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In melanocytic lesions the fraction of *BRAF*^{V600E} alleles is associated with sun exposure but unrelated to ERK phosphorylation

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BRAF^{V600E} mutation has been frequently reported in different types of melanocytic lesions, but its role in melanomagenesis is poorly understood, having been associated with either the proliferative-induced MAPK pathway activation or the acquisition of oncogene-driven senescence. The presence of _BRAF_ alterations has been related to sun exposure, although the molecular mechanisms underlying this event are only partly known. To elucidate the relationships among _BRAF/NRAS_ alterations, MAPK pathway activation, and sun exposure, we examined 22 acquired nevi and 18 cutaneus melanomas from 38 patients. Microdissected tissues from each lesion were subjected to _BRAF/NRAS_ mutation analysis by sequencing, allele-specific PCR and pyrosequencing assay. The same lesions were also examined for the expression of phosphorylated ERK1/2. Phototype and an accurate history of sun exposure were evaluated for each patient. _BRAF_^{V600E} mutation was detected in 50% of the acquired nevi and in 70% of the cutaneus melanomas in the absence of _NRAS_ alterations. The fraction of alleles carrying _BRAF_^{V600E} substitution was variable but strongly associated with sun exposure. In contrast, no relationship was evidenced between the presence of this mutation and patients' phototype, phosphorylated ERK1/2 expression, or Clark's level. Our findings indicate that in melanocytic lesions, _BRAF_^{V600E} mutation can affect a subset of the cells and is associated with the type and quantity of sun exposure. This mutation is independent of the nevo-melanoma progression and unrelated to ERK phosphorylation, suggesting that alternative mechanisms to the MAPK activation are also involved in this type of transformation.
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Cutaneus melanoma can be regarded as the consequence of the loss of controlling genetic mechanisms by disrupting initiating events due to environmental factors.

Somatic genetic alterations affecting the mitogen-activated protein kinase (MAPK) signaling pathway are believed to be determinant for the onset of this type of neoplasia. Among the genes controlling the MAPK pathway, *BRAF* has been found highly mutated in a variable percentage of nevi and melanomas.[1,2] However, at present, the exact contribution of these frequent mutations to the initiation and progression of the melanocytic lesions is still poorly understood. Most (>90%) of the reported *BRAF* alterations are oncogenic mutations, located in the region coding for the kinase domain. These alterations are prevalently due to T1799A transversion leading to a substitution of valine by glutamic acid at codon 600 (V600E). *BRAF*^{V600E} mutations have been shown to occur early during the progression from nevus to melanoma,[3,4] but the association of this alterations with prognosis is controversial.[5–7] Recent reports have also pointed out that the frequency of this oncogenic mutation varies remarkably according to the type of melanocytic lesions,[8–10] being the common acquired nevi and the cutaneus melanomas among the more

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affected ones. Alterations involving *NRAS*, another gene regulating the MAPK signaling pathway, have also been associated with melanomagenesis, although to a lesser extent. Mutations affecting this gene occur prevalently at codon 61 (NRASQ61) and have been reported in up to 18–25% of common acquired nevi and cutaneus melanomas.[8,11] Generally, activating mutations of *BRAF* and *NRAS* oncogenes are mutually exclusive. Similar to *BRAF* mutations, *NRAS* alterations have been found associated with the initiation of melanomagenesis, but not with the progression of the lesion or patient's survival.[3,11,12]

Both *BRAF* and *NRAS* oncogenic mutations are expected to activate the MAPK signaling pathway.[13] However, in melanomagenesis, this evidence is controversial, as some studies performed on different types of melanocytic lesions have shown that the MAPK pathway activation can be achieved independently of *BRAF* or *NRAS* alterations.[14] In addition, there are *in vitro* data supporting the involvement of $BRAF^{V600E}$ mutation in the induction of cell cycle arrest and senescence of nevocytes.[15]

Among the environmental factors related to the onset of cutaneus melanomas, exposure to sunlight has been generally accepted to have a major role.[16] Epidemiological data have shown that the type of exposure is determinant to the melanocytic transformation process, an intense intermittent sun exposure during childhood being particularly deleterious.[17] In addition to UVB, exposure to UVA is also now recognized to have a role in the development of melanoma. However, the molecular mechanisms by which UVB/UVA operate on cellular target genes are known only partly.[18] Some reports have shown a relationship between the type of sun exposure and the alterations affecting the *BRAF–RAS* pathway.[19–21] A higher frequency of *BRAF* mutations has been found in cutaneus melanomas arising in intermittent sun-exposed sites with respect to the lesions developing in mucosal membranes or unexposed sites.[22] On the contrary, *RAS* alterations are more common in skin sites subjected to chronic sun exposure. In a study carried out on 126 melanomas exposed to a variable degree of sunlight, Curtin *et al*[23] showed that the type of sun exposure activates distinct genetic pathway. Nevertheless, the relationship between sun exposure and *BRAF* mutation has been investigated mainly in melanomas, whereas it is almost unknown in nevi.

In the present report, we looked for the relationships among the presence of *BRAF*/*NRAS* alterations, the MAPK pathway activation, and sun exposure in 22 common nevi and 18 cutaneus melanomas, including three melanomas with underlying nevi. To this aim, data from an accurate *BRAF*/*NRAS* mutation analysis and the immunohistochemical assessment of phosphorylated ERK1/2 were associated with skin phototypes and detailed information about the history of sun exposure.

Tumor specimens, patients and methods

Tumor Specimens and Clinical Data

We collected archivial, paraffin-embedded tissue samples from 22 common acquired melanocytic nevi (3 junctional, 13 compound, and 6 dermal) and 18 primary cutaneus melanomas (two Clark's level II, four level III, six level IV, and two level V). The 22 common acquired nevi were obtained from 20 Italian patients (14 women and 6 men; median age: 40.6 years). Nevi N22A and N22B were excised from distant sites of the same patient, lumbar region and back, respectively. Similarly, N27A and N27B were removed from the neck and the hip of a second patient. Melanomas were obtained from 18 Italian patients (7 women and 11 men; median age: 55.4 years). M2, M3, and M10 were complex lesions in which melanoma and residual nevus were histologically identifiable. Clinical data of patients are reported on Tables 2 and 3.

After having obtained informed consent, the dermatologists (AZ and FP) interviewed the patients by using a standardized written questionnaire to collect clinical data and medical history. Some of the questions were focused on the quantity and quality of sun exposure that patients had undergone during their lifetime. These parameters were considered to give each case a numerical value. In-depth evaluation was made for cases of sunburn during childhood and adulthood, type of exposure during the day, length of exposure times, use of sunscreens, and the use of sun lamps (if extensive). The resulting scores are summarized in Table 1a. The phototype of the patients under study was also taken into consideration. Table 1b shows a classification of sun-reactive skin types according to Fitzpatrick.[24]

All melanomas were excised from skin areas that had been subjected to intermittent sun exposure. Among nevi, six cases (N1, N16, N21, N25, N28, and N31) were excised from unexposed skin areas. These nevi were given a value of 0 (Table 2).

This study was approved by the medical ethical committee of the IRCC, and carried out according to the principles of the Declaration of Helsinki.

Microdissection and DNA Extraction

Paraffin-embedded H&E-stained tissue sections from the patients were histologically evaluated for the presence of different components of the melanocytic lesion and contaminating normal cells (lymphocytes). A percentage of nevus-melanoma cells was assigned to each lesion. According to this percentage, nevi and melanomas were subjected to microdissection by means of optical microscope or laser microdissector (Leica AS-LMS, Leica Microsystems, Wetzlar, Germany). DNA extraction was performed as follows: cells were collected in Eppendorf tubes, deparafinized by xylol, rehydrated

Table 1 Sun exposure and phototype

Score	Type of sun exposure
(a) Sun exposure score	
0	Little or no photo exposure. Nevi excised from non-photo exposure skin areas.
1	Moderate photo exposure with suitable sunscreens. Negative anamnesis for sunburn during childhood or adulthood. Exposure to sun outside midday hours.
2	Prolonged photo exposure with or without suitable sunscreens. Negative anamnesis for sunburn during childhood or adulthood. Sun exposure including midday hours.
3	Relevant sunburn during childhood. Moderate and/or prolonged photo exposure but with suitable sunscreens during adulthood.
4	Relevant sunburn during childhood and adulthood. Prolonged photo exposure without suitable protections. Prolonged and continuous use of sun lamps.

Skin type	Sun sensitivity	Pigmentary response
(b) Phototype score[a]		
I	Very sensitive, always burn easily	Little or no tan
II	Very sensitive, always burn easily	Minimal tan
III	Sensitive, burn moderately	Tan gradually (light brown)
IV	Moderately sensitive, burn minimally	Tan easily (brown)
V	Minimally sensitive, rarely burn	Tan darkly (dark brown)
VI	Insensitive, never burn	Deeply pigmented (black)

[a]Classification of sun reactive skin types according to Fitzpatrick.[24]

and resuspended in 250 μl of lysis buffer containing 100 mM Tris-HCl (pH 8.5), 5 mM EDTA, 200 mMNaCl, 2% SDS, and 15 μl of proteinase K (20 mg/ml). After incubation for 1 day at 55°C, samples were precipitated with isopropanol, washed with 70% ethanol. According to the quantity of pellets, DNAs were resuspended in 30–50 μl of 10 mM Tris-HCl (pH 8.5).

Mutation Analysis

Sequencing and allele-specific PCR

Sequence analysis was based on *BRAF* and *NRAS* sequences (GenBank accession number NM_00433 and NM_002524 for *BRAF* and *NRAS*, respectively). *BRAF* exons 11 and 15 were subjected to PCR amplifications using primers as previously reported.[10] As far as *NRAS* exons 1 and 2 are concerned, we used the following primers: exon 1, forward 5′-TAAGGA TGGGGGTTGCTAGA-3′ and reverse 5′-TGCATAAC TGAATGTATACCCAAAA-3′; exon 2, forward 5′-TT GCATTCCCTGTGGTTTTT-3′ and reverse 5′-TGGT AACCTCATTTCCCCATA-3′. Briefly, for all exons, thermal cycling was carried out in a final volume of 50 μl containing 100 μM each dNTP, 10 pmole of primers, 3 μl of DNA extract and 5 U of Taq polymerase (Thermoprime Plus DNA Polymerase, Abgene, Epsom, UK). The amplification protocol that we used consisted of an initial denaturation of 4 min followed by 40 cycles with denaturation at 94°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 1 min and 30 s. PCR products were purified by MinElute Gel Extraction Kit (Qiagen, Hilden, Germany) and directly sequenced using ALF express sequencer and Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit (GE Healthcare, Little Chalfont, UK). A second round of sequence analysis was carried out on PCR products purified with the use of ExoSAP-IT (USB Corporation, Cleveland, OH, USA) using 3730 DNA Analyzer and Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). All lesions that resulted to be wild type for codon 600 were further analyzed by allele-specific PCR. To this aim, two different forward primers with substitution of a single base at the end of the primer (5′-GTGATTTTGGTCTAGCTACAGT-3′ and 5′-GTGATTTTGGTCTAGCTACAGA-3′) were designed to amplify the wild-type allele or *BRAF*-T1799A mutation, respectively; in both cases, the same reverse primer was used (5′-GGCCAAAATT TAATCAGTGGA-3′). DNAs were amplified with the two sets of primers on the following conditions: denaturation for 2 min at 94°C, 25 cycles at 94°C for 30 s, 62°C for 30 s and 72°C for 30 s, and a final elongation for 7 min at 72°C. PCR were carried out with two wild-type controls and a *BRAF*T1799A mutated sample used at different dilutions.

Mutation quantification

In the *BRAF*^{V600E} lesions, we assessed the percentage of alleles carrying the mutation by pyrosequencing analysis, as previously reported by Edlundh-Rose *et al.*[25] To perform this assay, which is based on ‘sequencing by synthesis’ principle, PCR amplifications were carried out with the following primers: 5′-biotin-CTTCATAATGCTTGCTCTGATAGG-3′ (forward) and 5′-GCATCTCAGGGCCAAAAA-3′ (reverse); samples were denaturated at 94°C for 5 min, amplified for 35 cycles consisting of 94°C for 30 s, 58°C for 30 s, and 72°C for 45 s and elongated at 72°C for 7 min. The 5′-biotinylated PCR products of the region including codon 1799 were immobilized onto streptavidin-coated paramagnetic beads (GE Healthcare), denaturated by 0.1 mol/l NaOH and released according to the manufacturer’s instructions using PyroMark Vacuum Prep Workstation (Biotage, Uppsala, Sweden). These reactions were performed in a 96-well plate using Pyro Gold Reagents (Biotage). The primed single-stranded DNA templates

Table 2 Clinicopathological data and *BRAF*^{V600E} mutations in the analysed nevi

Sample	*Sex*	*Age*	*Histotype*	*Site*	*BRAF V600E*	*Percentage of mutant alleles*	*pERK 1/2[a] expression*	*pERK 1/2 Score*	*Sun exposure score*	*Photo-type*	*Type of exposition*
N1	M	33	Intradermal	Head	wt[b]	/[c]	NA[d]	/	2	3a	Unexposed
N2	M	49	Junctional	Trunk	wt	/	NA	/	3	3b	Intermittent
N4	F	57	Compound	Thigh	yes	<25	Absent	/	NA	NA	Intermittent
N5	F	51	Compound	Shoulder	wt	/	NA	/	1	3a	Intermittent
N14	F	38	Junctional	Lumbar region	yes	<25	10–15%	+	3	2	Intermittent
N16	F	48	Intradermal	Head	wt	/	10–15%	+	2	3a	Unexposed
N17	F	23	Compound	Lumbar region	wt	/	Absent	/	3	2	Intermittent
N20	F	21	Compound	Back	yes	>25	5% cyt[e]	+	4	2	Intermittent
N21	F	51	Compound	Breast	wt	/	10%	+	0	3a	Unexposed
N22 A	M	41	Junctional	Lumbar region	yes	<25	Absent	/	3	3a	Intermittent
N22 B			Compound	Back	yes	<25					Intermittent
N23	F	26	Intradermal	Peri-umbilical	yes	25	Absent	/	4	4	Intermittent
N25	F	23	Compound	Breast	wt	/	Absent	/	2	4	Unexposed
N26	M	39	Compound	Back	yes	>25	Absent	/	4	3	Intermittent
N27 A	F	33	Intradermal	Neck	yes	>25	Absent	/	4	4	Intermittent
N27 B			Compound	Hip	yes	>25	Absent	/	4	4	Intermittent
N28	F	73	Junctional	Pubis	wt	/	NA	/	0	3	Unexposed
N29	F	38	Compound	Arm	wt	/	5% cyt	+	3	3	Intermittent
N31	M	36	Compound	Sovra-gluteal	wt	/	Absent	/	0	2	Unexposed
N32	M	58	Intradermal	Shoulder	wt	/	NA	/	1	4	Intermittent
N35	F	43	Compound	Leg	yes	>25	10%	+	3	4	Intermittent
N36	F	32	Intradermal	Lumbar region	yes	>25	Absent	/	NA	NA	Intermittent

[a]pERK1/2, phosphorylated ERK1/2.
[b]wt, wild type.
[c]/, not applicable.
[d]NA, not available.
[e]cyt, cytoplasmic immunostaining.

were subjected to real-time sequencing of the region including codon 600 by using the reverse primer 5′-CCACTCCATCGAGATT-3′. A titration series with different dilutions of a sample containing the V600E mutation was set up to evaluate the linear correlation between the height of the peaks and the percentage of mutant alleles in the heterozygous lesions. These percentages were calculated by using PSQ96MA software (Biotage) for allelic quantification.

Phosphorylated ERK1/2 Immunohistochemistry

The MAPK pathway activation was evaluated using an antibody to dually phosphorylated ERK1/2 protein on tissue sections. To this aim, Anti p44/42 MAPK (Thr202/Tyr204) mouse mAb was obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). Briefly, 3 μm formalin-fixed paraffin-embedded tissue sections were rehydrated, pretreated with 1:10 dilution Target Retrieval Solution High pH (Dako Cytomation, Carpinteria, CA, USA) for 15 min in microwave oven at 600 W and blocked in H_2O_2 for 5 min. Primary anti-p44/42 at 1:400 dilution was incubated for 1 h at room temperature and detected using IgG anti-mouse/rabbit-poly-HRP (Novocastra, Newcaste Upon Tyne, UK) for 30 min and visualized with DAB Chromogen. As active ERK1/2 localizes in the nucleus, but can also exhibit cytoplasmic targets, both cytoplasmic and nuclear immunostaining were considered, and the overall score was assessed as following: absent or weak, + (20% positive cells, low expression); medium, + + (25–50% positive cells, moderate expression); and strong, + + + (>50% positive cells, high expression). As previously reported by Saldanha *et al*,[8] the endothelium of peritumoral vessel was used as an internal positive control.

Statistical Analysis

The χ^2 test and Fisher's exact test (when appropriate) were used to infer proportions when assessing the presence of association between *BRAF* mutation, type of lesion, Clark's level, immunohistochemistry, exposure sites, phototype, and sun exposure. To avoid ignoring the available information on the ordinal nature of the variables, tests based on rank correlation coefficients (Spearman and Kendall's τ) were used. Student's *t*-test and Wilcoxon rank-sum test were also carried out for phototype and sun exposure. $P<0.05$ was considered significant. All calculations were done in R statistical programming language (http://www.R-project.org).

Results

BRAF/NRAS Mutations

BRAF mutations were detected in 11 of the 22 (50%) common nevi and in 13 of the 18 (70%) cutaneus

Table 3 Clinicopathological data and $BRAF^{V600E}$ mutations in the analysed melanomas

Sample	*Sex*	*Age*	*Histotype*	*Site*	*BRAF V600E*	*% Mutant alleles*	*pERK 1/2*[a] *expression*	*pERK 1/,2 Score*	*Sun exposure score*	*Phototype*
M2 MEL	F	38	Clark's level IV; 0.6 mm	Thigh	yes	<25	40%	++	3	2
M2 NEV			intradermal underlying nevus	Thigh	yes	<25	40% cyt[b]	++		
M3 MEL	M	46	Clark's level II; 0.3 mm	Arm	wt[c]	/	NA[d]	/[e]	2	3a
M3 NEV			intradermal underlying nevus	Arm	wt	/	NA	/		
M6	M	41	Clark Level IV; 2.2 mm	Thigh	wt	/	NA	/	3	3b
M7	M	33	Clark's level II; 0.4 mm	Back	wt	/	1-2%	+	2	3a
M10 MEL	M	42	Clark's level II; 0.3 mm	Shoulder	yes	<25	25%	++	3	3b
M10 NEV			compound underlying nevus	Shoulder	yes	<25	25%	++		
M11	M	77	Clark's level IV; 0.3 mm	Shoulder	yes	<25	25%; 10% cyt	++	NA	NA
M12	F	50	Clark' s level II; 0.2 mm	Leg	yes	>25	NA	/	2–3	3
M13	F	41	Clark′ s level III; 0.65 mm	Arm	yes	>25	50%	++	3	2
M14	M	62	Clark's level III; 0.65 mm	Thigh	yes	>25	NA	/	NA	NA
M18	M	81	Clark′s level IV; 9 mm	Back	yes	>25	35%; 35% cyt	++	4	3a
M20	M	70	Clark's level II; 0.3 mm	Chest	yes	>25	5%	+	3	3
M21	F	54	Clark' s level II; 0.5 mm	Shoulder	yes	>25	20%	+	4	3
M23 MEL1	M	51	Clark's level IV; 1.7 mm; *in situ* component	Thigh	wt	/	70%	+++	2	3a
M23 MEL2			Clark's level IV; 1.7 mm; invasive component	Thigh	wt	/				
M24	F	55	Clark's level III; 0.7 mm	Back	yes	>25	NA	/	NA	NA
M25	F	81	Clark' level V; 6 mm	Leg	yes	<25	1%	+	3	3b
M28	F	23	Clark' level III; 0.8 mm	Breast	wt	/	NA	/	2	2
M35	M	77	Clark' s level IV; 9 mm	Back	yes	>25	20%; 40% cyt	+++	3	2
M37	M	76	Clark's level V; 10 mm	Chest	yes	>25	90% cyt	+++	3	1

[a]pERK1/2, phosphorylated ERK1/2.
[b]cyt, cytoplasmic immunostaining.
[c]wt, wild-type.
[d]NA, not available.
[e]/, not applicable.

melanomas. All the alterations were due to GTG to GAG transversion on nucleotide 1799 (T1799A). This substitution leads to the oncogenic change V600E. No mutation was found in exon 11 of *BRAF* gene as well as in exons 1 and 2 of *NRAS* gene.

No further mutations were evidenced when the wild-type lesions were rechecked for the presence of T1799A by allele-specific PCR.

The sequencing analysis showed a variable level of the heights of the T/A peaks among the nevi and melanomas exhibiting *BRAF*^{V600E}. These different levels were suggestive of the presence of a variable number of alleles with this mutation (Figure 1). To assess this evidence, all samples were analyzed by pyrosequencing. Supporting the sequencing data, this method showed that the microdissected mutant lesions of both nevi and melanomas were characterized by different percentages of T1799A alleles, with a range included between 5 and 40%. In addition, we found that pyrosequencing detected less than 25% of *BRAF*^{V600E} alleles when the mutant peak of the corresponding sequences was lower than 50% of the wild-type signal (Figure 1b). The prevalence of the lesions characterized by less than 25% of *BRAF*^{V600E} alleles was 36 and 40% among the nevi and melanomas, respectively (Tables 2 and 3).

The presence of *BRAF*^{V600E} mutation was independent of the type of the microdissected lesion ($P=0.63$; Table 4a). For two patients (cases N22 and N27 in Table 2), we checked the nevi arisen in distant sites: *BRAF*^{V600E} mutation was detected in all lesions. In the first subject, (N22) the T/A transversion was found in the two nevi obtained from the lumbar region and from the shoulder; in the second case, (N27) *BRAF* mutation was detected in the nevi excised from both the neck and the hip. We also analyzed three lesions with underlying nevi (cases M2, M3, and M10 in Table 3). In these tissues, either *BRAF* mutation was found in the nevus and melanoma (cases M2 and M10) or both lesions were wild type (case M3). The level of the T/A substitution was similar in the two-microdissected components of cases M2 and M10 (Table 3). In the case of melanoma M23, which was laser microdissected into *in situ* and invasive components, both tissues resulted to be wild type (Table 3). As far as melanomas are concerned, no relationship was evidenced between the presence of *BRAF*^{V600E} mutation and Clark's level ($P=1$, Table 4c).

Phosphorylated ERK1/2 Immunohistochemistry

No correlation was evidenced between the presence of *BRAF*^{V600E} mutation and the expression of phosphorylated ERK1/2 ($P=0.72$, Table 4a). This lack of association was found in the common nevi as well as in the cutaneus melanomas ($P=0.8$ and $P=1$, Table 4b and c), and was independent of the level of ERK1/2 expression ($P=0.5$ and $P=0.19$, Table 4b and c). However, considerable differences in the staining of phosphorylated ERK1/2 were observed between the two types of lesion. In the nevi, a positive expression was detected in only six cases (N35, N29, N14, N16, N20, and N21) that showed a weak immunostaining in not more than 10–15% of the cells (score +, low expression) (Table 2). In two of these cases (N20 and N29), the ERK1/2 protein expression involved just 5% of the cells and was limited to the cytoplasmic compartment (Figure 2b). As a matter of fact, in 8 out of the 11 melanocytic nevi with *BRAF* mutation no positive expression of pERK1/2 was identified (Figure 2a). Interestingly, five of these lesions (N36, N23, N26, N27A, and N27B) were characterized by a higher level of mutant alleles (>25%) (Table 2). On the contrary, a considerable high expression of pERK1/2 was evidenced among melanomas consistent with a fully MAPK pathway activation (Figures 2c–f). In five of these lesions, about 50–90% of the cells were found positive for pERK1/2 immunostaining (score + + +; high expression), whereas in other five melanomas about 20–40% cells (score + +; moderate expression) were positive for the expression of this phosphorylated protein. In all these cases, we evidenced a nuclear staining variably associated with cytoplasmic expression (Figures 2c and d). In two combined lesions of melanoma with an underlying nevus (cases M2 and M10), we found a considerable level of pERK1/2 expression in both the nevus and the melanoma components (40% positive cells for M2 and 25% for M10). However, in the nevus, the staining was prevalently cytoplasmic, whereas it became mostly nuclear in the melanoma component. The pERK1/2 staining was independent of the level of T1799A substitution (Table 3). Similarly, a high percentage of positive cells (about 70%) for pERK1/2 expression was detected in both *in situ* and invasive components of case M23 characterized by the absence of *BRAF*^{V600E} mutation (Figures 2e and f). Finally, no correlation was found between phosphorylated ERK1/2 and Clark's level ($P=0.28$, Table 4c).

BRAF Mutations, Sun-Exposure and Phototype

We evidenced a strong association between the presence of mutation at codon 600 of *BRAF* and sun exposure, measured according to the score reported in Table 1 ($P<0.0001$, Table 4a). This association was highly significant in both nevi and melanomas ($P=0.0011$ and $P=0.002$, Table 4b and c). The strong association was confirmed when the lesions were taken into consideration according to the percentage of mutant alleles: wild type, <25 and >25% ($P<0.0001$, Table 4a). As far as nevi are concerned, we also found a significant correlation ($P=0.02$) between the presence of *BRAF*^{V600E} and the nevi excised from sun-exposed skin with respect to those derived from unexposed sites (Table 4b). By

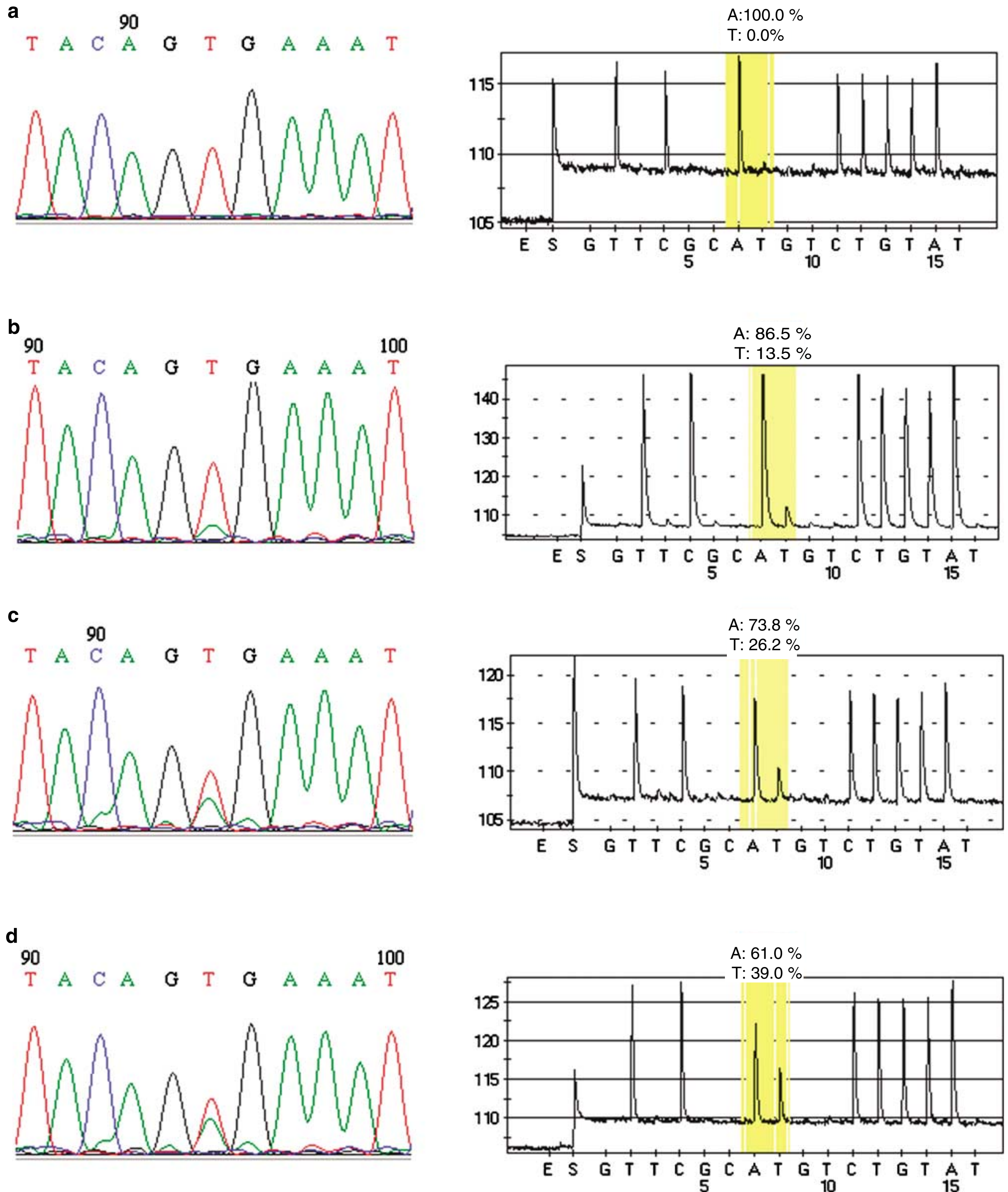

Figure 1 The comparison between sequencing and pyrosequencing analysis of some lesions with different levels of *BRAF*^{V600E} mutation (T1799A substitution). (**a**) A *BRAF* wild-type lesion; (**b**) the sequencing of a sample with the A peak lower than 50% of the T signal and its corresponding pyrogram characterized by the presence of <25% of the mutant alleles; (**c** and **d**) two lesions with the A signal higher than 50% and their pyrograms showing >25% of the mutant alleles. The pyrograms are reported in reverse.

Table 4 *BRAF*^{V600E} mutations, sun exposure and phototype

	Type of association	*Fisher's exact test*	*Wilcoxon t-test*
(a) All tested melanocytic lesions			
BRAF ^{V600E}	Type of lesion	0.63	
	pERK expression	0.72	
	Phototype	0.18	0.88
	Sun exposure	**<0.0001**	**<0.0001**
Percentage of *BRAF* ^{V600E} **alleles**[a]	**Sun exposure**	**<0.0001**	
(b) Nevi			
BRAF ^{V600E}	pERK expression	0.8	
	pERK expression level (−, +,++,+++)	0.5	
	Sun exposure	**0.0011**	**0.0003**
	Exposure sites	**0.02**	
(c) Melanomas			
BRAF ^{V600E}	Clark's level	1	
	pERK expression	1	
	pERK expression level (−, +,++,+++)	0.19	
	Sun exposure	**0.002**	**0.0003**
Clark's level	pERK expression level (−, +,++,+++)	0.28	

[a]The percentage of *BRAF* ^{V600E} alleles was considered as follows: 0% (wild-type), <25 or >25%.
Bold represents the parameters with a significant association ($P<0.05$) and their statistical *P*-values.

contrast, in both the two types of lesions, the patients' phototype was not associated with the substitution at codon 600 ($P=0.18$, Table 4a).

Discussion

In this study, we detected the presence of *BRAF*^{V600E} mutation in 50% of the common nevi and in 70% of the cutaneus melanoma.

Overall, our results evidenced that in both these types of lesions, *BRAF*^{V600E} substitution can affect a variable fraction of the alleles. It has been evidenced that, in junctional and small compound nevi, direct sequencing fails to detect the presence of *BRAF*^{V600E} when this affects less than 20% of the cells.[26] By combining microdissection and an accurate mutation analysis, including allele-specific PCR and pyrosequencing, we were able to perform a sensitive analysis and to quantify the percentage of these mutant alleles.

After sequencing, an underestimation of the presence of *BRAF*^{V600E} was ruled out in the wild-type lesions by performing allele-specific PCR analysis. In the mutant tissues, the quantification of T1799A allele was carried out by using pyrosequencing. This method has been proved to be a useful approach to quantitatively detect somatic mutations in genetic syndromes causing mosaicism[27] and it has been recently applied to perform *BRAF* and *NRAS* genotyping in melanomas.[28] In the present study, the percentage of alleles carrying T1799A ranged from 5 to 45%, suggesting that in some lesions only a fraction of the melanocytes are affected by this oncogenic substitution. However, even on homogeneous microdissected tissue, a direct association between the fractions of mutated alleles and cells cannot be claimed, as allelic imbalances due to chromosome 7q rearrangements could occur in melanomas. Moreover, although microdissection is a reliable and helpful method to separate different cell population in paraffin-embedded tissues, we cannot formally exclude the presence of contaminating non-melanocytic cells, particularly in nevi.

The percentage of alleles carrying *BRAF*^{V600E} substitution was found to be independent of the type of lesion. This supports the hypothesis of *BRAF* mutations as precocious events that are preserved throughout progression. This evidence was pointed out by the analysis of the three melanomas with residual nevi. In these cases, alleles carrying *BRAF* mutation were found in both components or, alternatively, were absent in each part of these lesions. Similarly, in another melanoma sample of this set (M23), no onset of V600E was evidenced in the transition from *in situ* to vertical growth phase. A role for this mutation as an initiating event is further supported by the lack of correlation between the presence of T1799A substitution and the Clark's level, a histological measure of melanoma progression. Accordingly, Omholt *et al*[3] showed that *BRAF* mutation can be detected equally in radial growth phase (RGP) and vertical growth phase (VGP) melanomas.

The fact that the percentage of mutated alleles does not increase in the nevus-melanoma transition implies that the acquisition of this mutation does not provide the cells with that proliferative advantage, which is necessary to overwhelm the remaining cell population. *BRAF*^{V600E} mutation is expected to constitutively activate the RAF/MEK/ERK pathway conferring the cells a proliferative and invasive potential. Nevertheless, in melanomas, the role of

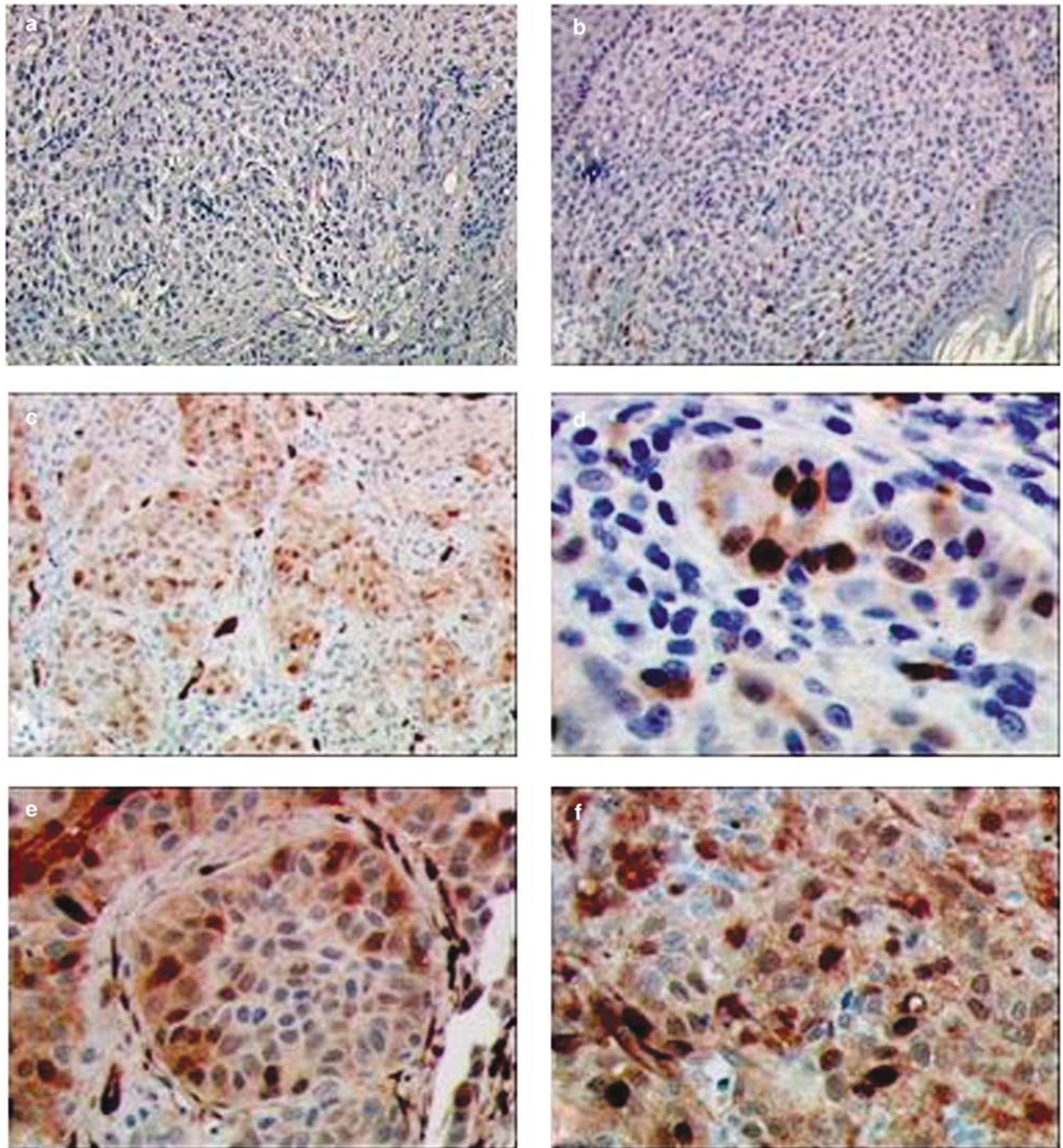

Figure 2 Phosphoprotein ERK1/2 immunohistochemistry. (**a**) Sections from nevus N23 negative for pERK1/2 staining; the same sample was characterized by >25% alleles carrying *BRAF*^{V600E} mutation. (**b**) Lesion N20 showed 5% of the cells positive for pERK1/2 (weak expression, +); staining was mainly cytoplasmic; this nevus was provided with >25% mutant alleles. (**c**) Melanoma M18 exhibited 35% positive cells for ERK1/2 staining (medium expression + +); the staining was both cytoplasmic and nuclear; pyrosequencing analysis of this lesion evidenced >25% mutant alleles. (**d**) Melanoma M18 at different magnifications. (**e**) *In situ* component of melanoma M23. (**f**) Invasive component of melanoma M23; both *in situ* and invasive components of this lesion were characterized by the presence of 70% positive cells (high expression + + +); the staining was found in cytoplasm as well as in nucleous; this lesion was wild type for *BRAF/NRAS* genes (magnification: **a** and **b**, ×10; **c**, ×20; **d–f**, ×40).

this commonly acquired *BRAF* mutation in the activation of the MAPK pathway is controversial. It has been shown that in nevi, the initial moderate proliferation supported by *BRAF*^{V600E} is followed by a cell cycle arrest, implying that this mutation is associated with an oncogene-driven senescence process.[15] In accordance with previous reports,[8,14] we did not find any association between this

substitution and the pERK1/2 immunohistochemical expression. This turned out to be clearly evident in nevi where only few cases showed a poor staining (5–10% positive cells), independently of the presence of *BRAF* mutation. Melanomas of our series generally displayed a stronger ERK1/2 expression (up to 50–70% positive cells), but this staining was not related to the presence of *BRAF*^{V600E} or to the fraction of the cells carrying this transversion. These results suggest that a full MAPK activation is achieved only when the lesion is at the stage of melanoma, when several other events can contribute to the activation of the RAF/MEK/ERK pathway. An overexpression of wild-type *BRAF* is one of the mechanisms underlying the activation of the MAPK pathway.[29] Alternatively, the MAPK signaling cascade can be modulated by the inhibition of MAPK phosphatases or by the suppression of RAF kinase inhibitors.[30,31] The phosphorylation of ERK can also be due to the interplay of different signaling events. Among these, in late-stage melanomas, there are an overexpression of *EGFR*,[32] and/or an increase of autocrine FGF signaling.[33]

It is well-known that a history of sunburn and intermittent exposure to sunlight may promote the development of melanoma.[17] In cutaneus melanomas, mutations of *BRAF* gene have been related to intermittent sun exposure.[22] Taking advantage of this finding, we collected an accurate history of the quantity and quality of sun exposure that each patient had undergone. By using this approach, we showed a strong association between the sun exposure, expressed as a score, and the presence of *BRAF*^{V600E} mutation. Thomas *et al*[34] have recently shown that the occurrence of *BRAF* mutations is associated with the early life UV exposure. In the present study, we found that there is a quantitative relationship between the level of sun exposure and the percentage of the alleles carrying V600E substitution. Our findings evidenced that the type of sun exposure is determinant for the onset of *BRAF*^{V600E} mutation not only in cutaneus melanomas of exposed skin but also in common nevi subjected to intermittent light exposure. Accordingly, we observed an absence of *NRAS* alterations that had been mostly reported in lesions excised from chronic sun-exposed sites. Interestingly, we found that the presence of this *BRAF* mutation is independent of the patient's phototype. Variant alleles of *MC1R*, the gene encoding the melanocortin-1 receptor, have been shown to contribute to the onset of *BRAF* mutations in sun-exposed melanomas.[35] However, this event may be partly unrelated to pigmentation, which is an important feature of skin phototype. As a matter of fact, Tadokoro *et al*[36] showed a relationship between melanin content and DNA damage induced by UV exposure.

BRAF^{V600E} alteration does not show the typical UVB signature mutations, which are characterized, through cyclobutane pyrimidine dimers and 6–4 photoproducts, by C/T and CC/TT transitions. Taking into consideration different variables such as nearby potential pyrimidine sites, the properties of specialized DNA polymerase, and biological selection, it has been suggested that V600E could arise from error-prone replication of UV-damaged DNA.[37] On the other hand, UVA absorption, being an important source of DNA oxidative damage, by generating reactive oxygen species (ROS), can greatly increase replicative errors of different types, including T/A base substitution. This oxidative damage is further implemented by inflammation processes, which is generally induced by UV sunburns.[38] Although, at present, it is difficult to assess how UV is involved in the onset of *BRAF* mutation, repeated events of intense sun exposure have been correlated with the accumulation of V600E in nevi and melanomas. This mechanism is consistent with our results showing a quantitative association between the sun exposure and the fraction of alleles carrying *BRAF*^{V600E}. A convincing example of this evidence is provided by those patients with two nevi obtained from distant, but sun-exposed sites. In these cases, both the lesions showed a similar fraction of V600E mutated cells.

In summary, our study shows that in acquired nevi and cutaneus melanomas, *BRAF*^{V600E} is an early event affecting a variable percentage of nevocytic and melanocytic alleles. The entity of this fraction depends on the type and history of sun exposure and is unrelated to the patients' phototype. According to our results, the onset of *BRAF*^{V600E} is independent of the progression and unrelated to the MAPK pathway activation, which is achieved only later, following the acquisition of other genetic alterations. As a consequence, in these lesions, *BRAF*^{V600E} can be regarded as a genetic hallmark of DNA damage induced by sun exposure. This mutation can lead to transformation by mechanisms alternative to cell proliferation.

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Disclosure/conflict of interest

The authors state no conflict of interest.

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