Intra-patient Heterogeneity of **BRAF** and **NRAS** Molecular Alterations in Primary Melanoma and Metastases

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**Mutations in MAPK signalling genes are driver events in melanoma, and have therapeutic relevance in the metastatic and adjuvant setting. This study evaluated the intra-patient heterogeneity of BRAF, NRAS and c-KIT mutational status between 30 primary melanomas and 39 related metastases, using molecular analysis and immunohistochemistry. BRAF mutations were identified in 46.7% of primary melanomas and 48.7% of metastases and NRAS mutations in 20% and 25.6%, respectively. Intra-patient heterogeneity was detected in 13.3% of patients for both BRAF and NRAS genes and was not associated with clinico-pathological characteristics of melanomas or metastases. High consistency was observed between immunostaining and molecular methods for **BRAF**<sup>V600E</sup> (k = 0.90; p < 0.001) and **NRAS**<sup>V618F</sup> (k = 0.87; p < 0.001). These findings demonstrate a relevant intra-patient heterogeneity between primary and metastatic lesions that is independent of clinical variables and methodological approach.**

**Key words:** melanoma; metastases; **BRAF; NRAS; c-KIT**; heterogeneity.

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**Melanoma is considered one of the most aggressive cancers, with an increasing incidence worldwide over recent decades (1). The treatment of metastatic disease has been a challenge in the recent past, with a low survival rate of approximately 20% at 5 years for stage IV patients (2–5). Nevertheless, the introduction of new therapeutic approaches, such as targeted therapy and immunotherapy, based on a growing understanding of molecular alterations involved in melanoma pathogenesis, has significantly improved outcomes for patients (6).**

The most relevant molecular pathway implicated in melanoma pathogenesis is the mitogen-activated protein kinase (MAPK) cascade, which is dysregulated in approximately 80% of melanomas (7). Within this pathway, mutations in **BRAF**, **NRAS** and **c-KIT** are considered driver events and have a strong clinical relevance for melanoma treatment (8–10). Activating **BRAF** mutations occur in approximately 50% of cutaneous melanomas, mainly at codon 600 in exon 15, with the most common mutation being the V600E change (identified in approximately 80% of cases) (8). Oncogenic **NRAS** mutations are found in 15–25% of cutaneous melanomas and are usually detected at codon 61, mainly with a glutamine to arginine/lysine/leucine substitution (Q61R/K/L) (8). Finally, approximately 3% of all melanomas carry somatic mutations in exons 11 (L576P) and 13 (K642E) of the **c-KIT** gene, but additional **c-KIT** aberrations might include mutations in exon 17 and gene amplifications (8). Mutations of **BRAF**, **NRAS** and **c-KIT** are usually identified by molecular methods (11), but immunohistochemical (IHC) analysis represents a useful option, being widely available, less labour-intensive and less expensive (11–14). However, there is no consensus so far on the best testing method (11).

Mutational concordance between primary melanoma and related metastases is a critical aspect that has become even more relevant with the introduction of therapies targeting specific mutations. This study evaluated the intra-patient heterogeneity of **BRAF**, **NRAS** and **c-KIT** mutational status in 30 primary melanomas and 39 related metastases, using molecular analysis and immunohistochemistry. Clinically meaningful intra-patient heterogeneity was found between primary melanoma and related metastases, independent of the technical approach, thus supporting the polyclonal model of melanoma progression.

**SIGNIFICANCE**

Cutaneous melanoma is one of the most aggressive and treatment-resistant tumours. Intra-patient concordance of mutations in genes, such as **BRAF**, **NRAS** and **c-KIT**, between primary melanoma and related metastases is a critical aspect that has become even more relevant with the introduction of therapies targeting specific mutations. This study evaluated the intra-patient heterogeneity of **BRAF**, **NRAS** and **c-KIT** mutational status in 30 primary melanomas and 39 related metastases, using molecular analysis and immunohistochemistry. Clinically meaningful intra-patient heterogeneity was found between primary melanoma and related metastases, independent of the technical approach, thus supporting the polyclonal model of melanoma progression.
higher heterogeneity rates have been reported for molecular approaches than for IHC-based methods (28). Few studies have focused on the intra-patient concordance of NRAS mutations between primary and metastatic lesions, with percentages of discordance ranging from 0 to 9% (17, 24, 26, 29). No data are available on c-KIT mutational heterogeneity in cutaneous melanoma.

This study aimed to investigate the mutational status of BRAF, NRAS and c-KIT genes in patients with metastatic melanoma in order to evaluate the intra-patient molecular heterogeneity between primary tumour and related metastases and to compare the consistency of mutational findings obtained by molecular and IHC analyses.

MATERIALS AND METHODS

Patients' and tissue samples

Patients with a histopathologically confirmed diagnosis of metastatic melanoma were recruited between January 2012 and December 2017 at the Department of Dermatology, University of L’Aquila, Italy. For each patient, tissues were retrieved from the primary melanoma and at least one metastasis. Patient’s clinical information including sex, age at diagnosis, anatomical site of primary melanoma and related metastases and AJCC stage 8th edition (30) were collected.

Haematoxylin-eosin-stained sections of primary and metastatic tissues were reviewed by 2 experienced pathologists (MDP and GC) to confirm the diagnosis. The following clinicopathological features of primary tumours and/or metastases were collected: anatomical location, histopathological variant, Breslow thickness, presence of ulceration, number of mitoses/mm², presence of melanoma-associated naevus and CSD (histologically defined according to the degree of solar elastosis).

Approval for this study was obtained from the Ethics Committee of the ASL-01 Avezzano, Sulmona, L’Aquila (protocol number 0012038/11). Written informed consent was provided by all patients. The study was performed according to the principles of the Declaration of Helsinki.

Molecular analysis

Somatic DNA was extracted from 5 formalin-fixed paraffin-embedded (FFPE) tissue sections (each 10-micron in thickness) obtained from the same tissue block used for IHC, by microdissection of marked tumour-rich areas from primary melanoma and related metastases and AJCC stage 8th edition (30) were collected.

Haematoxylin-eosin-stained sections of primary and metastatic tissues were reviewed by 2 experienced pathologists (MDP and GC) to confirm the diagnosis. The following clinicopathological features of primary tumours and/or metastases were collected: anatomical location, histopathological variant, Breslow thickness, presence of ulceration, number of mitoses/mm², presence of melanoma-associated naevus and CSD (histologically defined according to the degree of solar elastosis).

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IHC was performed on FFPE tissue sections of 4 μm thickness. The presence of BRAFV600E and NRASQ61K mutants and c-KIT expression were evaluated using the following monoclonal antibodies: BRAFV600E VE1 clone (Spring Bioscience, Pleasanton, CA, USA), NRASQ61K SP174 clone (Spring Bioscience), and CD117/c-Kit polyclonal (Spring Bioscience) at a dilution of 1:30, 1:80 and 1:100, respectively. Sections were freshly cut, dried at 60°C for 30 min, deparaffinized and rehydrated. Immunoreactions were performed on Ventana BenchMark GX automatic immune stainer (Ventana Medical Systems Inc., Tucson, AZ, USA) using the Ultra View Universal Alkaline Phosphatase Red Detection Kit, as previously reported (13). No chromogen was detected when positive staining was omitted. Positive controls were mounted on each section subjected to immunostaining. Negative controls were included in each run.

The evaluation of IHC status was performed independently by 2 observers (MDP and GC) blinded to the molecular mutational status; disagreement was resolved by consensus. Cytoplasmic staining of BRAFV600E VE1, NRASQ61K SP174 and CD117/c-Kit polyclonal antibodies in melanoma cells was considered as positive or negative and graded for intensity, according to previously published criteria (14, 31). Staining for BRAFV600E VE1 and NRASQ61K SP174 was considered positive if the percentage of stained melanoma cells was more than 10% and classified as homogeneous (staining in >95% of cells) or heterogeneous (staining in <95% of cells) (14). Negative staining was defined either as absence of any cytoplasmic labelling or staining of single interspersed melanoma cells (<10%). Intensity of staining was graded as weak, moderate or strong (14). CD117/c-Kit staining was assessed on the percentage of stained melanoma cells and strength of staining: 0, no staining; 1+, weak staining in isolated groups of melanoma cells; 2+, weak and widespread staining in <50% of melanoma cells; 3+, moderate staining in 50–75% of melanoma cells; 4+, strong staining in >75% of melanoma cells. Staining was classified as positive in the presence of moderate/strong membranous and cytoplasmic staining (3+/4+) and as negative if there was absence of cytoplasmic staining and if staining was weak and widespread (0/1+/2+).
Statistical analysis

For statistical analysis, categorical variables for primary melanoma were grouped as follows: anatomical site (head/neck, trunk, extremities, acral), histopathological subtype (superficial spreading melanoma [SSM], nodular melanoma), chronic sun damage (CSD or no CSD), presence of ulceration (yes, no), number of mitoses/mm² and presence of melanoma-associated naevus (yes, no). Synchronicity (defined as a metastasis diagnosed at the same time as the primary melanoma) and anatomical site (skin, lymph node, visceral, brain) were recorded for metastases.

Semi-quantitative data (age at diagnosis, Breslow thickness) were analysed by means of Student’s t-test or by medians with Mann–Whitney test. Univariate analysis by χ² test or by Fisher’s exact test was used to test the significance of mutation frequency according to clinico-pathological characteristics of melanoma patients and tumours. Molecular findings were used as the gold standard for statistical analysis. Cohen’s k coefficient test was used to measure the agreement between molecular and IHC methods in determining BRAF and NRAS mutational status. Samples harbouing the BRAF<sup>V600E</sup> mutation according to molecular analysis, that were wild-type on IHC VE1 staining, were not considered for Cohen’s k analysis. p-values less than 0.05 were considered statistically significant. Statistical analysis was performed using the statistical package SPSS 17.0 (SPSS Incorporated, Chicago, USA).

RESULTS

Patients’ and tumour samples

Overall, a total of 69 tumour tissues (30 primary melanomas and 39 related metastatic lesions) were collected from 30 patients with advanced melanoma (25 stage III and 5 stage IV). Enrolled patients included 17 males and 13 females, with a median age at first diagnosis of 65 years (range 25–84 years). In detail, 23 patients were diagnosed with 1 metastasis, 5 with 2 metastases and 2 with 3 metastases. Demographic and clinical characteristics of patients and tumour tissues are reported in Table I.

Twelve primary melanomas (12/30, 40.0%) were located on the extremities, 10 on the trunk (10/30, 33.3%), 5 (5/30, 16.7%) on the head/neck region and 3 (3/30, 10.0%) on acral sites. The majority of tumours were of the nodular histological subtype (23/30, 76.7%) followed by SSM (7/30, 23.3%); no acral lentiginous or lentigo maligna melanomas were diagnosed in our group of patients. Median Breslow thickness was 4.2 mm (range 0.6–30 mm). A pre-existing melanocytic naevus was associated in 36.7% (11/30) of primary melanomas.

The majority of metastatic tissues were collected from lymph nodes (23/39, 59.0%), followed by skin (11/39, 28.2%), brain (2/39, 5.1%), colon (1/39, 2.6%), liver (1/39, 2.6%) and parathyroid gland (1/39, 2.6%). Median time to first metastasis was 3 months (range 0–51 months). Metastases were synchronous in 17 (17/30, 56.7%) patients and metachronous in 9 (9/30, 30.0%); 4 (4/30, 13.3%) patients had both synchronously and metachronous metastases.

Molecular analysis

The distribution of BRAF, NRAS and c-KIT mutations in all analysed tissues is shown in Table II. BRAF mutations at codon 600 were detected in 33 of 69 (47.8%) tumour tissues, with 23 harbouring the BRAF<sup>V600E</sup> (23/33, 69.7%) and 10 the BRAF<sup>V600K</sup> (10/33, 30.3%) mutation. Distribution of BRAF mutations was similar between primary melanomas (14/30, 46.7%) and metastatic samples (19/39, 48.7%) (p = 0.42). Sixteen of 69 tumour tissues (16/69, 23.2%) carried NRAS mutations at codon 61 with the following genotypes: NRAS<sup>Q61R</sup> (8/16, 50.0%), NRAS<sup>Q61L</sup> (7/16, 43.7%) and NRAS<sup>Q61K</sup> (1/16, 6.3%). Among the 16 NRAS mutated tumours, 6 were primary melanomas (6/30, 20.0%) and 10 (10/39, 25.6%) metastatic samples (p = 0.61). Finally, 1 only missense c-KIT mutation, L802F mutation in exon 17, was detected in one (1/69, 1.4%) primary melanoma diagnosed on the upper extremity. A mutation in at least 1 of the 3 genes was present in 21 of 30 (70.0%) primary melanomas and 29 of 39 (74.3%) metastatic samples, for a total of 72.5% tissues (50/69). All tissues were mutually exclusive for BRAF, NRAS and c-KIT mutations (Table II). c-KIT gene amplification was carried out in 65/69 (94.2%) samples with 4 samples missing due to lack of DNA. An increased copy number (≥ 3 copies) was identified in 3 (3/69 4.3%) samples, with 2 of them being primary melanomas with a high level of chronic sun damage (CSD). All cases with c-KIT amplification carried concomitant BRAF<sup>V600E</sup> mutations.

| Table I. Demographic and clinical characteristics of melanoma patients and histopathological features of tumours and metastasis |
|---------------------------------|-----------------|
| Characteristics of patients (n = 30) |
| Sex, (n (%)) |
| Males | 17 (56.7) |
| Females | 13 (43.3) |
| Age, years, median (range) | 65 (25–84) |
| Metastases, (n (%)) |
| 2 | 15 (51.6) |
| 3 | 2 (6.7) |
| Synchronous/metachronous, (n (%)) |
| Synchronous | 17 (56.7) |
| Metachronous | 9 (30.0) |
| Stage, (n (%)) |
| III<sup>a</sup> | 25 (83.3) |
| IV | 5 (16.7) |
| Characteristics of primary melanoma (n = 30) |
| Breslow thickness, median (range) | 4.2 (0.6–30) |
| Histopathological subtype, (n (%)) |
| Superficial spreading melanoma | 7 (23.3) |
| Nodular melanoma | 23 (76.7) |
| Anatomical site, (n (%)) |
| Head/neck | 5 (16.7) |
| Trunk | 10 (33.3) |
| Extremities | 12 (40.0) |
| Acral | 3 (10.0) |
| Associated naevus, (n (%)) |
| Yes | 11 (36.7) |
| No | 19 (63.3) |
| Ulceration, (n (%)) |
| Yes | 20 (66.7) |
| No | 10 (33.3) |
| Solar elastosis, (n (%)) |
| Yes | 18 (60.0) |
| No | 12 (40.0) |
| Characteristics of metastases (n = 39) |
| Anatomical site, (n (%)) |
| Lymph nodes | 23 (59.0) |
| Skin | 11 (28.2) |
| Brain | 2 (5.1) |
| Visceral | 3 (7.7) |

<sup>a</sup>Stage III patients included 20 patients with nodal metastases and 5 patients with in-transit metastases without nodal involvement.
Table II. Summary of mutational patterns in primary and metastatic melanoma samples according to immunohistochemical and molecular analyses

| Patient | Age, years | Site | BRAF | NRAS | c-KIT | IHC* data | Molecular data | BRAF | NRAS | c-KIT | IHC* data | Molecular data | BRAF | NRAS | c-KIT | IHC* data | Molecular data |
|---------|------------|------|------|------|-------|-----------|---------------|------|------|-------|-----------|---------------|------|------|-------|-----------|---------------|
| 1       | 79         | T    | wt   | wt   | wt    | Q61L     | wt            | L    | wt   | m, het| Q61L     | wt            | L    | wt   | wt    | wt        | Q61L         |
| 2       | 81         | A    | s, hom| wt   | wt    | SK       | wt            | L    | wt   | wt    | SK       | wt            | L    | wt   | wt    | wt        | wt         |
| 3       | 46         | E    | s, hom| wt   | wt    | SK       | wt            | L    | wt   | s, hom| SK       | wt            | L    | wt   | wt    | wt        | wt         |
| 4       | 54         | T    | m, het| wt   | wt    | S        | wt            | L    | wt   | wt    | S        | wt            | L    | wt   | wt    | wt        | wt         |
| 5       | 69         | E    | wt   | wt, het| wt   | L        | wt            | L    | wt   | wt    | L        | wt            | L    | wt   | wt    | wt        | wt         |
| 6       | 72         | A    | wt   | wt    | wt    | Q61R     | wt            | L    | wt   | s, hom| L        | wt            | L    | wt   | wt    | wt        | wt         |
| 7       | 80         | T    | wt   | wt    | wt    | Q61L     | wt            | L    | wt   | wt    | Q61L     | wt            | L    | wt   | wt    | wt        | wt         |
| 8       | 65         | T    | s, het| wt   | V600E | L        | wt            | L    | wt   | wt    | V600E    | wt            | L    | wt   | wt    | wt        | wt         |
| 9       | 70         | E    | s, hom| wt   | V600E | wt        | wt            | L    | s, hom| wt    | V600E    | wt            | L    | s, hom| wt    | wt        | wt         |
| 10      | 57         | E    | s, hom| wt   | V600K | L        | wt            | L    | m, het| wt    | V600K    | wt            | L    | wt   | wt    | wt        | wt         |
| 11      | 70         | H    | wt   | wt    | wt    | SK       | wt            | L    | wt   | wt    | SK       | wt            | L    | wt   | wt    | wt        | wt         |
| 12      | 35         | E    | s, hom| wt   | V600E | L        | wt            | L    | s, het| wt    | V600E    | wt            | L    | s, het| wt    | wt        | wt         |
| 13      | 25         | E    | s, hom| m, het| V600E | L        | wt            | L    | m, het| wt    | V600E    | wt            | L    | m, het| wt    | wt        | wt         |
| 14      | 60         | E    | s, hom| wt   | V600K | L        | wt            | L    | m, het| wt    | V600K    | wt            | L    | m, het| wt    | wt        | wt         |
| 15      | 65         | T    | s, hom| wt   | V600K | L        | wt            | L    | m, het| wt    | V600K    | wt            | L    | m, het| wt    | wt        | wt         |
| 16      | 57         | E    | s, hom| wt   | V600K | L        | wt            | L    | w, het| wt    | V600K    | wt            | L    | w, het| wt    | wt        | wt         |
| 17      | 35         | E    | s, hom| m, hom| V600K | L        | wt            | L    | w, het| wt    | V600K    | wt            | L    | w, het| wt    | wt        | wt         |
| 18      | 42         | E    | s, hom| wt   | V600E | L        | wt            | L    | m, het| wt    | V600E    | wt            | L    | m, het| wt    | wt        | wt         |
| 19      | 45         | E    | s, hom| m, hom| V600K | L        | wt            | L    | m, het| wt    | V600K    | wt            | L    | m, het| wt    | wt        | wt         |
| 20      | 65         | T    | s, hom| wt   | L802F | L        | wt            | L    | w, het| wt    | L802F    | wt            | L    | w, het| wt    | wt        | wt         |
| 21      | 58         | H    | wt   | wt    | wt    | L        | wt            | L    | m, het| wt    | L        | wt            | L    | m, het| wt    | wt        | wt         |
| 22      | 76         | A    | wt   | m, het| wt    | B        | w, het        | L    | m, het| wt    | B        | wt            | L    | m, het| wt    | wt        | wt         |
| 23      | 65         | E    | wt   | wt    | wt    | B        | w, het        | L    | m, het| wt    | B        | wt            | L    | m, het| wt    | wt        | wt         |
| 24      | 76         | H    | wt   | wt    | wt    | B        | w, het        | L    | m, het| wt    | B        | wt            | L    | m, het| wt    | wt        | wt         |
| 25      | 69         | T    | wt   | wt    | wt    | Q61K     | wt            | L    | m, het| wt    | Q61K     | wt            | L    | m, het| wt    | wt        | wt         |
| 26      | 49         | T    | s, hom| wt   | V600E | L        | wt            | L    | s, hom| wt    | V600E    | wt            | L    | s, hom| wt    | wt        | wt         |
| 27      | 44         | T    | s, hom| wt   | V600E | L        | wt            | L    | s, hom| wt    | V600E    | wt            | L    | s, hom| wt    | wt        | wt         |
| 28      | 25         | E    | s, hom| m, hom| V600E | L        | wt            | L    | s, het| wt    | V600E    | wt            | L    | s, het| wt    | wt        | wt         |
| 29      | 44         | T    | s, hom| m, hom| V600E | L        | wt            | L    | s, het| wt    | V600E    | wt            | L    | s, het| wt    | wt        | wt         |
| 30      | 75         | H    | s, hom| m, hom| V600E | L        | wt            | L    | s, het| wt    | V600E    | wt            | L    | s, het| wt    | wt        | wt         |

*This patient also developed a third nodal metastasis that was wild-type for NRAS, both at immunohistochemical (IHC) and molecular analysis. **Staining intensity: strong (s); moderate (m); weak (w) and distribution: heterogeneous (het); homogeneous (hom) are indicated for IHC positive cases. A: acral site; amp: amplification (gene copy number ≥3); B: brain; E: extremities; H: head and neck; S: skin; V: visceral; L: lymph node; SK: skin; wt: wild type.

A significant association between mutational status and clinical characteristics of the primary melanoma and metastatic tissues was detected in 86.7% (77/88) patients, with the majority (21/30, 70%) of patients with BRAF mutation both at IHC and molecular analysis. **BRAF V600E mutation both at IHC and molecular analysis.*Staining intensity (s: strong; m: moderate; w: weak) and distribution (het: heterogeneous; hom: homogeneous) are indicated for IHC positive cases. A: acral site; amp: amplification (gene copy number ≥3); B: brain; E: extremities; H: head and neck; S: skin; V: visceral; L: lymph node; SK: skin; wt: wild type.
Considering the overall somatic profile of all BRAF/NRAS/c-KIT genes, intra-patient heterogeneity was present in 23.3% (7/30) of patients (6 with 1 metastasis and 1 with 2 metastases). Discordance rates were not associated with sex, synchronicity or anatomical sites of metastasis (Table SI1).

**Immunohistochemistry**

A total of 26 (26/69, 37.7%) samples showed positive immunostaining with anti-BRAF<sup>V600E</sup> VE1 antibody with homogenous staining in 19 of 26 (73.1%) tissues. Staining intensity was strong in 10 tumours (10/30, 33.3%) and moderate or weak in 8 (8/10, 80.0%). A total of 35 (35.9%) showed similar frequency of BRAF<sup>V600E</sup> positive staining (p = 0.46). Regarding NRAS, 10 tumour tissues (10/69, 14.5%) were positive for NRAS<sup>Q61R</sup> SP174 immunostaining and 59 were negative (59/69, 85.5%). Staining intensity was strong in 8 tumours (8/10, 80.0%), and moderate or weak in 1 (1/10, 10.0%) each. No significant difference was observed in the frequency of NRAS<sup>Q61R</sup> positive staining between the groups of primary melanomas (5/30, 16.7%) and metastatic lesions (5/39, 12.8%) (p = 0.45).

All 11 melanomas arising in association with a melanocytic naevus showed concordance for BRAF and NRAS between melanoma cells and naevoid cells, with 5 of them (5/11, 45.4%) showing positive immunostaining for BRAF<sup>V600E</sup> and 3 (3/11, 27.3%) for NRAS<sup>Q61R</sup>; the remaining cases were negative for both mutations (Fig. 2). Sixteen tumours (16/69, 23.2%) showed positive CD117/c-KIT expression, with 13 cases presenting a moderate and 3 cases a strong immunostaining. A trend for a higher prevalence of positivity was observed in the group of primary melanomas (9/30, 30.0%) than in metastatic samples (7/39, 17.9%), although not statistically significant (p = 0.06).

Considering the mutational findings obtained by IHC, the intra-patient BRAF<sup>V600E</sup> concordance between primary lesions and related metastases was present in 27/30 (90.0%) patients, including 10 (10/30, 33.3%) with BRAF<sup>V600E</sup>-positive lesions and 17 (17/30, 56.7%) negative. Intra-patient BRAF<sup>V600E</sup> heterogeneity was observed in 10.0% (3/30) of patients (Fig. 2). For NRAS<sup>Q61R</sup>, the majority of patients (29/30, 96.6%) showed an intra-patient concordant immunostaining between the primary lesion and related metastases, being 4 (4/30, 13.3%) consistent for NRAS<sup>Q61R</sup> positivity and 25 (25/30, 83.3%) for negative staining. A discrepant NRAS<sup>Q61R</sup> staining was observed in 1 patient (3.3%). Finally, intra-patient concordance of CD117/c-KIT expression between primary lesions and related metastases was present in 22/30 (73.3%) patients, including 3 (3/30, 10.0%) with positive and 19 (18/30, 63.3%) with negative tissues (Table II). Three patients with multiple metastases showed heterogeneity among their tumour tissues.
Correlation between molecular analysis and immunohistochemistry

**BRAF**<sup>V600E</sup> VE1 immunostaining was consistent with **BRAF** molecular findings in 56 of 59 (94.9%) tissues, with 23 (23/56, 41.1%) **BRAF**<sup>V600E</sup> mutated and 33 (34/56, 60.7%) wild-type (Table III). The 10 (10/69, 14.5%) samples harbouring a **BRAF**<sup>V600K</sup> mutation were indeed negative on IHC VE1 staining and were not included in this analysis. Discrepant findings were found in 3 of 59 (5.1%) samples: all cases were positive for immunostaining, but wild-type on molecular testing (both Sanger sequencing and competitive allele TaqMan™ PCR). Two discordant (2/3, 66.7%) cases (1 primary melanoma and 1 metastasis) had a moderate and heterogeneous **BRAF**<sup>V600E</sup> VE1 staining pattern, while the remaining sample (1 primary melanoma) presented a strong positive and homogeneous staining. **BRAF**<sup>V600E</sup> VE1 antibody sensitivity was 100.0%, specificity 91.7%, accuracy 94.9%. Overall, the agreement between molecular testing and IHC was “almost perfect” (Cohen’s kappa = 0.90; p < 0.001).

**NRAS**<sup>Q61R</sup> immunostaining and **NRAS** molecular analysis showed a high rate of consistency (60 of 61 cases, 98.4%), with 8 (8/60, 13.3%) tissues carrying the **NRAS**<sup>Q61R</sup> mutation and 52 (52/60, 86.7%) the wild-type genotype. Cases carrying other **NRAS**<sup>Q61</sup> mutations showed no IHC SP174 staining and were not included in the analysis. Only one primary melanoma resulted positive for IHC **NRAS**<sup>Q61R</sup> with weak and heterogeneous staining pattern, but wild-type at the molecular analysis. The sensitivity of the SP174 **NRAS**<sup>Q61R</sup> antibody was 100.0%, specificity 96.2%, and accuracy 96.7%. Overall, the agreement between molecular testing and IHC was “almost perfect” (Cohen’s kappa = 0.87; p < 0.001).

**DISCUSSION**

This study identified **BRAF** mutations in 46.7% of primary melanomas and in 48.7% of metastases and **NRAS** mutations in 20% and in 25.6%, respectively. The intra-patient molecular heterogeneity between primary melanoma and related metastases was detected in 13.3% of patients for both **BRAF** and **NRAS** genes and was not associated with clinico-pathological characteristics of melanoma or metastases. We demonstrated consistency of **BRAF**<sup>V600E</sup> and **NRAS**<sup>Q61R</sup> mutational findings obtained by molecular analysis and IHC immunostaining for both overall mutation frequencies and intra-patient heterogeneity with an “almost perfect” agreement.

Regarding **c-KIT** gene, no mutation was found in all 16 samples with a high level of CD117/c-KIT by IHC, while 2 of the 3 (66.7%) cases with **c-KIT** gene amplification showed increased expression of CD117/c-KIT.
molecular methods for very consistent findings between immunostaining and specific Taqman™ assays) and IHC. We observed molecular methods (Sanger sequencing and allele-comparing IHC approaches (12, 19, 23, 25, 27, 28). Therefore, we rate of heterogeneity for molecular methods than for to be due to the specific detection method, with a higher melanoma and related metastases has been hypothesized in the heterogeneity rate between primary melanoma and 33, 34). In the current study no differences were observed metastases (16). All our patients with multiple metastases in patients with 2 metastases, and 20% in patients with 3 metastases, i.e. 8% in patients with one metastasis, 18% and for NRAS Q61R in 95% of our samples, with the majority of them acquiring the mutation over time as part of disease progression. The intra-patient molecular heterogeneity between primary melanoma and related metastases has important implications in clinical practice when metastatic patients with discordant lesions need to be treated with targeted therapy. In addition, comparison between primary lesions and related metastases can provide insights into the processes involved in metastatic progression. We observed an intra-patient discrepancy in BRAF mutations between primary and metastatic lesions in 13.3% of patients, as evaluated by molecular methods, in line with the discordance rate of 13.4% and 15.5% reported in 2 recent meta-analyses (16, 27). A true biological manifestation of tumour heterogeneity, but also technical issues (molecular-based methods compared with IHC-based) have been hypothesized to explain these discrepancies (16, 27, 28). A higher mutational discordance rate has been reported with the increasing number of metastases, i.e. 8% in patients with one metastasis, 18% in patients with 2 metastases, and 20% in patients with 3 metastases (16). All our patients with multiple metastases (23.3%) showed a consistent BRAF status between primary melanoma and all metastatic tissues.

Regarding NRAS, the intra-patient discordance rate was reported to range from 3% to 14.3% of cases in few small studies (24, 26, 29), while no discrepancy was found in one study (17). A 13.3% discordant rate was observed in our patients, with the majority of them acquiring the NRAS mutation over time as part of disease progression.

Controversial results were reported for the rate of intra-patient BRAF or NRAS discrepancies according to the site of metastasis, with a suggested, but not confirmed, higher rate of discordance between primary melanoma and visceral metastases compared with lymph node metastases (17). The discrepancy rate for locoregional lymphatic metastases was indeed reported to range from 9.2% to 38% across 4 previous studies (19, 21, 26, 33), while for visceral metastases, including brain, from 13% to 50% (26, 33, 34). In the current study no differences were observed in the heterogeneity rate between primary melanoma and lymph node or visceral metastatic sites.

The intra-patient heterogeneity between primary melanoma and related metastases has been hypothesized to be due to the specific detection method, with a higher rate of heterogeneity for molecular methods than for IHC approaches (12, 19, 23, 25, 27, 28). Therefore, we compared BRAFV600E and NRASQ61R mutations using molecular methods (Sanger sequencing and allele-specific Taqman™ assays) and IHC. We observed very consistent findings between immunostaining and molecular methods for BRAFV600E mutation, as detected in 95% of our samples, and for NRASQ61R found in 98% of cases. Interpretation issues have been reported for weak and moderately stained lesions, as in 3 of our 4 discordant cases, since they have been considered either positive or negative in the literature, thus suggesting that caution is necessary in case of unclear staining (13, 35). In addition, a rare VE1 antibody cross-reactivity with an unknown epitope may also be a possible explanation for false-positive staining (36, 37). Overall, in our cases the intra-patient heterogeneity between primary melanoma and metastatic tissues does not seem to be attributable to methodological aspects, since it was similar for IHC and molecular methods, thus possibly reflecting the true biological heterogeneity during melanoma progression.

Few small studies have investigated the concordance rate of BRAFV600E and NRASQ61R mutations between naevus and melanoma in naevus-associated melanomas (14, 38–40). For BRAF gene, a concordance rate varying from 75% to 100% was reported in 4 studies (14, 38–40) and for NRAS, 91% of melanomas and associated naevi were concordant in one study (39). All 11 naevus-associated melanomas in our series showed a concordant of BRAFV600E and NRASQ61R status between melanoma and naevus cells by immunostaining.

This study has a few limitations. NF1 mutational analysis was not performed, since the clinical significance of NF1 mutations in melanoma is unknown and the interest as a potential therapeutic target is currently scarce. In addition, this study did not have adequate statistical power to evaluate intra-patient heterogeneity of c-KIT mutations, probably due to the low prevalence of acral lentiginous and mucosal melanoma subtypes. Finally, our results on intra-patient heterogeneity are mainly referred to lymph node metastasis due to the low number of visceral metastasis in our sample that might have underestimated the overall discordance rate. However, the evaluation of the discordance rate between primary melanoma and nodal metastases in disease-free patients is nowadays important for administration of targeted therapies in the adjuvant setting.

These findings confirm that a relevant intra-patient heterogeneity between primary melanoma and related metastases exists independently of the technical approach, thus supporting the polyclonal model of melanoma progression. In addition, IHC and molecular methods provided highly consistent results in the detection of BRAFV600E and NRASQ61R mutations, supporting IHC as a rapid and cost-effective screening method in melanoma, although a combined approach is necessary in cases with negative or doubtful immunostaining.

The authors have no conflicts of interest to declare.

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