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Mutational status of nevus associated-melanomas

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Abstract

Introduction—Melanoma origin has always been a debated subject, as well as the role of adjacent melanocytic nevi. Epidemiological and histopathological studies point to melanomas arising either *de novo* or from a nevus.

Methods—Sixty-one melanomas found in association with a preexisting nevus were microdissected, after careful selection of cell subpopulations and submitted to Sanger sequencing of the *BRAF*, *NRAS*, *C-KIT*, *PPP6C*, *STK19* and *RAC1* genes. Each gene was evaluated twice in all samples by sequencing or by sequencing and another confirmation method, allele-specific fluorescent polymerase chain reaction (PCR) and capillary electrophoresis detection, or by SNaPshot Analysis. Only mutations confirmed via two different molecular methods or twice by sequencing were considered positive.

Results—The majority of cases presented concordance of mutational status between melanoma and the associated nevus for all 6 genes (40/60; 66.7%). Nine cases presented concomitant *BRAF*

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Conflict of interest

The authors declare no conflicts of interest.

Author contribution

Dr(s)Shitara, Tell-Martí, Badenas, Enokihara, Alós, Larque, Michalany, Carrera, Malvehy, Bagatin, and Puig had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Dr(s) Puig. Acquisition of data: Dr(s)Shitara, Carrera, Malvehy, Alós, Larque, Michalany, Enokihara. Analysis and interpretation of data: Dr(s)Shitara, Tell-Martí, Badenas, Puig-Butille and Puig. Drafting of the manuscript: Dr(s)Shitara, Tell-Martí, Bagatin and Puig. Critical revision of the manuscript for important intellectual content: Dr(s) Shitara, Tell-Martí, Badenas, Enokihara, Alós, Michalany, Carrera, Malvehy, Bagatin, Puig-Butille and Puig. Statistical analysis: Dr Puig. Obtained funding: Dr(s) Puig, Shitara and Bagatin. Administrative, technical or material support: Dr(s) Carrera, Malvehy, Puig and Bagatin. Study supervision: Dr(s) Puig and Bagatin.

and *NRAS* mutations, including one case, in which both the melanoma and the adjacent nevus harbored V600E and Q61K double mutations. In two cases, both melanoma and associated nevus, located on acral sites were *BRAF* mutated, including an acral lentiginous melanoma.

Conclusions—This is the largest nevus-associated melanoma series molecularly evaluated to our knowledge. The majority of melanomas and adjacent nevi in our sample share the same mutational profile, corroborating the theory that the adjacent nevus and melanoma are clonally related and that melanoma originated within a nevus.

Keywords

melanoma; nevus; BRAF; NRAS; V600E

Introduction

Melanoma origin has always been a much debated subject, as well as the precursor role of pigmented lesions. Considering epidemiological and histological studies, melanoma may arise in association with a pre-existing nevus or *de novo*, without any associated lesion [1]. It is controversial whether nevus-associated melanoma and *de novo* melanomas have a different prognosis [2–6], but a prior nevus-associated melanoma seems to be associated with a 9-fold increased risk of presenting another nevus-associated melanoma [7]. According to histopathological studies, the incidence of nevus-associated melanoma may vary from 20–50% of all melanoma cases [2,5,8–18]. Some authors suggest that benign melanocytic lesions could correspond to precursor lesions in melanoma genesis [19–23]. Although the contiguity of nevus cells with melanoma, may provide some clues as to the etiopathogenesis of malignant melanoma, direct evidence of a causal relationship between the two is still lacking. The extent of genetic alterations has been shown to increase in parallel with the transitions from benign nevi to dysplastic nevi (DN) to melanoma, supporting multistep tumorigenesis [24]. Further evidence that nevi could correspond to initial steps in melanomagenesis are the facts that nevi and melanoma share genetic alterations and *BRAF* mutated melanomas are more likely to be associated with multiple melanocytic nevi than *BRAF*-wild type melanomas [25,26].

Although molecular studies of melanoma and nevus present in contiguity suggested that melanoma and nevus cells could be clonally related [22,27–30], there is still no consensus for the nevus-to-melanoma progression model [31]. While previous studies [22,27–32] differentiated nevus and melanoma cells using morphologic criteria only, it is important to point out that melanoma diagnosis may vary even among experts [33–35] and distinction of contiguous nevus cells and melanoma cells can be doubtful, especially in nevoid melanomas [35,36] and melanomas with adjacent dysplastic nevus [37,38]. Thus possible selection bias of cell populations in the previous studies cannot be ruled out when considering clonality between melanoma and adjacent nevus. A combination of morphologic and immunohistochemistry markers would be the most reliable diagnostic method for melanoma [39]. Therefore we sought to evaluate the presence of mutations in genes from well known melanomagenesis pathways, such as *BRAF*, *NRAS*, *c-KIT*, as well as new candidates as driver genes: *RAC1*, *PPP6C* and *STK19* [40], after careful selection of melanoma and nevus cell subpopulations in the largest series of nevus-associated melanomas, to our knowledge.

Materials and Methods

Cases of melanoma found in histological association with a nevus were evaluated. All Formalin-fixed, paraffin-embedded (FFPE) samples studied were collected from the archives of a dermatopathology referral center in São Paulo-Brazil and the Hospital Clínic of Barcelona-Spain. The clinico-pathological data was extracted from the histopathological history. New slides stained with hematoxylin and eosin (HE) were independently evaluated by experienced dermatopathologists (N.M., M.M.S.S.E. and L.A.) and a dermatologist (D.S.). The nevus cytology criteria used for differentiation from melanoma cells were described elsewhere [41–43]. Sixty-one cases (34 pairs from Brazil and 27 from Spain), which presented concordance for the diagnosis of nevus-associated melanoma by at least two of the evaluators and fulfilled the following criteria: undisputed distinction between nevus and melanoma and sufficient biological material from both melanoma and nevus cells for further analysis, were included.

All study participants gave their written informed consent to participate and the study was approved by the research ethic committees of the Federal University of São Paulo/ UNIFESP-EPM (number 188.713 of 24/01/2013) and the Hospital Clínic of Barcelona.

Immunohistochemistry

Available tumor tissue from 47 cases was stained using HMB-45 (Cell Marque, NL) and/or Ki-67 (Spring Bioscience, USA), Melan-A (Dako, DEN) as recommended by the manufacturer and slides were evaluated by two observers (D.S. and M.M.S.S.E.) in order to properly differentiate melanoma cells from nevus cells.

Laser Microdissection and DNA extraction

Contiguous nevus-melanoma specimens from FFPE archival tissue were sectioned (5 μm thick) on polyethylene naphthalate membranes (PEN Membrane slides®, LEICA, US) mounted on regular glass slides, according to manufacturer's instructions, and stained with HE. Melanoma and nevus cells were microdissected using LEICA microdissector (LMD6500®/ LEICA Microsystems) according to previous marking on correspondent HE slides. DNA was extracted using the QIAmp DNA Micro kit (QIAGEN® Sample & Assay Technologies, Spain).

Mutational screening of *BRAF*, *NRAS*, *C-KIT*, *PPP6C, STK19* and *RAC1* by Sanger sequencing

BRAF (exons 11 and 15), *NRAS* (exons 2 and 3), *c-KIT* (exon 11), *PPP6C* (exon 7), *RAC1* (exon 2) and *STK19* (exon 2) genes were amplified using nested PCR with 40–60ng of DNA for each reaction. PCR conditions were: denaturation at 95°C 5 min, 10 cycles (94°C 40sec, 60°C–55°C 40 sec, 72°C 1 min), followed by 25 cycles (94°C 40sec, 57°C 40 sec, 72°C 1 min) and extension at 72°C (10 min). The second PCR mixture was prepared using 1μl from the first PCR reaction. The nested PCR conditions were the same, except that the annealing temperature was 57°C. Nested primers were designed surrounding the exons (200–300bp long) with standard M13 forward (M13F: 5′ TGTAAAACGACGGCCAGT 3′) and M13 reverse (M13R: 5′CAGGAAACAGCTATGACC 3′) tails being included on the internal

primers to standardize the capillary sequencing. Nucleotide sequences of nested primers are reported in Tables 1a and 1b. The PCR products were cleaned up using 6μL of Exo-SAP-IT (Applied Biosystems, CA, USA) and amplification was confirmed by 2% agarose gel electrophoresis. The entire amplicons were sequenced using both primers M13RF and M13R with BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, CA, USA) on an ABI PRISM 3130xl automatic sequencer (Applied Biosystems, CA, USA) and analyzed using the SeqPilot 4.0.1 software (JSI Medical Systems).

BRAF p.V600E Mutational Analysis

The c.1799T>A (p.V600E) mutation was determined using allele-specific fluorescent PCR and capillary electrophoresis detection. Genomic DNA samples were amplified using primers described elsewhere [44]. PCR conditions were: denaturation at 95°C for 5 min; 35 cycles (94°C for 30 sec, 56°C for 40 sec, 72°C for 40 sec); extension at 72°C for 10 min. Amplicons were detected using capillary electrophoresis on ABI PRISM 3130xl and analyzed using GeneMapper Software (Applied Biosystems/Life Technologies, Grand Island, NY).

SNaPshot Analysis

The SNaPshot® Multiplex System was performed to confirm the results obtained by sequencing in the *BRAF*, *NRAS* and *c-KIT* genes[45]. We performed the multiplex PCR and extension PCR under the same conditions, using previously described primers [45].

Results

Table 2 summarizes the clinical characteristics for the 61paired cases (melanomas and their associated nevi) and their complete molecular screening results. Each gene was evaluated twice in all samples either by two different molecular methods or by Sanger sequencing twice and only confirmed mutations were considered as positive. The DNA extraction of nevus number 6 failed; therefore this pair was excluded from the analysis.

Clinical sample characteristics (Table 2)

Most melanomas were located on trunk and extremities (34/61 and 15/61 respectively). No mucosal melanomas were found. Most melanomas were superficial spreading melanomas (SSM) (39/61), one nodular melanoma, one acral lentiginous melanoma (ALM) and 18 were incipient melanomas which did not fulfill all the criteria for SSM. Two cases had incomplete pathological information.

Six associated nevi (10.0%) corresponded to congenital subtype, while 6 were junctional, 10 compound and 3 intradermal common nevi and in 36 cases, no subtype could be specified. Only one of these latter showed signs of dysplasia (1/36). Twenty-one melanomas were *in situ* melanomas, and most invasive melanomas were thin melanomas, with a mean Breslow thickness of 0.75mm.

Frequency of BRAF mutations in melanomas and associated nevi

BRAF exons 11 and 15 were evaluated in 60 paired cases. No mutation was detected in exon 11. In exon 15, we detected the V600E (c.1799T>A) mutation in 46.6% (28/60) of melanomas and in 51.7% (31/60) of nevi. Regarding *BRAF* mutational status, most paired cases were concordant (78.3%; 47/60; Fig. 1). In 38.3% of cases a V600E-mutated melanoma had a preexisting V600E-mutated nevus (23/60). Among the discordant cases, a V600E-mutated melanoma had an adjacent *BRAF*-WT nevus in 8.3% of cases (5/60), while in 13% a V600E-mutated nevus was associated with a *BRAF*-WT melanoma (8/60).

The V600E mutation was confirmed in all cases using allele-specific fluorescent PCR. With this technique we could also estimate the percentage of mutated DNA present in each sample. Focusing on the concordant *BRAF* V600E mutated cases (23 pairs), we observed the same percentage of V600E mutation in 39% of these cases (9/23). However, 11 melanomas showed a higher percentage of V600E mutation than their associated nevi (48%; 11/23) and 3 melanomas showed a lower percentage of V600E mutation in comparison with their associated nevi (13%; 3/23).

Frequency of *NRAS* mutations in melanomas and associated nevi

NRAS exons 2 and 3 were evaluated in 60 paired cases. No mutation was detected in exon 2, but in exon 3 we observed the Q61K (c.181C>A) mutation in 21.7% (13/60) of cases (Fig. 1). Regarding *NRAS* mutational status, 85% of cases were concordant, being 78% (47/60) WT and 7% mutated (4/60). However, in 10% of cases we detected a mutated melanoma adjacent to a WT nevus (6/60) and the remaining 5% were wild-type melanomas associated with a mutated nevus (3/60). Additionally, we observed a double mutation in *NRAS* exon 3 in two nevi (Table 2): in one case, melanoma and its associated nevus were both Q61K mutated but the nevus also harbored the Q70H (c.210A>T) variation. In the other case, an *NRAS* wild-type melanoma was associated with a Q61K mutated nevus that also harbored a D33D (c.99T>C) synonymous variation. These two nucleotide variations have not been previously reported.

Frequency of *C-KIT*, *PPP6C*, *STK19* and *RAC1* mutations in melanomas and associated nevi

No mutations were detected. All paired cases were wild-type for these genes.

Discussion

Driver mutations, being either the activation of an oncogene or the loss of a tumor suppressor allele, could trigger the establishment of a benign lesion. Thereafter the senescence program would control the tumor expansion, and another mutation would be necessary for tumor progression [30,46,47]. Molecular analysis of melanomas and so called “precursor lesions” separately, so far point to alterations in critical genes of the MAPK and the PI3-Kinase-Akt pathways, as well as in genes involved in cell cycle regulation, as molecular pathways involved in melanoma genesis in the majority of cases [48,49]. Indeed, most cases in this study show mutations in either *BRAF* or *NRAS* (67.2%; 41/61). The presence of *BRAF* V600E mutation in 31 of the studied nevi is in concordance with the

hypothesis that this mutation may be an early event in melanocytic tumorigenesis, even though alone it is not sufficient for progression to melanoma[50–55]. Most of the associated nevi included in this study were common melanocytic nevi. Since there is controversy regarding the diagnosis of dysplastic nevi [56,57], and since we only included cases in which distinction between melanoma and nevus cells was concordant among the observers, it may have led to a selection bias toward the inclusion of nevi with less dysplasia. A limitation observed by other authors [31]. Unfortunately, since most of the nevi observed in our study were not dysplastic, even though the majority of our cases depicted the same mutational status between melanoma and nevus, no conclusion regarding the Clark multi-step melanoma progression can be made.

In our study the majority of cases (78.3%; 47/60; Table 2; Fig. 1) presented the same *BRAF* mutational status for both melanoma and nevus (mut/mut; WT/WT), being mutated in 48% (23/47) of concordant pairs, similar to previous studies [27,29,31,58]. Considering these latter, the percentage of V600E mutation was similar in melanoma and nevus in 39% (9/23) of cases, while in 48% (11/23) the melanomas harbored a higher percentage of mutant cells compared to the adjacent nevus. The results support the hypothesis that the presence of V600E *BRAF* mutation confers an advantage for melanoma progression.

In a significant proportion of our cases (21.7%; 13/60) we observed that either the melanoma or the nevus was mutated while the counterpart was WT. It has been suggested that BRAF mutations are fully clonal in melanocytic nevi, so that it is expected that melanomas deriving from these nevi would be BRAF mutated as well [59]. Therefore cases in which only the nevus was mutated favors the possibility of the absence of a clonal relationship between these two lesions. In cases where the *BRAF* mutation was only present in the melanoma, the possibility of the melanoma arising in the nevus cannot be excluded, since the melanoma may have later acquired the mutation or mutant parts of the associated nevus may have been overgrown by the melanoma and therefore may not have been accessible for analysis.

Recently Kakavand et al., after obtaining 100% concordance of BRAF mutational status between melanomas and associated nevi, suggested that a higher percentage of discordant melanomas and associated nevi obtained by Sanger sequencing, might be related to the technical difficulty of micro-dissecting out melanoma and admixed nevus cells. This could eventually explain why in case 47, both melanoma and nevus were Q61K mutated, while only the nevus was V600E mutated. Nevertheless cases with only the melanoma BRAF and *NRAS* mutated (cases 30 and 32) and the presence of a nevus with mutation in *BRAF* while the melanoma harbored mutation in *NRAS* (case 42), are more difficult to explain by technical differences among mutation detection techniques, and could highlight the fact that in some cases the contiguity of melanoma and nevus correspond to mere fortuitous collision.

Although it has been referred that *BRAF* V600 and *NRAS* Q61R [60] are among the most recurrent nucleotide substitutions in melanoma, all *NRAS* mutations observed in this study were Q61K, being this mutation described in melanomas and congenital nevi [61]. Only one case in previous study [27–31] on melanoma and associated nevus described *BRAF* and *NRAS* mutations in the same lesion, while we found 9 cases with concomitant *BRAF* V600E and

NRAS Q61K mutations. A case, in which both melanoma and associated nevus harbored V600E and Q61K (case 35, Table 2; Fig. 2) and case 24, in which melanoma and nevus were V600E mutated, and only the melanoma acquired a Q61K mutation, further favors the possibility of melanoma and nevus cells being clonally related. *BRAF* and *NRAS* mutations are said to be mutually exclusive [49,62–66]. Mutation of both *BRAF* and *RAS*, may not provide an additional significant growth/survival advantage (epistatic theory) [67] or is not compatible with cell survival (synthetic lethality theory) [68,69]. Nevertheless there have been a few reports of primary melanomas harboring both mutations [70–73], with the hypothesis that these melanomas represent a mosaic with both mutations being mutually exclusive at a single cell level [70]. This mosaic explanation may explain the recurrence of melanomas after *BRAF* inhibition [74]. The presence of the Q61K mutation in both melanoma and nevus suggest that this mutation alone may not be sufficient for melanoma induction [75].

This is the first molecular study to evaluate the presence of *cKIT* among melanoma and associated nevus [22,27–32]. *KIT* has been related to acral, mucosal and chronic sun exposure melanomas [53]. We did not observe mutations in *cKIT*, not even among the Brazilian cases, even though solar exposure in Brazil is high. Interestingly, 2 cases located on acral sites were V600E mutated for both melanomas and nevi (Cases 50 and 61). One was an ALM (case 50) and the other was an *in situ* melanoma with no specified subtype (case 61). In the latter the nevus also harbored mutations in *NRAS* (D33D and Q61K), not present in the melanoma. Even though it was suggested that most acral melanomas would correspond to *de novo* melanomas [47], recently Lacruz et al described two cases of multiple *BRAF*-mutated acral melanomas. One of the cases presented a subungueal melanoma and a nevus-associated melanoma on the toe, both *BRAF* mutated. The findings of our study corroborate the idea that nevus-associated melanomas located on acral sites would not be related to *cKIT* as *de novo* acral melanomas, but instead to *BRAF*, as nevus-associated melanomas from other sites, such as trunk [76].

Only two cases from our sample were located on the head, one wild-type (Case 1), while in the other, only the melanoma harbored a Q61K mutation (Case 4). Both cases were *BRAF*-WT, concordant with the fact that *BRAF* is less frequent in high sun exposure areas, when compared to intermittent sun exposure areas [77]. It has been suggested that there may be more than one pathway to melanoma [19,78]. Roughly melanomas would be classified: 1) CSD melanomas (Chronic sun damage) related to chronic sun exposure, *KIT* or *NRAS* mutations and radial growth phase comprised of melanocytes arranged as solitary units with poor lateral circumscription; 2) non-CSD melanomas related to intermittent sun exposure, located on trunk and proximal extremities, association with nevus either directly adjacent or elsewhere, greater frequency of *BRAF* mutations and larger, slightly pigmented melanocytes arranged primarily in nests and that display upward intraepidermal scatter in their radial growth phase; 3) Acral melanomas with about 20% of mutations in *KIT* and 4) mucosal melanomas. The findings of the present study on 61 nevus-associated melanomas, that the majority of mutations observed affected *BRAF*, most cases were located on the trunk and proximal extremities and of the SSMM subtype and also the two acral nevus-associated melanomas were *BRAF* mutated, corroborate the idea that melanomas may have different

origins and that most nevus-associated melanomas may be classified amongst the non-CSD subtype.

Recently a list of genes with a statistically significant functional mutation burden was described after whole-genome sequencing. Among the most important were six well-known cancer genes (*BRAF*, *NRAS*, *PTEN*, *TP53*, *p16INK4a* and *MAP2K1*) and five new candidates for driver mutations, *SNX31*, *TACC1*, *PPP6C*, *RAC1* and *STK19* [40,79], the latter three harboring recurrent mutations. Despite the high rate of amplification in our study, we didn't find any mutations in the candidate driver genes (*RAC1*, *STK19*, *PPP6C*) evaluated. Mutations in *RAC1*, including the P29S mutation (UV-signature activating mutation)[40], and in *PPP6C* are more frequently observed in sun-exposed melanomas. Even though our sample was mostly comprised of chronic or intermittent sun exposed melanomas, no mutation in these genes was detected.

Considering altogether the molecular results for the 6 genes evaluated (*BRAF*, *NRAS*, *c-KIT*, *RAC1*, *PPP6C* and *STK19*), the majority of cases in our study presented concordance of mutational status between melanoma and nevus (40/60; 66.7%).

When evaluating the hypothesis of clonality between melanoma and nevus, the correct sampling of cells is vital, especially in nevoid melanomas and melanomas adjacent to dysplastic nevi. We carefully selected melanoma and nevus cells allying morphologic criteria and immunohistochemistry, since none of these criteria is 100% specific in the diagnosis of melanoma [39] and have included cases where the distinction between melanoma and nevus was concordant among the observers. Therefore, after minimizing the selection bias for the subpopulation of melanoma and adjacent nevus cells, the majority of melanomas and adjacent nevus share the same mutational profile regarding the most relevant genetic hallmarks described in melanoma genesis, corroborating the theory that these lesions may be clonally related and that melanoma most probably originated within the nevus. Nevertheless, at least for some cases, the possibility of a fortuitous collision event cannot be ruled out [18]. Even though we tested a broad panel of genes, the most frequently mutated genes in nevus-associated melanoma remain related to the MAPK pathway.

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What is known about this topic?

Epidemiological and histopathological studies point to melanomas arising either *de novo* or associated with a nevus, but the role of the adjacent benign lesion remains to be elucidated.

What does this study add?

The majority of melanomas and adjacent nevi share the same mutational profile, corroborating the theory that melanoma and the adjacent nevus are clonally related and that melanoma originated within a nevus

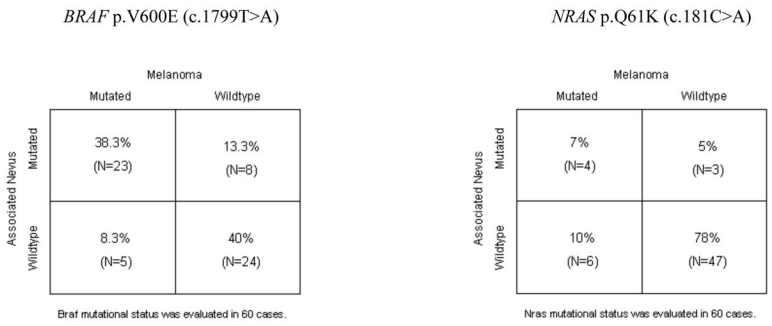

Figure 1.

Comparison of mutational status between melanomas and associated nevi, adapted with the numbers of the present study from Tschandl et al.

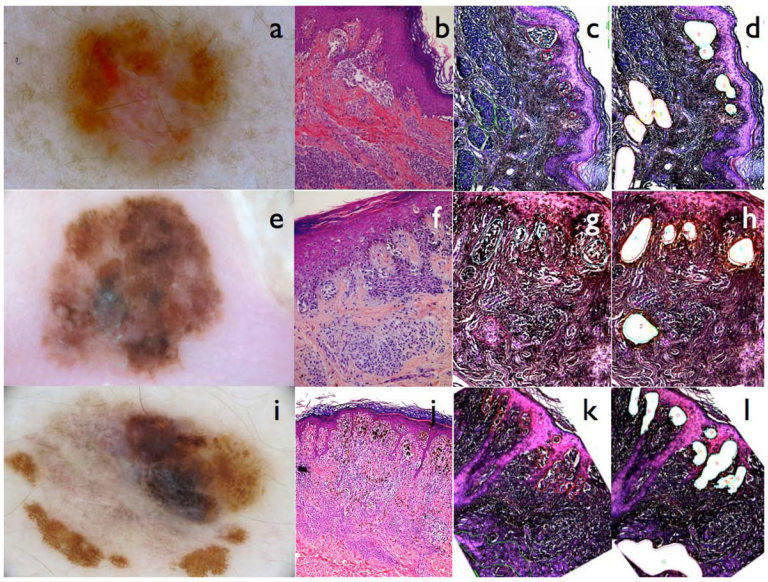

Figure 2.

Melanomas and associated nevi: Dermoscopy (a), Haematoxylin-eosin (b) and laser microdissection (c) (d) of case 35, melanoma and associated nevus both mutated for BRAF V600E and NRAS Q61K. Dermoscopy (e), HE (f) and laser microdissection (g)(h) of case 50, acral lentiginous melanoma and adjacent nevus both *BRAF* V600E mutated. Dermoscopy (i), HE (j) and laser microdissection (k)(l) of case 53, superficial spreading melanoma and adjacent nevus both *BRAF* V600E mutated. (c) and (k) Delimitation of melanoma cells in red and naevus cells in green. (g) Delimitation of melanoma cells in green and naevus cells in red. (d), (h) and (l) show the empty areas of melanoma and nevus cells after laser dissection.

Table 1a

Primers used for the amplification of *BRAF*, *NRAS*, *C-KIT*, *PPP6C*, *RAC1* and *STK19* genes.

Gene	Primer	Exon	Primer Sequence	Length of product (bp)
BRAF	Forward	11	5′ TTTCTTTTTCTGTTTGGCTTG	200
BRAF	Reverse	11	5′ ACTTGTCACAATGTCACCACA	200
BRAF	Forward	15	5′ TGCTTGCTCTGATAGGAAAA	204
BRAF	Reverse	15	5′ TCAGTGGAAAAATAGCCTCA	204
NRAS	Forward	2	5′ CGCCAATTAACCCTGATTAC	201
NRAS	Reverse	2	5′ AGAGACAGGATCAGGTCAGC	201
NRAS	Forward	3	5′ CCCCTTACCCTCCACACC	245
NRAS	Reverse	3	5′ AACACAAAGATCATCCTTTCAGA	245
C-KIT	Forward	11	5′ TGTTCTCTCTCCAGAGTGCTCTAA	291
C-KIT	Reverse	11	5′ AAACAAAGGAAGCCACTGGA	291
PPP6C	Forward	7	5′ AAACTCATCTGCAGAGCACA	271
PPP6C	Reverse	7	5′ AAGAAGAGGGCAGAAAAATG	271
RAC1	Forward	2	5′ TGTGATGTATATGCCTTGATTTT	254
RAC1	Reverse	2	5′ AGCAAAACAAATGGTCAAAG	254
STK19	Forward	2	5′ GACAAGTTGACGCTCCTTTC	270
STK19	Reverse	2	5′ AGAGGATCCGACTCCACAG	270

Table 1b

Primers used in nested PCR.

Gene	Primer	Exon	Primer sequence	Length of product (bp)
BRAF	Forward	11	5′ **TGTAAAACGACGGCCAGT**TTTCTTTTTCTGTTTGGCTTG	222
BRAF	Reverse	11	5′ **CAGGAAACAGCTATGACC**ACTTGTCACAATGTCACCACA	222
BRAF	Forward	15	5′ **TGTAAAACGACGGCCAGT**TGCTTGCTCTGATAGGAAAA	217
BRAF	Reverse	15	5′ **CAGGAAACAGCTATGACC**TCAGTGGAAAAATAGCCTCA	217
C-KIT	Forward	11	5′ **TGTAAAACGACGGCCAGT**TGTTCTCTCTCCAGAGTGCTCTAA	270
C-KIT	Reverse	11	5′ **CAGGAAACAGCTATGACC**AAACAAAGGAAGCCACTGGA	270
NRAS	Forward	2	**5′ TGTAAAACGACGGCCAGT**ACCCTGATTACTGGTTTCCA	253
NRAS	Reverse	2	**5′ CAGGAAACAGCTATGACC**GATCAGGTCAGCGGGCTA	253
NRAS	Forward	3	5′ **TGTAAAACGACGGCCAGT**CTTACCCTCCACACCCCC	274
NRAS	Reverse	3	**5′ CAGGAAACAGCTATGACC**CAAAGATCATCCTTTCAGAGAA	274
PPP6C	Forward	7	5′ **TGTAAAACGACGGCCAGT**CATCAACTAGTGCACGAAGG	210
PPP6C	Reverse	7	5′ **CAGGAAACAGCTATGACC**CGTTCTGGGAGGAATAACAC	210
RAC1	Forward	2	5′ **TGTAAAACGACGGCCAGT**TTTAGAGCTGTAGGTAAAACTTGC	148
RAC1	Reverse	2	5′ **CAGGAAACAGCTATGACC**GCAAAACAAATGGTCAAAGA	148
STK19	Forward	2	5′ **TGTAAAACGACGGCCAGT**AAGTTGACGCTCCTTTCGT	192
STK19	Reverse	2	5′ **CAGGAAACAGCTATGACC**GGATCAGGTGATGCCTCTT	192

Letters in bold correspond to the M13 tails sequence included on the nested primers.

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Table 2

Clinical characteristics and molecular screening results of melanomas and associated nevi

CASE	BRAF	NRAS	CKIT	PPP6C	STK19	RAC1	LOCAL	SUBTYPE	BRESLOW	CLARK	type of nevus
1	WT [A,B,*]	WT [A,B,#]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	HEAD	N/S	0.7	III	N/A
2	V600E [A,B,*]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	TRUNK	N/S	1.25	III	N/A
3	V600E [A,B,*]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	TRUNK	N/S	IS	I	N/A
4	WT [A,B,*]	WT[A,#],Q61K[B,#]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	HEAD	SSM	0.45	III	N/A
5	WT [A,B,*]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	EXT	N/S	IS	I	N/A
6	N/A[A],V600E[B,*]	N/A[A],WT[B]	N/A[A],WT[B]	N/A[A],WT[B]	N/A[A],WT[B]	N/A[A],WT[B]	EXT	NM	2.5	III	junctional
7	V600E[A],WT[B]	WT [A,B,#]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	TRUNK	N/S	IS	I	N/A
8	WT [A,B*]	WT [A,B,#]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	TRUNK	N/S	IS	I	N/A
9	WT [A,B*]	WT [A,B,#]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	TRUNK	N/S	IS	I	junctional
10	WT [A,B*]	WT [A,B,#]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	N/A	SSM	0.8	III	N/A
11	WT [A,B*]	WT [A,B,#]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	TRUNK	N/S	IS	I	compound
12	WT [A,B*]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	TRUNK	SSM	1.15	III	N/A
13	WT [A,B*]	WT [A,B,#]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	TRUNK	N/S	IS	I	compound
14	WT[A],V600E[B*]	WT [A,B,#]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	N/A	SSM	0.4	II	junctional
15	V600E [A,B*]	WT [A,B,#]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	TRUNK	SSM	0.4	II	N/A
16	V600E [A,B*]	Q61K[A,#], WT[B,#]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	TRUNK	SSM	0.5	II	compound
17	V600E [A,B*]	WT [A,B,#]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	EXT	SSM	0.35	II	N/A
18	WT [A,B*]	WT [A,B,#]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	TRUNK	SSM	0.75	III	compound
19	V600E [A,B*]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	TRUNK	N/S	IS	I	intradermal
20	WT [A,B*]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	TRUNK	SSM	0.7	III	compound
21	WT [A,B*]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	TRUNK	N/S	IS	I	compound
22	WT [A,B*]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	EXT	N/S	IS	I	junctional
23	V600E [A,B*]	WT [A,B,#]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	EXT	N/S	IS	I	congenital
24	V600E [A,B*]	WT[A,#],Q61K[B,#]	WT [A,B]	WT [A,B]	WT [A,B]	WT [A,B]	EXT	N/S	IS	I	junctional

CASE	BRAF	NRAS	CKIT	PPP6C	STK19	RAC1	LOCAL	SUBTYPE	BRESLOW	CLARK	type of nevus
25	V600E ^{A,B*}	WT $^{A,B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	N/A	SSM	0.45	II	junctional
26	V600E^{A},WT^{B}	WT $^{A,B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	TRUNK	SSM	0.5	II	compound
27	V600E^{A*},WT^{B*}	WT $^{A,B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	EXT	N/S	IS	I	N/A
28	WT^{A*},V600E^{B*}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	N/A	N/S	IS	I	N/A
29	V600E^{A*},WT^{B*}	WT $^{A,B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	TRUNK	SSM	IS	I	N/A
30	WT^{A*},V600E^{B*}	WT$^{A,\#}$,Q61K$^{B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	TRUNK	SSM	0.5	II	N/A
31	WT ^{A,B*}	WT $^{A,B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	TRUNK	SSM	IS	I	compound
32	WT^{A*},V600E^{B*}	WT$^{A,\#}$,Q61K$^{B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	EXT	SSM	IS	I	N/A
33	WT ^{A,B*}	Q61K $^{A,B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	TRUNK	N/S	IS	I	compound
34	V600E ^{A,B*}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	EXT	SSM	0.45	II	compound
35	V600E ^{A,B*}	Q61K $^{A,B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	EXT	SSM	0.5	III	N/A
36	WT ^{A,B*}	WT $^{A,B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	N/A	N/A	N/A	N/A	N/A
37	V600E ^{A,B*}	WT $^{A,B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	TRUNK	SSM	0.52	III	N/A
38	V600E^{A*},WT^{B*}	WT $^{A,B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	TRUNK	SSM	0.6	III	N/A
39	V600E ^{A,B*}	WT $^{A,B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	TRUNK	SSM	IS	I	N/A
40	WT ^{A,B*}	WT $^{A,B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	TRUNK	SSM	1.01	IV	N/A
41	V600E ^{A,B*}	WT $^{A,B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	TRUNK	SSM	0.8	III	congenital
42	V600E^{A*},WT^{B*}	WT$^{A,\#}$,Q61K$^{B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	TRUNK	SSM	0.7	III	N/A
43	WT ^{A,B*}	WT $^{A,B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	TRUNK	SSM	0.5	III	N/A
44	V600E^{A*},WT^{B*}	WT $^{A,B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	N/A	SSM	0.7	IV	N/A
45	WT ^{A,B*}	WT$^{A,\#}$,Q61K$^{B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	TRUNK	SSM	IS	I	congenital
46	WT ^{A,B*}	WT $^{A,B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	EXT	SSM	0.3	II	N/A
47	V600E^{A*},WT^{B*}	Q70H/Q61K $^{A,\#}$, Q61K$^{B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	TRUNK	SSM	0.9	III	N/A
48	WT^{A*},V600E^{B*}	WT $^{A,B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	TRUNK	SSM	0.8	III	congenital
49	WT ^{A,B*}	WT $^{A,B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	EXT	SSM	1.2	IV	intradermal
50	V600E ^{A,B*}	WT $^{A,B,\#}$	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	ACRAL	ACRAL LENTIGINOUS	1.3	IV	intradermal
51	WT ^{A,B*}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	WT ^{A,B}	TRUNK	SSM	1.2	IV	N/A

CASE	BRAF	NRAS	CKIT	PPP6C	STK19	RAC1	LOCAL	SUBTYPE	BRESLOW	CLARK	type of nevus
52	WT *A,B**	Q61K *A,B,#*	WT *A,B*	WT *A,B*	WT *A,B*	WT *A,B*	TRUNK	SSM	0.5	II	N/A
53	V600E *A,B**	WT *A,B,#*	WT *A,B*	WT *A,B*	WT *A,B*	WT *A,B*	N/A	SSM	0.85	III	N/A
54	V600E *A,B**	WT *A,B,#*	WT *A,B*	WT *A,B*	WT *A,B*	WT *A,B*	TRUNK	SSM	0.6	III	congenital
55	V600E *A,B**	WT *A,B,#*	WT *A,B*	WT *A,B*	WT *A,B*	WT *A,B*	EXT	SSM	1	III	N/A
56	V600E *A,B**	Q61K*A,#*, WT*B,#*	WT *A,B*	WT *A,B*	WT *A,B*	WT *A,B*	TRUNK	SSM	1.4	III	N/A
57	V600E *A,B**	WT *A,B,#*	WT *A,B*	WT *A,B*	WT *A,B*	WT *A,B*	N/A	SSM	0.55	I	N/A
58	WT *A,B**	WT *A,B,#*	WT *A,B*	WT *A,B*	WT *A,B*	WT *A,B*	EXT	SSM	0.5	II	N/A
59	V600E *A,B**	WT *A,B,#*	WT *A,B*	WT *A,B*	WT *A,B*	WT *A,B*	EXT	N/A	N/A	N/A	N/A
60	V600E *A,B**	WT *A,B,#*	WT *A,B*	WT *A,B*	WT *A,B*	WT *A,B*	TRUNK	SSM	1.2	IV	congenital
61	V600E *A,B**	D33D/Q61K*A,#*, WT*B,#*	WT *A,B*	WT *A,B*	WT *A,B*	WT *A,B*	ACRAL	N/S	IS	I	N/A

N/A: not available; N/S: not specified

A Nevus result;

B Melanoma result; EXT: extremities; SSM: superficial spreading melanoma; IS: in situ melanoma;

* Result determined by Sanger sequencing and V600E allele-specific fluorescent PCR;

Result determined by Sanger sequencing and SNaPshot method.