown to confer a dose-dependent increased melanoma risk (10, 11). A recent meta-analysis also demonstrated an increased risk of melanoma in airline flight and cabin crew, which is hypothesized to be related to higher levels of exposure to cosmic and UVR at high elevations (12).

A fraction of melanoma cases can be attributed to a genetic and/or hereditary predisposition. Clark et al. (1978) provided the first detailed documentation of what was then termed the 'B-K Mole Syndrome', named after the first letters of the names of the two probands, who had seven primary cutaneous melanomas between the two of them and extensive family histories of 'atypical' moles as well as melanoma (13). This hereditary melanoma predisposition syndrome, now known as the 'Familial atypical mole syndrome', is caused by germline mutations in the gene encoding cyclin-dependent kinase inhibitor 2A (CDKN2A) (14). This germline mutation is involved in approximately 8.5% of patients with multiple, sporadic primary melanomas and up to 14.1% of familial melanoma cases (15). Members of this syndrome are also known to have a predisposition for developing pancreatic malignancies and may also be at increased risk of lung and breast carcinomas (15).

Additional hereditary melanoma predisposition syndromes have been described, including the 'COMMON' familial syndrome/complex[ii]. This syndrome, typified most uniquely by the increased risk of metastatic uveal melanoma, mesothelioma, as well as characteristic nevoid melanoma-like melanocytic proliferations (NEMMPs), has been shown to be due to inactivating germline mutations in the BRCA1-associated protein (BAP-1) gene (16). BAP-1 encodes a tumor-suppressive deubiqutinating enzyme shown to interact with critical components of the epigenetic machinery, including the polycomb group repressive deubiquitnase complex, known to be involved physiologically in stem cell pluripotency and other critical developmental processes (17).

In 2002, genomic sequencing technologies allowed for the identification of frequent point mutations in BRAF, leading to valine-to-glutamic acid substitutions at codon 600 (BRAF V600E), at higher frequencies in melanoma than in other cancers (18). This finding would ultimately provide the basis for the timely development of a targeted inhibitor (Vemurafenib – 'V600E RAF inhibition), which as we ultimately learned, would unfortunately yield limited long-term

[ii] The name 'COMMON' symbolizes the heritable predisposition to cutaneous and ocular melanomas, characteristic melanocytic proliferations, and other internal neoplasms (16).

success when used alone, in large part due to the melanoma's ability to develop drug resistance (19). Accordingly, not unlike the combination therapy approach taken to prevent drug resistance in tuberculosis or to most effectively treat HIV infection, it has become clear that a 'cocktail' of targeted therapies may be required to achieve sustained therapeutic benefits in melanoma (20).

Closer examination of the initial study reporting frequent BRAF V600E mutations in melanoma, however, reveals that the control group for this study was composed of 'matched lymphoblastoid cell lines from the same individuals' – and, notably, not normal or non-malignant tissue controls (18). Furthermore, Pollock et al. (2003) would shortly thereafter report their discovery that benign dermal/compound as well as dysplastic nevi would harbor the very same BRAF V600E mutation at comparable and often higher frequencies than that found in melanoma (21). Indeed, whether therapeutically targeting an 'oncoprotein' present at similar frequencies in both malignant and non-malignant counterparts could provide sustained efficacy has yet to be realized. Moreover, these observations reinforce the need to examine and aggressively explore additional mechanisms that may be involved in malignant melanocytic transformation and melanoma progression.

Genomic sequencing technologies and personalized medicine: Advancing discoveries in melanoma pathobiology

In more recent years, next-generation sequencing technologies have enabled both targeted and whole genome sequencing of individual patient's cancers, including melanoma. These technological breakthroughs have helped to uncover novel pathogenic mechanisms and identify potential therapeutic targets, while at the same time laying the groundwork for personalizing current and future cancer treatments (22). However, these approaches also have their limitations, as highlighted by Lawrence et al. (2014), who point out that the elevated mutation rate in melanoma (which has a greater number of mutations than any other cancer type) reduces the statistical power to detect true 'driver' (as opposed to 'passenger') mutations (23). Thus, an estimated 5,300 melanoma samples are required to create a catalog of melanoma oncogenes/tumor suppressors mutated in at least 2% of patients, reinforcing the importance of inter-institutional collaborations to facilitate this level of resolution (23).

Epigenetic alterations in melanoma: A new frontier in cancer pathobiology

The mountains of genomic mutation data provided by present-day sequencing technologies present a wealth of information that, in aggregate, can seem overwhelming. However, using such data in a focused, 'targeted' fashion may shed light on the role of 'epigenetic' mechanisms in melanoma pathogenesis. Indeed, while enormous resources have been invested into our understanding of human and cancer genetics, variations in DNA sequences (or lack thereof) alone cannot explain certain fundamental biological observations. One, for instance, highlighted in the early 1900's by English developmental biologist Conrad Waddington (held by most to be the 'father' of epigenetics, **Figure 3**), is how specialized cells, such as fibroblasts and lymphocytes, stably maintain their distinct phenotypes throughout generations of cell divisions, despite sharing identical genotypes within one individual (24).

The past decade has witnessed an explosion of evidence implicating dysregulated epigenetic mechanisms, including DNA methylation/demethylation, histone modification, and non-coding RNAs (i.e. micro RNAs), in the pathogenesis of melanoma[iii] (25). This rapidly growing body of research also points to specific epigenetic mechanisms that may enable malignant, stem-like behavior, which is, arguably, one of the most challenging aspects of treating melanoma (25). In addition, our more detailed understanding of epigenetic mechanisms (DNA de-methylation, in particular) has enabled the discovery of epigenetic biomarkers that, as will be discussed, may potentially help enhance diagnostic and prognostic accuracy during the histopathologic evaluation of primary cutaneous melanoma and sentinel lymph node biopsies.

While many of the techniques required to study the basic biochemical and molecular aspects of epigenetics are challenging and in their infancy, next-generation sequencing technologies have already proven useful in detecting mutations in key epigenetic regulators in cancers such as melanoma. This approach was recently utilized to investigate mutations in epigenetic regulators in pediatric lymphomas and leukemias, revealing several novel epigenetic regulators of potential therapeutic and pathobiological interest (26). A similar strategy has also been undertaken for a component of this research thesis, [3] wherein targeted next generation sequencing data of a collection of patient melanoma samples is analyzed. As will be illustrated, this approach may shed light on novel pathogenic mechanisms and potential therapeutic targets

[iii] Please see Lee et al. 2014 (25) for comprehensive, up-to-date review of the evidence implicating epigenetic dysregulation in cutaneous melanoma.

in melanoma. Of note, the only FDA-approved therapies targeting epigenetic regulators to date are DNA methyltransferase (DNMT) inhibitors for the treatment of myelodysplastic syndromes and histone deacetylase (HDAC) inhibitors for cutaneous T-cell lymphoma (25).

DNA demethylation and 5-hydroxymethylcytosine: Epigenetic fidelity unraveled

It has been known for decades that the methylation mark on DNA is established by the enzymatic action of DNA methyltransferases (DNMTs) on cytosine residues (forming 5-methylcytosine, 5-mC, otherwise known as the 'fifth base'), particularly those preceding guanine (termed 'CpG', wherein 'p' signifies the inter-linking phosphodiester bond) (25). Regions enriched with CpG repeats, termed 'CpG islands', are present throughout the genome, although they most densely populate regions just upstream of gene promoters. There, and by convention, this covalent epigenetic mark encodes for and secures a transcriptionally repressed chromatin state. The timely addition and removal of this mark is critical to shaping the dynamic processes of cellular differentiation and programming during development in response to a changing microenvironment (27). In addition, 5-mC contributes to long-term gene silencing, as in the context of genomic imprinting, X-chromosome inactivation, and suppression of mobile genetic elements (28). Moreover, 5-mC is a heritable mark, thus enabling the sustained replication of differentiated cell types (27).

In contrast to DNA methylation, the reverse reaction (DNA de-methylation) is a process for which a mechanism has only recently been elucidated. A watershed moment in our understanding of DNA demethylation came in 2009 with the discovery of the ten-eleven translocase (TET) family (TET1, 2, and 3) enzymes[iv] (29-31). Experimental investigation revealed that the TET family enzymes function as Fe(II)/α-ketoglutarate-dependent oxygenase enzymes and perform a series of sequential, iterative oxidations on the methyl group of 5-mC, to form 5-hydroxymethylcytosine, the key intermediate and additional oxidative derivatives (**Figure 4**) (27, 30-32). The final derivative in this active DNA demethylation pathway (5-carboxylcytosine, 5-caC) may be excised by thymine DNA glycosylase and base excision repair, ultimately yielding cytosine from 5-methylcytosine (31). Prior to these discoveries, TET1 had

[iv] This discovery was made possible by the bioinformatics technology available today and the previous report of a novel, covalently modified form of the base uracil (glucosylated 5-hydroxymethyluracil, known as 'Base J') in Trypanosoma brucei (parasite responsible for African sleeping sickness) and an enzymatic paralogue of TET (JBP1 and JBP2) that had been known to oxidize the methyl group of 5-methyluracil (29).

already been suspect, owing to its known fusion to MLL (also known as KMT2A, which encodes a histone-lysine N-methyltransferase) in patients with acute myeloid leukemia (32, 33). In addition, TET2 mutations were soon found to be frequent events in myeloid lineage malignancies (34, 35).

'Loss' of 5-hydroxymethylcytosine: From bench to bedside

Three years after these landmark discoveries, Christine Lian et al. (2012) at the Brigham and Women's Hospital demonstrated that benign cutaneous nevi retained high levels of nuclear 5-hydroxymethylcytosine (5-hmC) immunopositivity whereas cutaneous melanomas demonstrated loss (36). In addition, it was observed that restoring the '5-hmC landscape' via TET2 overexpression (mouse-human melanoma xenograft) or IDH2 overexpression (zebrafish model) resulted in more indolent, less invasive melanomas; and, conversely, that those melanomas with the most profound loss of 5-hmC were associated with the worst clinical outcomes (36). As TET is an α-ketoglutarate (α-KG)-dependent enzyme, IDH overexpression is thought to enhance its function, whereas dysfunctional or mutant IDH (1 or 2) has been shown to increase oncometabolite (2-oxo-glutarate) production, which competitively inhibits the α-KG binding site on TET (37).

Since these discoveries, the 'loss of 5-hmC' has also been documented in malignancies of other organ systems, as has been its retention in corresponding normal, differentiated cells. For this reason, as well as based on additional evidence supporting its critical tumor suppressive function and the putative fidelity and/or editing capability made possible through DNA demethylation, TET has been regarded as a putative 'guardian of the epigenome' (25). Interestingly, in normal human epidermis, basal keratinocytes demonstrate weak positivity for 5-hmC, whereas keratinocyte nuclei progressively regain 5-hmC with increasing differentiation. A similar pattern of staining is seen in intestinal mucosal epithelium, suggesting that the absence of 5-hmC physiologically could be indicative of a state of replicative immortality and/or stem-like phenotype (38).

Figure 3. Photograph of English developmental biologist Dr. Conrad Waddington, who is regarded by most to be the 'Father of Epigenetics'. Image publically available via: http://www.che.ac.uk/what-we-do/conrad-waddington/

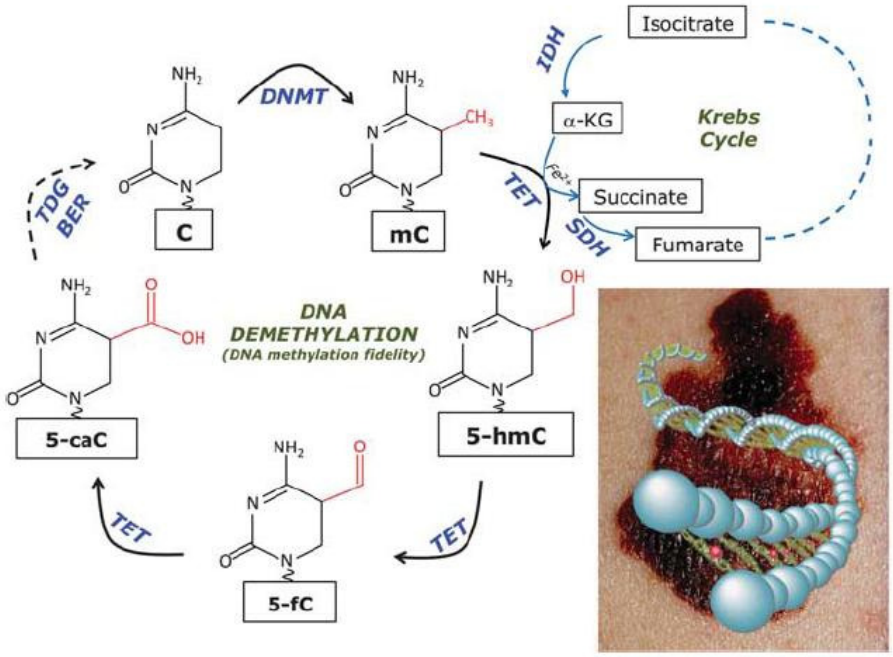


Figure 4. Active DNA demethlyation pathway. This pathway enables the removal of the methyl group from 5-methylcytosine through sequential, iterative oxidation of this methyl group by the Ten Eleven Translocase (TET) family enzymes, which requires Krebs cycle intermediate alpha-ketoglutarate (α-KG) as a co-factor. The 'loss' of the kinetically most stable intermediate, 5-hydroxymethylcytosine (5-hmC) is an epigenetic hallmark of melanoma (pictorially rendered, lower right).

Melanoma in focus: A troublesome diagnosis

While recent advancements and sophistication in our understanding of melanoma pathobiology and epigenetics provide reason for cautious optimism, melanoma remains among the most aggressive, treatment-resistant human cancers. In 2014, over 76,000 new melanoma cases and nearly 10,000 melanoma-related deaths are expected in the United States alone (39). While the detection of cutaneous melanoma is one of the professed duties of the clinical dermatologist (40) and has spawned subspecialization focused exclusively on pigmented lesions (41), all practitioners[v] and health care providers, as well as patients themselves (42), bear responsibility for recognizing this aggressive malignancy, particularly while in its early evolutionary stages, in order to insure appropriate, timely care.

While a blend of dermatologists, plastic surgeons, and general practitioners perform the initial diagnostic biopsy or therapeutic excision of suspicious pigmented lesions, the definitive diagnosis of melanoma and its many variants ultimately depends upon the careful histologic and microscopic evaluation by the pathologist. Dermatopathology, a subspecialty of both dermatology and pathology, and arguably one of the most academically challenging areas of practice within these two fields, is principally responsible for the pathologic evaluation of lesions clinically suspicious for melanoma at most academic medical centers and at a growing number of healthcare facilities in the community.

During the histopathologic evaluation of a melanocytic lesion, a mosaic of microscopic features and clues[vi] must be considered in order to arrive at the correct diagnosis. Distinguishing malignant melanoma and its many variants from benign mimics may be vexing and can even generate controversy amongst the most skilled dermatopathologists (43). Because incorrect assessments of such challenging diagnoses are often discovered through bad outcomes, it is not surprising that the most common reason for medical malpractice litigation in one study surveying surgical pathology cases from 1988 to 2005 was the alleged missed biopsy diagnosis of melanoma (44).

The introduction of functionally-significant epigenetic biomarkers (i.e. 5-hydroxymethylcytosine) to aid in making clinically and pathologically relevant histologic

[v] Particularly those practicing primary care, ophthalmology, plastic surgery, or gynecology, who are poised to detect melanoma in one of its many, anatomically and biologically unique forms.

[vi] Histopathologic features considered in the diagnosis of primary cutaneous melanoma include, and are not limited to: architecture, cytomorphology, the presence or absence of maturation, pagetoid spread, and/or continuous, contiguous replacement of the basal layer by atypical melanocytes, among others.

distinctions, as will be discussed, may enhance the accuracy and precision with which diagnoses are established. Two challenging areas in the pathologic evaluation of melanoma – one related to diagnosis and microstaging of primary lesions; the other related to evaluating for metastases, staging, and prognosis, will be examined. With regard to the former, [1] the role of immunohistochemical staining for 5-hydroxymethylcytosine in the evaluation of primary cutaneous melanomas demonstrating pseudomaturation or arising in association with a pre-existing nevus will be addressed. For the latter, [2] the diagnostic utility of immunohistochemical staining for 5-hydroxymethylcytosine during the evaluation of sentinel lymph node biopsies to distinguish between metastatic melanoma and nodal nevus deposits will also be explored.

Melanoma pseudomaturation and the pre-existing nevus: The challenge of histologically heterogeneous, malignant melanocytic lesions

The term ‘maturation’, in the context of a benign melanocytic lesion, generally refers to the gradual morphological changes observed as melanocytes involve progressively deeper layers of the dermis. In the most superficial dermis, melanocytic nevi are typically composed of cohesive nests of ‘type A’ cells, which are round to epithelioid in shape and often melanized. At intermediate dermal depths, nevic architecture and cytomorphology shifts towards smaller nests and cords of ‘type B’ cells, which have a smaller diameter and are generally devoid of melanin. Some nevus cells, when present within the deepest portions of the reticular dermis, differentiate into ‘type C’ cells, which have a spindled, fibroblast-like, neuroid or ‘schwannian’ appearance, occasionally forming structures that resemble Meissner’s tactile corpuscles (45). These morphologic changes likely reflect unique stages of melanocytic differentiation, governed by distinct genetic and/or epigenetic programs, and is emblematic of the unique biological and phenotypic ‘plasticity’ with which the neural crest-derived melanocyte has been naturally endowed. Of interest, some authors have proposed that nevus cell maturation is, in part, the result of losing interactions with keratinocytes when in their normal, physiologic microanatomic position and the associated changes to their microenvironment as the melanocytes progress deeper into the collagenous dermis (46).

When encountered in a melanocytic lesion, maturation typically signifies benignity. However, similar morphologic changes can also be seen in up to 8% of primary cutaneous melanomas (47). In this context, the terms ‘paradoxical maturation’, ‘maturation’, and

'pseudomaturation' have been used to refer to this phenomenon (47-49). Ruhoy et al. (2000) studied the largest cohort to date of 17 primary invasive melanomas and two cases of epidermotropic metastatic melanoma, each of which demonstrated pseudomaturation in the deepest component of the lesion (47). These investigators discovered that there was a statistically significant reduction in Ki-67, glycoprotein (gp)100, HMB-45, and tyrosinase levels in the 'pseudomaturing' areas, when compared to the deepest areas of conventional primary melanomas without pseudomaturation (47). Based on their findings, they proposed that pseudomaturation may reflect a less virulent state, at least within the pseudomaturing subpopulation of melanoma cells (47).

The clinical, pathologic, and prognostic significance of melanoma pseudomaturation remains incompletely understood. One specific area of practical diagnostic interest is in the potential impact of pseudomaturation on the interpretation and measurement of the Breslow's depth, which is recorded from the top of the epidermal granular cell layer to the deepest melanoma cell and is a major predictor of clinical outcome. When smaller, more nevoid yet potentially malignant cells that are cytologically distinct from the overlying overt melanoma cells exist in the deepest aspects of a lesion, it may be exceedingly difficult to differentiate between melanoma pseudomaturation versus concurrent benign nevus. Moreover, how such lesions are microstaged can vary amongst pathologists, resulting in inconsistencies in establishing critical prognostic parameters (i.e. Breslow's depth) and related clinical management. Further complicating this situation is that melanomas arising in association with a pre-existing nevus are also encountered in up to 30% of all primary cutaneous melanomas (50, 51).

Larson et al. (2014) recently demonstrated that benign nevi homogenously express high levels of nuclear staining for the epigenetic mark, 5-hydroxymethylcytosine (5-hmC), whereas dysplastic nevi and primary cutaneous melanomas exhibit partial to complete loss, respectively (36, 52). The loss of 5-hmC is thought to reflect a deficiency in Ten-Eleven Translocase (TET) family of active DNA de-methylation enzymes, as discussed above. Accordingly, the potential for 5-hmC immunoreactivity to uniquely distinguish between benign and malignant melanocytic proliferations makes it a useful, functionally-significant epigenetic biomarker to explore the pathobiologic significance of pseudomaturation in cutaneous melanoma.

Herein, [1] a unique cohort of primary cutaneous melanomas with pseudomaturation (MPM) has been obtained and 5-hmC immunoreactivity within these melanomas will be

explored. For comparison, additional cases of melanomas arising in association with pre-existing nevi (MPEN) will also be studied and examined.

Nodal nevus: A diagnostic pitfall of the sentinel lymph node evaluation

Melanoma patients meeting a defined threshold of histopathologic attributes in their primary cutaneous melanoma[vii] undergo sentinel lymph node biopsy in accordance with the American Joint Commission on Cancer (AJCC) guidelines (53). The implications of an accurate sentinel lymph node evaluation are not trivial, as completion lymphadenectomy, a procedure with significant potential for morbidity, customarily follows a positive sentinel lymph node diagnosis. Moreover, the sentinel lymph node status is the most important predictor of disease free survival in clinically node-negative melanoma patients (53). During the evaluation of sentinel lymph node biopsies, however, benign nodal nevi (also termed 'nodal melanocytic nevi', 'intranodal nevus', 'nevic rest', 'nevus cell aggregates', 'lymph-node nevus') can be encountered in up to 22% of lymphadenectomy specimens and represent one of the major diagnostic pitfalls in recognition of nodal metastases (54).

There are two prevailing theories to explain the origin of nodal nevi. The first, termed 'embolic transfer' (also referred to as 'mechanical transport' or 'benign metastasis'), proposes that melanocytes of benign cutaneous nevi gain entry into the lymphatics and are 'embolically' transferred to draining lymph nodes (54). Several observations support this theory. Firstly, the presence of nodal nevi has been shown to associate tightly with the presence of cutaneous nevi, particularly those in intimate spatial relationship with adnexal, neural, and lymphovascular structures in corresponding anatomic catchment areas (55). In addition, patients with nodal nevi are more likely to have had a primary cutaneous melanoma arising in association with a pre-existing nevus than those without nodal nevi (54). This observation has led some to hypothesize that benign nevic cells may be 'displaced' by their adjacent melanoma and, thereby, gain entry into the lymphatics (54).

The second theory suggests that nodal nevi arise from the arrested migration of neural crest progenitor cells during embryologic development (55). Nodal nevi have been found in lymph nodes draining anatomic regions devoid of cutaneous nevi, which may support this

[vii] Primary cutaneous melanomas bearing pathologic features including depth $\geq$ 1.0 mm, $\geq$ 1 mitosis/mm^2, or presence of ulceration, warrant sentinel lymph node biopsy, according to the most recent AJCC guidelines.

hypothesis, although it is known that the natural history of some common nevi is to undergo senescence and ultimately disappear (54). In addition, lymph nodes are known to contain microscopic collections of normal epithelial cell from the tissues they drain, such as that of the breast, salivary gland, mesothelium, thyroid, and urothelium, among others (56), arguing, by analogy, that mechanisms other than arrested migration during development may be responsible for the presence of nodal nevi. Recent studies demonstrate that the overall five-year survival for patients with nodal nevi does not differ significantly from that of patients with negative sentinel lymph nodes (57). In contrast, positivity for metastatic melanoma is an ominous and powerful predictor of disease progression and overall survival (53). Thus, distinguishing between nodal nevi and metastatic melanoma is most certainly a matter of clinical, diagnostic, and prognostic import.

Despite conventional dogma maintaining that nodal nevi reside within the lymph node capsule or trabeculae whereas metastatic melanomas are subcapsular, sinusoidal, or intraparenchymal (54), such microanatomic guidelines all too often break down and are insufficient for making a definitive distinction in routine practice. Cytomorphology may also be useful, but paradoxical mimics, such as the cellular enlargement of nevus cells due to activation or, conversely, 'small cell' differentiation of melanoma cells may limit the use of this parameter (58). In addition, routine immunohistochemical evaluation with melanocytic markers (i.e. S-100, MART-1, Melan-A, and SOX-10) cannot distinguish between the benign nodal nevus and metastatic melanoma, nor can HMB-45, which is also expressed in a substantial subset of nodal nevi (59).

The challenge of distinguishing benign nodal nevi from metastatic melanoma in sentinel lymph node biopsies has been extensively discussed in the literature (56, 60, 61), and a number of strategies have been proposed to aid in making this distinction. It has been suggested that immunohistochemical detection of p16, a component of the CDKN2A tumor suppressor gene involved in negative cell cycle regulation and known to be lost in familial melanoma, may be useful in distinguishing between nodal nevi and metastatic melanoma in sentinel lymph node biopsies (62). However, more recent studies demonstrate that p16 does not consistently differentiate between certain primary cutaneous melanomas subtypes and benign nevi, and for this reason, may not be a reliable or practical marker in routine practice (63). The use of fluorescence in situ hybridization (FISH) to detect the presence of certain chromosomal aberrations has also been reported to distinguish metastatic melanoma from nodal nevi (64). The

routine use of FISH in this context, however, has its own practical and economic limitations. Chen et al. (2013) have recently shown that the combined use of immunohistochemistry for the neural crest embryonic stem cell transcription factor, SOX2, and the intermediate filament protein, nestin, has predictive value in differentiating nevi from melanoma in sentinel lymph nodes (58). These efforts underscore the feasibility and importance of continued and aggressive exploration of functionally-relevant biomarkers that will be useful adjuncts in sentinel lymph node evaluation. Accordingly, herein and in addition to applications related to pseudomaturation, as outlined above, [2] the clinical application of 5-hmC to aid in the distinction between nodal nevi and metastatic melanoma in sentinel lymph node biopsies will be explored.

OBJECTIVES

The overall goals of this research endeavor are three-fold: [1] to investigate patterns of 5-hydroxymethylcytosine immunoreactivity in primary cutaneous melanomas demonstrating pseudomaturation as well as in those arising in association with pre-existing nevi; [2] to explore the diagnostic utility of 5-hydroxymethylcytosine (5-hmC) in distinguishing nodal nevus from metastatic melanoma in sentinel lymph node biopsies; and [3] to characterize the frequency and nature of mutations in epigenetic regulators in human melanoma specimens, obtained through the Dana-Farber Cancer Institute's Melanoma Program and the Brigham and Women's Hospital's Center for Advanced Molecular Diagnostics 'Oncopanel' Program.

MATERIALS AND METHODS

Ethics statement

Each component of this study was approved by the Institutional Review Board of the Brigham and Women's Hospital (Boston, MA), Dana Farber Cancer-Institute (Boston, MA), and/or the Royal Surrey County Hospital (Surrey, UK). In addition, informed consent was obtained from patients being evaluated and managed for primary or metastatic melanoma at the Dana Farber Cancer-Institute for targeted next-generation sequencing of their melanoma samples.

Acquisition of histopathologic samples for immunohistochemical study

A total of 52 cases of primary cutaneous melanomas (N_P=52) were retrieved from the pathology archives (2004-2014) of the Brigham and Women's Hospital and Royal Surrey County Hospital (Surrey, UK) via a collaboration with the European Organisation for Research and Treatment of Cancer (EORTC)[viii]. The cases were intentionally selected based on the initial diagnosis reporting either 'pseudomaturation/maturation' (MPM, n_{MPM}=24) and/or 'arising in association with a pre-existing nevus' (MPEN, n_{MPEN}=28). Detailed histopathologic data of each of the primary melanomas, including synoptic melanoma diagnostic/prognostic information, such as depth, ulceration, and mitotic rate, were obtained for each case. H&E-stained sections, prior diagnoses, and prognostic features were independently reviewed and confirmed by two study dermatopathologists (CGL, GFM).

In addition, separate sentinel lymph node (SLN) biopsy cases were retrieved from the archives of the Brigham and Women's Hospital Department of Pathology (2011-2014). A total of 28 sentinel lymph node biopsy cases (N_S=28) containing either histologically-confirmed metastatic melanoma (n_{MM}=18) or nodal nevi (n_{NN}=10) were obtained. There were no SLN cases in which one lymph node contained metastatic melanoma and another contained a nodal nevus. In addition, two 'equivocal' sentinel lymph node cases, whose diagnoses were debated, were also included in the study. One case (Case 1) featured scattered intraparenchymal MART-1 positive cells that contained minimally atypical nuclei that were only slightly larger than those of lymphocytes and with minimal nuclear atypia. The second case (Case 2) contained MART-1

[viii] Collaborations with the EORTC were facilitated by Dr. Martin C. Mihm, Jr. and Dr. Martin Cook.

positive cells predominantly within the capsule with regions consistent with involvement of an intracapsular angiolympatic space; these cells contained enlarged, somewhat atypical nuclei. Both cases resulted in extensive discussion among the dermatopathologists, with a consensus opinion favoring metastatic melanoma in both. Original H&E and routine S-100 and MART-1-stained sections were also obtained. These were independently reviewed and the prior diagnoses were also confirmed independently by two dermatopathologists (GFM and CGL). In addition, histopathologic data of the sentinel lymph node biopsies, including features such as the anatomic site of the biopsy, the microanatomic location and size of the focus, pattern of spread, among others were also recorded.

Immunohistochemistry protocol, clinical data acquisition, and quantitative analysis of primary cutaneous melanomas

Immunohistochemistry for 5-hydroxymethylcytosine (5-hmC) was performed on all cases of primary melanomas (N_P=52) with pseudomaturation (MPM) (n_{MPM}=24) or pre-existing nevus (MPEN) (n_{MPEN}=28) in accordance with Lian et al. (7). Sections were incubated overnight with rabbit-anti-5-hmC (Active Motif, Carlsbad, CA; 1:5,000 dilution), washed, and subsequently incubated with a peroxidase-linked anti-rabbit IgG (Vector Laboratories, Burlingame, CA; 1:200 dilution). The sections were then treated with the corresponding hydrogen peroxide substrate kit (Vector Laboratories, Burlingame, CA) and counterstained in hematoxylin and clarifying solution (Fisher Scientific Company, Kalamazoo, MI). Appropriate isotype-matched antibody controls and tissue controls were employed for all experiments.

5-hmC staining was scored based on previously published methodology (8). In brief, immunoreactivity was quantified based on the nuclear staining intensity (0-4; 0 = absent; 4 = dark brown reactivity involving the entire nuclear profile, and 1 through 3 representing semiquantitative intermediates [1 = faint tan; 2 = light brown; 3 = medium brown]. In addition, the percentage of 5-hmC-positive cells of melanocytic lineage, as assessed over representative 1-mm^2 fields, was also determined. Fields were selected based on the presence of key histologic features relevant to the study (i.e. pseudomaturation, pre-existing nevus, melanoma compartment), as assessed and determined by H&E examination alone. A total of five randomly selected but representative fields were examined in each histologic section. All semiquantitative immunoreactivity scoring was performed by one investigator (JJL) and a random subset of MPM

(n_R = 10) and MPEN (n_R = 12) cases was reviewed by a second investigator (CGL) to ensure concordance. Reviewers were blinded to the diagnosis of each case prior to their evaluation.

Clinical data was obtained for each case, when available. Clinical and histopathologic data between MPM and MPEN cases were compared using two-sample t-tests using StatPlus:Mac version 5.8.2.0 (AnalySoft, London, United Kingdom). Immunohistochemical staining scores were compared between specific foci of interest (i.e. melanoma, pre-existing nevus, melanoma pseudomaturation, etc.) by performing one-way ANOVA analysis using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA). All p-values were two-sided, with a p-value < 0.05 considered statistically significant.

Immunohistochemistry protocol, clinical data acquisition, and analysis of sentinel lymph node biopsy cases

Sentinel lymph node biopsy sections (N_S=28) were incubated overnight with a mixture of rabbit-anti-5-hmC (Active Motif, Carlsbad, CA) and mouse-anti-MART-1 antibodies (Covance, Princeton, New Jersey). The sections were washed and subsequently incubated with a mixture of secondary antibodies, including alkaline phosphatase-linked anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and peroxidase-linked anti-mouse IgG (Vector Laboratories, Burlingame, CA). The sections were then treated with their corresponding substrate kits (Vector Laboratories, Burlingame, CA). In addition, dual-labeling immunofluorescence was performed to complement immunohistochemistry as a means of two-channel identification of epitopes with nuclear staining of 5-hmC and membranous staining for MART-1. Instead of incubation with the secondary antibodies, these sections were incubated with a mixture of goat anti-rabbit IgG (Alexa Fluor, Grand Island, NY) and goat anti-mouse IgG (Alexa Fluor, Grand Island, NY). Appropriate isotype-matched antibody controls and tissue controls were employed for all experiments.

In addition, corresponding clinical information and histopathologic data of the patient's primary melanomas were also obtained for each sentinel lymph node case. Variables such as the patient's age, gender, location of primary, depth of primary melanoma (compiled based on initial biopsy and updated with re-excision data), mitotic rate, presence of ulceration, and the subsequent development of metastasis, among others were recorded.

The resulting quantitative and categorical data were analyzed using MedCalc version 13.2 (MedCalc Software, Ostend, Belgium). The primary melanoma clinical and histopathologic

data as well as the sentinel lymph node histopathologic data were sorted into two categories, based on whether they were associated with a nodal nevus or with a metastatic melanoma. Quantitative variables (i.e. age, Breslow's depth, # of foci within the sentinel lymph node, etc.) were compared between the two groups using a two-sample t-test; categorical variables were compiled into proportions (i.e. proportion of cases with ulceration in primary lesion, proportion of cases of that were intracapsular) and compared between the two groups using a two-sample z-test. All p-values were two-sided, with a p-value < 0.05 considered statistically significant.

Acquisition of patient melanoma tissue samples for next-generation sequencing and corresponding clinical data

Melanoma samples for next-generation sequencing (through 'Oncopanel', Center for Advanced Molecular Diagnostics Laboratory, Brigham and Women's Hospital) were obtained from patients with melanoma referred to the Dana-Farber Cancer Institute for evaluation and management. These patients were offered an opportunity to participate in this study. Informed consent was obtained to retrieve a sample of their tumor for our research. In order for a patient's data to be included in this study, the patient must not have previously received chemotherapy or radiation for the treatment.

In addition, for each case, patient clinical and demographic data (age, gender, primary versus metastatic, etc.) as well as detailed histopathologic data for each of the primary melanomas, including synoptic melanoma diagnostic/prognostic information, such as depth, ulceration, and mitotic rate, were obtained.

DNA isolation and next-generation sequencing

DNA was isolated from formalin-fixed melanoma tissue using standard methods. Samples were incubated in proteinase K overnight, followed by subsequent purification of the DNA (QIAamp DNA Mini Kit, QIAGEN, Gaithersburg, MD). DNA concentration was assessed using PicoGreen dsDNA detection (Life Technologies, Carlsbad, CA). Targeted next generation sequencing (NGS) was performed using a cancer genomic assay to detect mutations in the exonic regions of 275 cancer genes previously implicated in tumorigenesis and 91 intronic regions across 30 of the 275 genes (**Supplement 1**). The selection of this panel of genes was based on

desire to obtain mutational data on both well-known, established oncogenes as well as those that are primarily investigative. The complete coding sequence of the target genes was captured using a solution-phase Agilent SureSelect hybrid capture kit (AgilentTechnologies, Inc., Santa Clara, CA) and massively parallel sequencing was performed on an Illumina HiSeq 2500 sequencer (Illumina, Inc. San Diego, CA). Mutation calls were performed using Mutect and GATK software (Broad Institute, Cambridge, MA). While copy number variations were also obtained through this assay, this data was not available for analysis.

Data analysis was performed using an internally-developed bioinformatics Pipeline (Riker, REF) that was composed of reconfigured publically-available tools (GATK, MuTect, Indelocator, Oncotator) and internally-developed algorithms (VisCap Cancer [REF], Phaser, BreaKmer3). Reads obtained from pooled samples were demultiplexed using Picard (http://picard.sourceforge.net/command-line-overview.shtml), aligned to the Human Genome Reference Consortium reference sequence GRCh37p13 (BWA5) and duplicate reads were subsequently removed (Picard). GATK6 was used to refine the alignments near insertion/deletion (indel) sites. Single nucleotide variants (SNVs) were called using MuTect7 and indels were called using Indelocator (http://www.broadinstitute.org/cancer/cga/indelocator). Annotation was performed using Oncotator. Because tumor tissues were sequenced without a paired normal from the corresponding patients, additional informatics steps were taken to identify and account for common single nucleotide polymorphisms (SNPS): any SNP present at >0.1% in Exome Variant Server (NHLBI GO Exome Sequencing Project [ESP], Seattle, WA; URL: http://evs.gs.washington.edu/EVS/) or present in dbSNP was filtered. However variants also present in the COSMIC mutation database were rescued for manual review. Samples with a mean target coverage of <50X were failed and excluded from further analysis. Individual variants present at <10% allele fraction or in regions with <50X coverage were flagged for manual review and evaluated/interpreted by the reviewing laboratory scientists and molecular pathologists based on a variety of factors, including, but not limited to, overall tumor percentage, read depth, complexity of alteration, and evidence for associated copy number alterations.

Quantitative and additional analytics of somatic mutation data

Mutation data was queried with Microsoft Sequel and quantitatively analyzed with Microsoft Excel. Individual gene mutation frequencies were calculated based on the total number

of mutations as well as based on the total number of patients. Genes encoding proteins involved in DNA methylation/demethylation, histone modification, chromatin remodeling, or processing of non-coding RNAs (such as microRNAs) were categorized as 'epigenetic regulatory genes' (41 of 275 tested genes, 14.9%). Gene functions were determined by referencing the genetic database (accessible at: http://ghr.nlm.nih.gov/) provided by the United States National Library of Medicine and the National Institute of Health and supplemented by identifying reputable published literature available on PubMed documenting specific epigenetic function by a particular gene and/or its expressed protein. In addition, the type of mutation encountered (missense, nonsense, etc.) as well as whether or not the mutation reflected a UV signature mutation (i.e. C>T or CX>TX within a dinucleotide substitution for UVB; G>T or GX>TX within a dinucleotide substitution for UVA) was also recorded.

Quantified mutation data was subsequently analyzed and compared using MedCalc version 13.2 (MedCalc Software, Ostend, Belgium). Statistical methods were primarily descriptive and based on proportions and percentages. All p-values were two-sided, with a p-value < 0.05 considered statistically significant. In addition, to better understand the relationships between mutated epigenetic regulators, we performed Ingenuity Pathway Analysis (Qiagen, Redwood City, CA) to visualize direct relationships between specific epigenetic regulators.

RESULTS

I. Melanoma pseudomaturation or pre-existing nevus associated with primary cutaneous melanomas

Histopathologic and clinical outcome data

Clinical and histopathologic data, including age, gender, anatomic location, average depth, mitotic rate, etc. were summarized according to cases of melanoma with pseudomaturation (MPM) or melanomas arising in association with pre-existing nevi (MPEN) (**Table 1**). There was no significant difference in rate of metastasis or disease progression between the different classes of melanoma lesions studied, with an overall mean follow-up time of 23.6 months for MPEN cases and 77.2 months for MPM cases.

Review of conventional histology of melanomas with pre-existing nevi (MPEN; n_{MPEN}=28) revealed two cytomorphologically distinct zones. The first was occupied by a uniform population of large, malignant, epithelioid cells containing irregular and angulated nuclei with prominent nucleoli and coarsely clumped, vesicular chromatin patterns; and the latter by smaller cells containing round to ovoid nuclei with inconspicuous nucleoli, and evenly distributed, more delicate chromatin (nevus cells). In the majority of these cases, the nevic component was present deep to the melanoma, while in a minority of cases it was peripheral to or flanked the more central region containing the melanoma. Importantly, gradual transitions between overt melanoma and more nevic components were not encountered, and when present, infiltrating lymphocytes preferentially involved the zones of melanoma but not regions occupied by nevus cells. Melanomas with pseudomaturation (MPM, n_{MPM}=24), on the other hand, displayed a range of cytology, consisting of a gradual continuum with depth of invasion from more superficial melanoma cells (as described above) to smaller, more nevoid forms. While cells at the base of such lesions were considerably smaller than classical melanoma cells, unlike nevus cells, they contained nuclei with irregular and angulated profiles, frequently visible nucleoli, and displayed scattered mitoses. Additional histologic and clinical parameters are summarized in **Table 1**.

Table 1. Summary of clinical and histopathologic data of primary cutaneous melanomas

	MPM	MPEN
Total # cases	24	28
Average age (Range)	58.4 (36 to 99)	52.8 (15 to 90)
Gender	Male: 51.4% (13/24)	Male: 46.4% (13 of 28)
	Female: 45.8% (11/24)	Female: 53.6% (15 of 28)
Location of primary	H&N: 4.2% (1/24)	H&N: 7.1% (2 of 28)
	Upper extremity: 12.5% (3/24)	Upper extremity: 17.9% (5 of 28)
	Trunk: 45.8% (11/24)	Trunk: 53.6% (15 of 28)
	Lower extremity: 37.5% (9/24)	Lower extremity: 21.4% (6 of 28)
Average depth of primary in mm (Range)	0.89 (0.48 to 1.42)	0.97 (0.3 to 3)
Average mitotic rate per mm-sq (Range)	1.82 (0 to 15)	0.54 (0 to 2)
% Cases w/ nodal metastasis	20% (1 of 5 performed)	25% (3 of 12 performed)*
% Cases w/ distant or locoregional metastases	None	7.1% (2 of 28)
Average follow-up time in months (Range)	77.2 (20 to 99)	23.6 (8 to 37)

*Nodal nevus in 41.7% (5 of 12 SLN biopsies performed)

5-hydroxymethylcytosine immunoreactivity in MPEN versus MPM

Regions of conventional melanoma within all MPEN and MPM cases (N_P=52) demonstrated diffuse loss of 5-hmC (mean IHC intensity 0.255; mean percentage of positive cells 5.5%). In contrast, nevic regions associated with melanoma (n_{MPEN}=29) showed strong 5-hmC immunopositivity with mean IHC intensity score of 3.32 and mean percentage of positive cells of 78.9% (**Figure 5**). Regions of pseudomaturation in MPM cases (n_{MPM}=24) demonstrated a 5-hmC staining pattern that was intermediate to that of the conventional melanoma and associated nevus cells (**Figure 6**). Specifically, the mean IHC intensity score for regions of pseudomaturation in MPM cases was 1.62 with a mean percentage of positively-stained cells of 37%. All differences in 5-hmC staining within the melanoma, pre-existing nevus, and pseudomaturation compartments were significant ($p < 0.001$) (**Figure 7**).

Of note, two distinct patterns of 5-hmC immunoreactivity were observed in regions of pseudomaturation. Approximately 71% (17 of 24) of MPM cases showed a mean IHC intensity score and percentage of positive cells of 2.07 and 47.3%, respectively (**Figure 6**). In contrast, 29% (7 of 24) of MPM cases had a marked decrease in 5-hmC immunoreactivity (**Figure 8**), with mean IHC intensity score of 0.5, and mean percentage of positive cells of 4.2%, in a manner comparable to the overlying melanoma. Although the basis for this apparent dichotomy in 5-hmC immunoreactivity in areas of pseudomaturation is not entirely clear, it was noted that MPM cases with intermediate 5-hmC staining tended to be more superficial and less mitotically active than the MPM cases with markedly decreased staining (**Table 2**). These differences, however did not reach statistical significance in the limited number of cases under study.

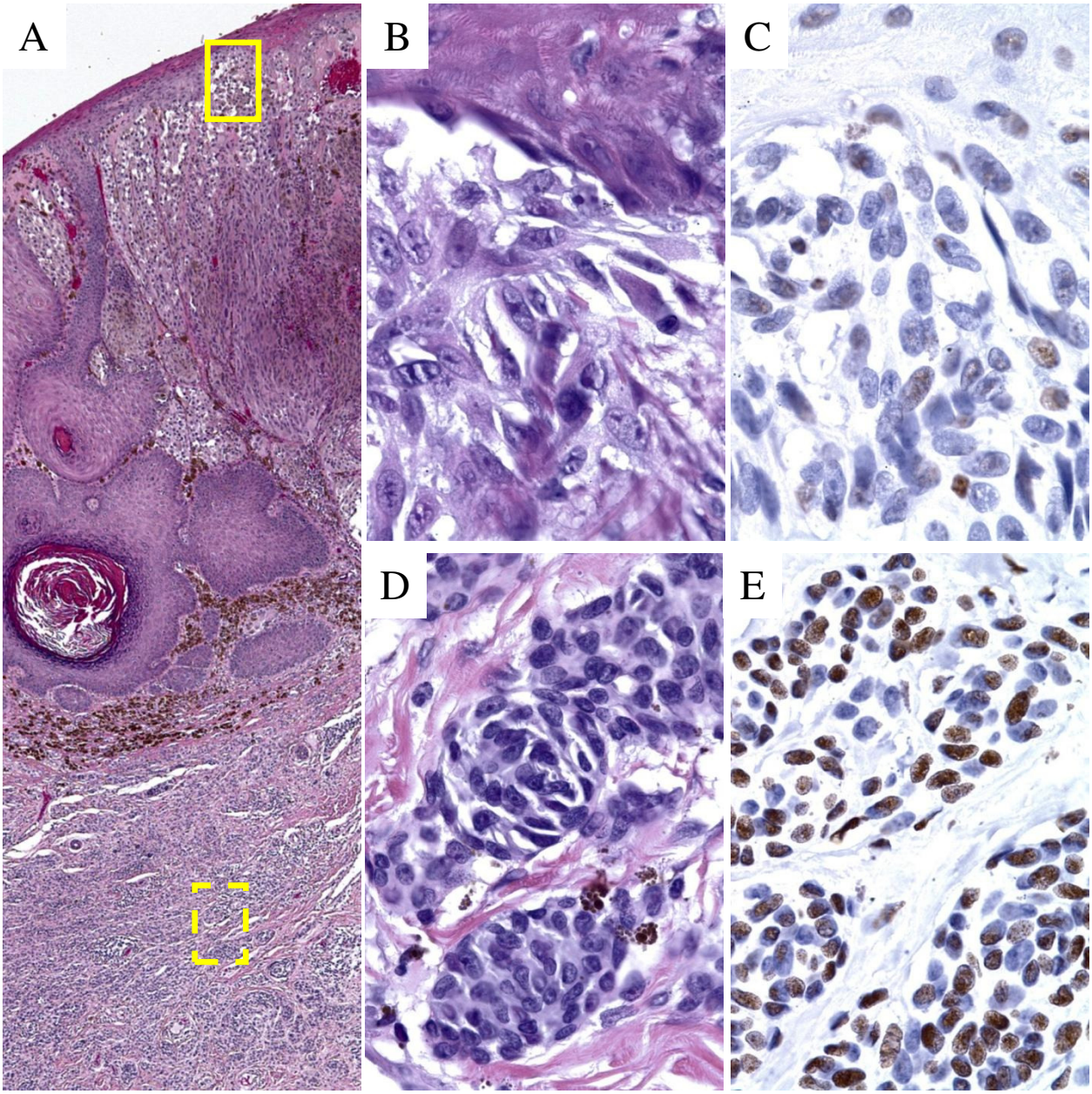


Figure 5. Melanoma arising in association with pre-existing nevus. (A) Melanoma composed of invasive sheets and cords of melanoma cells (solid outline) with cytomorphologically contrasting nevic component present deeper within the lesion (dashed outline) (H&E, 2X). (B) Discohesive nest of epithelioid melanoma cells with prominent nucleoli (H&E, 100X). (C) Nest of melanoma cells demonstrating loss of nuclear 5-hydroxymethylcytosine immunoreactivity (5-hmC, 100X). (D) Cytomorphologically distinct nests of benign nevic cells (H&E, 100X). (E) Nevic cells demonstrating strong, homogenous nuclear staining for 5-hmC (5-hmC, 100X).

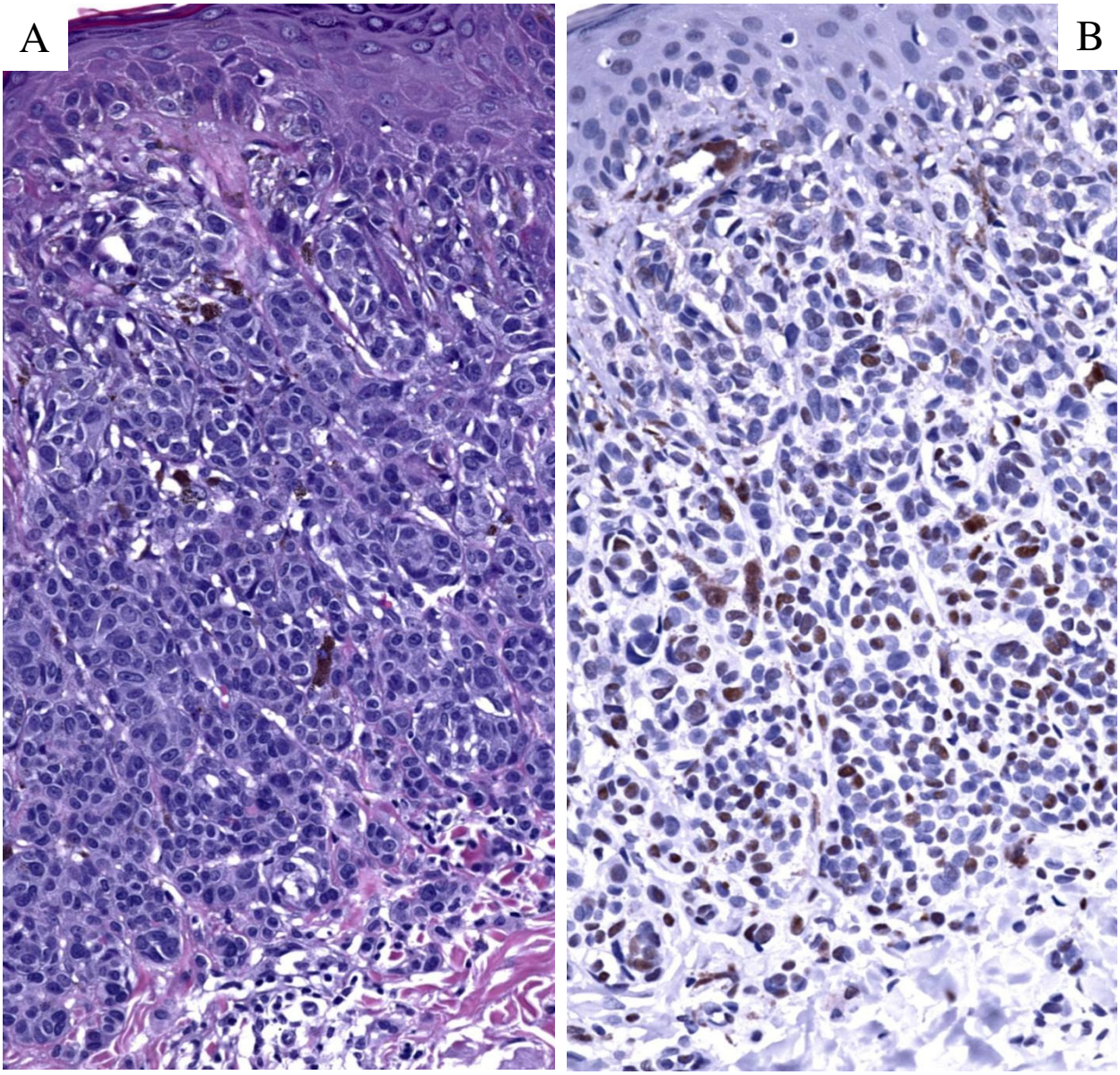


Figure 6. Melanoma pseudomaturation demonstrating intermediate 5-hydroxymethylcytosine immunopositivity. (A, left panel) Superficial spreading melanoma with invasive component demonstrating decrease in cell size with progressive depth (H&E, 20X). (B, right panel) Immunohistochemical staining for 5-hmC illustrates intermediate nuclear immunopositivity coinciding with increasing depth and decreasing cell size (5-hmC, 20X).

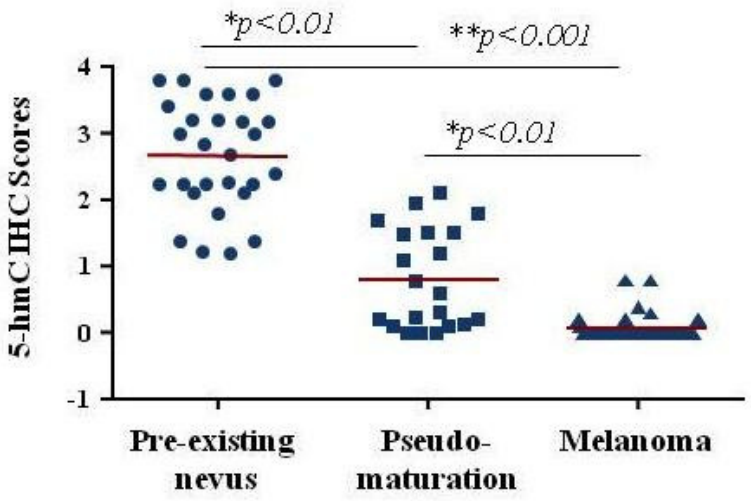


Figure 7. ANOVA analysis reveals statistically significant differences in 5-hydroxymethylcytosine (5-hmC) immunostaining in specific melanocytic compartments within primary cutaneous melanoma cases. Pre-existing nevi retained the strongest nuclear intensity for 5-hmC whereas bona fide melanoma compartments demonstrated loss. Regions of melanoma pseudomaturation demonstrated an intermediate increase in 5-hmC staining intensity that fell in between that of the nevus and that of the melanoma. Crimson bar indicates median 5-hmC staining intensity.

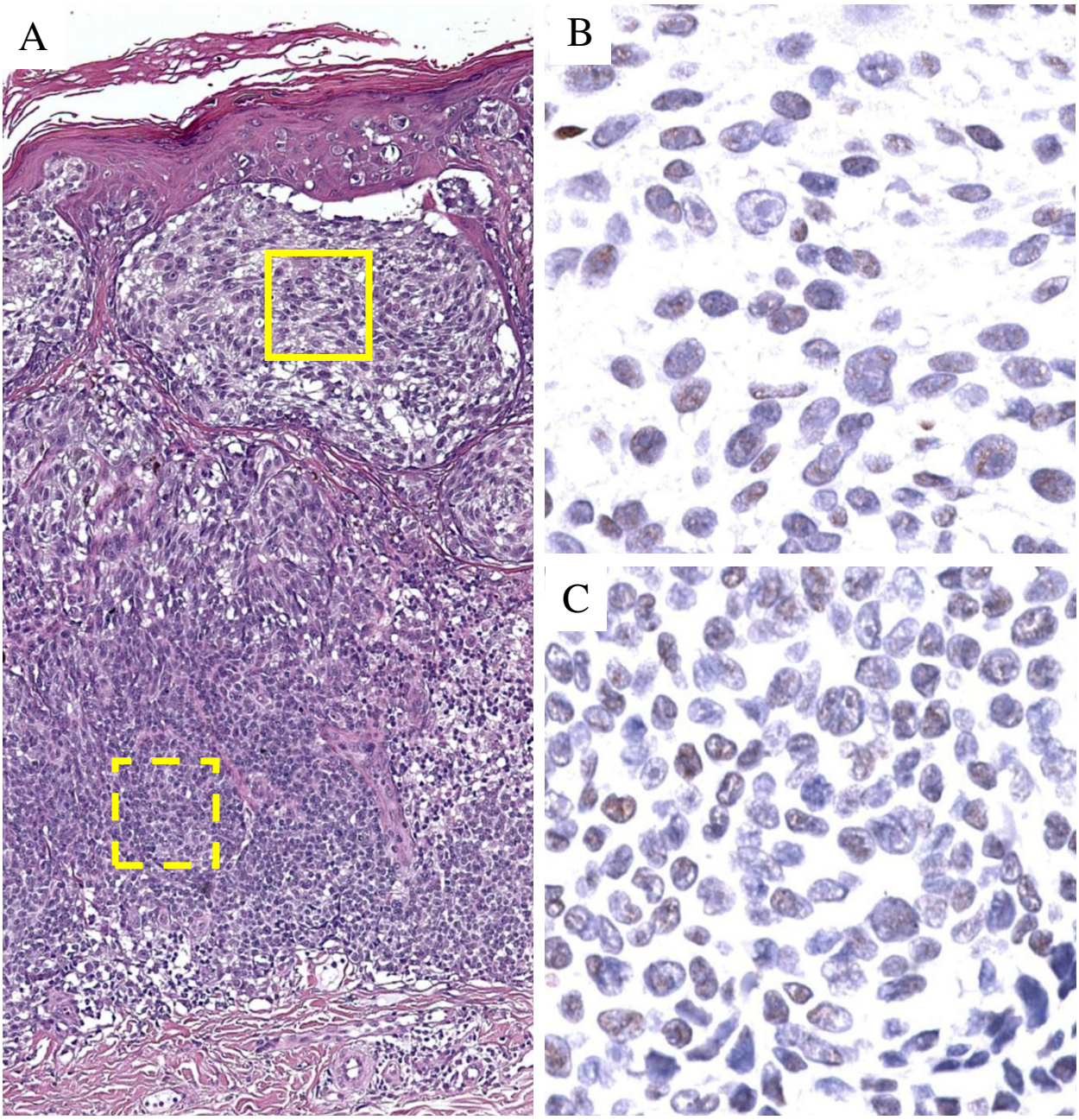


Figure 8. Melanoma pseudomaturation demonstrating significant loss of 5-hydroxymethylcytosine. (A) Junctional and superficial dermal nests of epithelioid melanoma cells demonstrating significant, gradual reduction in cell size with increasing dermal depth (H&E, 40X). (B) Larger melanoma cells within junctional nest, corresponding to box in part A, demonstrating loss of 5-hmC staining (5-hmC, 100X). (C) Pseudomaturing melanoma cells deeper in the dermis showing continued loss of 5-hmC (5-hmC, 100X).

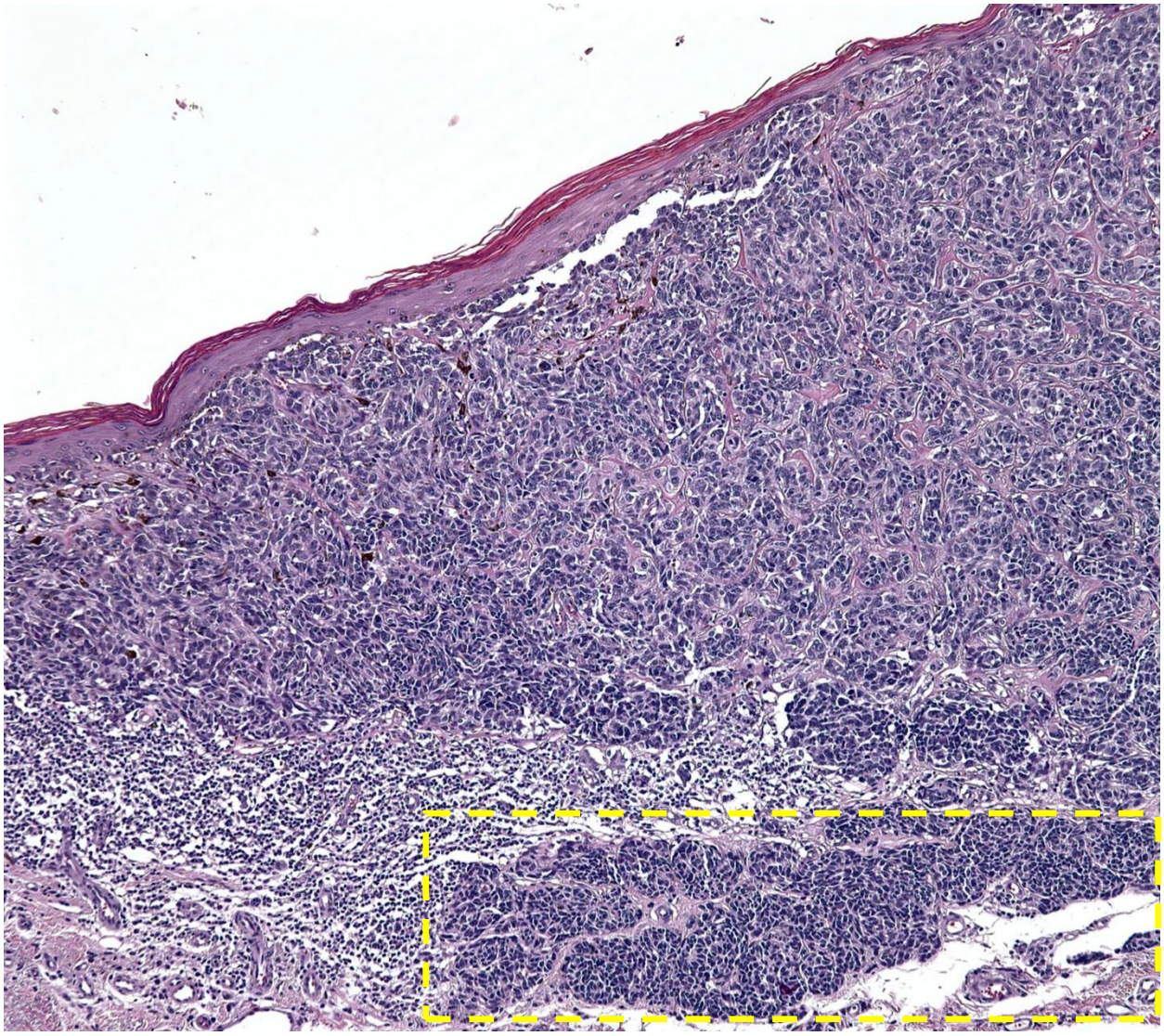


Figure 9.1. Melanoma with pseudomaturation and pre-existing nevus (H&E, 20X). Superficial spreading melanoma with invasive component demonstrating pseudomaturation with increasing dermal depth with a separate focus of nested nevic cells (box).

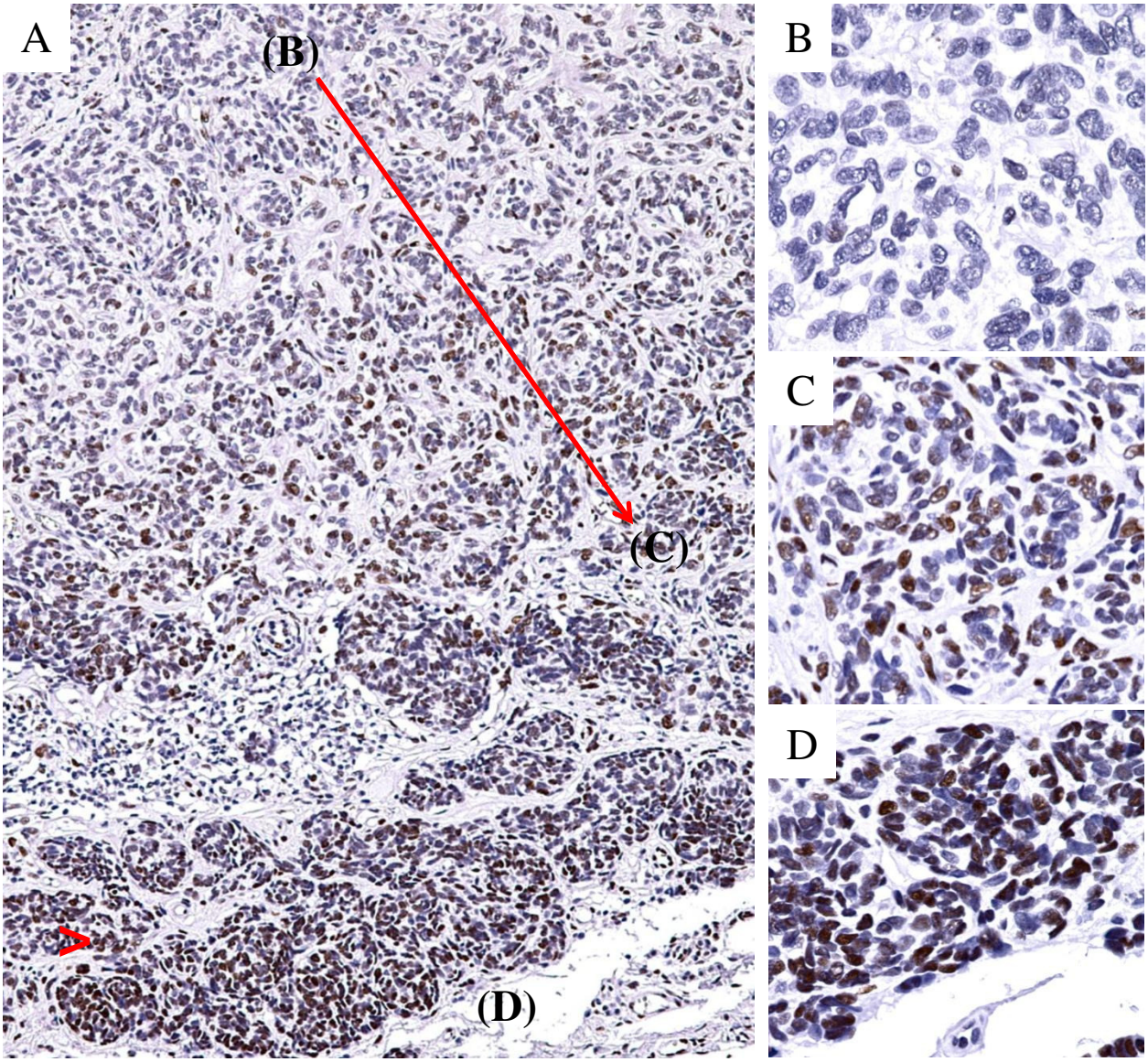


Figure 9.2. 5-hydroxymethylcytosine immunoreactivity in case of melanoma with pseudomaturation and pre-existing nevus. (A) Low-power view demonstrates gradual retention of intermediate 5-hydroxymethylcytosine immunopositivity with progressive dermal depth (arrow) and a separate nevic focus in the deepest aspect of the lesion demonstrating strong 5-hydroxymethylcytosine immunoreactivity (arrowhead) (5-hydroxymethylcytosine, 4X). (B) Melanoma cells in superficial dermis showing diffuse loss of 5-hydroxymethylcytosine (5-hydroxymethylcytosine, 100X). (C) Pseudomaturing melanoma cells deeper in the dermis showing weaker but intermediate 5-hydroxymethylcytosine immunopositivity (5-hydroxymethylcytosine, 100X). (D) Nevic cells with strong 5-hydroxymethylcytosine immunopositivity (5-hydroxymethylcytosine, 100X).

Table 2. Histopathologic comparison between pseudomaturing melanoma cases

	MPM w/ increased 5-hmC	**MPM w/ continued 5-hmC loss**
% of MPM cases	70.8 (17 of 24)	29.2% (7 of 24)
Average depth of primary in mm (Range)*	0.85 (0.4 to 1.2)	0.93 (0.5 to 1.19)
Average mitotic rate per mm-sq (Range)*	0.82 (0 to 7)	3.6 (0 to 15)

*Differences not statistically significant

Clinicopathologic data of primary cutaneous melanomas corresponding leading to sentinel lymph node biopsy

Clinical and histopathologic data of the primary cutaneous melanomas that led to subsequent sentinel lymph node biopsies containing either the unequivocal nodal nevus or melanoma are summarized in **Table 3**. Patients with either metastatic melanoma or nodal nevi did not differ significantly in age, gender, location of primary tumors, presence of ulceration, mitotic activity, presence of tumor-infiltrating lymphocytes, cytomorphology, or eventually having distant or locoregional metastases (average follow-up time, 15 months for both groups combined; range, 1-49 months). In addition, while only one of 18 (6%) of primary melanomas with nodal metastases was associated with a pre-existing/precursor nevus, 30% (3 of 10) of primary melanomas associated with such features were also found to have nodal nevi, although this difference did not reach statistical significance. However, cases of metastatic melanoma did have significantly deeper primary lesions (average Breslow's depth of 3.1 mm) than cases of nodal nevi (average Breslow's depth of 1.2 mm) ($p = 0.005$). The primary melanoma histopathologic data associated with the two 'equivocal' sentinel lymph node biopsy cases were subsequently included and comparatively analyzed but did not influence the statistical significance of the findings above ($p = 0.01$).

Detailed histopathologic and microanatomic data of sentinel lymph node biopsies

The histopathologic data of the sentinel lymph node biopsies containing either unequivocal nodal nevus or metastatic melanoma are summarized in **Table 4.** The most common location of melanoma metastasis within sentinel lymph nodes was to the lymph node parenchyma (intraparenchymal) in 83% (15 of 18) of cases, whereas a much smaller fraction of nodal nevi (30%, or 3 of 10 cases) shared the same location ($p = 0.017$). In contrast, 60% (6 of 10) nodal nevi were located within the capsule (intracapsular) whereas only 11% (two of 18) metastatic melanomas were at least partially intracapsular ($p = 0.021$). Cases of metastatic melanoma and nodal nevi did not differ significantly in the average number of foci (nodal nevi:

Table 3. Summary of clinical and histopathologic data of corresponding primary cutaneous melanomas.

	Nodal nevi	**Metastatic melanoma**
Total # cases	10	18
Average age (Range)	55.7 (25 to 84)	64.3 (29 to 92)
Gender	Male: 70% (7/10)	Male: 72% (13/18)
	Female: 30% (3/10)	Female: 28% (5/18)
Location of primary	H&N: 10% (1/10)	H&N: 22.2% (4/18)
	Upper extremity: 20% (2/10)	Upper extremity: 22.2% (4/18)
	Trunk: 40% (4/10)	Trunk: 44.4% (8/18)
	Lower extremity: 30% (3/10)	Lower extremity: 5.6% (1/18)
		Unknown: 5.6% (1/18)
Average depth of primary (Range) in mm	**1.216 (0.5 to 2.13)**	**3.124 (0.98 to 8.5)***
Average mitotic rate per mm-sq (Range)	4.7 (1 to 15)	8.3125 (0 to 21)
% Cases w/ ulceration	10% (1/10)	33.3% (6/18)
% Cases w/ TILs	40% (4/10)	55.5% (10/18)
% Cases w/ distant or locoregional metastases	0	38.9% (7/18)
Average follow-up time (Range) in months	17.1 (2 to 34)	9.7 (1 to 49)
Cytomorphology	Epithelioid: 50% (5/10)	Epithelioid: 72.2% (13/18)
	Spindle: 40% (4/10)	Spindle: 11.1% (2/18)
	Spitzoid: 10% (1/10)	Not specified: 16.7% (3/18)
% Cases w/ pre-existing/precursor nevus	30% (3 of 10)	5.6% (1 of 18)

Table 4. Summary of histopathologic data of sentinel lymph node biopsy cases.

	Nodal nevi	Metastatic melanoma
Anatomic location	Submandibular: 0% (0 of 10)	Submandibular: 5.6% (1 of 18)
	Cervical (Neck): 10% (1 of 10)	Cervical (Neck): 27.8% (5 of 18)
	Supraclavicular: 0% (0 of 10)	Supraclavicular: 5.6% (1 of 18)
	Axillary: 60% (6 of 10)	Axillary: 50% (9 of 18)
	Inguinal: 10% (1 of 10)	Inguinal: 11.1% (2 of 18)
	Femoral: 20% (2 of 10)	Femoral: 0% (0 of 18)
Location of focus	**Intracapsular: 60% (6 of 10)**	**Intracapsular: 11.1% (2 of 18)***
	Intratrabecular: 20% (2 of 10)	Intratrabecular: 0% (0 of 18)
	Subcapsular: 0% (0 of 10)	Subcapsular: 38.9% (7 of 18)
	Intraparenchymal: 30% (3 of 10)	**Intraparenchymal: 83.3% (15 of 18)***
Average # of foci (Range)	1.2 (1 to 3)	1.7 (1 to 6)
Average size of focus, in mm (Range)	0.35 (0.03 to 0.5)	1.56 (0.03 to 10)
Cytomorphology	Epithelioid: 100% (10 of 10)	Epithelioid: 94.4% (17 of 18)
		Spindled: 5.5% (1 of 18)
Pattern of spread	Nodular (aggregated): 70% (7 of 10)	Nodular (aggregated): 88.9% (16 of 18)
	Scattered (single-cell): 30% (3 of 10)	Scattered (single cell): 11.1% (2 of 18)

1.2, metastatic melanoma: 1.7), average size of foci (nodal nevi: 0.4 mm, metastatic melanoma: 1.6 mm), cytomorphology, or pattern of spread.

5-hydroxymethylcytosine immunoreactivity within sentinel lymph node biopsies

All (18 of 18) unequivocal metastatic melanomas showed complete loss of 5-hmC nuclear staining within MART-1-positive cells by both dual labeling immunohistochemistry and immunofluorescence evaluation (**Figure 9**). In contrast, all (10 of 10) unequivocal nodal nevi showed strong diffuse nuclear staining for 5-hmC within MART-1-positive cells by both approaches (**Figure 10**). Both ('Case 1' and 'Case 2') 'equivocal' cases demonstrated loss of 5-hmC nuclear reactivity, supporting a diagnosis of metastatic disease in both instances (**Figure 11, 12**). Occasional MART-1-negative cells expressing nuclear 5-hmC (usually lymphocytes) served as an internal positive control for loss of 5-hmC in nodal melanoma cells.

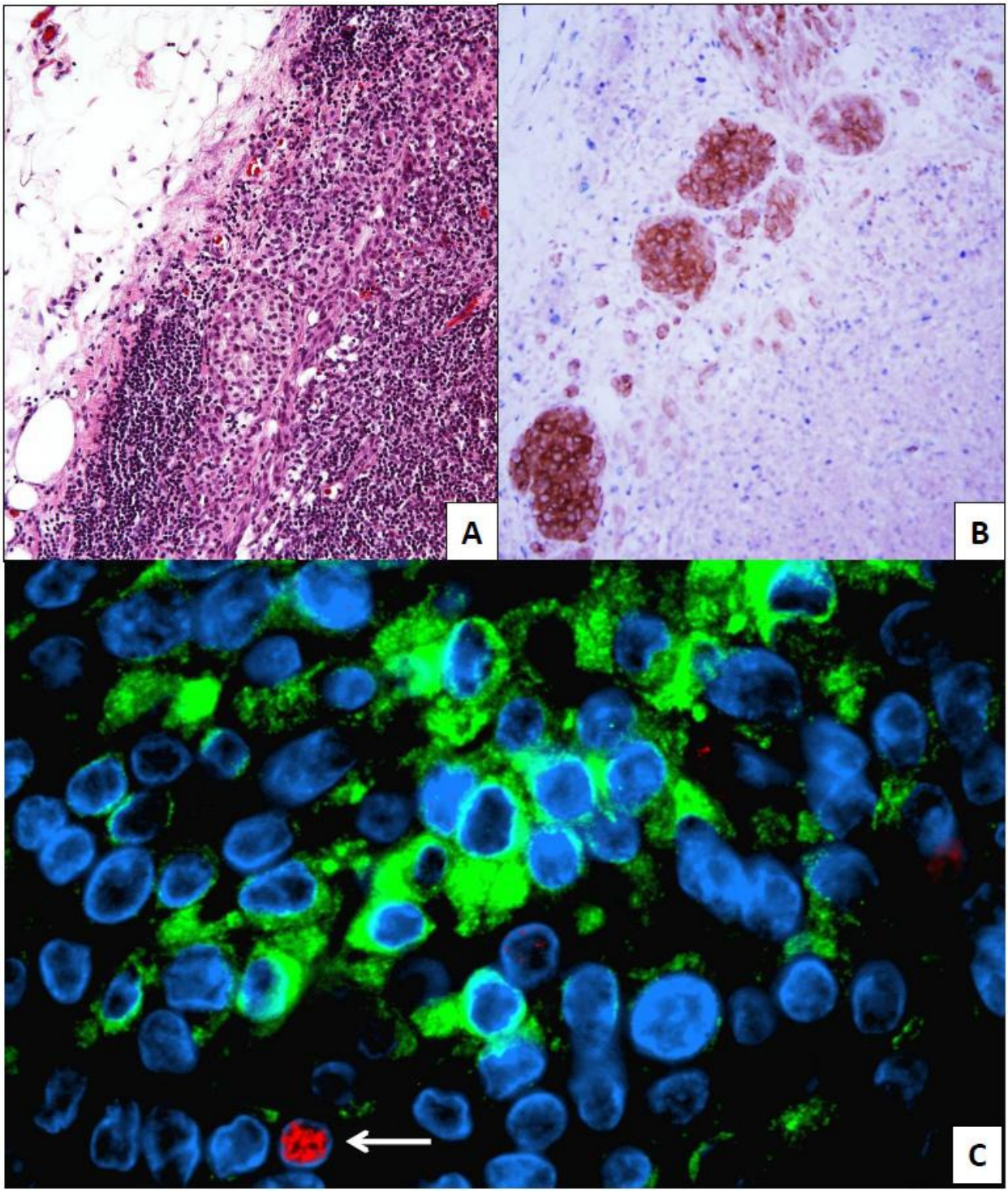


Figure 9. Metastatic melanoma. (A) Nests of epithelioid metastatic melanoma cells within the lymph node parenchyma (H&E, 20x). (B) Dual-label IHC for MART-1 (brown, cytoplasmic) and 5-hmC (blue, nuclear) exhibits nests of MART-1 positive cells showing loss of nuclear 5-hmC expression (20x). Note scattered mature lymphocytes showing 5-hmC positivity. (C) Dual-label DIF for MART-1 and 5-hmC shows loss of 5-hmC (red, nuclear) within the MART-1 (green, cytoplasmic) positive cells. Arrow indicates 5-hmC nuclear staining within lymphocyte, providing a positive internal control. Blue nuclear stain background provided by DAPI (100X).

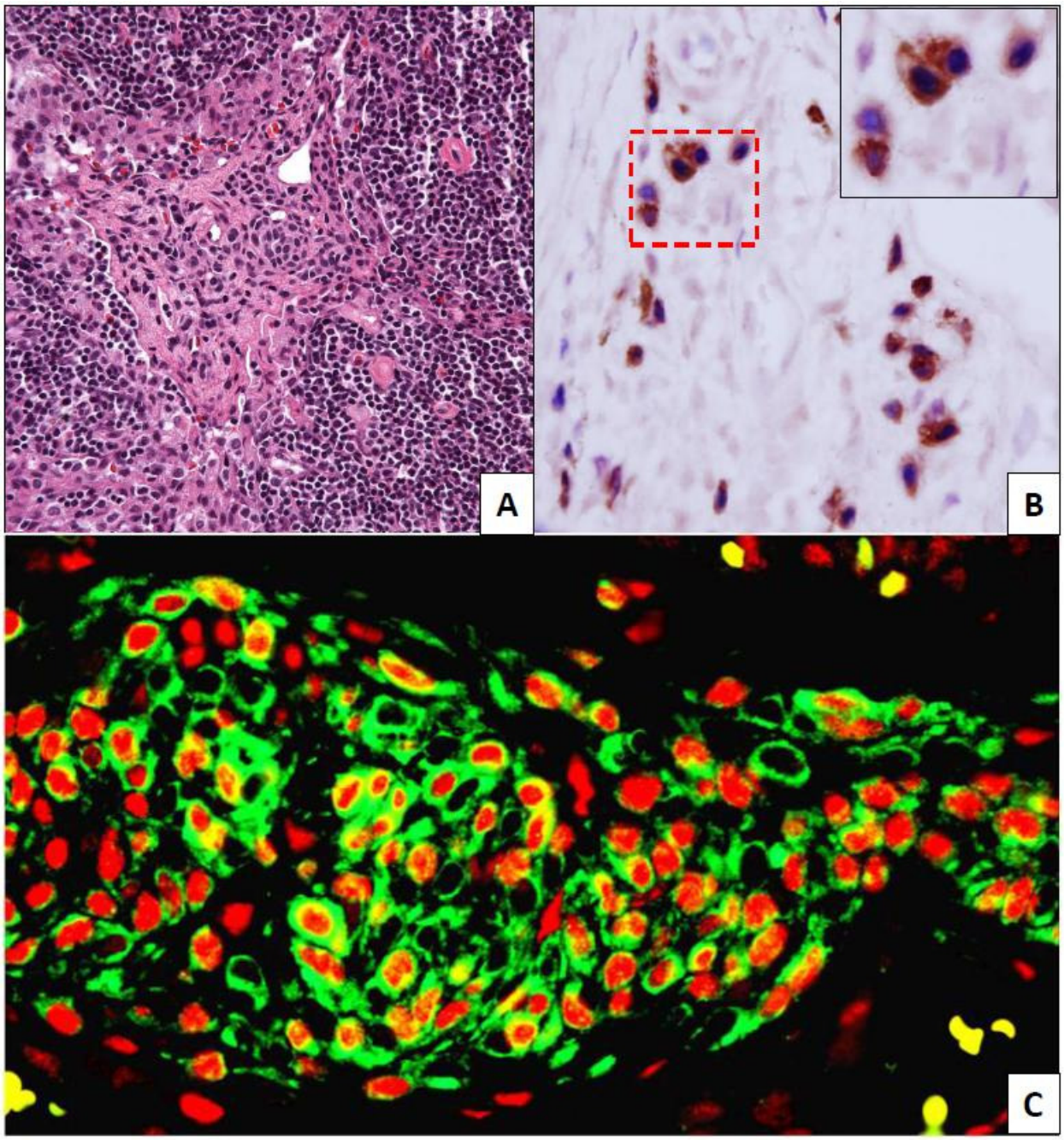


Figure 10. Nodal nevus. (A) Collection of bland-appearing melanocytes in nested pattern within lymph node trabeculum (H&E, 40x). (B) Dual-label IHC for MART-1 (brown, cytoplasmic) and 5-hmC (blue, nuclear) demonstrates retention of 5-hmC expression within MART-1 positive cells (100X). (Inset: higher magnification for emphasis). (C) Dual-label DIF for MART-1 (green) and 5-hmC (red) demonstrates retention of 5-hmC nuclear stain within vast majority of MART-1 positive cells (40X).

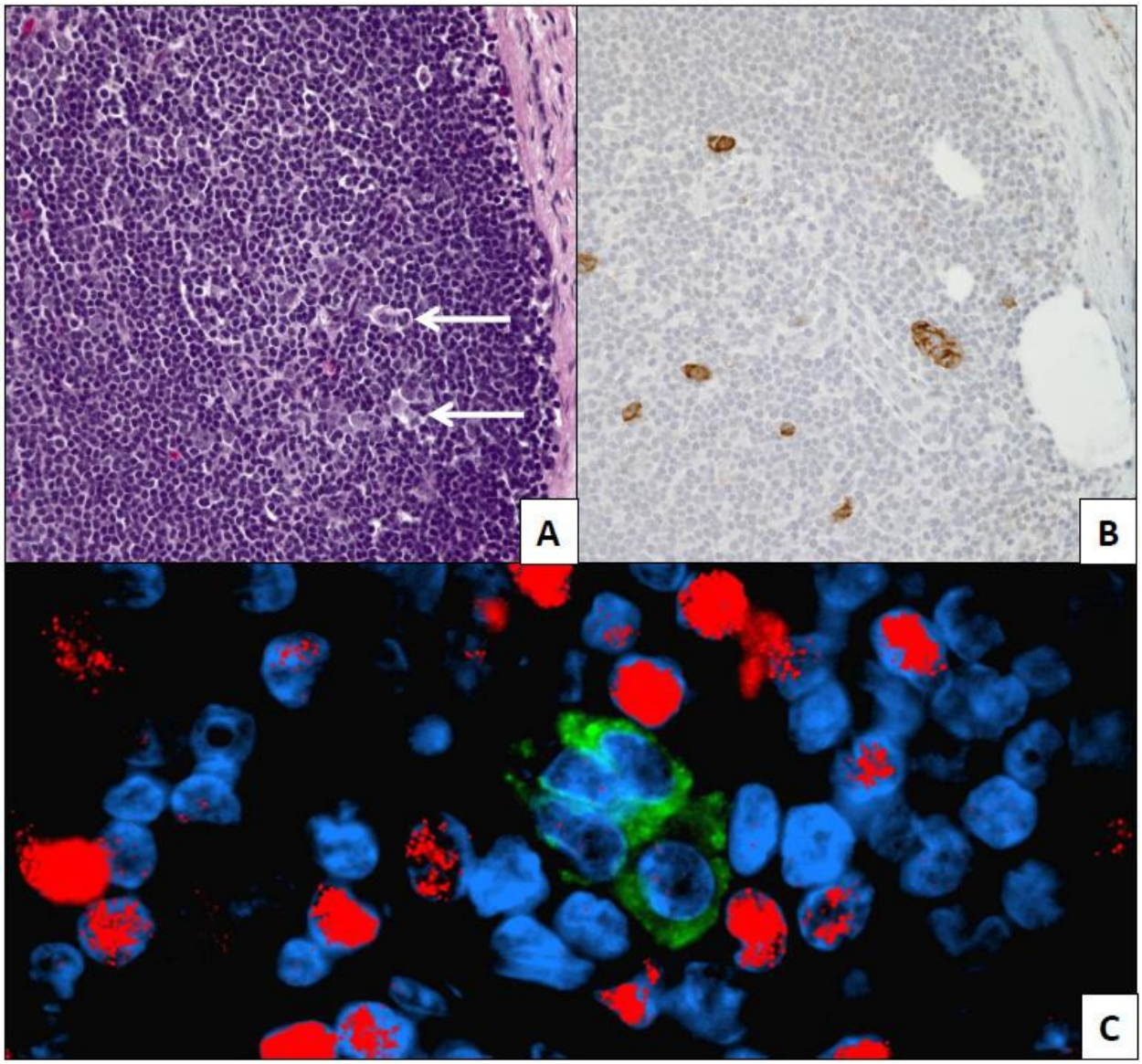


Figure 11. Case 1: Suspicious for intraparenchymal micrometastasis. (A) Lymph node parenchyma with rare, inconspicuous cells with epithelioid cytomorphology (white arrows) (H&E, 20x). (B) Single-label IHC for MART-1 (brown, cytoplasmic) highlights rare, scattered single and aggregated MART-1 positive cells containing nuclei slightly larger than those of lymphocytes (20x). (C) Dual-label DIF for MART-11 (green) and 5-hmC (red) demonstrates retention of 5-hmC nuclear staining within aggregate of MART-1 positive cells (100X).

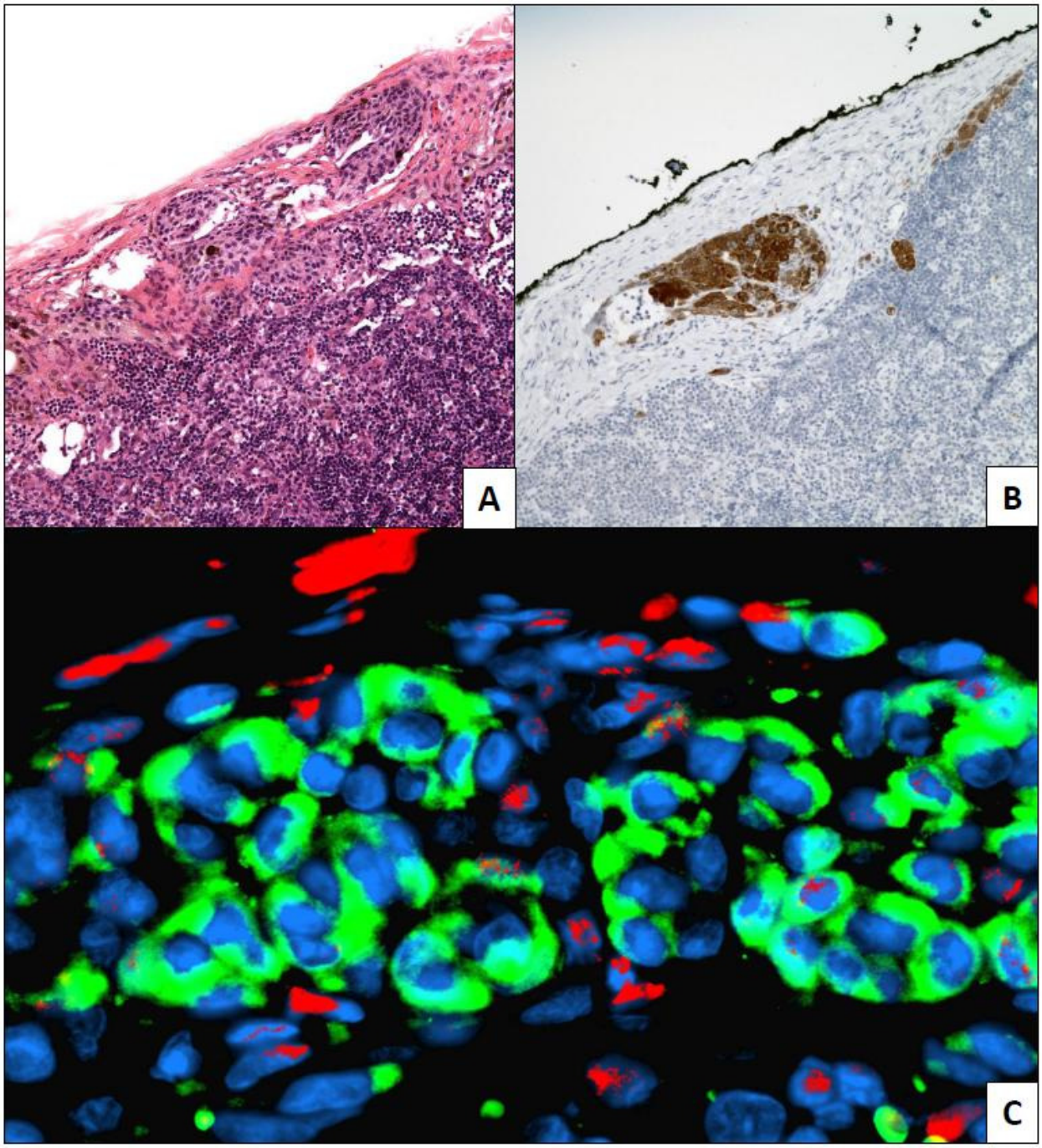


Figure 12. Case 2: Suspicious for intracapsular metastatic melanoma. (A) Metastatic melanoma cells present in spatial association with a capsular lymphovascular space (H&E, 20x). (B) Single-label IHC for MART-1 (brown) highlights MART-1 positive cells within the capsule and scattered subcapsular cells (20x). (C) Dual-label DIF for MART-1 (green) and 5-hmC (red) demonstrates loss of 5-hmC nuclear expression within MART-1 positive cells, consistent with metastatic melanoma (100X).

III. Targeted next-generation sequencing of patient melanoma samples

General somatic mutation distribution and characteristics

A total of 38 patient melanoma samples (N_M=38) were available for analysis. Of these, 22 samples were primary cutaneous melanomas (n_1=22) and the remaining 16 samples were obtained from locoregional and distant metastatic sites (n_2=16). Collectively across all 38 patient samples, a total of 740 non-silent mutations were identified in 204 of the 275 (74.2%) genes originally tested for. An average of approximately 20 mutations (median: 15.5, range: 3 to 132, standard deviation: 21.5) were identified in each patient melanoma sample. A graph summarizing the distribution of types of mutations is shown in **Figure 13**. The vast majority of mutations were missense mutations (84.7%, 627 of 740), followed by nonsense mutations (8.9%, 66 of 740), insertions or deletions resulting in frameshift (2.0%, 14 of 740), and splice site mutations (2.0%, 15 of 740). The largest percentage of nonsense mutations occurred in well-known tumor suppressor genes NF1 (12.1%, 8 of 66), CDKN2A (10.6%, 7 of 66), and TP53 (9.1%, 6 of 66).

The 40 most frequently mutated genes, as determined by number and frequency of total mutations, are graphically represented in **Figure 14**. BRAF (42.1%, or 16 of 38 patient samples), MECOM (36.8%, 14 of 38), NRAS (36.8%, 14 of 38), TP53 (31.6%, 12 of 38), MLL2 (29.0%, 11 of 38), as well as CDKN2A (29.0%, 11 of 38) were included in the most prevalently mutated genes. Among these, MECOM, BRAF, and MLL2 harbored the greatest number of total mutations (23, 19, 16, respectively). Of note, the BRAF V600E mutation was identified in seven (17.9%) of all patient samples.

High frequency of mutations identified in key epigenetic regulators

Interestingly, 22.3% (165 of 740) of all mutations occurred in genes encoding epigenetic regulators. Mutations in genes encoding histone-modifying proteins were the most common (64.2%, or 106 of 165 epigenetic gene mutations, which accounted for 14.3%, or 106 of all 740 identified mutations), including MECOM and MLL2, followed by chromatin remodeling proteins (24.2%, 40 of 165), DNA methylation/demethylation enzymes (9.1%, 15 of 165), and miRNA

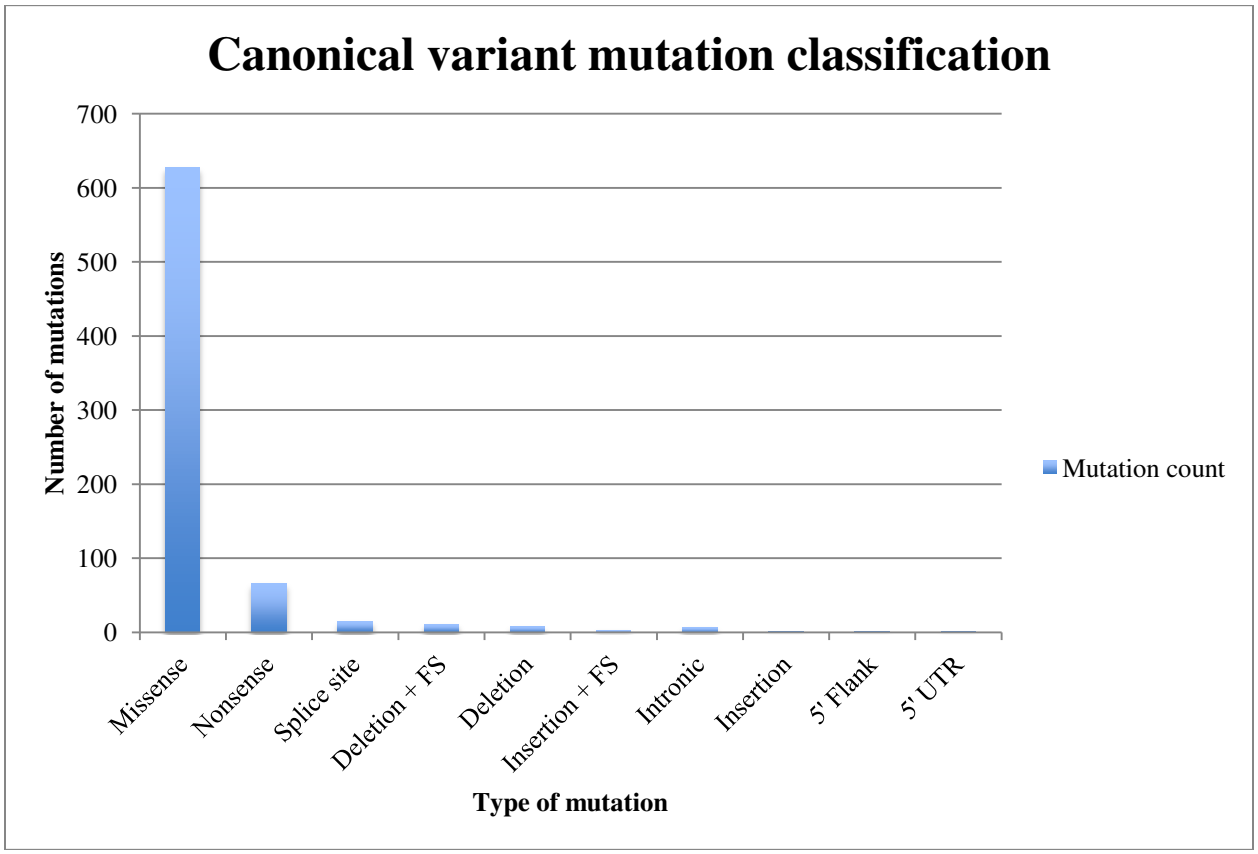


Figure 13. Bar graph summarizing distribution of mutation types in our 38 patient melanoma samples.

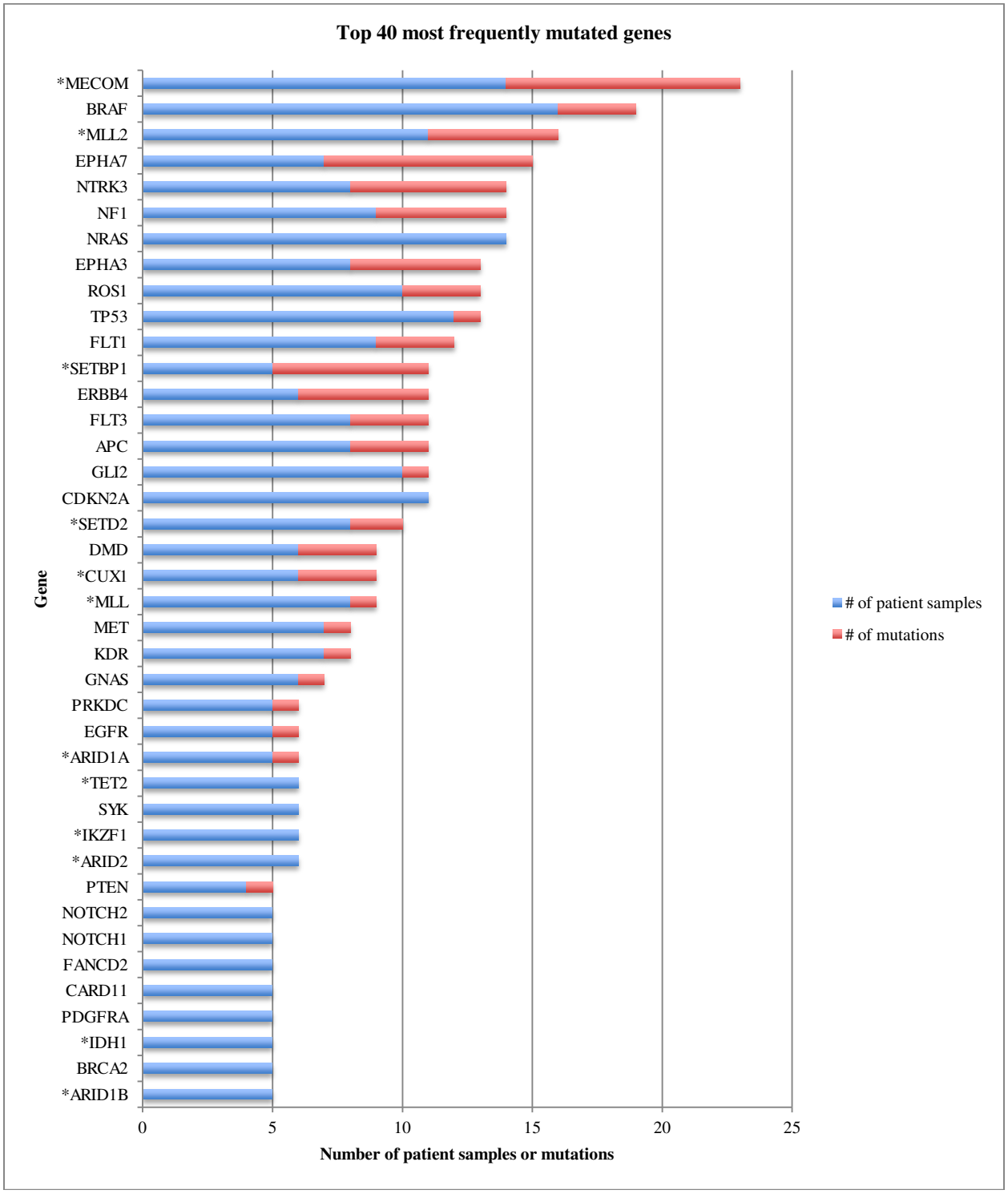

Top 40 most frequently mutated genes
*MECOM
BRAF
*MLL2
EPHA7
NTRK3
NF1
NRAS
EPHA3
ROS1
TP53
FLT1
*SETBP1
ERBB4
FLT3
APC
GLI2
CDKN2A
*SETD2
DMD
*CUX1
*MLL
MET
KDR
GNAS
PRKDC
EGFR
*ARID1A
*TET2
SYK
*IKZF1
*ARID2
PTEN
NOTCH2
NOTCH1
FANCD2
CARD11
PDGFRA
*IDH1
BRCA2
*ARID1B
Gene
0
5
10
15
20
25
Number of patient samples or mutations
# of patient samples
# of mutations

Figure 14. Chart summarizing the top 40 most frequently mutated genes. (*) indicates epigenetic gene.

processing (2.4%, 4 of 165). A summary of the frequency of mutated epigenetic genes by functional epigenetic category is illustrated in **Figure 15.** At least one mutation in an epigenetic regulator gene was found in 92.1% (35 of 38) of patients and 25 of these patients (65.7% of all samples) had more than one epigenetic regulatory gene mutated.

Of all the mutated genes identified, 17.2% (35 of 204) encoded epigenetic regulators, while only 14.9% (41 of 275) of the initial genes tested for were epigenetic in nature. Moreover, within the 40 most frequently mutated genes, 30.0% (12 of 40 genes) encoded epigenetic regulators (**Figure 14**). A two sample z-test comparing the proportion of epigenetic regulators within the 'top 40' (12 of 40) compared to that within the original panel of tested genes (41 of 275) revealed a statistically significant difference (p-value = 0.017, z-score = 2.4) between these two groups. In addition, 10 of 12 (81.2%) of the epigenetic genes within the 'top 40' were found to encode either histone-modifying proteins (e.g. MECOM, MLL2, SETD2, etc.) or subunits of chromatin remodeling complexes (e.g. ARID1B, ARID2). Two of 12 (16.7%) of these frequently mutated epigenetic genes encode enzymes involved in active DNA demethylation (TET2, IDH1). Moreover, 30.8% (4 of 13) of all genes containing an insertion or deletion resulting in frameshift encoded epigenetic regulators, including histone modifying enzymes (SETD2, CREBBP, MLL) and DNMT3A.

A summary graphic illustrating the landscape of epigenetic genes mutated within each patient melanoma sample as well as the basic clinical and histopathologic data for each case is presented in **Figure 16**. Our analysis did not reveal a tight correlation between prognostic, primary cutaneous melanoma histopathologic parameters such as depth and mitotic rate, and the number or percentage of mutations in epigenetic genes (data not shown). However, qualitative interpretation (**Figure 15**) reveals some suggestion that, at least among primary melanomas, there may be a clustering of mutations for genes encoding histone modifying enzymes (MECOM, MLL2, SETD2, and MLL) as well as chromatin remodeling complexes (ARID1B, ARID2) among the deepest primary melanomas (>2.5 mm). In addition, ingenuity pathway analysis revealed a complex interplay between several of our most frequently mutated epigenetic regulators (**Figure 17**).

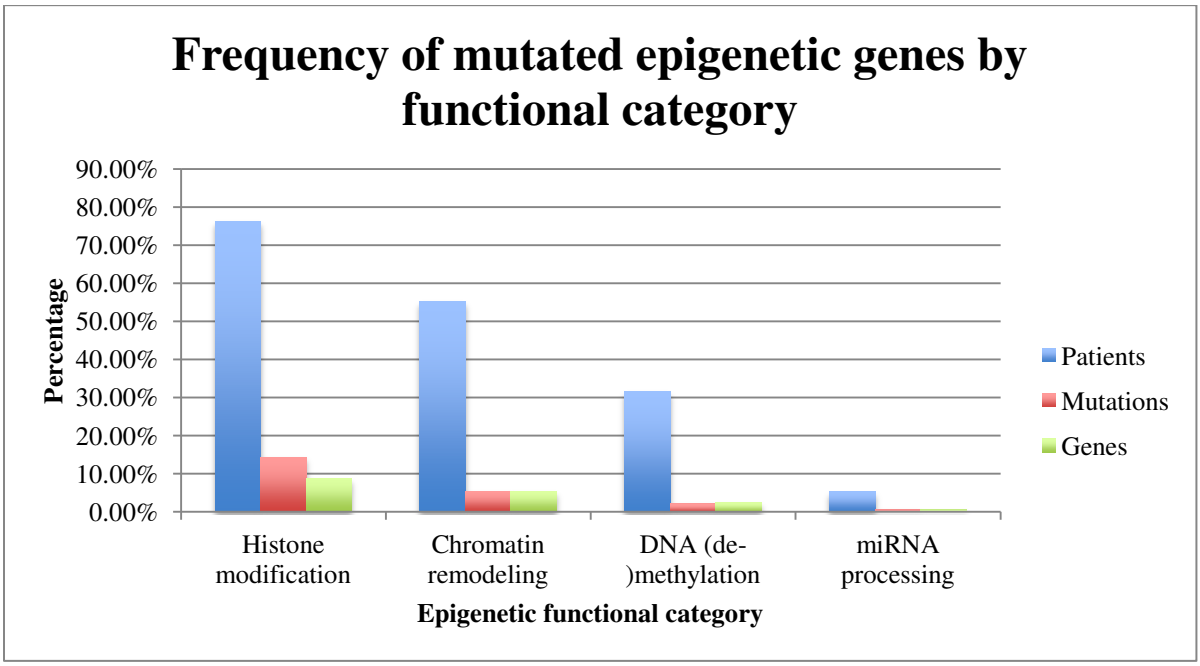
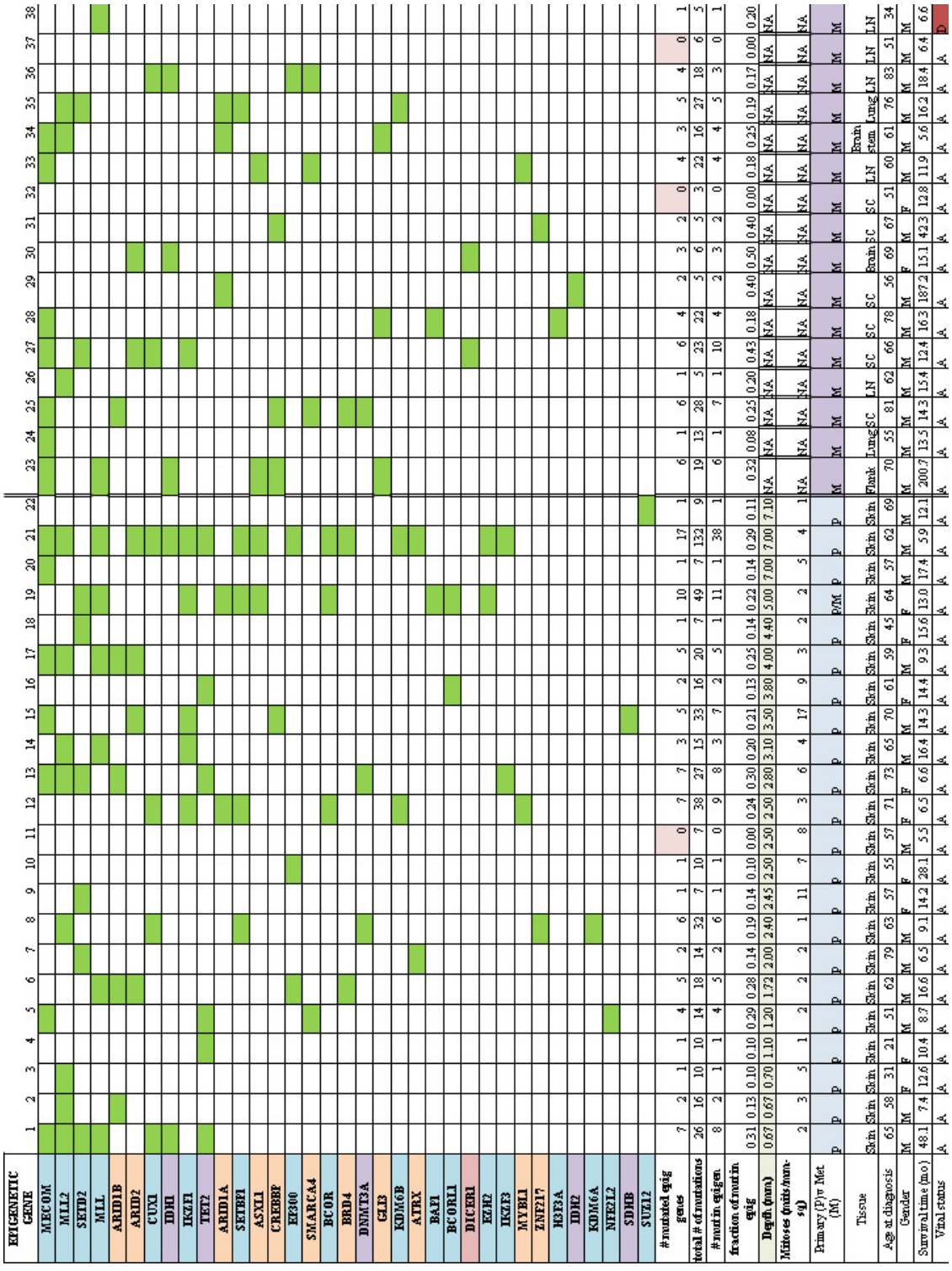


Figure 15. Frequency of mutated epigenetic genes organized by functional epigenetic category.

Figure 16. Landscape of mutations in epigenetic regulators, organized by patient melanoma sample.



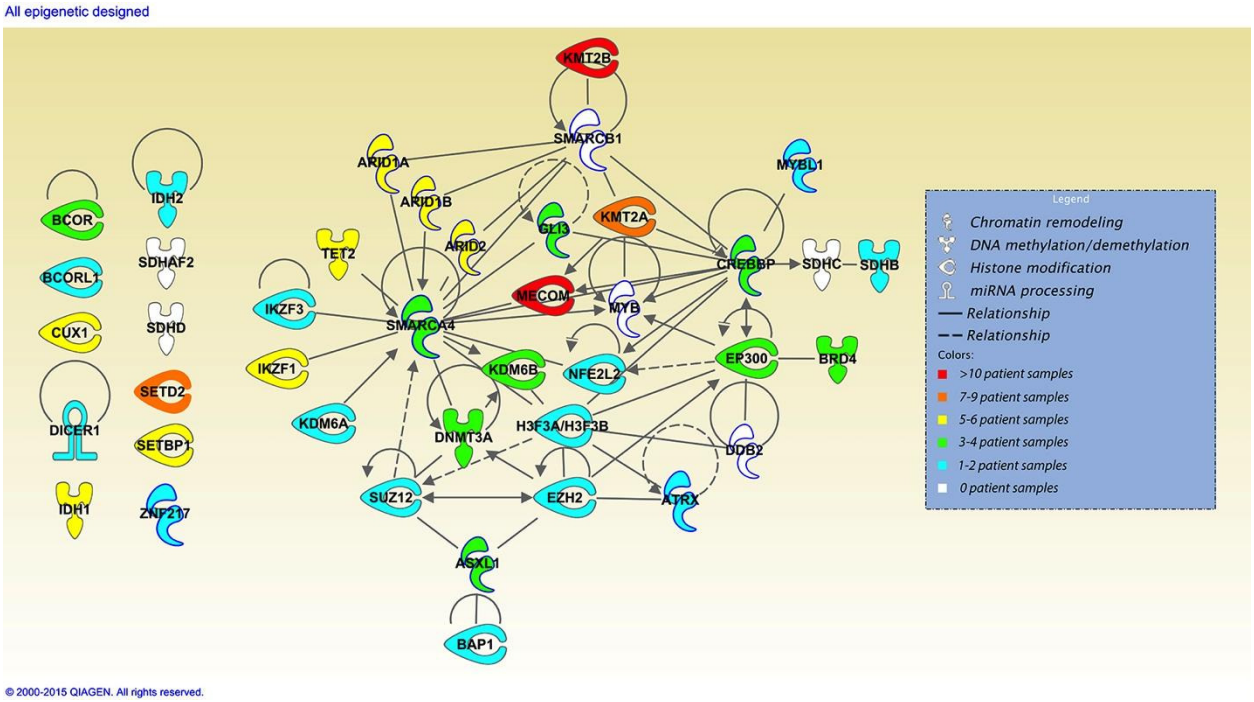


Figure 17. Ingeunity Pathway Analysis® of the 41 epigenetic regulators sequenced by our targeted next generation sequencing platform (Oncopanel, BWH). Unique shape indicates key epigenetic function while the color reflects the prevalence of mutations in that gene.

High frequency of UVB-signature mutations among key epigenetic regulators

Of all the mutations identified, 73.1% (541 of 740) resembled the mutation pattern associated with UVB-induced mutagenesis. A single C > T nucleotide substitution was the most common (70.3%, 520 of 740), followed by CC > TT tandem dinucleotide mutation (2.2%, 12 of 541), and C > T missense mutations within a dinucleotide substitution (1.7%, 9 of 541). In contrast, only 4.9% (36 of 740) of all mutations were G > T single nucleotide variant transitions, a signature of UVA-induced DNA damage. The genes with the greatest number of total UVB-induced mutations encoded epigenetic regulators, including central epigenetic regulator MECOM (82.6%, 19 of 23) and histone lysine methyltransferase MLL2 (100%, 16 of 16), and figures illustrating the spectrum of UV signature mutations across non-epigenetic and epigenetic genes are provided in **Figure 18** and **Figure 19**, respectively.

Collectively, non-epigenetic genes harbored an average of 2.4 UVB-associated mutations (median: 2, mode: 1, standard deviation: 2.5), whereas epigenetic genes harbored an average of 3.7 (median: 3, mode: 3, standard deviation: 3.9) (**Figure 20**). An unpaired, two-sample t-test comparing the average number of UVB-associated mutations present in non-epigenetic versus epigenetic genes revealed a statistically significant difference (p-value = 0.014) in the number of UVB-associated mutations between the two groups. Of note, 81.8% (9 of 11) CDKN2A mutations and 84.6% (11 of 13) TP53 mutations also resembled that associated with UVB damage. Interestingly, none of the NRAS mutations (0 of 14) and only 47.4% (9 of 19) of BRAF mutations resembled the UVB signature mutation.

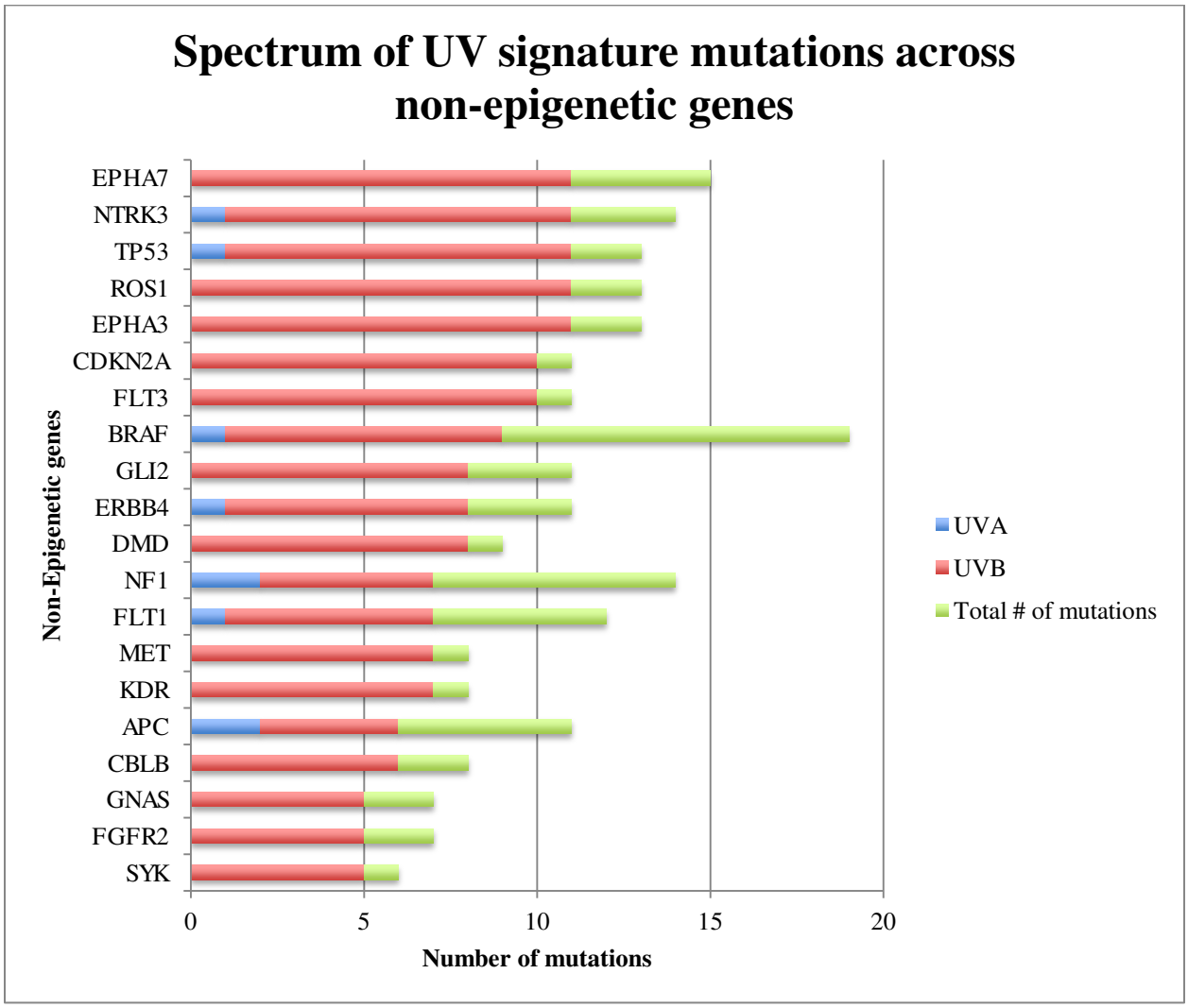


Figure 18. Spectrum of UV-signature mutations amongst non-epigenetic genes. Note the low percentage of UVB-signature mutations amongst BRAF.

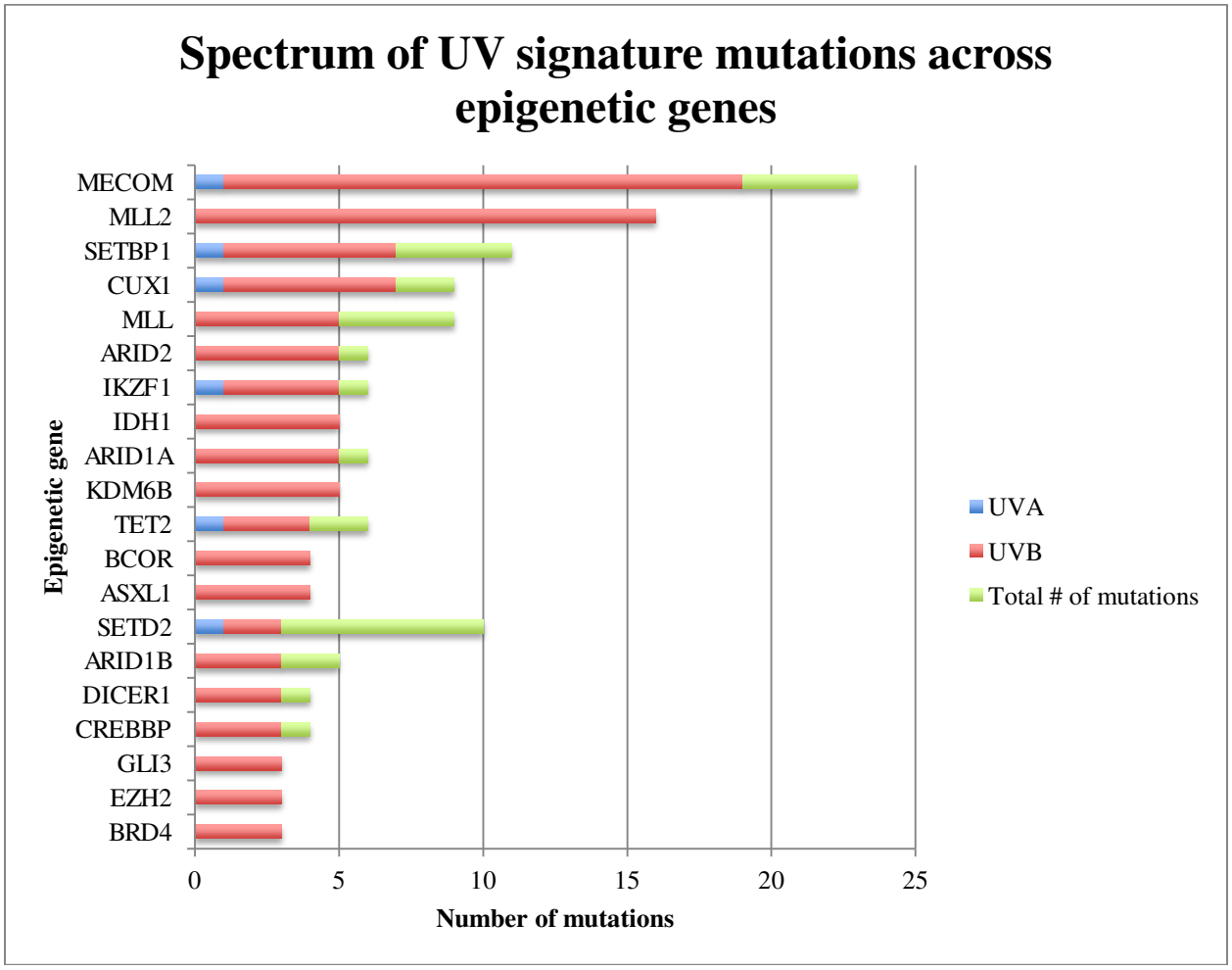


Figure 19. Spectrum of UV-signature mutations amongst epigenetic genes. Note the 100% UVB-signature mutations amongst histone lysine methyltransferase, MLL2.

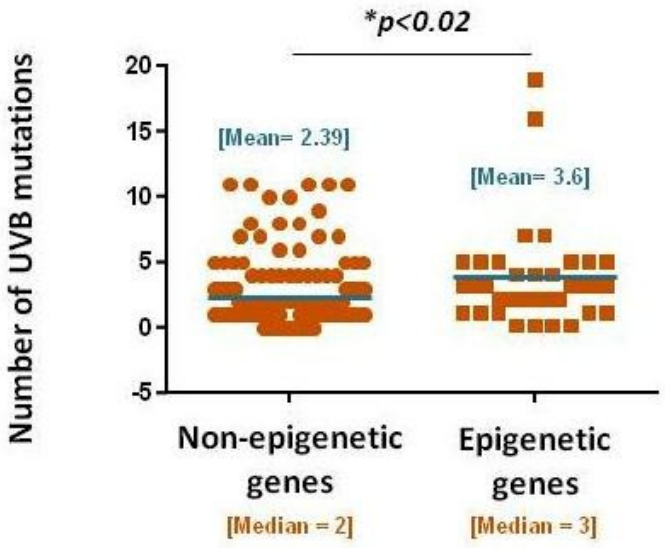


Figure 20. Distribution of UVB-signature mutations amongst non-epigenetic genes and epigenetic genes. Collectively, epigenetic genes harbored a significantly greater number of mean UVB-signature mutations than non-epigenetic genes. Turquoise bar reflects the median amongst both distributions.

DISCUSSION

5-hydroxymethylcytosine immunoreactivity highlights pseudomaturing subpopulation of melanoma cells while distinguishing pre-existing nevus from bona fide melanoma

Our analysis of primary cutaneous melanomas demonstrating pseudomaturation in primary melanomas (MPM) versus melanomas arising in association with pre-existing nevi (MPEN) provide further evidence for the 'loss of 5-hydroxymethylcytosine' as a useful biomarker in distinguishing bona fide melanoma from benign nevus cells, as well as in differentiating 'nevoid mimicry' in the former from true nevic elements. While prior studies demonstrate the use of 5-hmC to distinguish such components in separate cases of benign versus malignant melanocytic lesions (36, 65, 66), this collection of cases illustrates its potential utility in discriminating between pathobiologically distinct melanocytic components within a single lesion. Because persistence of nevus cells can closely resemble melanoma pseudomaturation or 'small cell differentiation' cytomorphologically, particularly when the deeper invasive component is involved, 5-hmC immunoreactivity may be a useful diagnostic adjunct in clarifying benign from malignant cells. In this manner, immunohistochemical staining for this epigenetic mark may enhance existing approaches to evaluating melanocytic lesions and refine approaches to measuring Breslow's depth in some of the more difficult diagnostic scenarios. The challenging case featured in **Figure 9.1** illustrates this point, wherein immunohistochemical staining for 5-hmC (**Figure 9.2**) elucidated three distinct melanocytic compartments within the dermal component of the lesion, providing adjunctive, functionally-relevant diagnostic evidence to aid in clarifying benign from malignant cells.

Our MPM cases revealed two unique patterns of 5-hmC immunostaining within the pseudomaturing regions. Approximately one third of cases demonstrated marked loss of 5-hmC within the smaller, pseudomaturing melanoma cells, in a manner akin to the 5-hmC loss in the larger, overt melanoma cells (**Figure 8**). In contrast, two-thirds of MPM cases demonstrated intermediate levels of 5-hmC immunoreactivity within the pseudomaturing areas, which fell in between that of the bona fide melanoma and the benign nevus cells (**Figure 6**). We have previously shown that restoring the '5-hmC landscape' via TET2 overexpression in xenograft models resulted in more indolent, less invasive melanomas and, conversely, that those melanomas with the most profound loss of 5-hmC were associated with the worst outcomes (36).

Taken together, our observations raise the possibility that pseudomaturing melanoma cells may reflect a more indolent subpopulation (47). Further studies are required, however, to evaluate the biological validity of this hypothesis.

'Loss of 5-hmC ' is thought to reflect dysfunction of the TET family of 5-methylcytosine (5-mC) hydroxylases, which perform the critical, sequential oxidation steps that convert 5-mC to 5-hmC and other derivatives along the recently described pathway of active DNA de-methylation (27). This critical epigenetic function is hypothesized to provide a viable mechanism that would enable the removal of the methyl group from 'incorrectly' methylated sites, which has earned TET the epithet, 'guardian' of DNA methylation fidelity (25). In addition, observations to date strongly suggest that the 'loss of 5-hmC' is highly specific to malignant as well as normal, regenerative, stem-like cells in most, if not all, human organ systems (38). 5-hmC immunostaining of normal epithelial tissues such as the skin, for instance, demonstrates that regenerative, basal keratinocytes are negative, yet as they differentiate and rise through the epidermal strata, keratinocyte nuclei progressively express 5-hmC (38). For these reasons, it is plausible that 5-hmC content may serve as an epigenetic biomarker of differentiation. Moreover, its 'loss' in the context of malignancy may be reflective of a biologically dedifferentiated state and/or increased replicative capacity. Such data, collectively, provide evidence supporting our hypothesis that intermediate 5-hmC immunoreactivity in the context of melanoma pseudomaturation may reflect a less virulent pathobiological state.

Recent studies have attempted to characterize the prevalence of somatic mutations involving TET2, a key member of the TET family, in cutaneous melanomas (67). However, this fraction, as confirmed through our own targeted sequencing analysis (**Figure 14**), is significantly less than the prevalence of dysfunctional TET2, as is indicated by the 'loss of 5-hmC in most, if not all, conventional primary cutaneous melanomas. Moreover, the subtle but quantifiable increase in 5-hmC immunoreactivity within the pseudomaturing components of our series of cutaneous melanomas all suggest that mechanisms beyond direct mutation to the TET gene itself are likely responsible for its dysfunction in this context, as suggested by previous studies (36). In fact, recent experimental data support that abnormal metabolic pathways and consequent oncometabolite accumulation can, alone, inhibit proper function of this critical epigenetic regulator, as discussed above (68, 69). Thus, it remains possible that epigenetic and/or metabolic mechanisms and/or microenvironmental influences may be, in part, responsible for the increased 5-hmC immunoreactivity within some pseudomaturing melanoma cells. Whether such changes

truly reflect a more indolent cellular state is yet to be determined. While our cohort of cases continues to be followed clinically, further studies are now indicated to explore these possibilities.

It is known that the cutaneous microenvironment differs at varying depths based on the presence or absence of certain cell-cell or cell-matrix interactions, cytokines, or growth factors (70). In addition, specific differences in the tumor microenvironment, such as localized hypoxia, have also been proposed to modify gene expression profiles to that associated with enhanced invasiveness and virulent behavior (71). Thus, it is tempting to speculate whether changes in the cutaneous microenvironment may play, in part via epigenetic mechanisms, an influential role in the biology and pseudomaturation observed in a subset of melanomas, as has been previously suggested (47). It is plausible that melanocytes harness a uniquely flexible epigenetic program, given their physiologic duty to modify gene expression in a timely fashion in response to environmental exposures and cues such as ultraviolet radiation. However, the role of epigenetic mechanisms in normal melanocyte physiology has yet to be established.

5-hydroxymethylcytosine distinguishes benign nodal nevus from metastatic melanoma in sentinel lymph node biopsies

Sentinel lymph node sampling is a standard staging procedure offered to patients with specific histopathologic attributes in their primary cutaneous melanoma. Most recently, the AJCC Melanoma Staging Committee and the National Comprehensive Cancer Network has expanded their criteria for considering sentinel lymph node biopsies to include those patients staged as T1b (0.76- 1.00 mm Breslow's depth with ulceration or mitoses $\geq 1/mm^2$) in addition to those staged as T2, T3, or T4 (> 1.00 mm in thickness with or without ulceration, collectively) (72). Despite the observed difference in average mitotic activity not reaching statistical significance within our cohort, patients having positive sentinel lymph nodes were, indeed, significantly more likely to have deeper primaries than those with only nodal nevi. This is a finding in keeping with the notion that sentinel lymph node positivity is a barometer that correlates with other prognostic attributes of melanoma virulence, such as depth (further reinforcing the importance of accurate Breslow's measurements in primary melanomas with pseudomaturation, as discussed above).

While our compiled histologic data indicate that melanomas metastatic to sentinel lymph nodes are characteristically intraparenchymal in location, in contrast to the intracapsular localization of benign nodal nevi, this general rule was far from absolute in our cohort. In fact, one of our cases of nodal nevi demonstrated clear-cut extension into the parenchyma from an intracapsular and intratrabecular location (**Figure 21A, B**), a histologic pattern that is typically worrisome for metastasis. Moreover, the nevus cells within the parenchyma were also larger than those within the capsule and trabeculae. However, strong 5-hmC nuclear immunoreactivity was retained within these cells (**Figure 21C**), consistent with their cytologic benignancy and architectural unity with the more conventional intracapsular/intratrabecular components. This case demonstrates that neither the microanatomic location nor the pattern of spread is sufficient, in itself, to distinguish true metastatic disease from nodal nevi. Our data also indicate that the size of the focus and the overall cytomorphology may, in some cases, also be inadequate for making a definitive distinction. The new AJCC guidelines additionally classifies patients having microscopic nodal metastases, even when only a single cell is identified, as stage III, based on data that associated such findings with reduced overall survival (53). Moreover, recent clinical data further support that even very small numbers of melanoma cells in SLNs may impact a patient's prognosis (73). In light of these recent findings and recommendations, adjunctive strategies to assist in the accurate detection of SLN metastasis and their distinction from benign nodal nevi or isolated nevic cells are critical.

We have demonstrated through immunohistochemistry and direct immunofluorescence that the epigenetic mark, 5-hmC, distinguishes benign nodal nevi from metastatic melanoma in sentinel lymph node biopsies. Our findings are in keeping with prior and more recent corroborative studies of 5-hmC expression in primary skin sections containing either benign nevi or malignant melanoma and our own cohort of melanomas arising in association with pre-existing nevi (36, 65, 74). The loss of 5-hmC has become a well-documented 'epigenetic hallmark' of human malignancy and has been shown to be an independent predictor of worse prognosis in melanoma as well as a number of other cancers (36, 38, 75-77).

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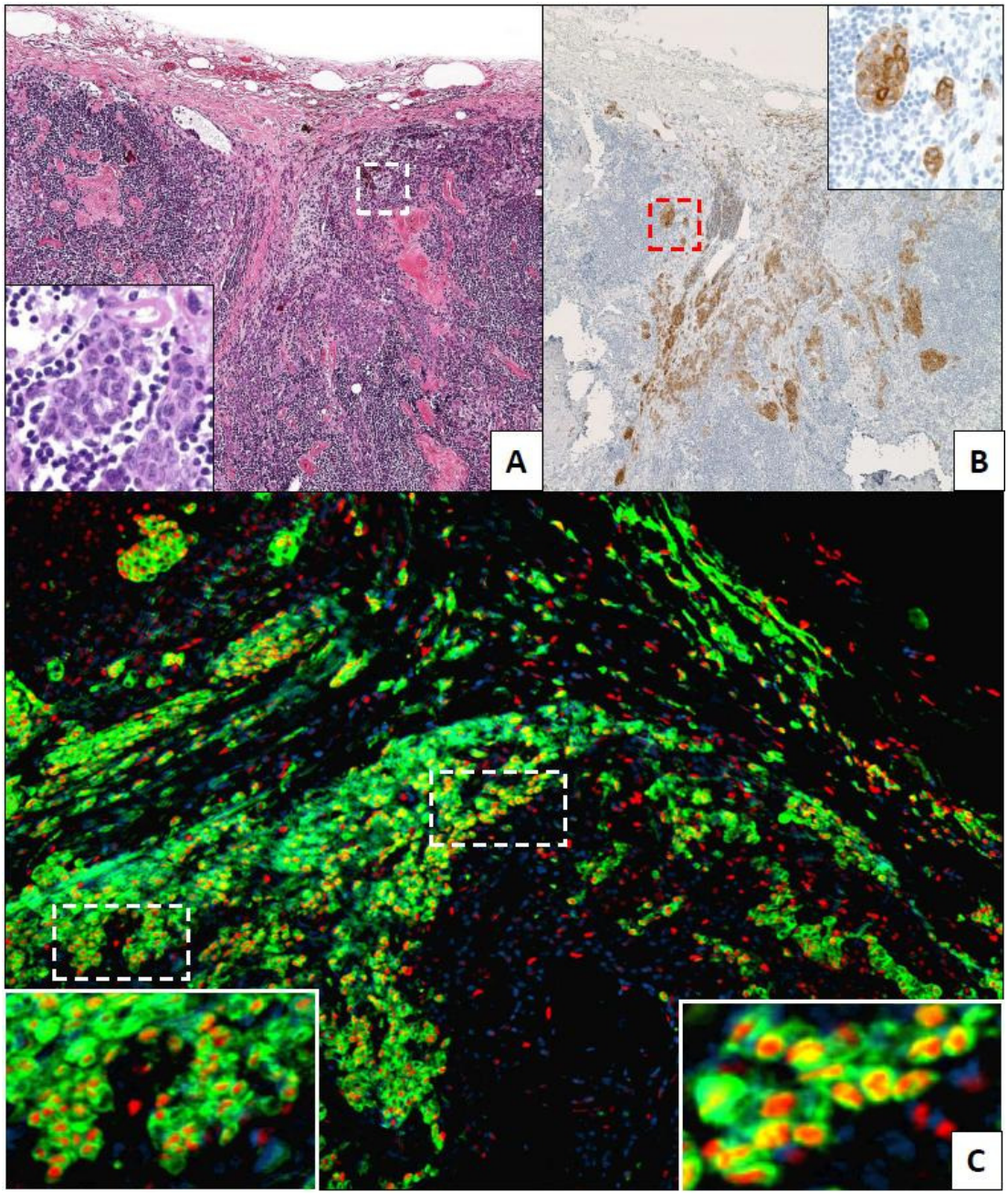

Figure 21. Intracapsular nodal nevus with extension into the parenchyma. (A) H&E (10X). Inset: High-power view of enclosed region of bland, nevic cytomorphology. (B) Single-label IHC for MART-1 (brown) highlights intraparenchymal collections of MART-1 positive cells (10X). Inset: High-power view of nests of MART-1 positive cells also within lymph node parenchyma. (C) Dual-label DIF for MART-1 (green) and 5-hmC (red) highlights retention of nuclear 5-hmC staining within MART-1 positive cells within the capsule as well as lymph node parenchyma, supporting the diagnosis of intracapsular and intraparenchymal nodal nevus (10X). Inset (left): High-power view of MART-1 and 5-hmC positive cells extending into lymph node parenchyma. Inset (right): High-power view of intracapsular nevic cells showing high intensity (red-yellow) nuclear 5-hmC staining.

existing nevi (36, 65, 74). The loss of 5-hmC has become a well-documented 'epigenetic hallmark' of human malignancy and has been shown to be an independent predictor of worse prognosis in melanoma as well as a number of other cancers (36, 38, 75-77).

From a strictly biologic standpoint, we took interest in the modestly higher sensitivity and specificity of 5-hmC in distinguishing metastatic melanoma from nodal nevi in sentinel lymph node biopsies compared to previously reported markers (58). Immunohistochemical investigation of 5-hmC expression in normal human tissues has demonstrated that regenerative, stem-like basal epithelia consistently shows 'loss of 5-hmC' similar to their malignant counterparts (38). Thus, the corresponding absence of TET enzyme and active DNA demethylation function may be a reflection of a more undifferentiated, 'stem-like' epigenomic cellular state. Our laboratory has reported that nestin and SOX2, markers of neural crest progenitor stem cells, are co-expressed in metastatic melanomas but much less commonly so in benign nevi (78). The recently reported expression of nestin and SOX2 in metastatic melanomas within sentinel lymph node specimens may, similar to the 'loss of 5-hmC', also reflect a 'less'-differentiated state, allowing for their distinction from nodal nevi. However, we hypothesize that the loss of 5-hmC and corresponding TET enzyme dysfunction may more directly reflect the 'upstream' epigenomic milieu of the undifferentiated, versatile cellular biology associated with a malignant, stem-like state that enables the re-expression of previously silenced markers, such as nestin and SOX2. In addition, studies have demonstrated that 'loss of 5-hmC' appears to be more pronounced in primary cutaneous melanomas that exhibit more aggressive behavior (36). Therefore, 5-hmC may be even more informative when used to evaluate for metastatic disease. For these reasons, it is plausible that 5-hmC may be more sensitive and specific for differentiating between benign and malignant melanocytic cells, particularly subsequent to metastatic spread of the latter to sentinel lymph nodes. Certainly, additional studies and further exploration of these proposed mechanisms and pathways are necessary before definitive conclusions can be drawn.

Interestingly, our data indicate a high concordance between initial histopathologic diagnoses and the corresponding 5-hmC staining profiles. Our investigation of two 'equivocal' biopsies, wherein the diagnosis of metastasis was favored but could not be definitively rendered, illustrates the potential utility of immunohistochemical staining with 5-hmC as a confirmatory test in diagnostically challenging scenarios. However, further immunohistochemical investigation of larger numbers of truly 'equivocal' sentinel lymph node biopsies, in concert with supporting clinical outcome and histological annotation, is warranted in order to further test the

routine clinical utility of predictive biomarkers such as 5-hmC. It is noteworthy that in Case 1, rare, scattered MART-1 positive cells were present within the lymph node parenchyma (**Figure 11A, B**). Such a collection could potentially represent either MART-1 positive cells of 'indeterminate lineage' (79) or true, 'single cell' micrometastasis. The 'scattered' pattern of spread may favor metastatic disease, but such a feature is not definitive nor is it pathognomonic. The loss of 5-hmC staining in this case supports the suspicion that this focus represented micrometastatic disease (**Figure 11C**). In addition, Case 2 illustrated a scenario wherein the MART-1 positive cells were predominantly intracapsular in location, a feature that is typically ascribed to nodal nevi (**Figure 12A, B**). The suspicion for metastasis in this case was raised based on the overall cytology and possible presence of some of these cells around and within an intracapsular lymphovascular space. Indeed, 5-hmC loss within these cells provides further confirmation for the diagnosis (**Figure 12C**).

Targeted next generation sequencing reveals landscape of mutations in epigenetic regulators with predilection for UVB-signature mutations in patient melanoma samples

The incidence of melanoma, unlike many other potentially preventable cancers, is steadily increasing worldwide, with an estimated 76,100 new cases diagnosed in the United States in 2014 alone (80). While representing less than 2% of all skin cancers, melanoma accounts for the vast majority of skin cancer deaths (80). Major risk factors for melanoma include those that are genetic and environmental in nature, such as a personal or family history of melanoma, the presence of five or more 'atypical' nevi, numerous (> 50) melanocytic nevi, fair-colored skin, and either a history of blistering sun burns during childhood/adolescence and/or a history of indoor tanning bed use (81). Approximately 10% of melanomas occur in a familial setting, and germline mutations involving a number of genes, including CDKN2A (9p21), CDK4 (12q14), BAP1 (3p21), TERT promoter (5p15) (82), and most recently POT1 (7q31) (83), have been demonstrated to predispose individuals to development of cutaneous melanoma in addition to other melanoma subtypes (BAP1 and metastatic uveal melanoma) and numerous atypical melanocytic nevi (CDKN2A, CDK4) (84). These observations collectively reinforce that 'melanoma' is a heterogeneous malignancy whose pathogenesis is complex, not infrequently unique, and multifactorial, involving both genetic and non-heritable (e.g. environmental) factors.

The most frequently mutated genes within our cohort resemble that reported in previous studies (22, 85) as well as data available on the Sanger Institute Catalogue Of Somatic Mutations in Cancer (COSMIC) online database (http://www.sanger.ac.uk/) (**Figure 14**) (86). Well-known oncogenes NRAS (1p13) and BRAF (7q34) as well as tumor suppressor genes CDKN2A and TP53 (17p13) were among the most frequently mutated genes within our cohort, in keeping with prior studies and datasets (22, 87). The latter two genes were also among the genes within our cohort containing the highest number and percentage of nonsense mutations, consistent with loss of their tumor suppressive function. While mutations to the TP53 gene have previously been considered a rare event in melanomagenesis (88), our findings corroborate data from others (22) in challenging this dogma.

In addition, a high frequency of mutations in genes encoding epigenetic regulators in both primary and metastatic cutaneous melanoma patient samples were identified. Genes encoding histone-modifying proteins (e.g. MECOM, MLL2, SETD2, etc.), subunits of chromatin remodeling complexes (e.g. ARID1B, ARID2), as well as units of the active DNA demethylation pathway (TET2, IDH1) were the most frequently mutated among this group. In particular, there were several novel standouts on our list of most commonly mutated epigenetic genes. MECOM (3q26) was one of the most frequently mutated genes (3.1%, 23 of 740 mutations; 36.8%, 14 of 38 patient samples) within our cohort of patient melanoma samples and higher than estimated by existing datasets (43).

MECOM (MDS1 and EV1 complex locus) encodes ecotropic viral integration site 1 (EVI1), an oncogenic zinc finger transcription factor known to be overexpressed in acute and chronic myeloid leukemia and whose over-activation has been tightly associated with poor patient survival (89, 90). Interactome analysis has demonstrated that the EVI1 oncoprotein exerts dynamic nuclear functions and is involved in a number of critical processes, including, but not limited to, transcription regulation, DNA repair, recombination, and mitosis (91). In addition, EVI1 has been shown to interact with multiple components of the epigenetic machinery, including DNA methyltransferases, histone modifying enzymes, and chromatin remodeling complexes, including the SWI/SNF nucleosome remodeler (92). Moreover, gene expression analyses has demonstrated a stem cell phenotype in EVI1-overexpressing acute myelocytic leukemia cells, leading some to speculate as to whether this oncoprotein could augment cancer stem cell self-renewal capacity in addition to facilitating disease progression and therapeutic resistance (92). Indeed, multiple lines of experimental evidence also suggest that EVI1 may be

involved in facilitating chemoresistance in human myeloid leukemias by inducing the CDKN1A/p21/WAF complex (93). Taken together, it is tempting to hypothesize whether MECOM/EVI1 could regulate the epigenetic machinery enabling stem cell-like properties in specific melanoma subpopulations. Additional studies are now indicated to investigate these possibilities to evaluate the potential of MECOM/EVI1 as a therapeutic target. The next step in our investigation, however, is to establish its mutational status in benign and dysplastic nevi.

MLL2 (or KMT2D, 12q13) was the second most frequently mutated epigenetic gene within our cohort. Remarkably, all 16 mutations identified bore the signature of UVB damage (C>T missense). MLL2 is a member of the myeloid/lymphoid or mixed-lineage leukemia (MLL) family genes and encodes a specific histone H3 lysine 4 (H3K4) methyltransferase, which provides an evolutionarily conserved epigenetic mark for active gene transcription (94). MLL2 was recently identified to extensively regulate the expression of a number of critical cell signaling pathways, including the p53 pathway and cAMP-mediated signaling, as well as the expression of the retinoic acid-responsive gene ASB2 (95). Moreover, and of particular interest to the biology of melanocytes and melanoma, MLL2 was recently found to associate with promoters and thereby regulate the expression of S100 alpha (S100A) genes (1q21), which are known to control cell cycle progression and differentiation within melanocytes (95). MLL2 has been frequently implicated in the pathogenesis of a number of human cancers (96-98) and our findings agree with recent data (87) suggesting the same may be true for melanoma.

In addition, our dataset and analysis also revealed a high frequency of UVB-associated mutations among epigenetic genes, in particular (**Figure 19**). The genes encoding the central epigenetic regulator MECOM/EVI1 (73.9%, 17 of 23) and histone lysine methyltransferase MLL2 (100%, 16 of 16) were found to harbor the greatest overall number of UVB-associated mutations. In addition, we found a significantly greater number of mean UVB mutations in epigenetic genes (3.7/gene) compared to non-epigenetic genes (2.4/gene) (**Figure 20**). Both within and outside of the familial/hereditary melanoma setting, ultraviolet light radiation (UVR) is thought to play a major role in the pathogenesis of most melanomas. While it is well-established that most of the mutational burden in melanoma is attributable to the mutagenic effects of UVR (99-101), our findings raise the possibility that UVR preferentially induces mutations in genes encoding epigenetic regulators. Indeed, whether epigenetic mechanisms are also involved in mediating the physiologic response of melanocytes to UVR has yet to be established. These speculations are in keeping with the proposed role of epigenetic mechanisms,

at large, in facilitating changes in gene expression in response to environmental cues (25, 102, 103) and could co-exist with established pathways known to be involved in the physiologic response of melanocytes to UVR, such as the p53-proopiomelanocortin (POMC) pathway (101). Of relevance, well-known oncogenes BRAF and NRAS had comparatively low frequencies (26.3% and 0%, respectively) of UVB-induced mutation, a finding that is also in keeping with previous studies. Taken together with the fact that such mutations are also present in benign nevi, their role in the pathogenesis of bona fide melanoma may be of limited relevance (22). In contrast to BRAF and NRAS, 81.8% of CDKN2A mutations, 84.6% of TP53 mutations, and 83.3% of IDH1 mutations resembled the UVB-induced mutation genotype in our cohort.

The presence of genomic evidence that epigenetic regulators may be involved in melanoma pathogenesis was recently put forth by Hodis et al. (2012), who found a high frequency of somatic mutations in chromatin-modifying proteins and other epigenetic regulators as well as a high frequency of UVB-induced, non-silent mutations in IDH1 and chromatin modifying-enzymes ARID2 (a component of the SWI/SNF chromatin-remodeling complex) and EZH2 (the histone lysine methylase component of Polycomb-group gene silencing complex) (22). Ding et al. (2014) also found a high frequency of truncation mutations to chromatin remodeling genes (ASXL3, MLL2, ARID2) in their cohort of metastatic melanoma cases (87). Our findings contribute additional data to this growing body of evidence that dysregulated epigenetic mechanisms and pathways may be more involved in the pathobiology of melanoma than has been previously recognized (**Figure 17**).

Broadly speaking, epigenetic mechanisms play a critical role in mediating heritable changes in gene expression as a result of specific environmental stimuli (104). A precise role for epigenetic regulation of gene expression in normal melanocyte physiology and the pigmentation response to UVR exposure, however, has yet to be established (105). Nonetheless, dysregulated epigenetic mechanisms have increasingly entered the limelight as important pathways in the development of melanoma as well as a number of other malignancies (106). Dysregulated promoter methylation, DNA demethylation and 5-methylcytosine hydroxylation, and non-coding RNAs have been demonstrated to impart virulent behavior and enable stem-like phenotypes in melanoma (25). Moreover, recent findings strongly implicate that somatic mutations in epigenetic regulators may play a critical role in mediating treatment resistance in pediatric acute lymphoblastic leukemia (107), further substantiating, by analogy, that such mechanisms may also play a similar role in melanoma. Moreover, evidence is accumulating that epigenetic

pathways may be intimately associated with metabolic derangements that occur alongside the malignant transformation (108). Our preliminary dataset indicates that somatic mutations to epigenetic regulators may be more common than previously appreciated. In total, we found that approximately one in five mutations occurred in a gene encoding an epigenetic regulator, with mutations to histone-modifying enzymes being the most common. Moreover, the overwhelming majority (92.1%, 35 of 38) of our patient samples harbored at least one mutation in an epigenetic regulatory gene with well over half of all patient samples (65.7%, 25 of 38) having more than one such gene mutated.

While this study provides novel insights into potential epigenetic mechanisms involved in the pathogenesis of melanoma, there are several limitations that must be acknowledged. Firstly, our modestly sized cohort may under- or overestimate the frequency of certain somatic mutations. Further collaboration with other academic institutions will be necessary to generate larger patient datasets. Secondly, the panel of epigenetic genes tested in our cohort is far from comprehensive, in view of the ever expanding family of epigenomic regulators (26), and additional sequencing platforms to test for these novel epigenetic genes should be prepared for further investigations. In addition, sequencing techniques do not detect chromosomal aberrations, which are known to occur and be involved in the pathogenesis of melanoma (109) and to distinguish benign melanocytic lesions from malignant melanoma (110). Nonetheless, our study demonstrates that clinical use of next generation sequencing can identify novel mutations in melanoma and may shed light on new, personalized, pathogenic mechanisms and unveil future targets of therapeutic interest (111). Because epigenetic defects, unlike genetic mutations, are potentially reversible, this area of investigation has tremendous potential for translational and therapeutic application.

CONCLUSION

Great advancements in science and technology have enhanced our understanding of the genetic and epigenetic basis of melanoma pathogenesis and have shed detailed, mechanistic insight into the astute clinical and pathologic observations made centuries ago by Drs. John Hunter and William Norris. In the diagnostic arena, immunohistochemical staining for the newly discovered epigenetic mark, 5-hydroxymethylcytosine (5-hmC), an intermediate of the active DNA demethylation pathway, may provide critical adjunctive evidence to support distinctions between benign and malignant melanocytic populations, as well as for those that may fall, uniquely, somewhere in between. In this study, 5-hmC distinguished unequivocal nodal nevi from metastatic melanoma with rather high sensitivity and specificity, while providing strong adjunctive evidence to support the diagnosis in more equivocal cases of micrometastatic disease. Moreover, this epigenetic biomarker highlighted a distinct subpopulation of pseudomaturing melanoma cells and, in addition to providing potential evidence in support of their pathobiological indolence, facilitated precision in obtaining Breslow's predictive measurement of invasion. Finally, targeted next-generation sequencing data revealed a high prevalence of mutations in epigenetic regulators with a quantifiable predilection for those associated with UVB damage in patient melanoma samples. In conclusion, this investigation provides further support for the use of epigenetic biomarkers to aid in challenging diagnostic scenarios and unlocks the door for further discoveries by unraveling novel epigenetic mechanisms that may be involved in melanoma pathogenesis. Continued investigation in this regard will have significant translational application and therapeutic relevance in the revolution towards precision medicine and personalized therapeutic targeting in melanoma treatment that is currently underway.

SUGGESTIONS FOR FUTURE WORK

There are many potential applications to further explore the diagnostic utility of 5-hydroxymethylcytosine (5-hmC) immunoreactivity. Investigating 5-hmC staining profiles in additional, diagnostically challenging melanocytic lesions, such as the Spitz nevus, atypical Spitz nevus, and 'Spitzoid' melanomas, as well as other 'borderline' lesions (i.e. melanocytic tumor of uncertain malignant potential, or 'MELTUMP'), may expand the diagnostic scenarios to which 5-hmC immunostaining may be clinically applied. In addition, 5-hmC staining profiles in non-melanoma skin cancers, such as the commonly-encountered squamous cell carcinomas and basal cell carcinomas as well as the less commonly-encountered cutaneous adnexal tumors, may enhance resolution to delineate the aggressive variants from the more indolent and, thereby, further guide clinical treatment strategies. 5-hmC may also have potential to enhance diagnostic accuracy and precision beyond the realm of dermatopathology, such as during the evaluation of cervical biopsies for low versus high-grade squamous intraepithelial lesions as well as in the evaluation of routine colonic mucosal biopsies, among others.

Based on findings from our next-generation sequencing data analysis, we are currently interrogating the mutational status of MECOM/EVI1 in benign and dysplastic nevi, as well as in additional cases of melanoma and several other variants. In addition, we also plan to investigate potential relationships between the TET-family enzymes and MECOM/EVI1. Should the mutational status of MECOM/EVI1 differ significantly between that of the benign/dysplastic nevus and melanoma (unlike BRAF), our next step will be to explore in-vitro and xenograft models interrogating the role of MECOM/EVI1 in melanoma pathogenesis and, potentially, its possible regulation of stem-like characteristics. Moreover, as stated above, additional targeted next-generation sequencing platforms should be developed to examine the mutational spectrum across additional epigenetic regulators in melanoma and other cancers. These proposed investigations have great potential to refine existing approaches to histopathologic diagnoses, contribute to the growing body of knowledge on epigenetic mechanisms in melanoma pathogenesis, and enhance diagnosis, prognosis, and therapeutic targeting in melanoma.

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SUPPLEMENTAL MATERIAL

Supplement 1. List of the 275 cancer gene exons sequenced in our cancer genomic assay as well as the 91 introns from 30 of these genes. Genes encoding epigenetic regulators have been bolded.

ABL1, AKT1, AKT2, AKT3, ALK, ALOX12B, APC, AR, ARAF, **ARID1A, ARID1B, ARID2, ASXL1,** ATM, **ATRX**, AURKA, AURKB, AXL, B2M, **BAP1**, BCL2, BCL2L1, BCL2L12, BCL6, **BCOR, BCORL1**, BLM, BMPR1A, BRAF, BRCA1, BRCA2, **BRD4**, BRIP1, BUB1B, CARD11, CBL, CBLB, CCND1, CCND2, CCND3, CCNE1, CD274, CD58, CD79B, CDC73, CDH1, CDK1, CDK2, CDK4, CDK5, CDK6, CDK9, CDKN1A, CDKN1B, CDKN1C, CDKN2A, CDKN2B, CDKN2C, CEBPA, CHEK2, CIITA, **CREBBP**, CRKL, CRLF2, CRTC1, CRTC2, CTNNB1, **CUX1**, CYLD, **DDB2**, DDR2, **DICER1**, DIS3, DMD, **DNMT3A**, EGFR, **EP300**, EPHA3, EPHA5, EPHA7, ERBB2, ERBB3, ERBB4, ERCC2, ERCC3, ERCC4, ERCC5, ESR1, ETV1, ETV4, ETV5, ETV6, EWSR1, EXT1, EXT2, **EZH2**, FAM46C, FANCA, FANCC, FANCD2, FANCE, FANCF, FANCG, FAS, FBXW7, FGFR1, FGFR2, FGFR3, FGFR4, FH, FKBP9, FLCN, FLT1, FLT3, FLT4, GATA3, GATA4, GATA6, GLI1, GLI2, **GLI3**, GNA11, GNAQ, GNAS, GPC3, GSTM5, **H3F3A**, HNF1A, HRAS, ID3, **IDH1, IDH2**, IGF1R, **IKZF1**, **IKZF3**, JAK2, JAK3, **KDM6A**, **KDM6B**, KDR, KIT, KRAS, LMO1, LMO2, LMO3, MAP2K1, MAP2K4, MAP3K1, MAPK1, MCL1, MDM2, MDM4, **MECOM**, MEF2B, MEN1, MET, MITF, MLH1, **MLL, MLL2,** MPL, MSH2, MSH6, MTOR, MUTYH, **MYB, MYBL1,** MYC, MYCL1, MYCN, MYD88, NBN, NF1, NF2, **NFE2L2**, NFKBIA, NFKBIZ, NKX2-1, NOTCH1, NOTCH2, NPM1, NRAS, NTRK1, NTRK2, NTRK3, PALB2, PARK2, PAX5, PDCD1LG2, PDGFRA, PDGFRB, PHF6, PHOX2B, PIK3C2B, PIK3CA, PIK3R1, PIM1, PMS1, PMS2, PNRC1, PRAME, PRDM1, PRF1, PRKAR1A, PRKCI, PRKCZ, PRKDC, PRPF40B, PRPF8, PSMD13, PTCH1, PTEN, PTK2, PTPN11, RAD21, RAF1, RARA, RB1, RBL2, REL, RET, RFWD2, RHPN2, ROS1, RPL26, RUNX1, SBDS, **SDHAF2, SDHB, SDHC, SDHD, SETBP1, SETD2,** SF1, SF3B1, SH2B3, SMAD2, SMAD4, **SMARCA4, SMARCB1**, SMC1A, SMC3, SMO, SOCS1, SOX2, SOX9, SRC, SRSF2, STAG1, STAG2, STAT3, STAT6, STK11, SUFU, **SUZ12**, SYK, TCF3, TCF7L1, TCF7L2, TERT, **TET2**, TNFAIP3, TP53, TSC1, TSC2, U2AF1, VHL, WRN, WT1, XPA, XPC, XPO1, **ZNF217**, ZNF708, ZRSR2.

Intronic regions of: ABL1, AKT3, ALK, BCL2, BCL6, BRAF, CIITA, EGFR, ETV1, EWSR1, FGFR1, FGFR3, FUS, IGH@, IGK@, IGL@, JAK2, MLL, MYC, NPM1, PAX5, PDGFRA, PDGFRB, RAF1, RARA, RET, ROS1, TRA@, TRB@, TRG@.