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# Heterogeneity of *BRAF*, *NRAS*, and *TERT* Promoter Mutational Status in Multiple Melanomas and Association with *MC1R* Genotype

## *Findings from Molecular and Immunohistochemical Analysis*

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Data on somatic heterogeneity and germline–somatic interaction in multiple primary melanoma (MPM) patients are limited. We investigated the mutational status of *BRAF*, *NRAS*, and *TERT* promoter genes in 97 melanomas of 44 MPM patients and compared molecular and immunohistochemical findings. We further evaluated the association of somatic alterations with the germline *MC1R* genotype. Mutations in *BRAF* gene were identified in 41.2% (40/97) of melanomas, in *NRAS* in 2.1% (2/97), and in *TERT* promoter in 19.6% (19/97). Distribution of *BRAF* mutations did not differ across multiple melanomas ($P = 0.85$), whereas *TERT* promoter changes decreased from first to subsequent melanomas ($P = 0.04$). Intrapatient discrepancy of *BRAF* mutations among multiple tumors was detected in 14 of 44 MPM patients (32%) and of *BRAF/NRAS/TERT* promoter genes in 20 of 44 (45%). We observed a high rate of agreement between allele-specific TaqMan assay and immunohistochemistry in $BRAF^{V600E}$ detection ($\kappa = 0.83$, $P < 0.01$) with 86 of 97 melanomas (88.7%) presenting similar *BRAF* status. Germline *MC1R* variants were identified in 81.4% (35/43) of MPM patients with no association of *MC1R* genotype with somatic mutations or with intrapatient concordance of somatic mutational profile. Our results support the genetic diversity of multiple melanomas and show that somatic heterogeneity is not influenced by inherited *MC1R* variants. Immunohistochemistry may be useful as an initial screening test. *(J Mol Diagn 2018, 20: 110–122; https://doi.org/10.1016/j.jmoldx.2017.10.002)*

Cutaneous melanoma is one of the most aggressive human cancers and is known for its rapid progression and poor prognosis in the advanced stages.[1] During the past few decades its incidence has been steadily rising among Caucasian populations and is predicted to continue increasing for at least two more decades.[2] Patients diagnosed with a single primary cutaneous melanoma have an approximately ninefold higher risk of developing subsequent melanomas compared with the general population.[3] The risk is highest within the first year after initial melanoma diagnosis, whereas it decreases but remains elevated for at least 20 years.[3,4] Multiple primary melanoma (MPM) accounts for 1.2% to 8.2% of all sporadic melanoma patients in Europe and for up to 23% in populations living in high incidence countries such as Australia.[3–6] MPMs are prevalent in men and in older patients[4–7] with subsequent melanomas being more frequently thinner[7,8] and contiguous to a dysplastic nevus compared with initial tumors.[4]

Melanoma arises through the gradual accumulation of genetic somatic abnormalities that involve critical signaling

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pathways such as the mitogen-activated protein kinase signal transduction cascade.[9] Oncogenic *BRAF* (v-raf murine sarcoma viral oncogene homolog B1) mutations occur in approximately 50% of cutaneous melanomas mainly at codon 600 in exon 15, with the most common mutation (approximately 90% of the cases) being the V600E change.[9] Activating *NRAS* (neuroblastoma RAS viral oncogene homolog) mutations, mostly affecting exon 2 at codon 61, have been reported in approximately 20% of cutaneous melanomas.[9] Although the somatic *BRAF* and *NRAS* mutational status is of great interest for melanoma treatment, there is no consensus so far on the best testing method.[10] Besides *BRAF* and *NRAS*, recent studies identified recurrent somatic mutations in the promoter region of the telomerase reverse transcriptase (*TERT*) gene occurring in early stages of melanoma development.[11,12]

The *MC1R* (melanocortin-1 receptor) gene is a key determinant of human pigmentation and specific germline allelic variants, defined R variants, show the strongest effect on melanoma susceptibility.[12] MPM patients have an increased probability to harbor two or more *MC1R* variants compared with patients with a single primary melanoma.[13–15] A synergistic association between somatic *BRAF* mutations and germline *MC1R* variants, with *MC1R* genotype conferring an increased risk of developing *BRAF*-mutated melanomas, was observed in Italian[16] and US patients[17] but not confirmed in other populations.[18–21] Recently, the entire somatic mutational load in melanoma has been shown to be influenced by germline *MC1R* variants with a higher somatic mutational burden in melanomas of patients carrying R variants compared with those of noncarriers, thus supporting a germline-somatic interaction.[22]

To date, only two studies investigated the somatic heterogeneity of multiple melanomas within the same patient, evaluating alterations in main candidate genes by molecular methods.[23,24] However, neither of the two analyzed the possible influence of germline mutations on the somatic diversity of MPM.

In this study, we investigated the mutational status of *BRAF*, *NRAS*, and *TERT* promoter genes in MPM patients to evaluate the intrapatient genetic heterogeneity of multiple melanomas and to compare the consistency of mutational findings obtained by molecular analysis and by immunohistochemistry. In addition, we explored the potential influence of germline *MC1R* genotype on the occurrence of somatic mutations.

## Materials and Methods

### Patient Selection and Melanoma Samples

Patients with histologically proven diagnosis of cutaneous MPM followed at the Department of Dermatology, University of L'Aquila (in 2010 to 2015), and at the Institute of Dermatology, Catholic University of the Sacred Heart, Rome, Italy (in 2014 to 2015), were included in the study. For each patient, synchronous (diagnosis of a second melanoma within 6 months from the first diagnosis) and metachronous multiple primary melanomas (including *in situ*) were retrieved.

Clinical information such as sex, age at diagnosis, anatomic site, family history of melanoma, personal and family history of other cutaneous or visceral neoplasms, and phenotypic characteristics such as hair color, eye color, number of melanocytic nevi, and presence or absence of clinically atypical nevi were collected for each patient.

Hematoxylin and eosin–stained sections of all melanomas were reviewed by two pathologists (G.C. and M.D.P.) to confirm the histopathologic diagnosis. Features of tumors, including histopathologic variant [superficial spreading melanoma (SSM), nodular melanoma, lentigo maligna melanoma, acral lentiginous melanoma, and others], Breslow thickness, and presence of melanoma-associated nevus were recorded. Representative sections from each melanoma were selected for immunohistochemistry (IHC), and tumor-rich areas were marked for molecular analysis.

Approval for this study was obtained from the local ethics committees (ASL L'Aquila-Teramo, protocol no. 30/CE15; Fondazione Policlinico Universitario Gemelli, protocol no. 25779/14). A written informed consent was signed by all patients. The study was performed according to the Helsinki Declaration.

### DNA Extraction and Molecular Analysis

Somatic DNA was extracted from five formalin-fixed, paraffin-embedded (FFPE) tissue sections (each of 10-μ thickness) by microdissection of marked melanoma tissue with the use of a QIAmp Micro tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The coding sequence and splice junctions of exon 15 of *BRAF* (NG_007873.2), exon 3 of *NRAS* (NG_007572.1), and core promoter region of *TERT* gene (NC_000005.9: 1,295,149 to 1,295,267, hg19 GRCh37) were screened by Sanger sequencing, and the $BRAF^{V600E}$, $BRAF^{V600K}$, and $NRAS^{Q61R}$ mutations were also analyzed by competitive allele-specific TaqMan PCR (castPCR Technology) to overcome the limit of detection of Sanger sequencing.

For germline analysis, the DNA was extracted from whole blood with the use of a QIAamp DNA-blood midi kit (Qiagen). Mutational screening of the entire open reading frame of *MC1R* (NG_012026.1) was performed by direct sequencing as above.

For Sanger sequencing analysis, PCR amplification of the regions of interest was performed in a Simply-Amp PCR-System (Thermo Fisher, Foster City, CA), using previously reported primers.[25–27] PCR experiments were performed with 1.25 U of AmpliTaq Gold-360 (Thermo Fisher) in a 50-μL volume, containing the 1X reaction buffer provided by the manufacturer, 1.6 mmol/L of $MgCl_2$, 200 μmol/L of each deoxynucleoside triphosphate, 0.2 μmol/L of each primer, and 30 ng of genomic DNA template. Five percent dimethyl sulfoxide or 5% glycerol (for *TERT* promoter

amplification) were added to the reaction solution. PCR amplification was performed as previously described.[27] Water controls and positive controls were run in parallel with DNA of samples. Sequencing of amplicons was performed with the 3500 Genetic Analyzer (Thermo Fisher). A nucleotide sequence was considered valid when the quality value was >20 (<1/100 error probability). The variants were detected using the Applied Biosystems Minor Variant Finder software version 1.0 (Thermo Fisher), specific to calling low-frequency somatic variants.

For competitive allele-specific TaqMan assay, PCRs containing 20 ng of DNA, 1X TaqMan Mutation Detection Assays (assay Hs00000111_mu for $BRAF^{V600E}$, Hs000000002_rm for $BRAF^{V600K}$, Hs000000083_rm for $NRAS^{Q61R}$), 1X TaqMan Genotyping Master Mix (Thermo Fisher), and water to reach the final volume of 20 μL were performed in 96-well plates with the use of the standard TaqMan protocol on 7500 Fast Real Time-PCR System (Thermo Fisher). Five DNA samples extracted from FFPE sections of melanomas positive for $BRAF^{V600E}$ or $BRAF^{V600K}$ and three extracted from FFPE sections of normal skin of unaffected individuals were used to set the ΔCt cutoff for experiments of mutation detection.

### Immunohistochemistry

Immunohistochemical analysis was performed on FFPE sections of 4-μm thickness, obtained from the same tissue block used for molecular analysis. $BRAF^{V600E}$ and $NRAS^{Q61R}$ mutants were evaluated with the following monoclonal antibodies: $BRAF^{V600E}$ VE1 clone (Spring Bioscience, Pleasanton, CA) and $NRAS^{Q61R}$ SP174 clone (Spring Bioscience) at a dilution of 1:30 and 1:80, respectively. For both $BRAF^{V600E}$ and $NRAS^{Q61R}$ IHC, sections were freshly cut, dried at 60°C for 30 minutes, deparaffinized, and rehydrated. Immunoreactions were performed on Ventana BenchMark XT immune stainer (Ventana Medical Systems Inc., Tucson, AZ) with the use of the ultraView Universal RED Detection Kit, as previously reported.[25] No chromogen was detected when primary antibody was omitted. Positive and negative controls were mounted on each section subjected to immunostaining. Glass slides for immunohistochemistry were gifted from USC Diagnostic (Rome, Italy).

The evaluation of IHC status was performed independently by two observers (G.C. and M.D.P.) blinded to the mutational molecular status; disagreement was resolved by consensus. Cytoplasmic staining of $BRAF^{V600E}$ VE1 or $NRAS^{Q61R}$ SP174 antibodies in melanoma cells was interpreted as positive or negative. In detail, negative staining was defined as absence of any cytoplasmic labeling either in single interspersed melanoma cells (<10%) or in cells of histiocytic/macrophage lineage; positive staining was classified as homogeneous (staining in >95% of cells) or heterogeneous (staining in <95% of cells) according to the percentage of cytoplasmic staining in melanoma cells, as previous described.[10,28] Intensity of the staining was graded as weak, moderate, or strong.[10]

### Statistical Analysis

Descriptive statistics and molecular and IHC data are given as means, medians, or proportions, as appropriate. Variables were categorized as follows: median age at melanoma diagnosis (≤40 years, >40 years), number of common melanocytic nevi (≤50, >50), number of primary melanomas (2, >2), occurrence of synchronous melanomas, histopathologic subtype (SSM or other subtypes), melanoma thickness (*in situ*, invasive; Breslow thickness ≤1 mm, >1 mm), and melanoma anatomic site (head/neck, trunk, extremities).

For *MC1R* analysis, we evaluate the association of germline variants with a patient's phenotypical characteristics as skin type (I/II, III/IV), hair color (red/blond, light brown, dark brown/black), and eye color (blue/green, light brown, dark brown).

The R142H, R151C, R160W, D294H, and I155T *MC1R* variants were considered as R variants, whereas all of the others were termed as r.[18] Synonymous variants were considered as wild type.

Molecular findings by allele-specific TaqMan assays were used as the gold standard for statistical analysis. Semiquantitative data (age at diagnosis, Breslow thickness) were analyzed by means of *t*-test or by medians with *U*-test. Univariate analysis by $\chi^2$ test or by Fisher exact test was used to test the significance of mutation frequency according to clinical characteristics of melanoma patients and clinicopathologic features of melanoma lesions. Odds ratio was calculated to determine the magnitude of differences among mutated and wild-type melanoma groups. Cohen κ coefficient test was used to measure the agreement between molecular and IHC methods in determining *BRAF* mutational status. In general, $P < 0.05$ was considered statistically significant. Statistical analysis was performed with the statistical package SPSS software version 17.0 (SPSS Incorporated, Chicago, IL).

## Results

### Patients and Melanoma Samples

Demographic and clinical characteristics of patients and related tumors are summarized in Table 1.

Samples of primary and subsequent melanomas were obtained from 44 MPM patients with stage I to II (24 men and 20 women; median age at first diagnosis, 49.0 years; range, 15 to 80 years). We analyzed a total of 97 melanoma tissues (35 patients [79.5%] with two paired tumor tissues, and nine [20.5%] with three tumor tissues; mean, 2.2 melanomas per patient). Synchronous melanomas occurred in 10 MPM patients (22.2%): nine patients with two melanomas and one patient with three melanomas. Metachronous lesions were diagnosed in 33 MPM patients (75.0%); one patient with three melanomas developed two synchronous tumors and the third one 2 years later. A family history of melanoma was recorded in nine MPM subjects (20.4%).

Fifty-seven melanomas (58.8%) were located on the trunk, 39 (40.2%) on the extremities, and 1 (1.0%) on the head/neck region. Twenty-seven MPM patients (61.4%) developed multiple melanomas at the same anatomic location. Almost all lesions were of the SSM subtype (97.0%); the median Breslow thickness was 0.50 mm (range, 0.2 to 2.0 mm), with 58 of 97 (59.8%) being *in situ* melanoma, 34 (35.1%) being melanomas with tumor thickness $\leq$ 1 mm, and 5 (5.1%) being $>$1 mm. No difference in Breslow thickness was observed, moving from the first to subsequent melanomas: median Breslow thicknesses were 0.50, 0.40, and 0.51 mm for first, second, and third melanomas, respectively ($P = 0.32$). Approximately 40% of melanomas (39/97, 40.2%) were associated with a preexisting melanocytic nevus.

## Somatic Molecular Analysis

Somatic molecular analysis was performed in all 97 melanoma tissues. *BRAF* mutations at codon 600 were detected by competitive allele-specific TaqMan assay in 40 of 97 melanomas (41.2%), with 37 tumors harboring the $BRAF^{V600E}$ mutation (37/40, 92.5%) and 3 the $BRAF^{V600K}$ (3/40, 7.5%). Sanger sequencing analysis identified 37 *BRAF*-mutated melanomas (38.1%) and 60 wild-type melanomas (61.9%) with three discordant cases.

Distribution of *BRAF* mutations was not different across multiple melanomas ($P = 0.85$), being 38.6% (17/44) in the subgroup of first melanomas, 45.4% (21/44) in second melanomas, and 22.2% (2/9) in third melanomas. The median age at melanoma onset was 41 years for $BRAF^{V600E}$-mutated melanomas compared with 56 years for *BRAF* wild-type melanomas ($P = 0.04$). No significant association was observed between *BRAF* mutational status and other clinicopathologic features (Table 2).

Two of the 97 melanomas (2.1%) carried *NRAS* mutations at codon 61 with the following genotypes identified by Sanger sequencing: Q61L (1/97, 1.0%) and Q61H (1/97, 1.0%). No $NRAS^{Q61R}$ substitution was detected.

*TERT* promoter mutations were found in 19 melanomas (19.6%): 17 of 19 melanomas (17.5%) harbored the $-126$ C$>$T (Chr.5 1.295.230) base change, and the remaining 2 (2.1%) the $-106$ C$>$T (Chr.5 1.295.210). *TERT* promoter analysis failed in three samples. A significant decrease of *TERT* promoter changes was observed moving from the first to subsequent melanomas ($P = 0.04$): 12 mutated first melanomas (12/44, 27.3%), 7 second melanomas (7/44, 15.9%), and no mutated third melanoma. The prevalence of *TERT* promoter mutations was significantly higher in patients older than 40 years ($P < 0.01$) and was associated with a positive family history of melanoma ($P = 0.02$) (Table 2).

A mutation in at least one of the three genes was present in more than one-half of the 97 samples (52/97, 53.6%), but none carried alterations in all genes simultaneously. All melanomas analyzed were mutually exclusive for *BRAF* and *NRAS* mutations and seven tumors (7.2%) carried both *BRAF* and *TERT* promoter alterations with no evidence of correlation between *TERT* promoter changes and $BRAF^{V600E}$ substitution ($\Phi$ coefficient, $-0.054$, $P = 0.60$).

**Table 1** Demographic and Clinical Characteristics of Melanoma Patients ($n = 44$) and Histopathologic Features of Tumors ($n = 97$)

| Characteristic | Value |
|---|---|
| Patients | |
| Sex | |
| Male | 24 (54.6) |
| Female | 20 (45.4) |
| Age, years | |
| Median (range) | 49 (15–80) |
| $\leq$40 | 17 (38.6) |
| $>$40 | 27 (61.4) |
| Nevus count* | |
| $\leq$50 | 12 (27.3) |
| $>$50 | 31 (70.4) |
| Clinically atypical nevi* | |
| No | 28 (63.6) |
| Yes | 11 (25.0) |
| Skin type | |
| I | 5 (11.4) |
| II | 22 (50.0) |
| III | 14 (31.8) |
| IV | 3 (6.8) |
| No. of primary melanomas | |
| 2 | 35 (79.5) |
| 3 | 9 (20.5) |
| Family history of melanoma | |
| Yes | 9 (20.4) |
| No | 35 (79.5) |
| Synchronous/metachronous† | |
| Synchronous | 10 (22.2) |
| Metachronous | 33 (75.0) |
| Tumors | |
| Breslow thickness | |
| *In situ* | 58 (59.8) |
| Invasive | 39 (40.2) |
| $\leq$1 mm | 34 (35.1) |
| $>$1 mm | 5 (5.1) |
| Median (range), mm | 0.5 (0.2–2.0) |
| Histopathologic subtype | |
| SSM | 94 (97.0) |
| NM | 1 (1.0) |
| LM/LMM | 1 (1.0) |
| Others (spitzoid) | 1 (1.0) |
| Anatomic site | |
| Head/neck | 1 (1.0) |
| Trunk | 57 (58.8) |
| Extremities | 39 (40.2) |
| Nevus association | |
| Yes | 39 (40.2) |
| No | 58 (59.8) |

Values are *n* (%) unless otherwise specified.

*Numbers do not add up to the total because of missing data.

†One patient with three melanomas developed two synchronous tumors and the third tumor 2 years later was excluded.

LM/LMM, lentigo maligna/lentigo maligna melanoma; NM, nodular melanoma; SSM, superficial spreading melanoma.

**Table 2** Frequency of *BRAF* and *TERT* Promoter Variants According to Clinical and Histopathologic Characteristics of Patients and Related Tumors

| Characteristic | All melanomas ($n = 97$) | *BRAF* | | | *TERT* promoter* | | |
|---|---|---|---|---|---|---|---|
| | | Wild-type ($n = 57$) | Mutations ($n = 40$) | *P* | Wild-type ($n = 75$) | Mutations ($n = 19$) | *P* |
| Patients | | | | | | | |
| Sex | | | | | | | |
| Male | 51 (52.6) | 28 (49.1) | 23 (57.5) | 0.42 | 37 (49.3) | 13 (68.4) | 0.26 |
| Female | 46 (47.4) | 29 (50.9) | 17 (42.5) | | 38 (50.7) | 6 (31.6) | |
| Age at diagnosis, years | | | | | | | |
| Median (range) | 49 (15–81) | 56 (17–81) | 41 (15–73) | 0.04 | 43 (15–81) | 53 (28–70) | 0.05 |
| ≤40 | 34 (35.1) | 15 (26.3) | 19 (47.5) | 0.03 | 42 (56.0) | 1 (5.3) | <0.01 |
| >40 | 63 (64.9) | 42 (73.7) | 21 (52.5) | | 33 (44.0) | 18 (94.7) | |
| Nevus count† | | | | | | | |
| ≤50 | 26 (26.8) | 15 (26.3) | 11 (27.5) | 0.98 | 19 (25.3) | 6 (31.6) | 0.43 |
| >50 | 69 (71.1) | 40 (70.2) | 29 (72.5) | | 54 (72.0) | 13 (68.4) | |
| Skin type | | | | | | | |
| I/II | 63 (64.9) | 39 (68.4) | 24 (60.0) | 0.39 | 52 (69.3) | 11 (57.9) | 0.34 |
| III/IV | 34 (35.1) | 18 (31.6) | 16 (40) | | 23 (30.7) | 8 (42.1) | |
| Family history of melanoma | | | | | | | |
| Yes | 18 (18.6) | 11 (19.3) | 7 (17.5) | 0.82 | 10 (13.7) | 7 (36.8) | 0.02 |
| No | 79 (81.4) | 46 (80.7) | 33 (82.5) | | 65 (86.6) | 12 (63.2) | |
| Tumors | | | | | | | |
| Breslow thickness | | | | | | | |
| *In situ* | 58 (59.8) | 36 (63.2) | 22 (55.0) | 0.42 | 44 (58.7) | 11 (57.9) | 0.95 |
| Invasive | 39 (40.2) | 21 (36.8) | 18 (45.0) | | 31 (41.3) | 8 (42.1) | |
| ≤1 mm | 34 (35.1) | 20 (35.1) | 14 (35.0) | 0.10 | 27 (36.0) | 7 (36.8) | 0.98 |
| >1 mm | 5 (5.1) | 1 (1.7) | 4 (10.0) | | 4 (5.3) | 1 (5.3) | |
| Median (range), mm | 0.5 (0.2–2.0) | 0.5 (0.2–1.0) | 0.5 (0.2–2.0) | 0.40 | 0.5 (0.2–2.0) | 0.5 (0.3–1.4) | 0.62 |
| Histopathologic subtype | | | | | | | |
| SSM | 94 (96.9) | 55 (96.5) | 39 (97.5) | 0.78 | 72 (96.0) | 19 (100.0) | 1.00 |
| Others‡ | 3 (3.1) | 2 (3.5) | 1 (2.5) | | 3 (4.0) | 0 (0) | |
| Anatomic site | | | | | | | |
| Head/neck | 1 (1.0) | 1 (1.7) | 0 (0) | 0.45 | 1 (1.3) | 0 (0) | 0.66 |
| Trunk | 57 | 31 (54.4) | 26 (65.0) | | 46 (61.3) | 10 (52.6) | |
| Extremities | 39 | 25 (43.9) | 14 (35.0) | | 28 (37.4) | 9 (47.4) | |
| Nevus-associated melanoma | | | | | | | |
| Yes | 39 | 21 (36.8) | 18 (45.0) | 0.53 | 32 (42.7) | 7 (36.8) | 0.64 |
| No | 58 | 36 (63.2) | 22 (55.0) | | 43 (57.3) | 12 (63.2) | |

Values are *n* (%) unless otherwise indicated.

**TERT* promoter amplification failed in three samples.

†Numbers do not always add up to the total because of missing data.

‡Others include nodular melanoma, lentigo maligna/lentigo maligna melanoma, and spitzoid melanoma.

SSM, superficial spreading melanoma.

None of the tumors harbored both $NRAS^{Q61}$ and *TERT* promoter mutations.

## Intrapatient Concordance of Somatic Mutational Profile

The somatic mutational pattern in melanomas of each MPM patient is shown in Table 3. Intrapatient *BRAF* concordance between first and subsequent melanomas by molecular analysis was observed in 68.2% (30/44) of patients, with 11 (11/30, 36.7%) being concordant for *BRAF*-V600 and 19 (19/30, 63.3%) for wild-type genotype. A discrepant somatic profile was detected in 20.4% (9/44) of patients. Five MPM patients (11.4%) diagnosed with three melanomas showed a partially inconsistent *BRAF* mutational status with concordance between two of the three melanomas. Concordance rate of somatic *BRAF* status did not differ by sex ($P = 0.95$), synchronous melanoma ($P = 0.08$), and location of MPM at the same or different body sites ($P = 0.31$).

Concerning *NRAS* mutational profile, a discordant *NRAS* mutational status was observed in both patients harboring the Q61 substitution, with the first melanoma being Q61 mutated and the subsequent melanomas being wild type.

*TERT* promoter concordance was present in 68.1% (30/44) of the patients, 27 (27/30, 90.0%) with the wild-type genotype and 3 (3/30, 10.0%) with *TERT* promoter mutations. Intrapatient discrepancy was observed in nine MPM patients (45%). Two patients (4.5%) showed a partially different *TERT* promoter mutational status. No significant association between the concordance rate of *TERT* promoter mutations and sex ($P = 0.25$), time of development ($P = 0.41$), and consistency in anatomic location ($P = 0.70$) was observed.

Regarding intrapatient concordance of the somatic profile of all three genes, approximately one-half of the patients (21/44, 47.7%; 17 patients with two melanomas and 4 patients with three melanomas) showed a consistent mutational pattern of *BRAF/NRAS/TERT* promoter between first and subsequent melanomas. Intrapatient discordance was present in 34.9% (15/44) of the patients, mainly because of differences in *BRAF* mutational status. The genetic alterations were partially different between the first and subsequent lesions with concordance in two of three melanomas in 11.4% (5/44) of MPM patients. A borderline association between concordance of *BRAF/NRAS/TERT* promoter mutations was observed for synchronous melanomas ($P = 0.05$).

### Immunohistochemical Analysis

A total of 45 of 97 melanomas (46.4%) showed positive immunostaining with anti-$BRAF^{V600E}$ VE1 antibody (Table 3 and Figure 1). Intratumoral homogenous expression of VE1 (percentage of stained cells >95%) was detected in 25 of 45 melanomas (55.5%), whereas a heterogeneous pattern was observed in 20 melanomas (20/45, 44.4%), with a percentage of stained cells, ranging from 10% to 80%. Staining intensity was strong in 19 of 45 tumors (42.2%), moderate in 18 tumors (40.0%), and weak in 8 tumors (17.8%). Prevalence of the $BRAF^{V600E}$-mutated protein was similar between the first and subsequent melanomas ($P = 0.58$). Intrapatient *BRAF* concordance in immunostaining between multiple melanomas was evident in 29 of 44 patients (65.9%), including 14 (14/29, 48.3%) $BRAF^{V600}$ and 15 (15/29, 51.7%) wild type.

All 39 melanomas arising in association with a melanocytic nevus showed concordance for *BRAF* staining between melanoma cells and corresponding nevus cells, being 18 $BRAF^{V600E}$ mutated (46.2%) and 21 wild type (53.8%) (Figure 2).

### Comparison Between Molecular and Immunohistochemical Findings

*BRAF* molecular results were consistent with IHC VE1 immunostaining in 86 of 97 melanomas (88.7%) with 37 $BRAF^{V600E}$ mutated (37/86, 43.0%) and 49 wild-type (49/86, 57.0%) melanomas (Table 3). Discordant findings were found in eight samples (8/97, 8.2%): all were wild type on both molecular testing methods but immunopositive with the VE1 antibody (Figure 3). Discordant cases showed weak (5/8, 62.5%) or moderately positive (3/8, 37.5%) VE1 staining. The staining pattern was heterogeneous in six melanomas (6/8, 75%), with a low fraction of tumor cells (from 10% to 40%) in four of the six and a higher percentage (approximately 80%) in two of the six, whereas it was homogeneous in the remaining two samples (2/8, 25%). The three samples harboring the $BRAF^{V600K}$ mutations according to molecular analysis were, as expected, wild type on IHC VE1 staining.

Overall, the agreement between molecular testing and IHC was high (Cohen $\kappa = 0.83$, $P < 0.001$).

$NRAS^{Q61R}$ SP174 immunostaining was negative in all 97 melanomas, including the two samples that showed Q61L and Q61H mutations on molecular analysis.

### Germline *MC1R* Mutational Analysis

Germline analysis of the *MC1R* gene was performed in 43 of 44 patients. Overall, 12 *MC1R* allelic variants were detected in 35 of 43 MPM patients (81.4%) (Supplemental Table S1), with 20 of 35 MPM patients (57.1%) carrying a single allelic variant and 15 of 35 MPM patients (42.8%) carrying two variants (all compound heterozygotes). The V60L substitution was the most prevalent variant and was detected in 10 of 35 patients (28.6%).

The presence of at least one *MC1R* variant was associated with a light phenotype (fair skin type and light hair color, $P = 0.01$ and $P < 0.01$, respectively), and this effect was mainly because of R substitutions ($P < 0.01$). Conversely, the presence of r variants was increased in darker phenotypes (skin type III/IV and light brown hair color, $P < 0.01$ for both). Carriers of R changes were diagnosed more frequently with melanoma on the trunk than wild-type patients ($P = 0.02$), whereas the r genotype was significantly associated with the occurrence of melanoma on the extremities ($P < 0.01$). The association of *MC1R* variants and clinicopathologic characteristics of patients and melanomas is described in Supplemental Table S2.

In addition, no significant association was found between germline *MC1R* variants with somatic *BRAF* and *TERT* promoter status (Supplemental Table S3) or with intrapatient concordance of somatic mutational profiles in multiple melanomas (Supplemental Table S4).

## Discussion

We identified *BRAF* mutations in 41.2% of multiple melanomas with a higher frequency in tumors of younger patients. *NRAS* and *TERT* promoter mutations were observed in 2.1% and 19.6% of the lesions, respectively, with *TERT* promoter changes decreasing moving from the first to subsequent melanomas. Overall, approximately one-half of the patients showed an inconsistent mutational pattern of *BRAF*, *NRAS*, and *TERT* promoter between their first and subsequent melanomas. In addition, we observed a good agreement between

**Table 3** Summary of Somatic Mutational Patterns in Multiple Melanomas According to Molecular and Immunohistochemical Analysis

| | First melanoma | | | | |
|---|---|---|---|---|---|
| | *BRAF* | | *NRAS* | | *TERT* |
| Patient | Molecular | IHC* | Molecular | IHC | Molecular |
| 1 | WT | WT | WT | WT | WT |
| 2 | WT† | WT† | WT† | WT† | WT† |
| 3* | V600E† | Het, moderate† | WT† | WT† | −126C>T† |
| 4 | V600E‡ | Het, weak | WT | WT | WT |
| 5 | V600E | Het, strong | WT | WT | WT |
| 6 | WT† | WT† | WT† | WT† | WT† |
| 7 | V600E | Het, strong | WT | WT | WT |
| 8 | WT | WT | WT | WT | WT |
| 9 | WT | WT | WT | WT | WT |
| 10 | V600E | Hom, strong | WT | WT | WT |
| 11 | V600E† | Hom, strong† | WT† | WT† | WT† |
| 12 | WT | WT | WT | WT | WT |
| 13 | WT§ | Hom, weak | WT | WT | WT |
| 14 | WT† | WT† | WT† | WT† | WT† |
| 15 | V600E | Hom, moderate | WT | WT | WT |
| 16 | WT | WT | WT | WT | WT |
| 17 | V600E | Het, moderate | WT | WT | WT |
| 18 | V600E† | Hom, strong† | WT† | WT† | WT† |
| 19 | WT | WT | WT | WT | WT |
| 20 | WT | WT | WT | WT | WT |
| 21 | V600E† | Hom, strong† | WT† | WT† | WT† |
| 22 | V600E† | Hom, strong† | WT† | WT† | −126C>T† |
| 23 | WT† | WT† | WT† | WT† | WT† |
| 24 | V600E | Het, moderate | WT | WT | −126C>T |
| 25 | WT† | WT† | Q61L† | WT† | WT† |
| 26 | V600E† | Hom, moderate† | WT† | WT† | WT† |
| 27 | WT§ | Het, moderate§ | WT | WT | WT |
| 28 | WT† | WT† | Q61H† | WT† | WT† |
| 29 | V600E† | Hom, strong† | WT† | WT† | WT† |
| 30 | WT† | WT† | WT† | WT† | −126C>T† |
| 31 | V600E† | Hom, strong† | WT† | WT† | −126C>T† |
| 32 | V600E† | Hom, strong† | WT† | WT† | −126C>T† |
| 33 | WT | WT | WT | WT | WT |
| 34 | V600K | WT | WT | WT | WT |
| 35 | WT† | WT† | WT† | WT† | −126C>T† |
| 36 | WT† | WT† | WT† | WT† | WT† |
| 37 | WT† | WT† | WT† | WT† | −126C>T† |
| 38 | WT†,§ | Het, weak†,§ | WT† | WT† | −106C>T† |
| 39 | WT§ | Hom, moderate§ | WT | WT | −126C>T |
| 40 | WT | WT | WT | WT | NA |
| 41 | WT | WT | WT | WT | −126C>T |
| 42 | WT | WT | WT | WT | NA |
| 43 | WT | WT | WT | WT | −126C>T |
| 44 | WT | Het, weak | WT | WT | WT |

(*table continues*)

*Staining intensity and distribution are indicated for IHC-positive cases.

†Molecular intrapatient mutational discordance of *BRAF*, *NRAS*, and *TERT* promoter.

‡Samples with discrepancy in *BRAF* status by TaqMan versus Sanger methods. These samples were $BRAF^{V600E}$ for TaqMan assay and BRAF WT for Sanger sequencing.

§Discrepant molecular and IHC findings.

Het, heterogeneous immunostaining; Hom, homogeneous immunostaining; IHC, immunohistochemistry; NA, not applicable; WT, wild-type.

**Table 3** (*continued*)

| Second melanoma | | | | | Third melanoma | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *BRAF* | | *NRAS* | | *TERT* | *BRAF* | | *NRAS* | | *TERT* |
| Molecular | IHC | Molecular | IHC | Molecular | Molecular | IHC | Molecular | IHC | Molecular |
| WT | WT | WT | WT | WT | | | | | |
| V600E[†] | Het, moderate[†] | WT[†] | WT[†] | WT[†] | WT[†] | WT[†] | WT[†] | WT[†] | WT[†] |
| V600E[†] | Het, moderate[†] | WT[†] | WT[†] | WT[†] | | | | | |
| V600E | Hom, strong | WT | WT | WT | | | | | |
| V600E | Het, moderate | WT | WT | WT | | | | | |
| V600E[†] | Hom, moderate[†] | WT[†] | WT[†] | WT[†] | WT[†,§] | Het, weak[†,§] | WT[†] | WT[†] | WT[†] |
| V600E | Het, moderate | WT | WT | WT | | | | | |
| WT | WT | WT | WT | WT | WT | WT | WT | WT | WT |
| WT | WT | WT | WT | WT | | | | | |
| V600E | Hom, strong | WT | WT | WT | | | | | |
| V600E[†] | Hom, strong[†] | WT[†] | WT[†] | −126C>T[†] | | | | | |
| WT | WT | WT | WT | WT | | | | | |
| WT | WT | WT | WT | WT | WT | WT | WT | WT | WT |
| V600E[†,‡] | Het, weak[†] | WT[†] | WT[†] | WT[†] | | | | | |
| V600E | Hom, strong | WT | WT | WT | | | | | |
| WT | WT | WT | WT | WT | WT | WT | WT | WT | WT |
| V600E | Het, moderate | WT | WT | WT | V600E | Hom, weak | WT | WT | WT |
| WT[†] | WT[†] | WT[†] | WT[†] | WT[†] | | | | | |
| WT | WT | WT | WT | WT | | | | | |
| WT | WT | WT | WT | WT | | | | | |
| V600E[†] | Hom, strong[†] | WT[†] | WT[†] | WT[†] | WT[†] | WT[†] | WT[†] | WT[†] | WT[†] |
| V600E[†] | Hom, strong[†] | WT[†] | WT[†] | WT[†] | | | | | |
| V600E[†] | Het, moderate[†] | WT[†] | WT[†] | WT[†] | | | | | |
| V600E[‡] | Het, moderate | WT | WT | −126C>T | | | | | |
| WT[†] | WT[†] | WT[†] | WT[†] | WT[†] | | | | | |
| WT[†] | WT[†] | WT[†] | WT[†] | WT[†] | | | | | |
| WT[§] | Het, weak[§] | WT | WT | WT | | | | | |
| WT[†] | WT[†] | WT[†] | WT[†] | −126C>T[†] | | | | | |
| V600E[†] | Hom, strong[†] | WT[†] | WT[†] | −126C>T[†] | | | | | |
| V600E[†] | Hom, strong[†] | WT[†] | WT[†] | WT[†] | | | | | |
| WT[†] | WT[†] | WT[†] | WT[†] | WT[†] | | | | | |
| WT[†] | WT[†] | WT[†] | WT[†] | WT[†] | | | | | |
| WT | WT | WT | WT | WT | | | | | |
| V600K | WT | WT | WT | WT | | | | | |
| V600K[†] | WT[†] | WT[†] | WT[†] | WT[†] | | | | | |
| V600E[†] | Hom, moderate[†] | WT[†] | WT[†] | WT[†] | | | | | |
| V600E[†] | Hom, moderate[†] | WT[†] | WT[†] | WT[†] | WT[†] | WT[†] | WT[†] | WT[†] | WT[†] |
| WT[†,§] | Het, moderate[†,§] | WT[†] | WT[†] | WT[†] | V600E[†] | Hom, strong[†] | WT[†] | WT[†] | WT[†] |
| WT | WT | WT | WT | −126C>T | | | | | |
| WT | WT | WT | WT | −106C>T | | | | | |
| WT | WT | WT | WT | −126C>T | | | | | |
| WT | WT | WT | WT | WT | | | | | |
| WT | WT | WT | WT | NA | | | | | |
| WT | WT | WT | WT | WT | | | | | |

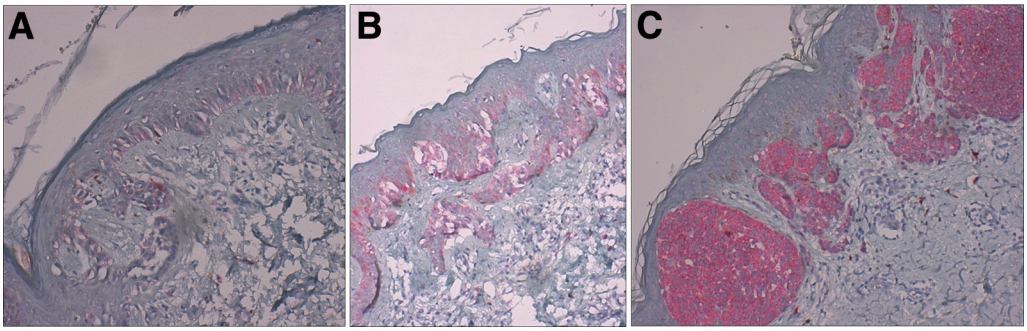


**Figure 1** Representative cases of $BRAF^{V600E}$ (VE1) immunostaining. **A:** Weak staining intensity with heterogeneous distribution. **B:** Moderate staining intensity with heterogeneous distribution. **C:** Strong staining intensity with homogeneous distribution. Original magnification, ×20.

molecular and IHC findings for $BRAF^{V600E}$ and $NRAS^{Q61R}$ mutations. Germline *MC1R* variants were identified in 81.4% of MPM patients, but no correlation between *MC1R* variants with somatic *BRAF* status or with intrapatient concordance of somatic mutational profile was found.

Signaling activation of the mitogen-activated protein kinase pathway has been shown to drive the growth of most cutaneous melanomas, with somatic mutations of both *BRAF* and *NRAS* identified in approximately 70% of the lesions.[9] The overall frequency of *BRAF* mutations found in our sample (41.2%) was in line with two large series of melanomas[29,30] and with other Italian cohorts.[16,17,23,31] Similarly, the prevalence rate of the different $BRAF^{V600E}$ and $BRAF^{V600K}$ mutations was consistent with that previously reported.[9] Concerning clinical characteristics, our results confirm that *BRAF*-mutated melanomas are associated with younger age and frequently occur on the trunk.

Interestingly, we found that all 39 nevus-associated melanomas showed concordant immunostaining of the *BRAF* between melanoma and nevus cells. A concordance rate of 100% has been also reported by Kakavand et al[32] in 29 cases. A slightly lower degree of concordance of 75% and 80% was described in two additional studies, including 21 and 46 nevus-associated melanomas, respectively.[33,34] The high concordance rate of $BRAF^{V600E}$ mutations between melanoma and its nevus counterpart has been interpreted as a causal relationship between the two lesions.

*NRAS* mutations (Q61L and Q61H) were identified in only two melanomas, and none was found to carry the most common Q61R change. This low frequency (2.1%) was not unexpected because of histopathologic features of our samples. Most lesions were of the SSM subtype and thin in thickness, although *NRAS* mutations are most frequently described in melanomas with aggressive features (ie, nodular subtype, greater thickness, more frequent ulceration, and higher tumor mitotic rate).[29,35–37]

Telomerase activation by *TERT* promoter mutations is a common and early event in melanoma progression with a rate ranging from 22%[38] to 71%,[39] thus representing the second most prevalent mutated gene after *BRAF*.[40] In our study, mutations of *TERT* promoter were identified in 19.6% of the tumors, occurring more frequently in older patients and in patients with a positive family history of melanoma. Similar to *NRAS* mutations, *TERT* promoter mutations have been associated with nodular histologic type, high tumor thickness, and advanced pathologic tumor stage.[11,38,40,41] We did not identify a significant co-occurrence of *TERT* promoter mutations and $BRAF^{V600E}$ substitution, as reported in Austrian and Spanish populations.[42,43] The absence of significance in our study might be a consequence of the characteristics of the studied population, which is not representative of the overall melanoma population.

Regarding genetic heterogeneity of multiple melanomas, our data confirm the complexity of melanoma molecular development within the same patient. In detail, we observed an intrapatient discrepancy of *BRAF* mutations among multiple lesions in 32% of MPM patients. The same rate (32.8%) was reported by Colombino et al[23] in a series of multiple melanomas from 112 Italian patients. The inconsistence of the *BRAF* status in MPM patients might have implications in clinical practice if MPM subjects with discordant multiple primary melanomas need to be molecularly classified for therapy with *BRAF* inhibitors. In addition, when we considered the mutational status of all three genes, *BRAF/NRAS/TERT* promoter, we observed an intrapatient discordance of 45%. A higher discordance rate of the genetic profile of the same genes was recently reported in 61% of MPM patients in a German study that included 237 multiple melanomas from 96 patients.[24] Differences in the clinicopathologic features of analyzed melanomas and the higher frequency of *NRAS* and *TERT* promoter mutations identified in the German study could explain the different findings.

Because *BRAF* and *MEK* inhibitors are becoming prominent therapeutic options for melanoma treatment, it is crucial that melanomas with *BRAF* and *NRAS* mutations are identified with methods that are sensitive, accurate, and fast. We compared *BRAF* and *NRAS* mutation analysis with the use of molecular methods, such as allele-specific TaqMan assays, and IHC with anti-$BRAF^{V600E}$ VE1 and anti-$NRAS^{Q61R}$ SP174 antibodies. Consistent mutational findings between

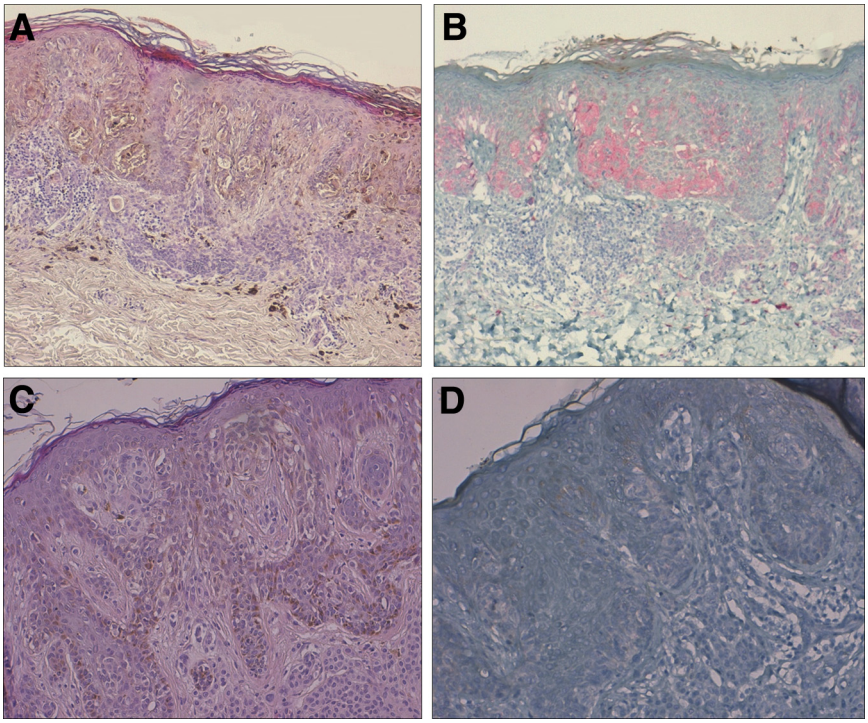


**Figure 2** $BRAF^{V600E}$ (VE1) staining in melanoma and associated nevus. **A** and **B:** Lesion with concordant positive $BRAF^{V600E}$ in melanoma and nevus: hematoxylin and eosin (H&E) staining (**A**) and $BRAF^{V600E}$ (VE1) staining (**B**). **C** and **D:** Lesion with concordant BRAF wild type in melanoma and associated nevus: H&E staining (**C**) and negative $BRAF^{V600E}$ (VE1) staining (**D**). Original magnification: ×10 (**A** and **B**); ×20 (**C** and **D**).

molecular analysis and $BRAF^{V600E}$ VE1 immunostaining were observed in 89% of our tumors. Previous studies reported different concordance rate for $BRAF^{V600E}$ VE1 detection compared with DNA-based assays[44]; however, comparison between studies is difficult because of differences in the molecular approaches. Overall, we detected 8.2% of discrepant cases, being $BRAF^{V600E}$ positive by IHC stain but negative by molecular analysis. They showed a weak-to-moderate immunostaining and most stained heterogeneously and with low tumor cell content. Interpretation issues have been raised for weak-to-moderately stained lesions because they are considered either positive or negative by different investigators, thus suggesting that caution must be used in case of unclear weak staining.[45–48] However, the low proportion of tumor cell content in our series, including a high percentage of thin melanomas, could favor IHC with respect to molecular methods. Of note, the occurrence of antibody cross-reactivity in unknown epitopes could be an additional explanation for our discrepant cases.

At the germline level, we confirmed the high prevalence of *MC1R* polymorphisms in MPM patients (81.4%), with the V60L being the most frequent variant detected, as observed in other Italian studies, including ours.[14,16,27,49,50] *MC1R* R variants were identified in 45% of subjects and associated with a light phenotype, supporting the role of *MC1R* in human pigmentation. Regarding body site, *MC1R* variants, especially R changes, were prevalent in patients with melanoma on the trunk. Similar results were observed in Swedish melanoma patients,[51] whereas R variants were associated with melanoma on the arms in a multicenter study.[52] Differences in body site grouping and *MC1R* variants categorization make it difficult to compare our results with those of previous studies.[14,53]

We demonstrated that *MC1R* variants did not influence intrapatient concordance of mutational profile among multiple melanomas and somatic *BRAF* or *TERT* promoter mutational status. As in our series, germline *MC1R* variants were shown not to be associated with somatic *BRAF* mutations in different populations.[18–21] However, two earlier Italian studies,[16,17] including one from our group, reported opposite results, probably reflecting differences in inclusion criteria, restricted to invasive melanomas and mainly including melanomas on chronically exposed sites. Finally, we could not confirm previous findings to show that *MC1R* polymorphisms, both R and r variants, are determinants for developing *TERT* promoter mutated melanomas, probably because of differences in melanoma thickness and rate of *MC1R* and *TERT* mutations.[54]

Beyond *MC1R*, other melanoma predisposing genes (*CDKN2A*, *CDK4*, *POT1*, *MITF*) were previously screened in this cohort of MPM patients,[27] but the low frequency of mutations detected did not allow us to evaluate their influence on the somatic mutational status.

The main limitation of our study is that we mainly included thin melanomas; therefore the results may not be extrapolated to invasive and more aggressive melanomas. However, multiple melanomas are known to have lower thickness than the initial one.[4,5] Regarding methodologic

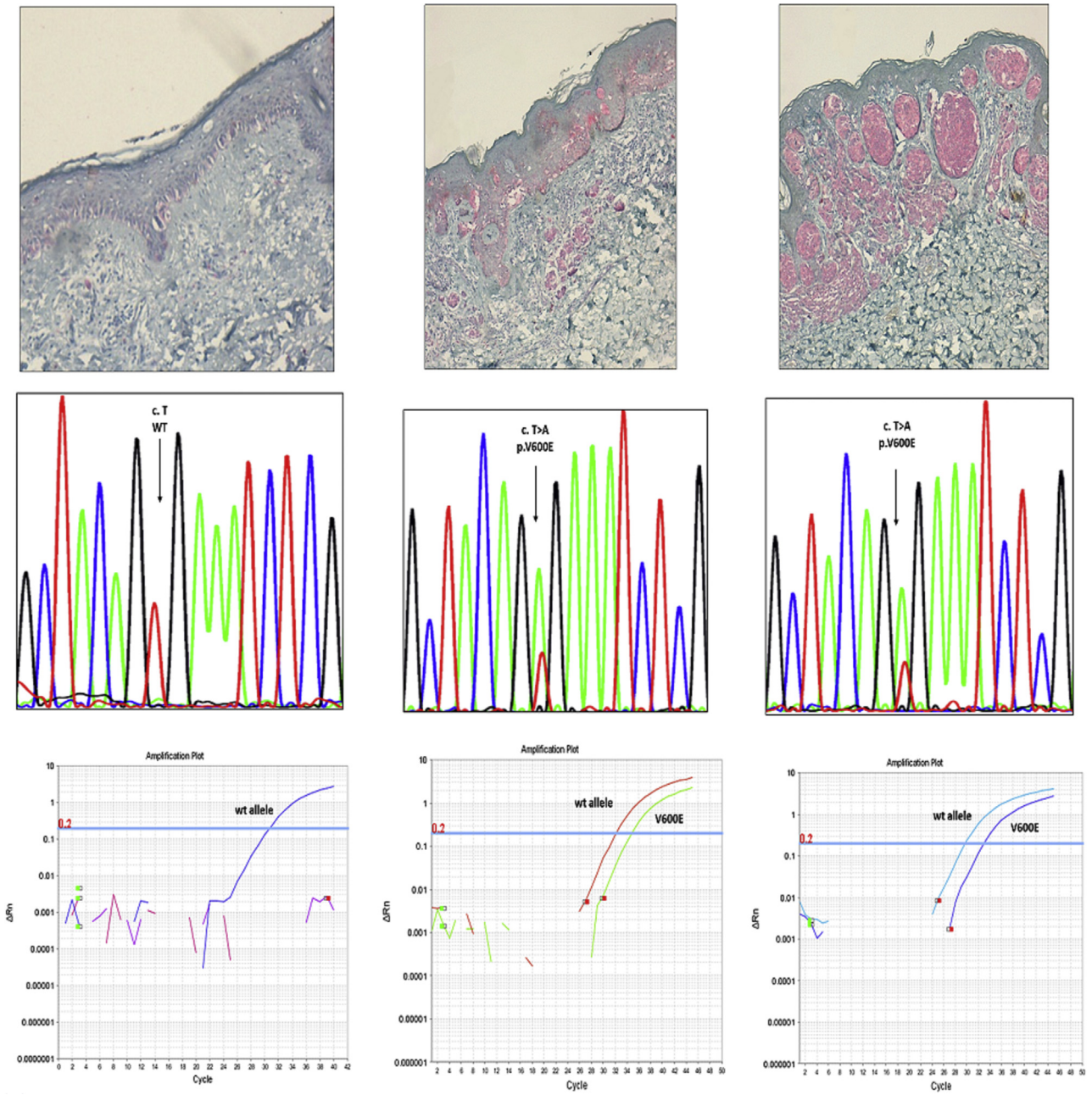


**Figure 3** Illustrative cases of melanomas that show concordant or discordant findings between molecular analysis and immunohistochemistry. **Left column:** Melanoma showing BRAF wild type by molecular testing but weak and heterogeneous staining by $BRAF^{V600E}$VE1 antibody. **Middle column:** Melanoma with the $BRAF^{V600E}$ mutation by molecular testing and moderately and heterogeneously immunostained by immunohistochemistry (IHC). **Right column:** Melanoma $BRAF^{V600E}$ mutated according to molecular testing with strong and homogeneous immunostaining by IHC. Original magnification, ×10. A, adenine; c., chromosome nucleotide; T, thymine; WT, wild-type.

aspects, the false-positive samples identified by IHC compared with molecular methods could suggest a technical issue. However, we used the allele-specific TaqMan assay technique that allows detection of a low number of mutated tumor cells. In addition, we limited the false immunostaining or the incorrect interpretation using the ultraView Universal RED Detection Kit that reduces the background artifacts, easily distinguishing between positive staining and melanin pigmentation.[48]

## Conclusions

Our results provide further evidence of the genetic diversity of multiple melanomas and demonstrate that the somatic heterogeneity is not influenced by inherited *MC1R* variants. In addition, our data suggest that a multiple analytical approach with both IHC and molecular methods may not be required to reach the correct classification of melanoma in clinical practice, supporting IHC for initial somatic genetic testing.

## Acknowledgment

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## Supplemental Data

Supplemental material for this article can be found at *https://doi.org/10.1016/j.jmoldx.2017.10.002*.

## References

1. Lo JA, Fisher DE: The melanoma revolution: from UV carcinogenesis to a new era in therapeutics. Science 2014, 346:945–949
2. Nikolaou V, Stratigos AJ: Emerging trends in the epidemiology of melanoma. Br J Dermatol 2014, 170:11–19
3. Bradford PT, Freedman DM, Goldstein AM, Tucker MA: Increased risk of second primary cancers after a diagnosis of melanoma. Arch Dermatol 2010, 146:265–272
4. Murali R, Goumas C, Kricker A, From L, Busam KJ, Begg CB, Dwyer T, Gruber SB, Kanetsky PA, Orlow I, Rosso S, Thomas NE, Berwick M, Scolyer RA, Armstrong BK; GEM Study Group: Clinicopathologic features of incident and subsequent tumors in patients with multiple primary cutaneous melanomas. Ann Surg Oncol 2012, 19:1024–1033
5. Savoia P, Osella-Abate S, Deboli T, Marenco F, Stroppiana E, Novelli M, Fierro MT, Bernengo MG: Clinical and prognostic reports from 270 patients with multiple primary melanomas: a 34-year single-institution study. J Eur Acad Dermatol Venereol 2012, 26:882–888
6. Youlden DR, Youl PH, Soyer HP, Aitken JF, Baade PD: Distribution of subsequent primary invasive melanomas following a first primary invasive or in situ melanoma in Queensland, Australia, 1982–2010. JAMA Dermatol 2014, 150:526–534
7. Ferrone CR, Ben Porat L, Panageas KS, Berwick M, Halpern AC, Patel A, Coit DG: Clinicopathological features of and risk factors for multiple primary melanomas. JAMA 2005, 294:1647–1654
8. Siskind V, Hughes MC, Palmer JM, Symmons JM, Aitken JF, Martin NG, Hayward NK, Whiteman DC: Nevi, family history, and fair skin increase the risk of second primary melanoma. J Invest Dermatol 2011, 131:461–467
9. Wangari-Talbot J, Chen S: Genetics of melanoma. Front Genet 2013, 3:330
10. Bruno W, Martinuzzi C, Andreotti V, Pastorino L, Spagnolo F, Dalmasso B, Cabiddu F, Gualco M, Ballestrero A, Bianchi-Scarrà G, Queirolo P, Grillo F, Mastracci L, Ghiorzo P; Italian Melanoma Intergroup (IMI): Heterogeneity and frequency of BRAF mutations in primary melanoma: comparison between molecular methods and immunohistochemistry. Oncotarget 2017, 8:8069–8082
11. Heidenreich B, Nagore E, Rachakonda PS, Garcia-Casado Z, Requena C, Traves V, Becker J, Soufir N, Hemminki K, Kumar R: Telomerase reverse transcriptase promoter mutations in primary cutaneous melanoma. Nat Commun 2014, 5:3401
12. Kanetsky PA, Rebbeck TR, Hummer AJ, Panossian S, Armstrong BK, Kricker A, Marrett LD, Millikan RC, Gruber SB, Culver HA, Zanetti R, Gallagher RP, Dwyer T, Busam K, From L, Mujumdar U, Wilcox H, Begg CB, Berwick M: Population-based study of natural variation in the melanocortin-1 receptor gene and melanoma. Cancer Res 2006, 66:9330–9337
13. Goldstein AM, Landi MT, Tsang S, Fraser MC, Munroe DJ, Tucker MA: Association of MC1R variants and risk of melanoma in melanoma-prone families with CDKN2A mutations. Cancer Epidemiol Biomarkers Prev 2005, 14:2208–2212
14. Pastorino L, Bonelli L, Ghiorzo P, Queirolo P, Battistuzzi L, Balleari E, Nasti S, Gargiulo S, Gliori S, Savoia P, Abate Osella S, Bernengo MG, Bianchi Scarrà G: CDKN2A mutations and MC1R variants in Italian patients with single or multiple primary melanoma. Pigment Cell Melanoma Res 2008, 21:700–709
15. Puntervoll HE, Yang XR, Vetti HH, Bachmann IM, Avril MF, Benfodda M, Catricalà C, Dalle S, Duval-Modeste AB, Ghiorzo P, Grammatico P, Harland M, Hayward NK, Hu HH, Jouary T, Martin-Denavit T, Ozola A, Palmer JM, Pastorino L, Pjanova D, Soufir N, Steine SJ, Stratigos AJ, Thomas L, Tinat J, Tsao H, Veinalde R, Tucker MA, Bressac-de Paillerets B, Newton-Bishop JA, Goldstein AM, Akslen LA, Molven A: Melanoma prone families with CDK4 germline mutation: phenotypic profile and associations with MC1R variants. J Med Genet 2013, 50:264–270
16. Fargnoli MC, Pike K, Pfeiffer RM, Tsang S, Rozenblum E, Munroe DJ, Golubeva Y, Calista D, Seidenari S, Massi D, Carli P, Bauer J, Elder DE, Bastian BC, Peris K, Landi MT: MC1R variants increase risk of melanomas harboring BRAF mutations. J Invest Dermatol 2008, 128:2485–2490
17. Landi MT, Bauer J, Pfeiffer RM, Elder DE, Hulley B, Minghetti P, Calista D, Kanetsky PA, Pinkel D, Bastian BC: MC1R germline variants confer risk for BRAF-mutant melanoma. Science 2006, 313:521–522
18. Thomas NE, Kanetsky PA, Edmiston SN, Alexander A, Begg CB, Groben PA, Hao H, Busam K, Ollila DW, Berwick M, Conway K: Relationship between germline MC1R variants and BRAF-mutant melanoma in a North Carolina population-based study. J Invest Dermatol 2010, 130:1463–1465
19. Hacker E, Nagore E, Cerroni L, Woods SL, Hayward NK, Chapman B, Montgomery GW, Soyer HP, Whiteman DC: NRAS and BRAF mutations in cutaneous melanoma and the association with MC1R genotype: findings from Spanish and Austrian populations. J Invest Dermatol 2013, 133:1027–1033
20. Scherer D, Rachakonda PS, Angelini S, Mehnert F, Sucker A, Egberts F, Hauschild A, Hemminki K, Schadendorf D, Kumar R: Association between the germline MC1R variants and somatic BRAF/NRAS mutations in melanoma tumors. J Invest Dermatol 2010, 130:2844–2848
21. Hacker E, Hayward NK, Dumenil T, James MR, Whiteman DC: The association between MC1R genotype and BRAF mutation status in cutaneous melanoma: findings from an Australian population. J Invest Dermatol 2010, 130:241–248
22. Robles-Espinoza CD, Roberts ND, Chen S, Leacy FP, Alexandrov LB, Pornputtapong N, Halaban R, Krauthammer M, Cui R, Timothy Bishop D, Adams DJ: Germline MC1R status influences somatic mutation burden in melanoma. Nat Commun 2016, 7:12064
23. Colombino M, Sini M, Lissia A, De Giorgi V, Stanganelli I, Ayala F, Massi D, Rubino C, Manca A, Paliogiannis P, Rossari S, Magi S, Mazzoni L, Botti G, Capone M, Palla M, Ascierto PA, Cossu A, Palmieri G; Italian Melanoma Intergroup (IMI): Discrepant alterations in main candidate genes among multiple primary melanomas. J Transl Med 2014, 12:117
24. Egberts F, Bohne AS, Krüger S, Hedderich J, Rompel R, Haag J, Röcken C, Hauschild A: Varying mutational alterations in multiple primary melanomas. J Mol Diagn 2016, 18:75–83
25. Massi D, Simi L, Sensi E, Baroni G, Xue G, Scatena C, Caldarella A, Pinzani P, Fontanini G, Carobbio A, Urso C, Mandalà M: Immunohistochemistry is highly sensitive and specific for the detection of NRASQ61R mutation in melanoma. Mod Pathol 2015, 28:487–497
26. Chen YL, Jeng YM, Chang CN, Lee HJ, Hsu HC, Lai PL, Yuan RH: TERT promoter mutation in resectable hepatocellular carcinomas: a strong association with hepatitis C infection and absence of hepatitis B infection. Int J Surg 2014, 12:659–665

27. Pellegrini C, Maturo MG, Martorelli C, Suppa M, Antonini A, Kostaki D, Verna L, Landi MT, Peris K, Fargnoli MC: Characterization of melanoma susceptibility genes in high-risk patients from Central Italy. Melanoma Res 2017, 27:258−267
28. Tetzlaff MT, Pattanaprichakul P, Wargo J, Fox PS, Patel KP, Estrella JS, Broaddus RR, Williams MD, Davies MA, Routbort MJ, Lazar AJ, Woodman SE, Hwu WJ, Gershenwald JE, Prieto VG, Torres-Cabala CA, Curry JL: Utility of BRAF V600E immunohistochemistry expression pattern as a surrogate of BRAF mutation status in 154 patients with advanced melanoma. Hum Pathol 2015, 46:1101−1110
29. Lee JH, Choi JW, Kim YS: Frequencies of BRAF and NRAS mutations are different in histological types and sites of origin of cutaneous melanoma: a meta-analysis. Br J Dermatol 2011, 164:776−784
30. Kim SY, Kim SN, Hahn HJ, Lee YW, Choe YB, Ahn KJ: Meta-analysis of BRAF mutations and clinicopathologic characteristics in primary melanoma. J Am Acad Dermatol 2015, 72:1036−1046.e2
31. Colombino M, Lissia A, Capone M, De Giorgi V, Massi D, Stanganelli I, Fonsatti E, Maio M, Botti G, Caracò C, Mozzillo N, Ascierto PA, Cossu A, Palmieri G: Heterogeneous distribution of BRAF/NRAS mutations among Italian patients with advanced melanoma. J Transl Med 2013, 11:202
32. Kakavand H, Crainic O, Lum T, O'Toole SA, Kefford RF, Thompson JF, Wilmott JS, Long GV, Scolyer RA: Concordant BRAFV600E mutation status in primary melanomas and associated naevi: implications for mutation testing of primary melanomas. Pathology 2014, 46:193−198
33. Tschandl P, Berghoff AS, Preusser M, Burgstaller-Muehlbacher S, Pehamberger H, Okamoto I, Kittler H: NRAS and BRAF mutations in melanoma-associated nevi and uninvolved nevi. PLoS One 2013, 8: e69639
34. Yazdi AS, Palmedo G, Flaig MJ, Puchta U, Reckwerth A, Rütten A, Mentzel T, Hügel H, Hantschke M, Schmid-Wendtner MH, Kutzner H, Sander CA: Mutations of the BRAF gene in benign and malignant melanocytic lesions. J Invest Dermatol 2003, 121: 1160−1162
35. Edlundh-Rose E, Egyházi S, Omholt K, Månsson-Brahme E, Platz A, Hansson J, Lundeberg J: NRAS and BRAF mutations in melanoma tumours in relation to clinical characteristics: a study based on mutation screening by pyrosequencing. Melanoma Res 2006, 16: 471−478
36. Ellerhorst JA, Greene VR, Ekmekcioglu S, Warneke CL, Johnson MM, Cooke CP, Wang LE, Prieto VG, Gershenwald JE, Wei Q, Grimm EA: Clinical correlates of NRAS and BRAF mutations in primary human melanoma. Clin Cancer Res 2011, 17: 229−235
37. Thomas NE, Edmiston SN, Alexander A, Groben PA, Parrish E, Kricker A, Armstrong BK, Anton-Culver H, Gruber SB, From L, Busam KJ, Hao H, Orlow I, Kanetsky PA, Luo L, Reiner AS, Paine S, Frank JS, Bramson JI, Marrett LD, Gallagher RP, Zanetti R, Rosso S, Dwyer T, Cust AE, Ollila DW, Begg CB, Berwick M, Conway K; GEM Study Group: Association between NRAS and BRAF mutational status and melanoma-specific survival among patients with higher-risk primary melanoma. JAMA Oncol 2015, 1:359−368
38. Pópulo H, Boaventura P, Vinagre J, Batista R, Mendes A, Caldas R, Pardal J, Azevedo F, Honavar M, Guimarães I, Manuel Lopes J, Sobrinho-Simões M, Soares P: TERT promoter mutations in skin cancer: the effects of sun exposure and X-irradiation. J Invest Dermatol 2014, 134:2251−2257
39. Huang FW, Hodis E, Xu MJ, Kryukov GV, Chin L, Garraway LA: Highly recurrent TERT promoter mutations in human melanoma. Science 2013, 339:957−959
40. Griewank KG, Murali R, Puig-Butille JA, Schilling B, Livingstone E, Potrony M, Carrera C, Schimming T, Möller I, Schwamborn M, Sucker A, Hillen U, Badenas C, Malvehy J, Zimmer L, Scherag A, Puig S, Schadendorf D: TERT promoter mutation status as an independent prognostic factor in cutaneous melanoma. J Natl Cancer Inst 2014, 106:dju246
41. Roh MR, Park KH, Chung KY, Shin SJ, Rha SY, Tsao H: Telomerase reverse transcriptase (TERT) promoter mutations in Korean melanoma patients. Am J Cancer Res 2017, 7:134−138
42. Ofner R, Ritter C, Heidenreich B, Kumar R, Ugurel S, Schrama D, Becker JC: Distribution of TERT promoter mutations in primary and metastatic melanomas in Austrian patients. J Cancer Res Clin Oncol 2017, 143:613−617
43. Nagore E, Heidenreich B, Rachakonda S, Garcia-Casado Z, Requena C, Soriano V, Frank C, Traves V, Quecedo E, Sanjuan-Gimenez J, Hemminki K, Landi MT, Kumar R: TERT promoter mutations in melanoma survival. Int J Cancer 2016, 139:75−84
44. Anwar MA, Murad F, Dawson E, Abd Elmageed ZY, Tsumagari K, Kandil E: Immunohistochemistry as a reliable method for detection of BRAF-V600E mutation in melanoma: a systematic review and meta-analysis of current published literature. J Surg Res 2016, 203:407−415
45. Heinzerling L, Kühnapfel S, Meckbach D, Baiter M, Kaempgen E, Keikavoussi P, Schuler G, Agaimy A, Bauer J, Hartmann A, Kiesewetter F, Schneider-Stock R: Rare BRAF mutations in melanoma patients: implications for molecular testing in clinical practice. Br J Cancer 2013, 108:2164−2171
46. Ihle MA, Fassunke J, König K, Grünewald I, Schlaak M, Kreuzberg N, Tietze L, Schildhaus HU, Büttner R, Merkelbach-Bruse S: Comparison of high resolution melting analysis, pyrosequencing, next generation sequencing and immunohistochemistry to conventional Sanger sequencing for the detection of p.V600E and non-p.V600E BRAF mutations. BMC Cancer 2014, 14:13
47. Busam KJ, Hedvat C, Pulitzer M, von Deimling A, Jungbluth AA: Immunohistochemical analysis of BRAF(V600E) expression of primary and metastatic melanoma and comparison with mutation status and melanocyte differentiation antigens of metastatic lesions. Am J Surg Pathol 2013, 37:413−420
48. Uguen A, Talagas M, Costa S, Samaison L, Paule L, Alavi Z, De Braekeleer M, Le Marechal C, Marcorelles P: NRAS (Q61R), BRAF (V600E) immunohistochemistry: a concomitant tool for mutation screening in melanomas. Diagn Pathol 2015, 10:121
49. Peris K, Fargnoli MC, Pacifico A, Surrenti T, Stolz W, Wolf P, Soyer HP, Chimenti S: CDKN2A and MC1R mutations in patients with sporadic multiple primary melanoma. J Invest Dermatol 2004, 122:1327−1330
50. Landi MT, Kanetsky PA, Tsang S, Gold B, Munroe D, Rebbeck T, Swoyer J, Ter-Minassian M, Hedayati M, Grossman L, Goldstein AM, Calista D, Pfeiffer RM: MC1R, ASIP, and DNA repair in sporadic and familial melanoma in a Mediterranean population. J Natl Cancer Inst 2005, 97:998−1007
51. Höiom V, Tuominen R, Käller M, Lindén D, Ahmadian A, Månsson-Brahme E, Egyhazi S, Sjöberg K, Lundeberg J, Hansson J: MC1R variation and melanoma risk in the Swedish population in relation to clinical and pathological parameters. Pigment Cell Melanoma Res 2009, 22:196−204
52. Taylor NJ, Busam KJ, From L, Groben PA, Anton-Culver H, Cust AE, Begg CB, Dwyer T, Gallagher RP, Gruber SB, Orlow I, Rosso S, Thomas NE, Zanetti R, Rebbeck TR, Berwick M, Kanetsky PA: Inherited variation at MC1R and histological characteristics of primary melanoma. PLoS One 2015, 10:e0119920
53. Stratigos AJ, Dimisianos G, Nikolaou V, Poulou M, Sypsa V, Stefanaki I, Papadopoulos O, Polydorou D, Plaka M, Christofidou E, Gogas H, Tsoutsos D, Kastana O, Antoniou C, Hatzakis A, Kanavakis E, Katsambas AD: Melanocortin receptor-1 gene polymorphisms and the risk of cutaneous melanoma in a low-risk southern European population. J Invest Dermatol 2006, 126:1842−1849
54. Nagore E, Reyes-Garcia D, Heidenreich B, Garcia-Casado Z, Requena C, Kumar R: TERT promoter mutations associate with MC1R variants in melanoma patients. Pigment Cell Melanoma Res 2017, 30:273−275