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Copy number variation in archival melanoma biopsies versus benign melanocytic lesions

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Abstract.

BACKGROUND: Skin melanocytes can give rise to benign and malignant neoplasms. Discrimination of an early melanoma from an unusual/atypical benign nevus can represent a significant challenge. However, previous studies have shown that in contrast to benign nevi, melanoma demonstrates pervasive chromosomal aberrations.

OBJECTIVE: This substantial difference between melanoma and benign nevi can be exploited to discriminate between melanoma and benign nevi.

METHODS: Array-comparative genomic hybridization (aCGH) is an approach that can be used on DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissues to assess the entire genome for the presence of changes in DNA copy number. In this study, high resolution, genome-wide single-nucleotide polymorphism (SNP) arrays were utilized to perform comprehensive and detailed analyses of recurrent copy number aberrations in 41 melanoma samples in comparison with 21 benign nevi.

RESULTS: We found statistically significant copy number gains and losses within melanoma samples. Some of the identified aberrations are previously implicated in melanoma. Moreover, novel regions of copy number alterations were identified, revealing new candidate genes potentially involved in melanoma pathogenesis.

CONCLUSIONS: Taken together, these findings can help improve melanoma diagnosis and introduce novel melanoma therapeutic targets.

Keywords: Array comparative genomic hybridization, melanoma, FFPE

1. Background

In the United States, skin cancer is the most common of all cancers [1]. Most cases of skin cancer are non-melanoma skin cancer. In fact, melanoma accounts for less than 2% of skin cancer cases [1]. Melanoma is a cancer that arises from the malignant transformation of epidermal melanocytes, pigment-synthesizing cells of the skin. When melanoma escapes early detection, it becomes one of the most aggressive and

highly lethal forms of cancer. Although it accounts for the minority of skin cancers, a large majority (75%) of skin cancer related-deaths are accounted for by melanoma [1,2]. The incidence and mortality of melanoma has increased dramatically in the last few decades [3]. The American Cancer Society estimates that about 73,870 people in United States will be diagnosed with melanoma in 2015 and about 9,940 people are expected to die from the disease. Importantly, the 5-year survival rate of melanoma depends on the stage of the disease when it is diagnosed. It can be as high as 98% when the melanoma is detected early before it spreads to the lymph nodes or other organs. When melanoma reaches the lymph nodes, the 5-year survival rate drops to 62%, and to 15% when melanoma spread to other organs [2]. Different factors are re-

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sponsible for an increased risk of melanoma development. These factors include physical characteristics such as red hair, blue eyes, light complexion and presence of pigmented lesions. Moreover, environmental factors such as sun exposure are associated with an increased risk of melanoma. Yet, the genetic factors and a strong family history of the disease are the most important factors contributing to an increased risk of melanoma [4,5].

The pigment-producing cells, melanocytes, can give rise to benign (melanocytic nevi) or malignant (melanoma) neoplasms. Early diagnosis of melanoma still the most effective way for long term survival and saving melanoma patients' lives from the disease [6]. In the majority of cases, dermatopathologists can correctly diagnose and differentiate a melanocytic nevus from a malignant melanoma. However, dermatopathologists are aware of the diagnostic difficulties of a subset of melanocytic tumors that cannot be easily classified as benign or melanoma. These tumors have ambiguous histopathological features that overlap between melanocytic nevi and melanoma, where some benign melanocytic nevi, due to secondary changes, show unusual attributes that are more associated with melanoma diagnosis. Therefore, the pathology of melanocytic neoplasms remains as one of the most challenging and controversial areas in diagnostic histopathology [7]. The uncertainty and discordance among expert dermatopathologists in diagnosing melanocytic neoplasms have been shown in several studies [8–14]. The diagnostic uncertainty and the ambiguity of some melanocytic tumors results in melanoma misdiagnosis, which in turn can lead to melanoma overdiagnosis accompanied with increase in medical costs and unnecessary surgeries and stress. Conversely, melanoma underdiagnoses results in negligence of a lethal disease [6] that would have been imminently curable if resected earlier.

Histopathological examination of hematoxylin and eosin (H&E)-stained tissue sections remains the main approach for evaluating melanocytic tumors. However, due to the histopathological ambiguity of some melanocytic neoplasms, molecular diagnostic techniques have emerged in the field of dermatopathology as ancillary tests that can help in the diagnosis of melanoma. These molecular tests have shown promise in improving the differential diagnosis of melanoma. One of these molecular diagnostic techniques that has been used intensively in melanoma diagnosis is immunohistochemical staining for melanocytic markers such as Melan-A (A103), S-100 and HMB-45 [15–17].

More recently, cytogenetic analyses have been developed and become popular methods in the area of distinguishing melanoma from benign nevi. For instance, using fluorescence *in situ* hybridization (FISH) assays as an adjunctive test in the diagnosis of ambiguous melanocytic tumors have been increasingly utilized in dermatopathology laboratories. Several studies have shown the potential of the FISH assay as a successful discriminatory test that can distinguish between problematic melanocytic lesions [7,18–21]. Currently, the most commonly used FISH assay employs a 4-probe panel targeting 4 loci (RREB1, MYB, centromere 6, and CCND1) on 2 different chromosomes. The 4-probes FISH has shown a sensitivity and specificity of 86.7% and 95.4% respectively [18]. Recent study has shown an improvement of the FISH assay by incorporating new probes that target 4 different chromosomes (CDKN2A on 9p21, RREB1 on 6p25, MYC on 8q24 and CCND1 on 11q13) with increased sensitivity and specificity to 94% and 98% respectively [7].

Although the FISH assay was introduced as a diagnostic tool in the field of differential diagnosis of melanoma fairly recently, the principle of developing this assay was based on findings that existed over a decade ago. After the *emersion* of comparative genomic hybridization as a novel technique that can screen the entire genome for copy number changes in one experiments in 1992 [22], several studies (by Bastian and others) have revealed that the majority of melanomas differ from benign nevi in their genetic makeup. These studies demonstrated gain or loss of specific chromosomal segments and showed that the majority of melanomas harbor recurrent chromosomal copy number aberrations. With some exceptions such as in Spitz nevi, these chromosomal rearrangements are rarely detected in melanocytic nevi [23–26]. Frequent genomic alterations known to occur in melanoma include gains at 1q, 6p, 7p, 7q, 8q, 17q and 20q in conjunction with deletions at 6q, 8p, 9p, 9q, 10p, 10q, 11q and 21q [23–25]. These fundamental differences in the pattern of genetic alterations between melanomas and benign nevi established the idea that copy number variations can be diagnostically valuable for histopathologically ambiguous melanocytic neoplasms. Therefore, developing diagnostic assays targeting these genetic differences, such as FISH assays, would help improve the differential diagnosis and prognosis of melanoma.

Utilizing CGH as a research tool has been decidedly a huge advancement in the cancer research field. As previously mentioned, the use of CGH has en-

hanced our knowledge of the genetic alterations occurring in melanocytic tumors. However, in melanoma these genetic alterations tend to be broad copy number events spanning large genomic regions. Rationally, the frequent existence of changes in these genomic regions in melanoma, but not in the benign nevi, indicates the presence of critical melanoma-related genes within these regions. The task of uncovering such genes remains a challenge. Yet, significant progress in CGH technology and the development of newer, high-density, genome-wide single-nucleotide polymorphism (SNP) arrays have simplified this task and make it achievable. The high-resolution microarrays allow for detection of more precise and smaller regions of specific copy number changes. The effectiveness of using these arrays in accurate identification of copy number alterations has been shown in various cancer studies [27–31]. Applying this high-resolution technique in melanoma has shown more detailed and recurrent amplifications and deletions of genomic regions containing important cancer genes. Among these genes are CDKN2A and PTEN in the statistically significant deleted regions; BRAF, EGFR and CCND as the most frequently amplified genes [4, 21,32,33]. Furthermore, good examples of the ability of these high-resolution microarrays in revealing potential cancer genes were demonstrated in identifying the current melanoma biomarker MITF gene [34] and the melanoma metastatic gene NEDD9 as well [35]. Therefore, these advances in array CGH technology have provided great opportunities to detect novel and previously unrecognized key driver genes that can help improve melanoma prognosis, diagnosis, and even aid development of targeted therapies.

Here, we report our investigation for chromosomal aberrations that can help in the identification of genomic targets for melanoma diagnosis and therapy. To detect genome-wide statistically significant copy number events, we analyzed high-density single-nucleotide polymorphism (SNP) array data of 41 melanoma samples compared with 21 benign nevi. We utilize a statistical method called genomic identification of significant targets in cancer (GISTIC) that allow of detection of genomic regions that have a high probability to contain driver cancer genes [36,37]. GISTIC has been actively used in different cancer studies and has helped in the detection of different amplified and deleted genes [38–42]. The 41 melanoma samples analyzed by GISTIC show 8 statistically significant amplifications and 32 deletions, some of which are previously known in melanoma.

2. Methods

2.1. Specimen collection and preparation

Following IRB review, total of 62 specimens (41 melanoma plus 21 benign nevi) were selected from a large archive of FFPE skin biopsies collected at a national dermatopathology laboratory (Dermatopathology Laboratory of Central States, DLCS, Dayton, OH). Patients ranged in age from 14 to 90 years (Table 1). The cohort of biopsy specimens used in this study was collected between 2001 and 2013, making their age range between 2 and 14 years. Specimens were stored in a temperature-controlled environment. Melanomas were all between stages II and V, and were verified by a qualified dermatopathologist. Upon review, melanoma specimens with less than 60% tumor tissue (such as specimens with a brisk lymphocytic infiltrate) were excluded from study.

De-identified retrospective clinical data were obtained from clinical databases and patient health records at DLCS. For all experiments, 10 μ m thick sections were taken for each sample from paraffin blocks by using a microtome with disposable blades. Care was taken to avoid contamination between the specimens by wearing gloves when handling the blocks, cleaning the microtome after cutting a block, and using fresh blades for each specimen. The sections were placed in a warm water bath and then mounted on slides to be air dried. The first and last slides from each block were stained with H&E to verify that the region of interest (consisting of cellular material) had not been exhausted. Tissue was then scraped from slides using sterile scalpel blades into 1.5 mL microcentrifuge tubes or Screw-Cap microTUBES (Covaris, Woburn, MA, USA) for DNA extraction. The number of sections taken per sample varies between methods, as described below.

2.2. DNA Isolation from FFPE tissues

DNA isolation was carried out using column-based methods. Qiagen QIAamp DNA FFPE tissue kit (Qiagen, Valencia, CA, USA) or adaptive focused acoustics (AFA)- based extraction using the Covaris truXTRAC FFPE DNA kit (Covaris).

For the Qiagen method, 24 sections of 10 μ m thickness FFPE tissues were used as suggested by the manufacturer with some modifications as follows. Deparaffinization of FFPE tissues was performed by incubating twice in 1 mL xylene, then in a descending con-

Table I
Clinicopathological data for specimens used in this study

#	Age at diagnosis	Sex	Location	Clark's level	Type
1	77	Male	Right Arm	IV	Superficial spreading type
2	28	Male	Right Ear	I	Malignant melanoma in situ
3	80	Male	Forehead	IV	Desmoplastic type
4	75	Female	Upper Back	IV/V	Superficial spreading type
5	21	Female	Right Shoulder	I	Malignant melanoma in situ
6	40	Male	Back	IV	Malignant melanoma
7	60	Female	Left Leg	IV	Nodular spitzoid type
8	57	Female	Right Arm	I	Malignant melanoma in situ
9	81	Male	Left Auricular	IV	Superficial spreading type
10	93	Male	Right Groin	IV	Nodular malignant melanoma
11	60	Female	Left Leg	IV	Nodular spitzoid type
12	89	Male	Right Shoulder	III/IV	Malignant melanoma
13	63	Female	Right Forearm	IV	Superficial spreading type
14	76	Female	Right Cheek	IV	Superficial spreading type
15	57	Male	Left Arm	IV	Superficial spreading type
16	45	Female	Left Back	IV	Ulcerated nodular malignant melanoma
17	60	Male	Left Shin	IV	Spitzoid type
18	77	Male	Right Elbow	IV	Invasive malignant melanoma
19	27	Female	Right Neck	IV	Posterior invasive polypoid malignant melanoma
20	69	Male	Left Temple	IV	Superficial spreading type
21	74	Male	Left Neck	IV	Superficial spreading type
22	67	Female	Right Back	IV	Malignant melanoma with verticle growth
23	53	Male	Right Arm	IV	Nodular malignant melanoma
24	68	Female	Left Arm	I	Residual malignant melanoma
25	54	Female	Anterior Thigh	IV	Superficial spreading type
26	72	Male	Right Shoulder	III	Superficial spreading type
27	80	Male	Forehead	IV	Desmoplastic type
28	48	Female	Right Forearm	V	Nodular variant
29	40	Male	Back	IV	Malignant melanoma
30	74	Female	Right Arm	II	Superficial spreading type
31	63	Male	Back	IV	Superficial spreading type
32	45	Female	Left Arm	III	Superficial spreading type
33	75	Male	Right Chest	III-IV	Desmoplastic type
34	40	Female	Upper Back	III-IV	Nodular type
35	85	Female	Right Cheek	IV/V	Malignant melanoma
36	87	Male	Right Cheek	V	Malignant melanoma
37	90	Male	Left Back	IV	Superficial spreading type
38	55	Female	Middle Back	II/III	Superficial spreading type
39	28	Female	Left Shoulder	III	Invasive spitzoid malignant melanoma
40	42	Male	Right Pretibial	III	Superficial spreading type
41	75	Male	Left Back	IV	Melanoma with regression
42	34	Male	Right Arm	—	Dermal nevus
43	44	Female	Right Buttock	—	Dermal nevus
44	55	Male	Left Back	—	Dermal nevus
45	19	Female	Left Back	—	Dermal nevus
46	39	Male	Left Neck	—	Dermal nevus
47	35	Male	Left Flank	—	Dermal nevus
48	34	Female	Right Neck	—	Dermal nevus
49	44	Male	Right Groin	—	Dermal nevus
50	27	Female	Right Back	—	Dermal nevus
51	62	Female	Right Eyebrow	—	Dermal nevus
52	48	Female	Left Neck	—	Dermal nevus
53	75	Female	Left Shoulder	—	Dermal nevus
54	34	Female	Left Deltoid	—	Dermal nevus
55	33	Female	Right Back	—	Dermal nevus
56	38	Female	Middle Back	—	Dermal nevus
57	52	Female	Right Back	—	Dermal nevus
58	41	Female	Right Neck	—	Dermal nevus
59	14	Female	Right Shoulder	—	Dermal nevus
60	16	Female	Left Back	—	Dermal nevus
61	21	Male	Right Back	—	Dermal nevus
62	60	Female	Right Neck	—	Dermal nevus

centration of ethanol (100, 75%, then 50%). The tissues were then incubated in 300 μ L Qiagen buffer ATL plus 40 μ L proteinase K (20 mg/mL, 5 PRIME Inc., Gaithersburg, MD, USA) for 72 hours. An additional 30 μ L proteinase K was added at 24 hours, and another 30 μ L at 48 hours. After 72 hours digestion, samples were washed in Qiagen DNeasy Mini Spin Columns with buffers AW1 and AW2. An extra wash with AW2 buffer was used to further reduce the carry-over of solvents. Finally, samples were eluted in 100 μ L ATE buffer.

The second extraction method used adaptive focused acoustics (AFA) technology as described previously [43]. This was performed using FFPE tissues with 10 μ m thickness for 8–10 sections, depending on the size of the tissue, to obtain approximately 5 mg of tissue. The extraction was performed according to the protocol suggested by Covaris in the truXTRAC FFPE DNA kit. Slides were warmed on a heat block to 37°C for 30 seconds. FFPE tissues were then scraped from the slides, avoiding paraffin, using Covaris SectionPicks. Sections were collected into Screw-Cap microTUBES by using FFPE SectionPicks provided by Covaris. AFA was performed per manufacturer's instructions ("protocol C") on a Covaris M220 Focused-Ultrasonicator. Homogenized tissues were then digested for 2 hours in Covaris proteinase K at 56°C. DNA was finally isolated from lysates using the columns of the Covaris truXTRAC FFPE DNA kit and eluted in 100 μ L Covaris BE buffer. When needed, DNA was concentrated by speedvac without heat.

2.3. Quality control

A Nanodrop spectrophotometer was used to quantify DNA concentration as well as determine the A^{260}/A^{230} and A^{260}/A^{280} ratios. For a more accurate quantitation, the Qubit® dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA) was used to check the concentrations of dsDNA for all samples.

Genomic DNA fragment sizes were first estimated by agarose gel electrophoresis of 250 ng DNA using 1% agarose gels (90 mM Tris – borate, 2 mM EDTA, 1% agarose). Samples with visible DNA fragments as large as 23,000 base pairs (bp) were processed further by randomly amplified polymorphic DNA PCR (RAPD-PCR) to directly determine the ability of each sample to produce high molecular weight amplicons ("amplifiability"). Non-specific primers and PCR conditions (below) were used to produce multiple amplicons from each sample. Agarose gel electrophoresis

was used to determine the range of product sizes and only samples with amplicons larger than 500 bp were used for microarray analysis.

RAPD-PCR reactions were carried out in a 20 μ L volume containing 25 ng DNA and using 10 μ L of GoTaq 2X Green Master Mix (Promega, Madison, WI USA). PCR was performed in 0.2 mL tubes in a GeneAmp PCR System 9700 thermocycler (Life Technologies, Carlsbad, CA, USA).

Primers used for RAPD-PCR were generated by Eurofins MWG Operon Inc. (Huntsville, AL, USA). Sequences for the primer pairs and cycling parameters were as follows: 5'-AATCGGGCTG-3' and 5'-GAAACGGGTG-3', 94°C for 2.5 minutes, then 45 cycles of 1 minute 94°C, 1 minute 55°C and 2 minutes 72°C, then 7 minutes 72°C and holding at 4°C; or 5'-TGTGCCCAGTGAAGACTCAG-3' and 5'-GAGTGA GCGGAGAGGGA ACT-3', 45 cycles of 94°C for 1 minute, 35°C for 1 minute, and 72°C for 2 minutes. PCR products were resolved on 3% TBE agarose plus SYBR Safe dye (Life Technologies). Gels were visualized with a GE ImageQuant LAS-3000 camera (GE Healthcare Life Sciences, Piscataway, NJ, USA).

2.4. Microarrays

DNA copy number analysis was performed by hybridizing the extracted and qualified gDNA to Affymetrix SNP6.0 microarrays (Affymetrix, Santa Clara, CA, USA). All samples passed RAPD-PCR quality control. 0.5 μ g of genomic DNA was processed using the SNP 6.0 protocol and microarrays by Affymetrix with some modifications to the standard protocol. The input amount of DNA was increased from 250 ng per restriction enzyme (Nsp1 and Sty1) to 500 ng each. The number of PCR reactions was doubled from the suggested three for Sty1 and four for Nsp1 to six for Sty1 and eight for Nsp1. It is important to note that the number of reactions was increased; the number of cycles in each reaction remained the same. The additional PCR reactions were combined as in the standard protocol. PCR cleanup was performed using isopropanol extraction (refer to Affymetrix User Bulletin 2: Improvements to step 7 of the SNP Assay 6.0, PCR cleanup using an isopropanol precipitation method, P/N 702968 Rev. 1). Hybridization and scanning of the arrays followed the manufacturer's protocol to generate SNP6.0 CEL files.

2.5. Data analysis

The Copy Number Inference Pipeline is a method in GenePattern from the Broad Institute (Massachusetts Institute of Technology, Cambridge, MA) that takes Affymetrix SNP6 CEL files and process them in a pipeline consisting of different modules to generate segmented copy number calls for each sample [44,45]. DNA copy number was estimated probe set-wise by comparing the normalized signal from 41 melanoma samples to data generated from 21 benign samples that were used as a reference. Briefly, the pipeline first uses SNPFileCreator_SNP6 module to normalize all of the SNP arrays by adjusting the raw intensity values from the SNP6 array so that they can be compared with other arrays. The second step is to convert intensity measurements into copy number calls by using the CopyNumberInference module. Then the copy number noise was calculated and copy number calls were de-noised with the RemoveCopyNumberOutliers module that removes probes that are outliers, which have radically different copy number calls than their hg19-adjacent neighbors. The Tangent normalization algorithm then reduces the noise further by subtracting out variation seen in a pre-defined set of a panel of more than 3000 blood normals from the Cancer Genome Atlas (TCGA). This exclusion of the germline CNVs is particularly important for algorithms that identify somatic alterations that are statistically significant such as GISTIC. The copy number data then were segmented by using the CBS (Circular Binary segmentation) algorithm that identifies regions in the genome that, in spite of noise, probably have a uniform underlying copy number. It compresses the values from a set of adjacent probes into a single value for that interval [46]. Lastly, the segmented copy number data were analyzed with GISTIC to identify significant melanoma genetic aberrations.

Genomic Identification of Significant targets in Cancer (GISTIC2.0) is a statistical method (also within GenePattern) that identifies likely somatic copy number alterations that drive cancer pathogenesis by evaluating the frequency and amplitude of observed copy number events that are more frequent than would be expected by chance [36,37]. GISTIC assigns G^{AMP} and G^{DEL} scores to each locus. The G score represents the frequency of a genetic aberration (amplifications or deletions) seen at that locus across a set of samples, multiplied by the average (increase or decrease) in the log2 ratio in the region of aberration. A peak region is reported at each statistically signifi-

cant genomic region of aberration and known genes located at that region are listed. GISTIC performs false discovery rate control where G-scores are compared against a null model, and regions with q-values below 0.25 are considered statistically significant. GISTIC analysis was performed using the default parameters with some modifications. Based on the segmented data values, we set the amplifications and deletions threshold for log2 ratios to > 0.1 and < -0.3 , respectively. The segmented copy number data (in log2 ratio) were capped to 2, where regions with log2 values greater than 2 are replaced with 2 and regions with log2 values less than -2 are replaced with -2 . The confidence level used to calculate the region containing a driver was 99%. GISTIC also employed a length-based filtering of arm-level events where all copy number events occupying more than 98% of a chromosome arm were removed. Using this length-based filter greatly increases the sensitivity of GISTIC to detect focal events [36]. When calculating for significant deletions, segments that contain a number of markers less than or equal to 10 were joined to the neighboring segment that is closest in copy number. The Genomic coordinates were mapped to the human genome build 19 (<http://genome.ucsc.edu>).

3. Results and discussion

3.1. Amplifications identified by GISTIC

21q22.1

This region has not been shown to be amplified in melanoma before. Only one gene was reported in this region in our findings, which is ERG. ERG “Avian v-ets erythroblastosis virus E26 oncogene” is a proto-oncogene that belongs to the ETS transcription factor gene family that plays a role in embryonic development, cell proliferation, angiogenesis, and apoptosis [47,48]. Overexpression of ERG has been found in different cancers including Ewing sarcoma, acute myeloid leukemia, and meningiomas [47]. Moreover, the oncogenic role of ERG is prominent in the TMPRSS2-ERG fusion gene in prostate cancer [49–53]. These recent findings have declared the role of this gene in cancer. In this study, a high focal amplification targeting the ERG gene on chromosome 21 was identified. This focal amplification strongly suggests that ERG could be an important driver gene in melanoma.

11q14.1

This region has just one gene, which is DLG2. DLG2 “discs, large homolog2” is a member of membrane-associated guanylate kinases family that has important roles in tissue developments, cell-cell communications, cell polarity control, and cellular signal transductions [54]. A study found DLG2 was upregulated in renal oncocytoma, a benign tumor of the kidney, which explains its potential role as an oncogene [55]. Interestingly, this chromosomal location 11q14.1 is located close to CCND1 at 11q13, an amplified region in melanoma that is used as a FISH target to distinguish between melanoma and benign nevi [7].

10q11.21

This peak represent a focal amplification of this region that spanned the RET gene. RET “Rearranged during transfection” is a proto-oncogene that encodes a transmembrane receptor tyrosine kinase [56–58]. RET has been shown to have an important role in human cancers [57,58], and its amplification and overexpression have been reported in different types of cancers such as thyroid cancer [59], lung cancer [60], breast cancer [56], and pancreatic cancer [61]. Furthermore, RET has been shown to be involved in activation of several important signaling pathways including PI3K, Ras/MAPK, Jun N-terminal kinase “JNK” and PLC-dependent pathways [58,62].

In melanoma, a study on human melanoma cell lines has showed a correlation between the expression of RET and melanomagenesis. Also, the study showed that inhibition of RET signaling suppressed all proliferation and invasion in melanoma [62]. Another study reported the involvement of RET in melanoma development in RET-mice and human melanoma cells [63]. These two studies besides the focal region of amplification centered on the RET gene in our study indicate the potential importance of RET in melanoma. Therefore, our study suggest that RET could be one of the driver genes in melanoma tumorigenesis.

6p24.3

Gain of 6p is a common chromosomal imbalance in several human cancers, which indicate the importance of genes involved in this region in cancer pathogenesis [64]. In melanoma, gain of 6p is one of the most common chromosomal abnormalities that was reported in several studies [5,6,25,26,35,64,65]. Identifying important genes in this large gain still a challenge. In melanoma, the minimal region of 6p gain has not been characterized [65]. In our study, this peak at 6p24.3

represents a partial gain of 6p (22.74 Mb). Interestingly, several important genes in melanoma were identified in this peak. NEDD9, one of the main melanoma metastasis genes [35], RREB1, one of the FISH assay targets that is used to distinguish between melanoma and benign nevi [7], and DEK, an oncogene that was reported to have a dual and selective roles in proliferation and apoptotic resistance in melanoma [65]. Another important gene in this region is E2F3 gene, an oncogene that has an important role in tumorigenesis in bladder cancer [66]. Therefore, against the large background of 6p gain that is common in melanoma, the partial gain of this part of chromosome 6p in our analysis, with known genes in melanoma located in this part, minimizes the broad gain of 6p to a smaller region that is highly associated with melanoma.

1q31.2

This peak represents gain of the entire long arm of chromosome 1 (1q). Gain of the long arm of chromosome 1 represents a common genetic alteration in melanoma [5,6,25,26,64]. A CGH study has shown that Patients with 1q and 6p gain had a lower overall survival rate in comparison with patients without these gains, which implies that 1q and 6p gains could give a prognostic differences [67]. Several important genes in cancer, and more specifically in melanoma, are located on this genomic region. This includes AKT3 [68,69], MDM4 [70], and ABL2 [71]. Our study confirms that gain in chromosome 1q as one of the most important genetic alterations in melanoma.

8q24.23

Gain of 8q is another hallmark in melanoma that was reported in many different studies [4,6,25,26,72]. In our findings, the peak on chromosome 8 represent high amplification of a part of the long arm of chromosome 8, which is 8q24. A study has suggested that targeting this region 8q24 by FISH assays could be a useful prognostic marker in melanoma cancers [73]. A later study that sought to improve the sensitivity and specificity of FISH assay for discriminating melanoma from nevi has confirmed and included the 8q24 region as one of four FISH targets with high discriminatory power to differentiate between melanoma and benign nevi [7]. The most prominent gene in this region is MYC, a proto-oncogene that encodes a nuclear phosphoprotein transcription factor that plays a role in different cellular processes, such as proliferation, cell cycle progression, metabolism, differentiation and apoptosis [74]. MYC amplification has been

shown in different cancers including prostate [75] and breast cancer [76]. In melanoma, it has been shown that melanomas with gain of 8q24 have elevated cytoplasmic and membranous expression of MYC in comparison with melanomas without gain of 8q24, where they had significantly decreased MYC expression. This elevated expression of MYC seems to play a role in the aggressive clinical behavior of melanomas [77]. This is another common genetic alteration in melanoma that is confirmed in our study.

7p12.3 & 7p14.3

Gain of the p arm of chromosome 7 is one of the most common copy number gains in melanoma [6,25,26,78]. On the other hand, the q arm in melanoma is known with the activating point mutation of the BRAF oncogene [79]. Although the gain of 7p is common in melanoma, targets in this arm still undescribed [78]. Here, two focal amplifications were identified in our study, 7p12.3 and 7p14.3 regions. For the 7p12.3 region, GISTIC has reported this region with no known genes, but TNS3 is the nearest known gene to that region. Despite what gene was found here, this chromosomal region has gotten much attention in cancer studies. Amplification of this region has been reported in different types of cancers including amplification of 7p12.3 in pancreatic cancer [80] and rectal cancer [81]. Also amplifications in the 7p12 band has been shown in acute lymphoblastic leukemia [82] and osteosarcomas [83]. The attention to this region is mainly because the oncogene EGFR “Epidermal growth factor receptor” is located in this band [84]. EGFR is known to play a role in metastasis, cellular proliferation, invasion, and in cancer progression in general [85]. Gain of 7p12 band has been associated with gain of EGFR gene as in squamous cell carcinoma of the lung [86] and gastric cancer [87]. In melanoma, a study that used two different cytogenetic approaches (FISH and aCGH) has found a frequent amplification of the region 7p12.3 among melanoma samples [88]. Other studies that focused on the expression level of EGFR on 7p12.3 in melanoma have shown a correlation between high expression of EGFR and gain in copy number of this region, which might explain the role of this amplification in development of malignant melanomas [89,90].

The other region, 7p14.3, was reported with amplification of just one gene, AAA1 NPSR1-AS1. Very little is known about this antisense RNA. However, these findings do not exclude the possibility that other genes located at 7p14 can be influenced by this gain of this region. An example, the gene NPSR1, a G protein cou-

pled receptor, is also located on 7p14.3. Overexpression of this gene has been reported to activate some cancer-related pathways [91].

The importance of our results lies in showing a focal amplification of 7p12-p14 region in chromosome 7p as a statically significant copy number gain, instead of showing a broad copy number change that encompasses the entire arm, which implies the importance of this region on chromosome 7p in melanoma.

3.2. Deletions identified by GISTIC

8p23.2

This region was reported by GISTIC with the lowest q value among all other deletions. Loss or decrease in copy number in chromosome 8p has been observed in melanoma [4,25,26] and other cancers such as prostate cancer [92] and breast cancer [93]. Despite the frequent deletion of this chromosomal arm in melanoma, the molecular drivers of the 8p loss remain uncharacterized. Here, our study shows a minimal region of deletion within chromosome 8p. This focal deletion of 8p23.2 harbors the tumor suppressor gene CSMD1. Deletion of this minimal region with the tumor suppressor gene CSMD1 has been reported in many different cancers, including colorectal cancer [94], liver cancer [95], ovary cancer [96], and more common in the head and neck squamous cell carcinoma (HNSCC) and correlates with poor prognosis [97,98]. In melanoma, a recent study of Tang et al. has reported that CSMD1 functions as a tumor suppressor gene in melanoma cells. They found that the level of CSMD1 mRNA and protein in melanoma cells was lower than in normal skin cancer. Also, they showed that CSMD1 expression decreased proliferation and migration, and increased apoptosis and G1 arrest in A375 melanoma cells in vitro. Furthermore, the survival rate of mice with tumors expression CSMD1 was significantly higher than mice with tumors that did not express CSMD1. Moreover, the study showed that CSMD1 exhibits antitumor activity through activation of Smad pathway [99]. That study and our study provide CSMD1 as a candidate biomarker gene in melanoma.

4p16.3

Deletion of the short arm of chromosome 4 is common in several cancers including breast cancer [100], colon cancer [101], gastric cancer [102], and lung cancer [103]. However, deletion of this chromosomal arm does not seem to have been observed previously in

melanoma. Yet, loss of the chromosome 4 has been reported in melanoma before [4]. In this study, a novel minimal deletion of the 4p16.3 telomeric region was identified. This region spanning 1.05 Mb included 26 genes, 6 of which are zinc finger genes. An interesting gene that is also mapped to this region is the atypical fibroblast growth factor receptor (FGFRL1). Deletion or LOH of the FGFRL1 has been investigated intensively in bladder cancer, where a recent study [104] has investigate the role of FGFRL1 as a candidate tumor suppressor in cancer. The reasons that were proposed to consider FGFRL1 as a candidate tumor suppressor were that FGFRL1 acts as a decoy receptor preventing activation of conventional FGFRs due to its lack of the intracellular tyrosine-kinase domain, also it interacts with the negative regulator of the MAPK signaling pathway SPRED1, and it is ability to promote cell adhesion ~~by promoting cell adhesion and~~ could therefore prevent tumor development and spreading by enhancing cell-cell adhesion and inhibiting invasion and metastasis [104]. Moreover, FGFRL1 has been shown to be down regulated in ovarian tumor [105] and to reduce cell proliferation in response to FGF2 when ectopically expressed in the osteosarcoma cell line MG-63 [104].

6q26

Loss of the long arm of chromosome 6 is well-known genetic alteration in melanoma [4–6,25,26]. Yet, few drivers have been pinpointed in this region of loss. One of the melanoma biomarkers in this chromosomal arm is MYB (6q23) that is used in FISH assays [7]. Here, rather than detecting the broad loss of the chromosome 6q that is known in melanoma, a high significant of deletion in a narrow region was detected (in fact, the broad deletion of 6q was reported in the GISTIC figure, but another higher and smaller peak emerged from that broad deletion, which is 6q26, indicating that this small deletion is the most significant minimal deletion in 6q). This region 6q26 contains the tumor suppressor gene PARK2. Inactivation of PARK2 due to copy number loss has been identified in various human cancers including esophageal adenocarcinoma, glioma, non-small cell lung cancer, lung adenocarcinoma, ovarian cancer, pancreatic adenocarcinoma, and in skin cutaneous melanoma (3.5%) (reviewed in [106]). The copy number loss is the primary mode of alteration that inactivates PARK2 [107]. This gene was studied as an important tumor suppressor gene for several reasons. First, its frequent deletion in many cancers, as was mentioned above. Second, it

is involvement in many crucial cellular processes and pathways, such as controlling cell cycle progression. A study on a large group of tumors has reported the PARK2 as master regulator of G1/S cyclins, where it mediates the coordination of different classes of cyclins and therefore regulates the cell cycle. The study showed that PARK2 targets cyclin D and cyclin E for degradation, therefore inactivation of PARK2 results in the accumulation of cyclin D and acceleration of cell cycle progression [107]. Moreover, the mRNA and the protein expression of PARK2 have been shown to be downregulated in many different cancers, and the low mRNA expression correlates with increased lymph node metastasis, higher tumor grade, and worse overall survival in ccRCC [106].

The other gene that was reported in this narrow deletion is PACRG, which is located 670 bp upstream of PARK2 and transcribed from the opposite DNA strand. PACRG has been shown to be downregulated in leukemias, glioblastoma, and astrocytic tumors [108]. A study on ccRCC has reported that the mRNA and protein expression of PACRG and PARK2 together was significantly downregulated compared with the nonmalignant tissue [108].

So, this focal deletion in our study, different than the large deletion of chromosome 6q that is frequently detected in melanoma, seems to be important in cancer development.

11q13.1

Deletion of chromosome 11q is commonly known in melanoma [4,23,25,109] and other types of cancers such as breast cancer [110], lung cancer [111], and neuroblastoma [112]. Different studies and biological evidences support the existence of melanoma tumor suppressor genes on chromosome 11q, and deletion of this chromosome in melanoma has been reported to be associated with advanced tumor stage, younger age at presentation, poorer prognosis, and metastasis to the brain [109]. In our study, a focal deletion of ~94 kb region was defined in this chromosomal arm. This narrow region contains the ovarian tumor domain-containing Ub aldehyde-binding protein 1 (OTUB1). OTUB1 is expected to play broad functions in cells [113]. However, a recent study has shown that Otub1 is positive regulator of the tumor suppressor p53 [114]. The study showed that Otub1 plays a critical role in p53 stabilization and activation in cells in response to DNA damage, and that through suppression the MDM2-mediated p53 ubiquitination. Further, overexpression of Otub1 results in marked apoptosis

and inhibition of cell proliferation in a p53-dependent manner. Also, Inhibition of Otub1 markedly impaired p53 activation induced by DNA damage [114]. Therefore, a recent review article concluded that Otub1 may act as a tumor suppressor, and more studies are needed to determine if Otub1 is downregulated in human cancers [113].

2p21

This deletion centered on the gene PKDCC, which has a developmental role [115] but no suspected significance in cancer. It also overlaps a portion of a long noncoding RNA LOC102723824, which has not been characterized. Interestingly, this region includes the first exon of EML4. Multiple fusion products of EML4 and ALK have been characterized in non-small cell lung cancer [116–119].

5q35.3

Although several genes fall within this region, few may be of interest in cancer. SQSTM1 is an activator of NF- κ B [120], which plays a complex role in cancer etiology. It is thought that early inhibition of NF- κ B may help cancer cells evade the immune system, although late inhibition is known to promote cancer cell survival [121].

Deletions on chromosome 5 do not seem to be common genetic alterations in melanoma. One study has observed deletion of chromosome 5q in melanoma, but indicated that deletion of this chromosome has not been associated with harboring any putative TSGs in melanoma [33]. However, loss of this region (5q35.3) has been reported in a number of tumors such as non-small cell lung carcinoma [122], and breast tumors [123], which implies the potential importance of genes within these region in cancer development.

6p22.1

Deletion of chromosome 6p has been reported in various types of tumors [124]. Yet, this is not true in melanoma, where amplification of this chromosomal arm is well known, as mentioned above. However, here, a very narrow focal deletion was reported, spanning only one gene, MAS1L. This deletion does not overlap with the amplified region that was reported here (6p24.3). Interestingly, homozygous deletion at 6p22.1 has been shown in different cancers such as gliomas [124] and high frequent LOH of this region was reported in cervical cancer [125]. Although MAS1L does not seem to have a known role in tumorigenesis, frequent deletion of this region in different cancers suggest a potential importance for genes in this region.

5p15.33

As was mentioned above, deletions on chromosome 5 in melanoma are not common. However, here, a very interesting minimal region of deletion was identified on the chromosome 5p. Since deletion or LOH of 5p15 is common in different cancers [126], several studies aimed to identify the minimal deletion of this region. A study on sporadic gastric carcinomas found high frequent LOH at 5p15.33, and an obvious genotype-phenotype correlation on 5p15.33 was observed [127]. Moreover, other studies on cervical carcinoma and sporadic colorectal cancer have reported 5p15.3 as the minimal deletion on 5p [128,129]. Further, 5p15.3 has been proposed to contain one or more tumor suppressor genes [126]. In our study, this narrow region (1.05 Mb) was reported harboring 18 genes, 3 of which are important putative tumor suppressor genes AHRR, SDHA, and NKD2.

ARHH: a study has reported AHRR as a tumor suppressor gene in multiple human cancers. This study found a consistent downregulation of AHRR mRNA in human malignant tissue from different origins including colon, breast, lung, stomach, cervix and ovary. Moreover, they found that silencing of ARHH enhances tumor growth *in vitro* and *in vivo* through deregulation in cell cycle control and protects against apoptosis and enhances angiogenic potential, migration and invasion in tumor cells. Furthermore, ectopic expression of AHRR resulted in growth inhibition and reduced angiogenic potential. The study concluded that AHRR plays an important role in suppressing tumor formation in humans [126].

SDHA: a study on paraganglioma “known with RET mutation” has reported SDHA as a tumor suppressor gene. SDHA is not well studied, but the study, through immunohistochemistry and transcriptome analysis, indicated that SDHA acts as a tumor suppressor gene through activation of a pseudo-hypoxic pathway [130].

NKD2: in a very recent study on osteosarcoma (OS), NKD2 was shown to be a negative regulator of WNT signaling pathway. The study showed that decreased expression of NKD2 is associated with highly aggressive OS state. Also, overexpression of NKD2 in metastatic human and mouse OS cells significantly decreases cell proliferation, migration, and invasion ability *in vitro* and significantly diminishes OS tumor growth and metastasis *in vivo*. Therefore, the study showed NKD2 as a novel suppressor of OS tumor growth and metastasis in both mouse and human [131].

16q23.3

Loss of chromosome 16q, and particularly 16q23 has been reported in different cancers [132–134]. In melanoma, different studies have shown loss of chromosome 16q [6,135]. Moreover, a CGH study on primary and metastatic melanoma has reported the loss of this minimal deletion of 16q23, but no gene was specified in this region [136]. In our study, chromosome 16q was reported with a focal deletion centered at the 16q23.3 band. This narrow region harbors the putative tumor suppressor gene CDH13, and two non-coding genes *has*-mir-3180 and miR-3182. The involvement of CDH13 in various cancers was reviewed recently by Andreeva and Kutuzov [137]. Downregulation of CDH13 has been reported in broad range of cancers, including melanoma cell lines, and nondetection of CDH13 transcript in all examined breast cancer and most other cancer cell lines supports its role as a tumor suppressor. Further, the review discussed association between downregulation of CDH13 and poor prognosis in various carcinomas. CDH13 re-expression in most cancer cell lines inhibits cell proliferation and invasiveness, increase susceptibility to apoptosis, and reduce tumor growth *in vivo* models. Reporting this minimal deletion containing CDH13 as the most significant loss in chromosome 16q across the melanoma samples might imply the importance of this gene in melanoma tumorigenesis.

17q11.2

This is a well-known frequent region of deletion in different cancers, and that because it harbors the famous tumor suppressor gene NF1, the RAS inhibitor. NF1 is the gene that encodes RAS GTPase-activating protein, so affecting this gene by deletion or mutation affects RAS-MAPK signaling. NF1 has been reported to work as a tumor suppressor in melanoma [138]. Loss of NF1 function in melanoma has been shown in several studies [139–141]. These findings confirm the frequent inactivation of the NF1 tumor suppressor in melanoma.

12q24.31

This is focal deletion was reported on chromosome 12q, which has been found to be amplified in melanoma [23]. Yet, this particular region of deletion (12q24) has also been reported in melanoma [136, 142], which indicated the tendency of this region to be lost in melanoma cells. In our results, this minimal deletion was reported containing NCOR2 gene. Interestingly, NCOR2 (also known as SMRT) is a tu-

mor suppressor that has been reported in different cancers. SMRT was shown to be involved as a novel tumor suppressor in non-Hodgkin lymphomas [143]. Also, down-regulation of SMRT in multiple myeloma has been shown to jeopardize several gene functions that play an important role in apoptosis, therefore, restoration of SMRT activity might correct the overexpression of antiapoptotic genes [144]. Lastly, a recent study has reported that SMRT is an activator of p53 transcription [145].

20q11.21

Here, a very short region of deletion in the chromosome 20q was reported. This short deletion contains just one gene called BPIFB1, known also as LPLUNC1. Recent studies have reported this gene as an important tumor suppressor gene in NPC (Nasopharyngeal Carcinoma) [146]. The study reported that LPLUNC1 inhibited NPC cell proliferation *in vitro* and tumor formation *in vivo*. LPLUNC1 also delayed cell cycle progression from G1 to S phase and inhibited the expression of cyclin D1, cyclin-dependent kinase 4 (CDK4) and phosphorylated Rb. LPLUNC1 inhibited the expression of certain mitogen-activated protein (MAP) kinases (MAPK) kinases and cell cycle-related molecules. Western blotting confirmed that the expression of MEK1, phosphorylated ERK1/2, phosphorylated JNK1/2, c-Myc and c-Jun were inhibited by LPLUNC1, suggesting that the MAPK signaling pathway is regulated by LPLUNC1 [146]. Another study reported that LPLUNC1 *inhibits* NPC cell proliferation through inactivation Stat3. Induction of LPLUNC1 overexpression inhibited NPC cell proliferation, induced NPC cell arrest, promoted NPC cell apoptosis even after IL-6 stimulation, and inhibited the growth of implanted NPC tumors *in vivo*, which were associated with decreasing cyclin D1 and Bcl-2 expression and the Janus kinase 2 (JAK2)/Stat3 activation, but enhancing Bax and p21 expression [147].

6q14.1

The gene SH3BGRL2 is a paralog to SH3BGRL. While the latter has been shown to contribute to Rel-mediated transformation when inactivated [148], so such role has yet been identified in the former.

10q23.2

10q is a known deletion in melanoma [4]. Loss of 10q23 is common in melanoma and loss of this region (more specifically 10q23.3) has been associated

with inactivation of the tumor suppressor gene PTEN in melanoma [4,149,150].

This region also contains the last exon of WAPAL, a gene which is a component of the cohesin complex. Loss of WAPAL function potentially prevents the release of cohesin from sister chromatids [151] or interferes with DNA repair [152].

5q35.1

Only one gene was reported at this focal deletion, which is the potassium channel subfamily M regulatory beta subunit 1, KCNMB1. There is no known importance of this gene in oncogenesis. However, this region has been reported to be frequently deleted in lung cancer, indicating the presence of important genes with tumor suppression function at this region [122, 153,154].

16p13.3

Deletion of 16p has been reported in melanoma [26, 155]. Here, a small (~752 Kb) telomeric deletion that contains 40 genes. Here, at least two known tumor suppressors can be identified, AXIN1 and ARHGDI1.

AXIN1 is a WNT pathway tumor suppressor that is essential for beta catenine degradation, and it is inactivation has reported in various tumors [156,157]. Furthermore, AXIN has been shown to act as a tumor suppressor through stimulating p53 function [158].

ARHGDI1 is a metastasis suppressor that has been shown to contribute to cancer cell invasion and metastasis, and also has been shown to be involved in mouse melanoma B16 cells [159].

13q13.3

Deletion of this particular region 13q13.3 has been reported in different cancers such as breast cancer [160, 161]; and lung cancer [162]. Here, this region was reported with one gene DCLK1. Some studies have reported deletion of this region with this gene such as in testicular primary seminoma [163], and in pleomorphic sarcoma of bone [164]. A study on melanoma has reported a large deletion of 13q12-34 [135].

3q13.31

ZBTB20 is involved in NF- κ B signaling and promotes the innate immune response [165] (cf. SQSTM1 also deleted). It is also a negative regulator of Sox9 [166]. Sox9 has been shown to induce cell cycle arrest and apoptosis in melanoma via antagonism of Sox10 [167]. Therefore, deletion of Sox9 has the potential to promote melanoma initiation and progression.

10p15.3

Loss of 10p15.3 is a common deleted region in different cancers including colorectal cancers [168] and lung cancer [153]. In melanoma, loss of 10p is common genetic alteration (with loss of 10q as well as loss of the whole chromosome) [4,33,169]. Microarrays studies on melanoma have reported deletion of 10p15.3 [33,150]. In our study, this region of deletion was reported with the RNA editing enzyme gene ADARB2. ADARB2 RNA level has been reported to be 99% decreased in brain tumors and ADARB2 reduction correlates with grade of malignancy of glioblastoma multiforme, the most aggressive of brain tumors [170].

3p22.1

This is a very narrow deletion centered at the gene VIPR1. This region of deletion is common in non-small cell lung cancer [171]. VIPR1 has been reported to be a tumor suppressor in lung adenocarcinoma, where it is significantly downregulated, and deletion of 3p22 is the mechanism that leads to its downregulation [172].

1p32.3

This deletion encompasses the entire gene ACOT11, a lipid transfer protein. ACOT11 has been shown to be significantly methylated in bladder cancer, and the degree of methylation was associated with tumor stage [173]. Any specific biological function of ACOT11 in cancer remains to be determined.

11p15.4

This region is adjacent to a domain on 11p15.5 known to play a role in Wilms and rhabdomyosarcoma [174], adrenocortical carcinoma [175], and lung [176], ovarian [177] and breast cancers [178].

Interestingly, 11p has been reported with reduced copy number in melanoma [25].

21q22.3

Loss of 21q22.3 has been shown to be associated with melanoma [179]. Within this large deletion, the transient receptor potential channel gene TRPM2 has been shown to increase the susceptibility of melanoma to apoptosis and necrosis [180]. An antisense transcript of TRPM2 is up-regulated in melanoma (ibid). Intriguingly, the related gene TRPM1 (melastatin, at 15q13.3) has been known to be downregulated in highly metastatic melanoma [181]. 21q22.3 deletion is also observed in prostate cancer [182].

18p11.31

Here, only one gene was reported, LAMA1. It is one of the genes that encode laminins, which are major proteins in the basal lamina. They influence cell differentiation, migration, and adhesion. Also they can play a role in invasive behavior of tumor cells [183]. LAMA1 has been reported to be hypermethylated and underexpressed in pancreatic tumor samples compared to normal samples [184]. Moreover, downregulation of LAMA1 has been reported in ovarian cancer cell lines [183].

1q21.3

This deletion is almost exclusive to the gene KCNN3 (SK3). This potassium channel is better known for its role in neuron firing, but it has also been shown to play a role in the motility of breast cancer [185] and melanoma [186] cells. It was shown that KCNN3 was not expressed in normal melanocytes, but that induced expression of KCNN3 in melanoma cells increased migration. This work was done in cultured melanoma cell lines; the frequency of SK3 overexpression in melanoma is unknown *in situ*, but our data would suggest that SK3 overexpression could be an artifact of cell culture.

2p21

This deletion includes the last exon of PPM1B. PPM1B is thought to act as a phosphatase toward IKK β , thereby attenuating the activity of NF- κ B [187]. This locus as a whole is involved in 2p21 Deletion Syndrome (OMIM #606407), which is not known to contribute to an elevated prevalence of any cancers.

9p21.3

Although deletion of chromosome 9 was also reported in our result, a more focused deletion was also reported in chromosome 9 centered at 9p12.3. This region contains the well-known tumor suppressor gene CDKN2A, and other tumor suppressors such as CDKN2B, MTAP and ELAVL2. CDKN2A is the one of the highest-penetrance melanoma susceptibility *gene* [188,189] and *it is* deletion or loss of expression in melanoma is well-known [188–191]. As was mentioned above, other important genes are also located in this region. CDKN2B is one of the genes located at 9p21 and has been shown to be deleted in melanoma [6,192] and other cancers such as cutaneous T-cell lymphoma [193]. Interestingly, codeletion of a cluster of genes involving CDKN2A, CDKN2B, MTAP and in some cases ELAVL2 has been

shown in different cancers. For instance, deletion of CDKN2A, CDKN2B, MTAP and ELAVL2 in myeloid leukemia [194] and CDKN2A, CDKN2B and MTAP in B- lineage acute lymphoblastic leukemia [195], head and neck squamous cell carcinoma [196], mesotheliomas [197] and in glioblastoma [198]. Deletion of this large 9p21 segment is a frequent genetic alteration in variety of cancers causing inactivation of critical tumor suppressor genes and therefore plays a very important role in development of many human cancers, including melanoma [199,200]. Our results confirm the frequent involvement of 9p21 deletion in melanoma samples.

However, 9p21 is not a high discriminatory loci, where *it is* heterogeneous deletion can also be seen not just in melanoma but in melanocytic nevi as well. In contrast, homozygous deletion of 9p21 seems to be more unique to melanoma [201,202].

22q13.32

This deletion encompasses over 150 annotated genes, and is frequently deleted in human breast and colon cancers [203]. Within this region are at least three genes with some demonstrated connection to cancer: BIK, PRR5, and PANX2. BIK is an important player in the activation of Bax to induce apoptosis, and has been found to be deleted in several human cancers [204]. Interestingly, overexpression of Bik induces apoptosis in melanoma cells, and BIK expression in a xenograft model delayed melanoma tumor growth [205]. PRR5 is suspected tumor suppressor gene in breast cancer [203] and a component of mammalian target of rapamycin complex-2 (MTORC2) [203] although little is known about its function. PANX2 acts as a tumor suppressor in glioma cells [206]. The related pannexin family members PANX1 and PANX3 show reduced expression in basal and squamous cell carcinomas [207]; however, PANX1 may be a driver of melanoma [208]. Much remains to be understood about how these pore channel proteins play roles in tumorigenesis. 22q13.32 has also been found deleted in 25% of fibrolamellar hepatocellular carcinomas [209].

2q33.2

Two caspases falls into this region. CASP8 lies just downstream of death receptors in the cell-extrinsic apoptosis pathway. As such, it has been found to be mutated or lost in many cancers (reviewed in [210]). CASP10 mutation has been observed in gastric cancer [211] and non-Hodgkin's lymphoma [212].

Fig. 1. Focal amplifications determined by GISTIC. Chromosome position is arranged along the x-axis and GISTIC q-value is shown on the y-axis. The default cutoff of 0.25 for genes discussed here is indicated in green. The locations of genes not specifically implicated as gained in melanoma previously are depicted in bold.

Fig. 2. Focal deletions determined by GISTIC. Chromosome position is arranged along the x-axis and GISTIC q-value is shown on the y-axis. The default cutoff of 0.25 for genes discussed here is indicated in green. The locations of genes not specifically implicated as lost in melanoma previously are depicted in bold.

RAPH1, also found within this deletion, has been observed as lost in oral squamous cell carcinoma [213], head and neck squamous cell carcinoma [214], esophageal squamous cell carcinoma [215], lung cancer [216], and neuroblastoma [217]. Reduced expression of ABI2, also found in this region, is observed in breast and ovarian cancers [218]. Many studies have reported ABI2 as a tumor suppressor, where Abi2 promotes Abl-mediated phosphorylation of Cdc2 and inactivation of Cdc2 kinase activity, leading to suppression of cell growth [219,220].

13q34

This deletion has been observed previously in several cancers, but not to our knowledge in melanoma. Notably, 13q34 is lost in 45% of cutaneous anaplastic large cell lymphoma [221], 67% of chronic lymphocytic leukemias [222], and 8% of Burkitt lymphomas [223]. It has also been noted missing in some cervical squamous cell carcinomas [224–226], breast cancer cases showing centrosome abnormalities [227], one case of chronic lymphocytic leukemia [228], esophageal squamous cell carcinoma [229], bladder

Table 2

Significant copy number variation. Regions of variation are ordered by q-value (an upper bound on the expected fraction of false positives). Location of each CNV is given as genomic location and coordinates. The number of known genes within each region was determined from the hg19 assembly of the human genome. Genes of interest (those known to play a role in cancer, or specifically in melanoma) are featured, and references to the relevant studies are given in the Results section

Rank	Genomic location	Peak region	GISTIC q-value	Gene count	Known in cancer	Known in melanoma
Amplifications						
1	21q22.1	chr21:39861045-39866904	2.22E-7	1	ERG	—
2	11q14.1	chr11:83358080-83366882	2.56E-7	1	DLG2	—
3	10q11.21	chr10:43622934-43623811	5.92E-4	1	RET	—
4	6p24.3	chr6:1-22748031	1.17E-2	118	NEDD9, DEK, E2F3	6p, NEDD9, RREB1, DEK
5	7p14.3	chr7:34477091-34483332	1.17E-2	1	—	7p
6	1q31.2	chr1:114755790-249250621	1.30E-1	1206	AKT3,MDM4, ABL2	1q
7	8q24.23	chr8:99387730-146364022	1.30E-1	284	MYC	MYC
8	7p12.3	chr7:46879412-46880294	1.30E-1	1	7p12	7p
Deletions						
1	8p23.2	chr8:1986591-6269441	1.5489E-7	3	CSMD1	8p
2	4p16.3	chr4:1-1110173	1.74E-04	23	FGFRL1	—
3	6q26	chr6:161767520-163770210	1.74E-04	4	PARK2, PACRG	6q
4	11q13.1	chr11:63680046-63774300	1.74E-04	3	OTUB1	11q
5	2p21	chr2:42180913-42397164	2.03E-04	1	PKDCC, EML4	—
6	5q35.3	chr5:179219193-179388308	1.04E-03	6	SQSTM1	5q
7	6p22.1	chr6:29427138-29498496	1.36E-03	1	6p22.1	—
8	5p15.33	chr5:1-1054830	2.56E-03	18	SDHA, AHRR, NKD2	—
9	16q23.3	chr16:82658729-83542835	3.40E-03	3	CDH13	16q
10	17q11.2	chr17:29704122-29872630	7.98E-03	2	NF1	NF1
11	12q24.31	chr12:124452696-125266431	1.18E-02	4	NCOR2 (SMRT)	12q24
12	20q11.21	chr20:31830310-31946860	1.43E-02	1	BPIFB1 (LPLUNC1)	—
13	6q14.1	chr6:80412926-80513550	2.10E-02	1	SH3BGRL2	6q
14	10q23.2	chr10:88278583-88520408	2.67E-02	2	WAPAL	10q
15	5q35.1	chr5:169759991-169932228	2.77E-02	1	—	5q
16	16p13.3	chr16:1-752680	2.80E-02	40	AXIN1, ARHGDIG	16p
17	13q13.3	chr13:36341439-36742905	3.05E-02	1	DCLK1	13q12-34
18	3q13.31	chr3:114054708-114463757	4.46E-02	3	ZBTB20	—
19	10p15.3	chr10:1208827-1601334	5.09E-02	2	ADARB2	—
20	3p22.1	chr3:42451413-42590432	5.27E-02	1	VIPR1	—
21	1p32.3	chr1:54870183-55085182	5.81E-02	1	ACOT11	—
22	11p15.4	chr11:3076258-3249828	5.81E-02	3	11p15.5	11p
23	21q22.3	chr21:45878481-48129895	5.81E-02	50	21q22.3	21q22.3
24	18p11.31	chr18:6929284-7231471	6.72E-02	1	LAMA1	—
25	1q21.3	chr1:154596123-154898074	7.09E-02	1	KCNN3	—
26	2p21	chr2:44460525-44590065	7.09E-02	3	PPM1B	—
27	9p21.3	chr9:20651454-25679201	7.09E-02	34	CDKN2A, CDKN2B	CDKN2A, CDKN2B
28	22q13.32	chr22:41481652-51304566	7.09E-02	154	BIK, PRR5, PANX5	—
29	2q33.2	chr2:200814810-206486163	1.10E-01	45	RAPH1,CASP8, ABI2	—
30	13q34	chr13:109280035-115169878	1.47E-01	42	ING1, COL4A1, COL4A1	—
31	15q26.1	chr15:91642272-93012556	1.51E-01	3	SLCO3A1	—
32	Chr.9	chr9:1-141213431	1.91E-01	953	Chr.9	Chr.9

Table 3

Summary of broad and focal copy number variations

Broad chromosomal copy number	Focal chromosomal copy number
Amplifications:	
1q, 6p, 8q	7p12.3, 7p14.3, 10q11.21, 11q14.1, 21q22.1
Deletions:	
2q33, all 9, 13q34, 15q26, 16p13, 22q13	1q21.3, 1p32.3, 2p21, 3q13.31, 3p22.1, 4p16.3, 5q35.1, 5q35.3, 5p15.33, 6q14.1, 6q26, 6p22.1, 8p23.2, 9p21.3, 10q23.2, 10p15.3, 11q13.1, 11p15.4, 12q21.31, 13q13.3, 15q26.1, 16q23.3, 17q11.2, 18p11.31, 20q11.21

carcinoma [230], and cutaneous T-cell lymphomas [226]. Among the genes found in this region are ING1, COL4A1, and COL4A2. ING1 is down-regulated or lost in several cancers [231,232]. ING1 (Inhibitor of growth 1) is a well-known tumor suppressor that is known to be involved in cell growth control, apoptosis, cell proliferation, senescence, and DNA replication and repair [232,233]. COL4A1 and COL4A2 are suspected tumor suppressor genes [230].

15q26.1

SLCO3A1, found here in its entirety, is another known regulator of NF- κ B [234] (along with PPM1B, SQSTM1, and ZBTB20) deleted in our melanoma specimens. Overexpression of SLCO3A1 was shown to induce NF- κ B transcriptional activity (ibid). It has also been suspected of serving to transport anticancer drugs out of the cell [235], but this has not been demonstrated to our knowledge. Deletion of SLCO3A1 has been observed in acute lymphoblastic leukemia [236].

Chromosome 9

This peak (with the highest q value) represents loss of the entire chromosome 9. The incidence of this genetic alteration is frequent in bladder cancer [237,238]. CGH study on primary cutaneous melanoma by Bastian et al. has reported the loss of chromosome 9 in melanoma samples [26]. Moreover, another study on sporadic and familial melanomas has shown the loss of entire copies of chromosome 9 [239].

4. Conclusions

Here, we have compared melanoma to benign nevi rather than to normal skin or controls taken from peripheral blood. The reasoning here is that the differences of interest are between the lesions being compared for diagnostic purposes in the clinic. That is, we are more interested in the cytogenetic differences that may set apart an ambiguous melanoma from a benign lesion than the differences between melanoma and no lesion at all. This method does not, therefore, detect nevus-associated copy number variation. In this study we identified several important regions of genomic amplification (Fig. 1) and deletion (Fig. 2) in melanoma. These are summarized in Table 2. Several regions encompass broad areas of copy number variation (detailed in Table 3), but others represent more focal events. Some of the affected genes in these regions have previously identified roles in melanoma,

and more have been observed in various other cancers. Taken together, these results support much previous work in melanoma etiology and may serve to develop other diagnostic or therapeutic strategies for this disease.

GISTIC provided a robust and unbiased analysis to identify somatic copy number alterations in melanoma samples. The identified regions of aberrations represent the most statistically significant differences between the clinical melanoma and benign nevi specimens. Therefore, these results can be exploited to improve current diagnostic techniques and provide more sensitive methods to discriminate between problematic melanomas and benign nevi neoplasms. Currently, the most commonly used FISH assay employs a 4-probe panel targeting 4 loci (RREB1, MYB, centromere 6, and CCND1) on 2 different chromosomes. The 4-probes FISH has shown a sensitivity and specificity of 86.7% and 95.4% respectively [18]. Recent study has shown an improvement of the FISH assay by incorporating new probes that target 4 different chromosomes (CDKN2A on 9p21, RREB1 on 6p25, MYC on 8q24 and CCND1 on 11q13) with increased sensitivity and specificity to 94% and 98% respectively [7]. Due to the technical limitations of FISH, the justification for adding a locus to the analysis must overcome the consequences of removing another. CGH or aCGH itself, as here, could be developed as diagnostic tools for melanoma and indeed this is described in the literature [21]. A third potential approach is the addition of CNV observed here to qPCR-based assays like the duplex ratio tests described by Moore et al. [240]. This has the advantage of being amenable to FFPE specimens and being imminently expandable to incorporate new loci.

Moreover, GISTIC detected key genomic regions whose genes seem to play important roles in melanoma pathogenesis. Some of these novel identified genes (especially in significant region of amplifications) are potential candidates for molecular targeted therapies. In fact, proposed therapies have been introduced targeting some of the novel genes identified in our analysis, such as targeting the RET gene with small receptor tyrosine kinase inhibitors in thyroid cancer (specifically for identified activating mutations in RET) and other cancers [241–243]. Another gene that is being targeted for cancer therapy is ERG transcription factor, the gene with the most significant copy number gain in our analysis. Several studies have reported their attempts in developing drugs that target the ERG transcription factor, mostly in prostate cancer [244,245]. Further validation of the results described here would suggest the expansion of such trials to include melanoma.

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