Aus der Klinik und Poliklinik für Dermatologie und Allergologie-Klinik
der Ludwig-Maximilians-Universität München
Direktor: Prof. Dr. med. Lars E. French

"**Morphologische Charakterisierung der BRAF mutierten Melanome mittels Immunhistochemie, Dermatoskopie und konfokaler Laserscanmikroskopie**"

Dissertation
zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von
Cristel Ruini

aus Scandiano, Italien

2020

**Mit Genehmigung der Medizinischen Fakultät
der Universität München**

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Tag der mündlichen Prüfung: 19.05.2020

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München, 09.03.2019

Dott. Cristel Ruini
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List of abbreviations

DEJ: dermo-epidermal junction, dermo-epidermale Junktion

IHC: Immunohistochemistry, Immunhistochemie

KLSM: konfokale Laserscanmikroskopie

µm: microns, mikrometer

RCM: reflectance confocal microscopy

Publications to be included in the cumulative dissertation

1) Ponti G, Tomasi A, Maiorana A, Ruini C, Maccaferri M, Cesinaro AM, Depenni R, Manni P, Gelsomino F, Giusti F, Garagnani L, Pellacani G. **BRAFp.V600E, p.V600K, and p.V600R Mutations in Malignant Melanoma: Do They Also Differ in Immunohistochemical Assessment and Clinical Features?** Appl Immunohistochem Mol Morphol. 2016 Jan;24(1):30-4.

2) Ruini C., Manfredini M., Pellacani G., Desmond Mandel V., Tomasi A., Ponti G. **Confocal microscopy characterization of BRAFV600E mutated melanomas.** Melanoma Res. 2015 Aug;25(4):367-71.

Morphological characterisation of BRAF mutated melanomas by means of immunohistochemistry, dermoscopy and reflectance confocal microscopy

Introduction

Melanoma is the most lethal cutaneous malignancy, with increasing incidence rate in the general population, even at young age.(1, 2) When diagnosed in early stage, surgical excision with safety margins according to valid guidelines is usually curative. However, its early invasiveness and high frequency of genomic alterations make early metastatic spreading common especially in thicker high risk tumors. Luckily, the therapeutic scenario of advanced unresectable and metastatic melanoma underwent striking changes in the last few years, thanks to the approval of new molecular targeted therapies and immunotherapies. Between the Seventies and 2011, the only available treatment was Dacarbazine chemotherapy, followed by IL-2, however no randomized controlled trial had reported a significant improvement in survival rates.(3, 4) The advent of anti-CTLA4 Antibody Ipilimumab, followed by anti-BRAF and anti-MEK molecular targeted therapies and anti-PD1 antibodies dramatically improved life expectancy of patients with advanced and metastatic melanoma from an average 9 months to an as yet undefined timeframe.(5, 6) Recently, 5-year overall survival rates of up to 55% under the combined molecular targeted therapies Dabrafenib/Trametinib and of up to 60% under the immune-checkpoint inhibitors Nivolumab/Ipilimumab have been reported.(6)

One of the main milestones for the development of new melanoma therapies was the identification of the significant role of the MAP-Kinases signalling pathway (identified with the cascade of RAS, RAF, MEK and ERK) leading to increased survival of malignant melanocytic cells through their unrestrained proliferation (7) Estimated 40-60% of

melanomas harbour a BRAF mutation, most of them V600E (ca. 75%), a substitution of glutamic acid for valine at codon 600. The most frequent non-V600E mutation is BRAF-V600K, corresponding to a Leucine substituting a Valine at codon 600 (15–20%). More rarely occurring mutations are BRAF-V600D, BRAF-V600R and BRAF-V600M (1–2%).(8-10)

BRAF mutated melanomas are eligible for a molecular-targeted therapy using so called "BRAF inhibitor drugs" including Dabrafenib, Vemurafenib and Encorafenib, nowadays mostly combined with a "MEK inhibitor drug" such as Trametinib, Cobimetinib or Binimetinib, in order to reduce side effects and to avoid mechanisms of resistance. In 2018, the combination of Dabrafenib and Trametinib has also been approved in an adjuvant setting for stage III melanoma.(6, 11-13)

Interestingly, BRAF-V600K mutated melanomas seem to be less responsive to BRAF inhibitor therapy, compared to their BRAF-V600E mutated counterparts.

BRAF inhibitors were the first effective molecular targeted therapy for malignant melanoma.

For this reason, mutational profiling has become of outmost importance to establish from the very beginning the best treatment options in patients with advanced melanoma. Current guidelines recommend testing every advanced melanoma (in the German guidelines from stage IIIB) for BRAF mutation, since BRAF inhibitors are available for both, the adjuvant and therapeutic setting(14). Mutation testing is performed on primary (and eventually also metastatic) tumour tissue samples.

Gene mutation status is nowadays mainly determined using molecular technologies after DNA extraction such as PCR (= real-time polymerase chain reaction), SNaPshot, Sanger sequencing, together with other validated methods in the field of molecular pathology. Concerning BRAF mutations, BRAF Exon 15 PCR amplification and sequencing of genomic DNA is the gold standard for BRAF analysis in melanoma samples. However, molecular genetic tests are expensive and time consuming, can be only performed in selected pathology departments and

are not worldwide available. Financial issues play an important role, since equipment and reagents for molecular analysis are quite expensive. Since molecular pathology tests require tumour samples which are fixed in formalin and embedded in paraffin, the meanwhile worldwide available immunohistochemistry technique (IHC) represents an appealing alternative method.

Since different melanoma subtypes can be distinguished based on genetic mutations such as BRAFV600E, corresponding morphological patterns might also be hypothesized. Reflectance confocal microscopy (RCM) and dermoscopy were used as in-vivo techniques, while immunohistochemistry (IHC), cheaper than PCR and available in most countries, was selected as ex-vivo method.

Dermoscopy

Dermoscopy refers to the examination of skin lesions using skin surface or epiluminescence microscopes.(15, 16) Dermoscopes are handheld devices using visible non-polarized and polarized light to visualize skin structures up to 40x magnification. They are portable and relatively cheap. Skin examination through dermoscopy requires a liquid (water, immersion oil or alcohol-based disinfection spray) to reduce the reflectivity of the skin and enhance the transparency of the stratum corneum. Digital dermoscopes with a magnification of up to 400x are also available. A non-invasive examination of so-called dermoscopic patterns consents an analysis of epidermis, dermo-epidermal junction and papillary dermis, based on the distribution of melanin, keratin and vascular structures.(17) Dermoscopy is now the gold-standard for the first level diagnostic of skin cancer. Numerous studies in the last thirty years reported an increase of sensitivity from 60% up to 99% for diagnosing melanoma(18-20). Cochrane meta-analyses have recently confirmed a superiority of dermoscopy in comparison to simple clinical examination while diagnosing melanoma and non-melanoma skin cancer. (21) Numerous formal algorithms for optimizing melanoma diagnosis with dermoscopy have

been published so far, although they appear to be more of use for training purposes and less for expert observers(19-22). Main dermoscopic patterns associated to malignant melanocytic lesions are(17, 18, 22-24):

– Blue-whitish veil

– Multiple irregular dots

– Pseudopods

– Radial streaming

– Scar-like depigmentation

– Peripheral black dots/globules

– Multiple colours

– Broadened pigment network

– Focal sharply cut-off border

– Inverse network

– Irregular vessels

Reflectance confocal microscopy

Reflectance confocal microscopy is a second-level diagnostic tool to dermoscopy; it allows visualization of skin structures to a near histologic resolution, avoiding unnecessary excisions.(25) The commercially available RCMs (Vivascope1500 and 3000-handheld device, MAVIG GmbH, Munich, Germany) use a diode laser with a near-infrared wavelength of 830 nm at a low (<25 mW) laser power that does not harm human tissues. The imaging depth is limited to the papillary dermis (around 200–300 μm).(26) The machine generates basic images with a 500 μm x 500 field of view; those can be arranged either in a 2D mosaic grid of contiguous horizontal images (VivaBlock) or in a vertical sequence that is captured in depth (VivaStack). With the handheld device, it is possible to examine difficult to access skin regions, directly exploring large skin areas, with a little wider field of view (800 μm x 800 μm). An

Image Control System automatically adjusts laser power for best image quality, so that the entire field can be quickly and directly explored. A dermoscopic camera is also integrated to permit a live navigation of the scanned areas.

RCM has proven its utility in diagnosing melanoma compared to dermoscopy especially in difficult lesions.(27) It has a reported sensitivity of 93,5% and specificity of 78,8% for diagnosing pigmented melanomas; a sensitivity of 67% and specificity of 89% were described for amelanotic melanomas.(28-30)

Main RCM morphologic criteria associated to malignant melanoma are: (31-35)

<u>In the superficial epidermal layers (stratum granulosum-spinosum):</u>

- Disarranged epidermal pattern with atypical honeycombed pattern

- Pagetoid spread of cells (round and dendritic)

<u>At the dermo-epidermal junction:</u>

- Non-edged papillae

- Dense and sparse nests

- Junctional thickening

- Pagetoid cells

- Sheet-like distribution of atypical melanocytic cells

<u>In the superficial dermis:</u>

- Dense and sparse nests

- Cerebriform nests

Immunohistochemistry

Immunohistochemistry (IHC) is an immunofluorescence technique for detecting cellular antigens in tissue sections, worldwide used in the daily routine for infectious and neoplastic

diseases.(36) IHC is based on the identification of specific tissue antigens (Ag) through specific antibodies (Ab), mainly Immunglobulines G. These are marked by a colored histochemical reaction and become visible by light microscopy or fluorochromes with ultraviolet light. Nowadays, extremely sensitive methods are available even for multiple tissue sections at the same time (microarray technology).(37)

Melanomas tend to mimic the histologic features of different tumours, such as neuroendocrine tumours, lymphomas, sarcomas, poorly differentiated carcinomas and germ cell tumours. For this reason, IHC is of outmost importance in their differentiation in conventionally stained sections(38). Established IHC markers for melanoma are:

- S100
- HMB45
- MART-1/Melan-A
- Tyrosinase
- MITF
- NKI/C3
- Vimentin

IHC together with conventional histology of Hematoxylin & Eosin (H&E) stained tissue samples is the gold standard for the diagnosis of melanoma.(39)

Also genetic mutated proteins can be targeted by specific antibodies. IHC in this case has the advantage of allowing the direct visualization of protein distribution inside the specimen by light microscopy to analyse tissue heterogeneity. Moreover, it is able to visualize even single mutated cells. Various BRAF-V600E specific antibodies are commercially available and have been validated with a reported sensitivity of 94% and a specificity of 95%.(40-45).

Aim of this study was to identify morphological correlates able to differentiate BRAF mutated melanomas compared to their wild type counterpart, based on alternative methods such as RCM, dermoscopy and IHC.

Objectives

This cumulative work is focused on the attempt to describe morphological features able to distinguish BRAFV600E mutated melanomas from wild type melanomas. This might help to find interesting hints for screening melanoma mutational status prior to excision through non-invasive methods such as dermoscopy and RCM or right after surgery in the histopathology laboratory, while proceeding to conventional evaluation. Both works were pilot studies on small samples.

In the paper "**Confocal microscopy characterization of BRAFV600E mutated melanomas"(46)** we analysed eight BRAFV600E mutated melanomas (six primary and two metastases), paired with age-, sex- and tumour thickness- matched wild-type controls, that had been imaged with dermoscopy and in-vivo confocal microscopy prior to their excision. Two physicians expert in dermoscopy and confocal microscopy evaluated six typical dermoscopy features ("irregularly distributed globules and dots", "blue–gray blotches", "irregular vessels", "white regression", "peppering") and six common RCM parameters ("pleomorphic pagetoid cells", "architectural disarrangement", "dischoesed junctional nests", "bright particles", "collagen bundles", "plump bright cells"). Such melanoma-associated dermoscopic and confocal patterns belong to the list of widely recognized morphologic criteria in the clinical practice and international textbooks, scientific literature and consensus guidelines.(17, 18, 23, 24, 34, 35, 47) A statistical analysis to calculate the frequency for each parameter in BRAFV600E mutated melanomas was performed. Aim of this study was to observe whether specific morphological patterns were more common in BRAF mutated

melanomas compared to their wild-type counterpart, in order to pre-screen the mutational status of patients before the excision and ideally plan a targeted therapy if necessary.(46, 48) Analogously, in the article **"BRAFp.V600E, p.V600K, and p.V600R Mutations in Malignant Melanoma: Do They Also Differ in Immunohistochemical Assessment and Clinical Features?"**(48) we selected eighteen melanomas with known BRAF mutational status (six V600E, seven V600K, one V600R and four wild type) determined through Sanger sequencing for which full tissue samples of the primary tumour were available. We then performed IHC with BRAFV600E (clone: VE1, 1:100) (Spring Bioscience, Pleasanton, CA) antibodies. The results were separately evaluated by two pathologists, blinded to the mutational status. The aim was to determine whether IHC was able to recognize BRAFV600 mutated melanomas, in order to provide a cost-effective and quicker method to screen melanomas for their mutational status without using expensive and time-consuming molecular pathology.

State of art

The articles discussed in this cumulative dissertation were published in 2015(46) and 2016(48), respectively. Pozzobon and colleagues (49) had retrospectively analyzed dermoscopic findings in a cohort of 72 BRAF mutated melanomas in 2014. They described peppering, already known as expression of regression and melanophages in the dermis, as an expression of immune reaction in BRAF mutated melanomas. In 2017, Bombonato and colleagues associated irregular peripheral streaks and ulceration to BRAF mutated melanomas and dotted vessels to wild type melanomas. They could not find any statistically significant association between RCM features and mutational status. (50) In 2018, a Spanish study came to different results, identifying blue-whitish veil as the only relevant parameter in the differentiation of BRAFV600E mutated and wild type melanomas.(51) Our study was the first to look for a RCM correlation of BRAF mutated melanomas.

Concerning IHC, the need for cheaper and quicker alternative methods to molecular pathology was, since the advent of effective molecular targeted therapies, clear. The most recent studies on the topic report a good overall concordance of IHC (89% to 95%) with PCR associated methods, however limited to BRAFV600E.(40, 41, 52).

Results

Most common dermoscopic features in BRAFV600E mutated melanomas were irregularly distributed dots and globules and blue-gray blotches (62%), followed by irregular vessels, white regression, and peppering (50%). Most common RCM patterns were: "pleomorphic pagetoid cells", "disarrangement at the dermo-epidermal junction" (DEJ), "decohesed junctional nests", and "bright particles at the DEJ" (75%).

Peppering in dermoscopy and plump bright cells in RCM were more common in BRAFV600E melanomas than in wild-type ones (63% and 37%, respectively). Additionally, we could associate the RCM detected inflammation at the DEJ (collagen bundles, plump bright cells, bright particles) to the dermoscopic feature "peppering".

In IHC, V600E specific antibody stained all melanomas harbouring V600E and, interestingly, the only melanoma harbouring V600R mutation, but was not able to recognize BRAF V600K mutated melanomas. All V600E and wild type cases were correctly identified by the 2 pathologists and the overall concordance rate between protein expression and BRAFV600E mutations was 100% (6/6). The single BRAFV600R mutated melanoma had a strong cytoplasmic staining. The 7 BRAF p.V600K-mutated melanomas were reported as negatively stained by both observers.

Also prognostic data analysis was in line with similar studies, recording the worst prognosis for patients with V600K mutated melanomas.

Conclusions

The BRAF mutational profiling of melanomas is nowadays necessary to classify patients based on potential adjuvant and therapeutic strategies. Today, the gold standard for the mutational analysis are molecular technologies such as high-resolution melting (HRM) analysis, together with sequencing, Sanger bidirectional sequencing, pyrosequencing, and next-generation sequencing (NGS) with special gene-panels. Above-mentioned methods are however quite pricey and timewasting, require trained technicians, conspicuous tissue samples, and are not worldwide available.

The search for morphological predictors of mutational status remains an actual topic, especially concerning cost-effective screening methods.

Dermoscopy and confocal microscopy are related to each other and might provide useful information in the non-invasive initial categorization of melanoma patients potentially harbouring a BRAFV600E mutation.

Histopathological examination represents the gold standard in the diagnosis of melanocytic tumours. It is thus proven that RCM is useful in the characterization of cellular and structural alterations in nevi and melanomas, with specific histological correlations. A preliminary study could also find peculiar RCM features in subjects with multiple melanomas harbouring CDKN2A and MC1R genetic variants.

RCM is a valuable tool especially in those lesions where dermoscopy alone is not able to make a diagnosis of melanoma with enough confidence. The potential ability of RCM to evaluate main morphologic patterns in melanomas is evident also in the cases where dermoscopy cannot identify them. For example, regression features (corresponding to "bright and thickened hyperrefractive collagen fibers and plump bright cells) could be seen also in those

melanomas where peppering (a dermoscopic correlate for inflammation and fibrosis) was not found. Those parameters were, in fact, seen in the histological examination.

At the same time, ICH might be efficiently used for the preliminary search of BRAFV600E mutations in subjects with high risk or metastatic melanomas potentially benefiting from a systemic therapy with BRAF inhibitors. Compared to molecular pathologic methods, IHC is easier to perform by trained technicians and can be performed in the same time window for different antibodies such as S100. It allows the direct visualization of mutational distribution in the tumour sample and therefore tissue heterogeneity. It is cheaper and can be performed in most pathology departments worldwide. Hence, it represents a cost-effective and rapid screening method for BRAFV600E mutations in melanomas. However, IHC should be followed by molecular techniques in V600E-negative melanomas, since the commercially available antibodies are mainly not able to identify V600K and other rare nonV600E BRAF mutations.

Both studies are limited by the small sample size, so that they should be considered preliminary and further developments in larger, prospective cohorts are needed.

Zusammenfassung (Deutsch)

Hintergrund

Seit dem Aufkommen molekularer zielgerichteter Therapien ist die Mutationsprofilierung von malignen Melanomen von größter Bedeutung geworden. Melanome können anhand des Vorhandenseins von BRAF-Mutationen unterschieden werden. Schätzungsweise 40-60% der Melanome weisen eine BRAF-Mutation auf, die meisten davon V600E. Aktuelle Richtlinien empfehlen, jedes fortgeschrittene Melanom auf BRAF-Mutationen zu testen, da BRAF-Inhibitoren sowohl im adjuvanten als auch im therapeutischen Setting verfügbar sind. Molekularpathologische Techniken sind der Goldstandard für den Nachweis von BRAF-Mutationen, insbesondere die Ampflizierung und Sequenzierung von BRAF Exon 15 aus genomischer DNA mittels PCR. Solche Tests sind jedoch teuer und zeitaufwändig. Da verschiedene Melanom-Subtypen anhand genetischer Mutationen wie BRAFV600E unterschieden werden können, können entsprechende morphologische Muster angenommen werden. Ziel dieser Studie war es, BRAF-mutierte Melanome im Vergleich zu ihrem Wildtyp-Korrelat auf der Grundlage alternativer Methoden wie konfokaler in-vivo-Mikroskopie (RCM), Dermatoskopie und Immunhistochemie (IHC) weiter zu charakterisieren.

Methoden

Acht mutierte BRAFV600E-Melanome (sechs primäre und zwei Metastasen), gepaart mit alters-, geschlechts- und tumourdickenangepassten Wildtyp-Kontrollen, wurden mit Dermoskopie und konfokaler in-vivo-Mikroskopie analysiert.

Auf der anderen Seite wurden 18 Melanome mit bekanntem BRAF-Mutationsstatus (BRAFV600E, V600K, V600R und Wildtyp) zusätzlich durch Immunhistochemie mit Anti-BRAF-Antikörpern bewertet.

Ergebnisse

Die häufigsten dermatoskopischen Merkmale bei mutierten BRAFV600E-Melanomen waren unregelmäßig verteilte Punkte und Schollen sowie grau-blaue homogene Areale (62%), gefolgt von unregelmäßigen Gefäßen, weißer Regression und Peppering (50%). Die häufigsten RCM-Muster waren pleomorphe pagetoide Zellen, Störungen der dermo-epidermalen Junktion (DEJ), atypische junktionale Melanozytennester und helle Partikel an der DEJ (75%).

Peppering in der Dermatoskopie und plumpe helle Zellen in RCM wurden häufiger in mutierten primären BRAFV600E-Melanomen als in Wildtyp-Melanomen gefunden (63% vs. 37%).

In der IHC färbte der V600E-spezifische Antikörper alle Melanome, die eine V600E- und V600R-Mutation enthielten, konnte jedoch keine BRAF V600K-mutierten Melanome erkennen.

Schlussfolgerungen

Dermatoskopie und konfokale Mikroskopie zeigen eine gute Korrelation zueinander und könnten nützliche Informationen für das nicht-invasive, preliminäre Screening und die Charakterisierung von BRAFV600E-mutierten Melanomen liefern.

Gleichzeitig könnte die Immunhistochemie als erster Schritt zum Nachweis der BRAFV600E-Mutation bei der Auswahl von Patienten mit fortgeschrittenen Melanomen als Kandidaten für eine systemische Therapie mit BRAF-Inhibitoren wirksam eingesetzt werden. IHC sollte von molekularen Techniken bei p.V600E-negativen Melanomen gefolgt werden, um p.V600K und andere seltene Nicht-V600E-BRAF-Mutationen zu entdecken.

Schlüsselworte: Malignes Melanom, BRAF Mutation, Immunhistochemie, konfokale Laserscanmikroskopie, nicht-invasive Diagnostik.

Abstract (English)

Background

Since the advent of molecular targeted therapies, mutational profiling of malignant melanoma has become of outmost importance. Melanomas can be distinguished based on the presence of BRAF mutations. Estimated 40-60% of melanomas harbour a BRAF mutation, most of them V600E. Current guidelines recommend testing every advanced melanoma for BRAF mutation, since BRAF inhibitors are available for both adjuvant and therapeutic setting. BRAF Exon 15 PCR amplification and sequencing of genomic DNA now represents the gold standard for BRAF analysis in melanoma samples. Molecular genetic tests are however expensive and time consuming. Since different melanoma subtypes can be distinguished based on genetic mutations such as BRAFV600E, corresponding morphological patterns might be hypothesized. Aim of this study was to further characterize BRAF mutated melanomas compared to their wild type counterpart based on alternative methods such as in-vivo confocal microscopy (RCM), dermoscopy and immunohistochemistry (IHC).

Methods

Eight BRAFV600E mutated melanomas (six primary and two metastases), paired with age-, sex- and tumour thickness- matched wild-type controls were analysed with dermoscopy and in-vivo confocal microscopy.

On the other side, eighteen melanomas with known BRAF mutational status (BRAFV600E, V600K, V600R and wild-type) were additionally evaluated through immunohistochemistry with anti-BRAF antibodies.

Results

Most common dermoscopic features in BRAFV600E mutated melanomas were irregularly distributed dots and globules and gray-blue blotches (62%), followed by irregular vessels, white regression, and peppering (50%). Most common RCM patterns were pleomorphic

pagetoid cells, disarrangement at the dermoepidermal junction (DEJ), decohesed junctional nests, and bright particles at the dermal–epidermal junction (75%).

Peppering in dermoscopy and plump bright cells in RCM were more frequently found in BRAFV600E mutated primary melanomas compared to wild-type ones (63% and 37%, respectively).

In IHC, V600E-specific antibody stained all melanomas harbouring V600E and V600R mutation, but was not able to recognize BRAF V600K-mutated melanomas.

Conclusions

Dermoscopy and confocal microscopy are related to each other and might provide useful information in the non-invasive initial categorization of melanoma patients potentially harbouring a BRAFV600E mutation.

At the same time, IHC might be a useful tool for the initial diagnosis of a BRAFV600E mutation in subjects with high risk or metastatic melanomas potentially benefiting from a systemic therapy with BRAF inhibitors. After the IHC screening, molecular techniques shall be used in V600E wild type melanomas, in the search for less frequent non-V600E BRAF mutations.

Keywords: malignant melanoma, BRAF mutation, immunohistochemistry, reflectance confocal microscopy, non-invasive diagnostics.

BRAFp.V600E, p.V600K, and p.V600R Mutations in Malignant Melanoma: Do They Also Differ in Immunohistochemical Assessment and Clinical Features?

published in:

BRAFp.V600E, p.V600K, and p.V600R Mutations in Malignant Melanoma: Do They Also Differ in Immunohistochemical Assessment and Clinical Features?

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Introduction: Although the detection of *BRAF* p.V600E mutation by immunohistochemistry was clearly described in melanoma, discordant evidences were reported for the detection of p.V600K and p.V600R mutations. The aim of the study was to evaluate the efficacy of *BRAF*p.V600E, p.V600K, and p.V600R detection by immunohistochemistry in melanoma.

Materials and Methods: Immunohistochemistry with VE1 antibody was performed on 18 tissue samples of metastatic melanomas with known *BRAF* mutational status.

Results: The concordance rate of immunohistochemistry was 100% for p.V600E mutation. In contrast, the 7 p.V600K-mutated melanomas were scored as negative. p.V600K-mutated melanomas were significantly associated with older age, male sex, and worst clinical outcome.

Conclusions: Immunohistochemistry could efficaciously be adopted as a first step for the detection of *BRAF*p.V600E mutation in the initial selection of patients with advanced melanomas as candidates for *BRAF* inhibitors. It should be followed by molecular techniques in p.V600E-negative melanomas, for the specific search of p.V600K and other non-p.V600E *BRAF* mutations.

Key Words: *BRAF*p.V600K mutation, *BRAF*p.V600E immunohistochemical assessment, malignant melanoma, *BRAF*p.V600R mutation, *BRAF* inhibitors

(*Appl Immunohistochem Mol Morphol* 2016;24:30–34)

About 40% to 60% of malignant melanomas are *BRAF* mutated.[1,2] The most common mutations are *BRAFp.V600*E (80%) and *BRAFp.V600*K (5-30%).[3]

These mutations result (mainly p.V600E) in an enhanced *BRAF* kinase activity and an increased phosphorylation of downstream targets, particularly MEK. Nowadays, the evaluation of somatic *BRAF* mutations is required for molecular-targeted treatments of metastatic melanoma. In fact, *BRAF* inhibitors targeting common p.V600E mutations have become increasingly popular because of their high objective response rate and few side effects. *BRAF* inhibitors have a clinical activity also in melanoma patients harboring a non-p.V600E *BRAF* mutation, particularly p.V600K and p.V600R.[4–6] *BRAF*p.V600M-D are quite rare and they were not included in the trials for *BRAF*-selective inhibitors.[5]

The ability to recognize *BRAFp.V600*E mutation changes according to the methods used for mutation testing. At present time, the cobas 4800 *BRAFp.V600* Mutation Test, approved by FDA and specifically created to detect *BRAFp.V600*E mutation, is able to detect 70% of *BRAFp.V600*K mutations.[7]

Although the immunohistochemical assessment of p.V600E mutation was clearly reported in melanoma and other tumors,[8,9] discordant evidences were reported for p.V600K *BRAF* mutations.[2,10] Different authors reported that none of the non-p.V600E cases, including p.V600K, stained positive with the antibody[8,9]; in contrast, Routhier et al[10] and Heinzerling et al[11] described 2 p.V600K melanomas with positive VE1 staining.

The aim of this paper was to assess the efficacy of *BRAF*p.V600E and p.V600K detection by immunohistochemistry in melanoma and the distinctive clinical features of the most common *BRAF* mutations.

PATIENTS AND METHODS

We selected 35 patients with *BRAF*-positive advanced melanoma, diagnosed at the University of Modena and Reggio Emilia from January 2010. Among these, 18 patients with known *BRAF* mutational status (12 males, 6 females) were included (Table 1). The 22 remaining tissues could not be IHC tested because of nonavailability of enough sample or missing informed consent. We decided to analyze the patients' data and include them in the study because of their epidemiological importance.

TABLE 1. Correlation of *BRAF* Mutation Status With Monoclonal VE1 Immunostaining Evidences

ID	Sex	Age (y)	BRAF Mutation Types	VE1 Staining Pathologist 1	VE1 Staining Pathologist 2
1	M	69	V600E	3+	3+
2	F	51	V600E	3+	3+
3	M	51	V600E	3+	3+
4	M	50	V600E	3+	3+
5	F	67	V600E	3+	3+
6	M	66	V600E	3+	3+
7	M	75	V600K	0	0
8	M	48	V600K	0	0
9	M	62	V600K	0	0
10	M	74	V600K	0	0
11	M	69	V600K	0	0
12	F	51	V600K	0	0
13	M	82	V600K	0	0
14	M	61	V600R	2+	2+
15	F	68	WT	0	0
16	M	68	WT	0	0
17	F	70	WT	0	0
18	F	41	WT	0	0

BRAF mutations were detected as following: 25 V600E (c. 1799 T > A; codon GTG > GAG); 2 *BRAF* V600 "E2" (c.1799_1800TG.AA:p. Val600Glu); 7 V600K (c.1798_1799GT.AA:p.Val600Lys); 1 V600R (c.1798_1799GT.AG:p.Val600Arg).

F indicates female; M, male.

Tumor samples were collected according to the principles of the Declaration of Helsinki after obtaining informed consent from each patient. The study was approved by the institutional review board.

IMMUNOHISTOCHEMISTRY

Eighteen melanomas referred for genotyping assay at our Molecular Diagnostic Laboratory were retrieved from the pathology database, all with known *BRAF* mutational status: p.V600E (n = 6), p.V600K (n = 7), p.V600R (n = 1), and WT (n = 4).

Immunohistochemistry with anti-*BRAF* antibody was performed on 5-μm-thick whole tissue sections of formalin-fixed, paraffin-embedded tissue in a automated immunostainer (Benchmark XT, Ventana) and primary antibodies against *BRAF*p.V600E (clone: VE1, 1:100) (Spring Bioscience, Pleasanton, CA). All slides were double-blind reviewed by 2 independent observers (A.M.C. and M.M.). The VE1 antibody staining was scored as negative when there was no staining or only isolated nuclear staining. Positive staining was considered by diffuse and moderate (2+) to strong (3+) cytoplasmic staining. Staining results were interpreted as negative when there was no staining (0+), or also slight/faint/barely perceptible staining or staining of only single cells (1+).

BRAF MUTATION ANALYSIS

Two pathologists reviewed all H&E-stained slides of primary melanomas. The tumor area of interest was detected and marked on each specimen. DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Basel, Switzerland) and tested for *BRAF* mutations in 2 different laboratories.

Sanger sequencing analyses was performed on all samples to determine *BRAF* mutational status. The PCR product were checked for the right fragment length, purified and sequenced as previously described.[4] Data were manually edited with the sequencing analysis software (Applied Biosystems, Darmstadt, Germany).

RESULTS

Average ages were 59 years for patients with p.V600E and 66 years for patients with p.V600K (Table 1). *BRAF*p.V600K-mutated patients had a worse prognosis compared with their V600E counterparts (Table 2).

The correlation between immunohistochemistry for VE1 antibody and *BRAF* mutational status is shown in Table 1. V600E-specific antibody stained 6 p.V600E-mutated melanomas, but no *BRAF* wild-type or p.V600K-mutated melanomas. All p.V600E and wild-type cases were correctly identified by the 2 pathologists and the overall concordance rate between protein expression and *BRAF*p.V600E mutations was 100% (6/6). The single *BRAF*p.V600R-mutated melanoma had a strong cytoplasmic staining. The 7 *BRAF* p.V600K-mutated melanomas were reported as negatively stained with VE1 by both observers (Table 1).

In all cases, the interobserver agreement was almost total. Cross reactivity was not observed (Fig. 1).

DISCUSSION

Our study shows that, in contrast to *BRAF*p.V600E mutation, *BRAF*p.V600K is not detected by immunohistochemistry with VE1 antibody. Our analysis confirms the high sensitivity and specificity of VE1 antibody in detecting p.V600E mutation, as seen in recently published studies.[8–10] Although isolated instances of VE1 reactivity in p.V600K-mutant melanoma have been reported, our findings clarify the value of this antibody as a screening tool for V600E mutations.[10,11]

Even though our results are based on a small sample size and further studies are still needed to set appropriate diagnostic standards, we can still highlight some key concepts.

Although *BRAF* inhibitors are efficacious on tumors with p.V600E and p.V600K mutations, the 2 tumor types shall be considered as distinct entities with slightly different age of onset and clinical behavior.[2,3]

Regarding the response to treatment, the clinical trials with vemurafenib/dabrafenib including patients with p.V600K mutations reported worse outcomes and a strong trend for shorter overall survival (OS) among p.V600K melanoma patients compared with those with *BRAF*p.V600E mutation.[2]

Recently published studies demonstrated that patients with *BRAFp.V600K* mutation are characterized by an older age of cancer onset, an increased risk for brain and lung metastases, and a shorter time from diagnosis to

TABLE 2. Clinicopathologic Features in Melanoma Patients Treated With *BRAF* Inhibitors

ID	BRAF Mutation Types	Sex	Age (y)	Location	TNM	ECOG (t_0)	Time to Metastasis (y)	Treatment Duration (mo)	Objective Response	Adverse Effects	Time to Progression (mo)	Follow-up (mo)	Status	ECOG (t_1)
1	V600E	M	69	Arm	T2bN0M0	0	15	6	Partial	Keratoacanthomas	6	11	Dead	—
2	V600E	F	51	Leg	T2aN0M0	0	10	14	Partial	Arthralgia (I)	14	15	Alive	0
3	V600E	M	51	Trunk	TxNxMx	0	—	6	Partial	—	6	14	Alive	0
4	V600E	M	50	Trunk	TxNxMx	0	2	2	—	Fatigue (II)	3	3	Dead	—
5	V600E	F	67	Trunk	T2aN0M0	0	—	12	Partial	—	In response	13	Alive	0
6	V600E	M	66	Trunk	T1N0M0	0	1	6	Partial	—	14	6	Dead	—
7	V600K	M	75	Head	T1NxM0	1	8	6	Partial	—	6	8	Dead	2
8	V600K	M	48	Scalp	T4bN3M1	0	0.5	13	Partial	—	13	14	Alive	0
9	V600K	M	62	Scalp	T3bN2bM0	0	0	—	—	—	0	35	Alive	0
10	V600K	M	74	Trunk	T2N1M0	1	4	—	—	—	6	6	Dead	2
11	V600K	M	69	Chest	T2aN0M0	2	2.5	0.3	—	—	—	3	Alive	4
12	V600K	F	51	Leg	T1N0M0	0	0	—	—	—	—	4	Alive	0
13	V600K	M	82	Scalp	T3N0M0	1	—	3	Partial	Nausea (II), headache (I)	3	3	Dead	1
14	V600R	M	61	Scalp	T2aN1M0	0	4	2	Partial	—	In response	2	Alive	0

F indicates female; M, male.

metastasis and death. Reports of melanoma patients with *BRAF*p.*V600*E and p.V600K mutations recognized significant differences in sex, age, primary melanoma location, interval from the time of initial melanoma diagnosis to diagnosis of stage IV disease, and OS after the diagnosis of the stage IV disease. p.V600K mutation was significantly associated with older age, male sex, head and neck primary melanoma site, higher degree of chronic sun damage, and short OS from the time of diagnosis of stage IV disease.[3]

Our case series confirm that p.V600K-mutated melanomas arise at an older age (59 vs. 66 y old) and is predominant in males. In this case, it arises in the head and neck area. Their biological behavior is more aggressive and with tendency to systemic disease, compared with their p.V600E counterparts.

Our experience confirms, according to recent literature on the same topic, the efficacy of VE1 in the recognition of *BRAF*p.V600R mutation[12] (Table 1, Fig. 1); although just 1 melanoma belonging to our cohort was p.V600R mutated, the clear staining positivity led us to the hypothesis that the protein conformational change is similar enough to the one induced by p.V600E to be bound by VE1 antibody. However, the risk of misidentifying p.V600R as p.V600E by IHC is of limited clinical relevance. p.V600R mutation is less common than the others, but it is the third most common mutation occurring in 5% to 7% of patients with *BRAF*-mutant melanoma and there are evidences that the melanoma patients carrying this mutation can be successfully treated with oral *BRAF* inhibitors.[6,12]

It is known that it is very useful to screen all patients with advanced melanoma (unresectable stage III and IV) and high risk of recurrence (stage IIIb and IIIc) for *BRAF*p.*V600* mutations other than p.V600E, so that a higher number of patients might benefit from *BRAF* selective inhibitors. With this regard, *BRAF*p.*V600*K mutation, which is present in about 20% of melanomas, should be investigated with a specific antibody at immunohistochemistry or through direct sequencing. In contrast to p.V600E and p.V600R, in fact, VE1 antibody is not able to recognize p.V600K.

To sum up, p.V600K-mutated melanomas seem to constitute a specific clinical and pathologic entity, showing different features in comparison with their p.V600E counterparts. This becomes evident if we consider the different sensitivity to VE1 antibody at immunohistochemistry, which requires the use of distinct antibodies for detecting p.V600K mutation.

Moreover, we realized that the immunohistochemical screening for *BRAF* mutations, which is less expensive and less time consuming than molecular sequencing, can be efficaciously used in the preliminary screening of the great majority of *BRAF*p.V600E-mutated melanomas. This preliminary analysis could be then further enriched through the use of a specific antibody for p.V600K mutation and, eventually, direct sequencing for other *BRAF* mutations in case of negative immunohistochemical staining.
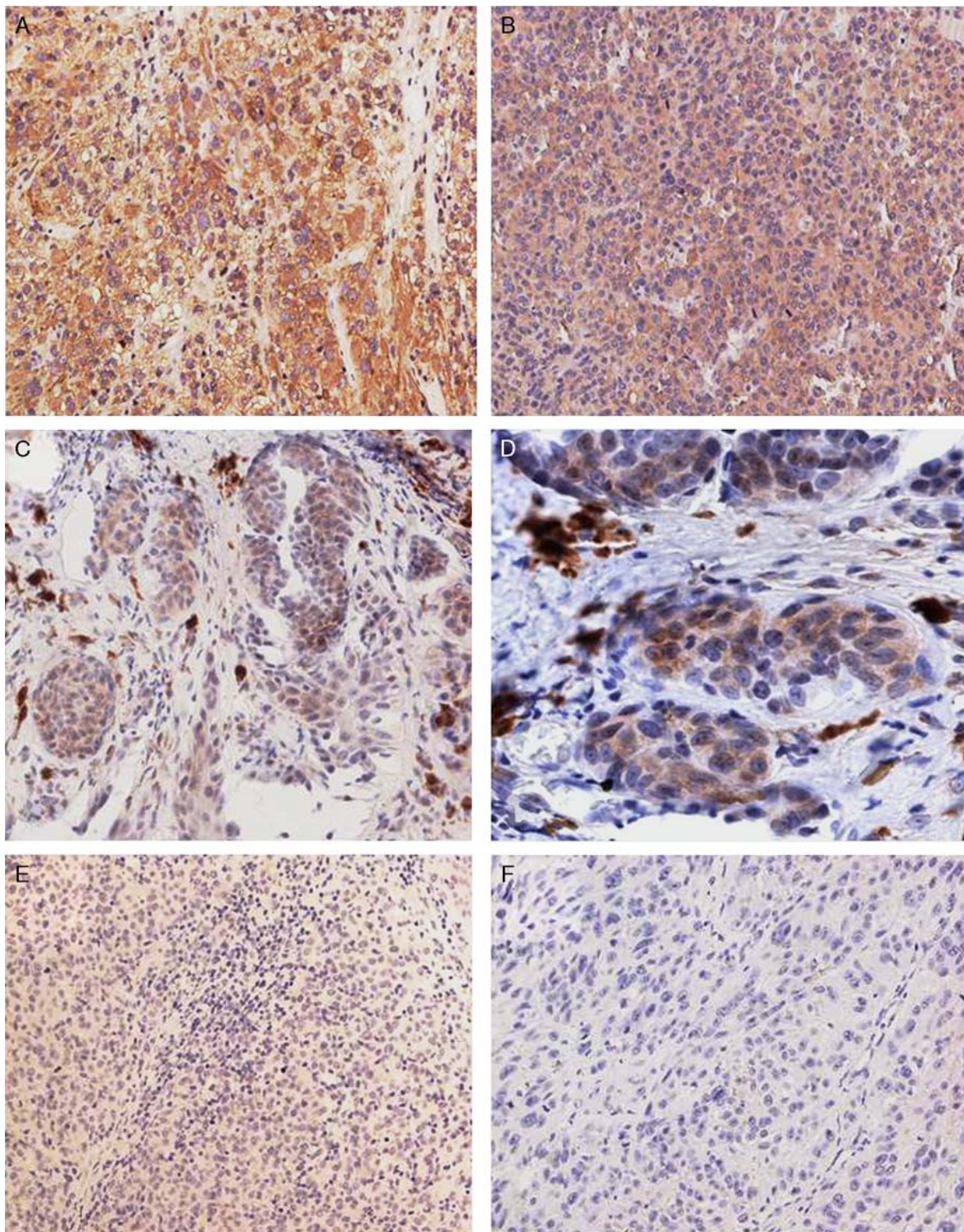
FIGURE 1. Immunohistochemistry with anti-*BRAF* p.V600E-specific VE1 antibody. p.V600E-mutated case with strong positive cytoplasmic staining of melanoma cells (A); p.v600E-mutated case with strong positive cytoplasmic staining of melanoma cells (B); p.V600R-mutated case with positive cytoplasmic staining of melanoma cells (×20) (C); p.V600R-mutated case with positive cytoplasmic staining of melanoma cells (×40) (D); p.V600K-mutated case with negative cytoplasmic staining (E); *BRAF* wild-type (WT) case with negative cytoplasmic staining (F).

ACKNOWLEDGMENT

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Confocal microscopy characterization of BRAFV600E mutated melanomas

published in:

Ruini C., Manfredini M., Pellacani G., Desmond Mandel V., Tomasi A., Ponti G. **Confocal microscopy characterization of BRAFV600E mutated melanomas.** Melanoma Res. 2015 Aug;25(4):367-71.

Impact factor (2015): 2.219

Confocal microscopy characterization of *BRAFV600E* mutated melanomas

Cristel Ruini[a,c], Marco Manfredini[a], Giovanni Pellacani[a], Victor D. Mandel[a], Aldo Tomasi[b] and Giovanni Ponti[b]

Thanks to modern techniques, molecular signatures for melanoma are now identifiable and have opened new horizons in the treatment of metastatic disease with molecular-targeted therapies. We distinguish different melanoma subtypes on the basis of genetic mutations such as *BRAFV600E* and we can therefore hypothesize the existence of corresponding morphological patterns that might be detected *in vivo* by noninvasive diagnostic tools such as dermoscopy and confocal microscopy. Eight *BRAFV600E* mutated melanomas (six primary and two metastases) were collected, matched in terms of age, sex, and thickness wild-type controls, and analyzed. In this preliminary study, regression, corresponding to fibrosis and melanophages in the dermis, was the predominant pattern and was also observed confocally when dermoscopy showed no peppering. In particular, confocal microscopy could not only detect regression but also provided a semiquantitative analysis of its grade through the count of melanophages. Confocal microscopy can be proposed as a useful tool in the preliminary screening and characterization of *BRAFV600E* mutated melanomas, providing new insights for patients' screening and follow-up. *Melanoma Res* 25:367–371 Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.

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Introduction

Thanks to the development of increasingly more sophisticated molecular techniques, it has been possible to identify molecular signatures for cancers. In the case of melanoma, the last few years have witnessed the identification of molecular mutations involved in the tumorigenesis and the subsequent discovery of molecular-targeted therapies. Such agents are highly selected for specific mutations and provide higher response rates and fewer adverse events than traditional agents. Around 40–60% of malignant melanomas harbor a mutation of the *BRAF* gene [1], mostly *V600E*; however, *V600K*, *V600R*, and *V600D* mutations are also known. Specific *BRAF* inhibitors such as vemurafenib and dabrafenib have shown promising results in the molecular-targeted treatment of *BRAF* mutated metastatic melanoma [2,3]. Other newly available tailored therapies are those with *C-KIT* and *MEK* inhibitors [4]. Because different molecular alterations categorize distinct melanoma subtypes, corresponding to a molecular signature and distinctive clinical features, it is reasonable to hypothesize the existence of diverse morphological patterns [5]. In particular, melanomas with common genetic mutations might share certain morphological features detectable with the noninvasive diagnostic tools used widely in common clinical practice in selected centers, such as dermoscopy and reflectance confocal microscopy (RCM). Recently published scientific articles have reported the analysis of the main dermoscopic differences in melanocytic lesions with certain genetic mutations in some research centers [6–8], but the use of RCM has not been reported before. In particular, Pozzobon *et al.* [7] found that 'peppering', the dermoscopic pattern expressing regression and the presence of melanophages in the dermis, is the most common dermoscopic pattern in *BRAF* mutated melanomas compared with wild-type melanocytic cancer. We present our preliminary personal experience in the evaluation of *BRAFV600E* mutated melanomas with RCM compared with wild-type melanomas matched for age, sex, and thickness.

Methods

Among patients with *BRAFp.V600E* mutated melanomas diagnosed in our clinic from January 2010, dermoscopic and RCM images of eight melanomas (six primary melanomas and two metastases, each from different patients) stored in our databases were available for evaluation. Six age-matched, sex-matched, and thickness-matched primary melanomas and two metastasis controls (each from different patients) with no known *BRAF* mutations (wild-type) were also selected. Dermoscopic and RCM patterns (Table 1) of both cases and controls lesions were assessed. Dermoscopic images were obtained using a digital camera Konica Minolta Dimage Z10 3.2 MegaPixels (Konica Minolta Holdings Inc., Tokyo, Japan) equipped

Table 1 Clinical, dermoscopic, and RCM features of *BRAFp.V600E* mutated melanoma patients

ID	BRAF mutation types	Sex	Age	Location	TNM	Status	Breslow thickness	Histologic presence of regression	Globules irregularly distributed	Blue-gray blotches	Irregular vessels	White regression	Dots irregularly distributed	Peppering	Pleomorphic pagetoid cells	Disarrangement at the DEJ	Dischoesed junctional nests	Bright particles	Collagen bundles	Plump bright cells
1	V600E	Male	81	Head	T3aN2aM0	Dead	3.0	Present	0	1	0	0	1	0	1	1	1	1	0	1
2	V600E	Male	50	Scalp	T4aN1a M1c	Alive	Metastases	Not defined	1	0	0	0	1	0	0	0	0	0	1	0
3	V600E	Male	39	Scalp	T4bN3M1c	Dead	10.0	Present	0	0	1	1	1	1	1	1	1	2	1	1
4	V600E	Female	45	Trunk	T2aN3 M1c	Alive	8.0	Absent	1	1	0	0	1	0	0	1	1	1	0	1
5	V600E	Female	41	Trunk	T3aN2aM0	Alive	4.0	Absent	0	0	1	1	1	1	0	0	0	0	0	1
6	V600E	Male	83	Scalp	T4aN3 M1c	Alive	7.0	Absent	1	1	1	1	1	0	1	1	1	2	1	2
7	V600E	Female	51	Trunk	T2aN0M0	Alive	1.1	Absent	1	1	0	0	0	1	0	1	0	1	0	1
8	V600E	Male	80	Scalp	T2aN2cM1a	Alive	Metastases	Not defined	1	1	0	1	0	0	1	0	1	0	1	0
9	WT	Female	42	Trunk	T4N0 M0	Alive	2.0	Absent	0	0	0	0	1	0	0	0	0	0	0	0
10	WT	Male	85	Arm	T4N0 M0	Alive	6.0	Absent	1	1	0	1	0	0	1	1	1	1	0	1
11	WT	Male	48	Shoulder	T1N0 M0	Alive	0.6	Absent	1	0	0	0	0	0	1	1	1	0	0	0
12	WT	Female	52	Leg	T2bN0 M0	Alive	0.5	Absent	1	1	0	1	0	0	1	1	1	0	0	0
13	WT	Female	65	Trunk	T2aN0M0	Alive	0.35	Absent	0	0	1	0	1	0	1	1	0	1	0	1
14	WT	Male	58	Scalp	T2bN0 M0	Alive	7.0	Present	1	0	0	0	0	0	0	1	0	0	1	0
15	WT	Female	63	Scalp	T4aN3 M1c	Dead	Metastases	Not defined	1	1	1	1	1	0	0	1	1	0	0	0
16	WT	Male	72	Leg	T4aN3 M1c	Alive	Metastases	Not defined	1	0	0	0	0	0	0	0	1	0	1	0

All lesions were histologically proven. Main dermoscopic and RCM features were analyzed by two expert evaluators. RCM patterns were selected among the most representative for melanocytic lesions with dermoscopic correlates.
Globules irregularly distributed, blue–gray blotches, irregular vessels, white regression, dots irregularly distributed, peppering, pleomorphic pagetoid cells, aspecific junctional pattern, decohesed junctional nests, collagen bundles: absent: 0, present: 1.
Bright particles, plump bright cells: absent: 0, mild: 1, abundant: 2.
DEJ, dermoepidermal junction; RCM, reflectance confocal microscopy.

with a dermatoscope DermLite Photo (3Gen, San Juan Capistrano, California, USA). Confocal imaging was performed using a near-infrared reflectance-mode confocal laser scanning microscope (Vivascope1500; Caliber ID, Rochester, New York, USA). The instrument uses a diode laser at 830 nm with power less than 16 mW at the tissue level and a × 30 water-immersion lens. Instruments and acquisition methods have been described elsewhere [9]. For the evaluation of dermoscopic and confocal images, both semiquantitative evaluations and quantitative measurements were obtained. Two expert dermoscopists and confocalists evaluated six dermoscopic patterns and six RCM parameters that were selected to better characterize invasive melanomas from the wider group of known dermoscopic and RCM known descriptors (Table 1) [10]. Absolute and relative frequencies were calculated for each parameter to characterize *BRAFp.V600E* mutated melanomas. All lesions were histologically proven and examined by an expert dermatopathologist in our department. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Basel, Switzerland) and tested for BRAF mutations in our laboratory. Sanger sequencing analyses were carried out on all samples to determine BRAF mutational status. The PCR products could be directly used for sequencing analyses, whereas conventionally amplified PCR products were checked for the right fragment length by agarose gel electrophoresis and purified using MicroSpin S-300 HR Columns (Amersham Pharmacia Biotech, Freiburg, Germany) or polyethylene glycol precipitation. Purified PCR products were sequenced using the BigDye Terminator v1.1 Cycle Sequencing Kit, sequences were run on an ABI Prism 3130 automated sequencer, and data were edited manually using sequencing analysis software (all from Applied Biosystems, Darmstadt, Germany).

Results

The group of patients with *BRAFp.V600E* mutated primary melanomas included four men and two women, ranging in age from 36 to 81 years, mean age 53 years. The two patients with *BRAFp.V600E* metastases included one man and one woman, ranging in age from 49 to 78 years, mean age 63.5 years. The group of patients with wild-type *BRAF* primary melanoma included six patients, three men and three women, ranging in age from 42 to 85 years, mean age 58 years, whereas the patients with wild-type melanoma metastases included one man and one woman, ranging in age from 63 to 72 years, mean age 67.5 years.

The relative frequencies of the dermoscopic and RCM parameter of *BRAFp.V600E* mutated primary melanoma and of wild-type *BRAF* primary melanoma are shown in Table 1. In *BRAFp.V600E* mutated primary melanoma, the dermoscopic descriptors globules blue–gray blotches and dots irregularly distributed were each present in 62%

Fig. 1
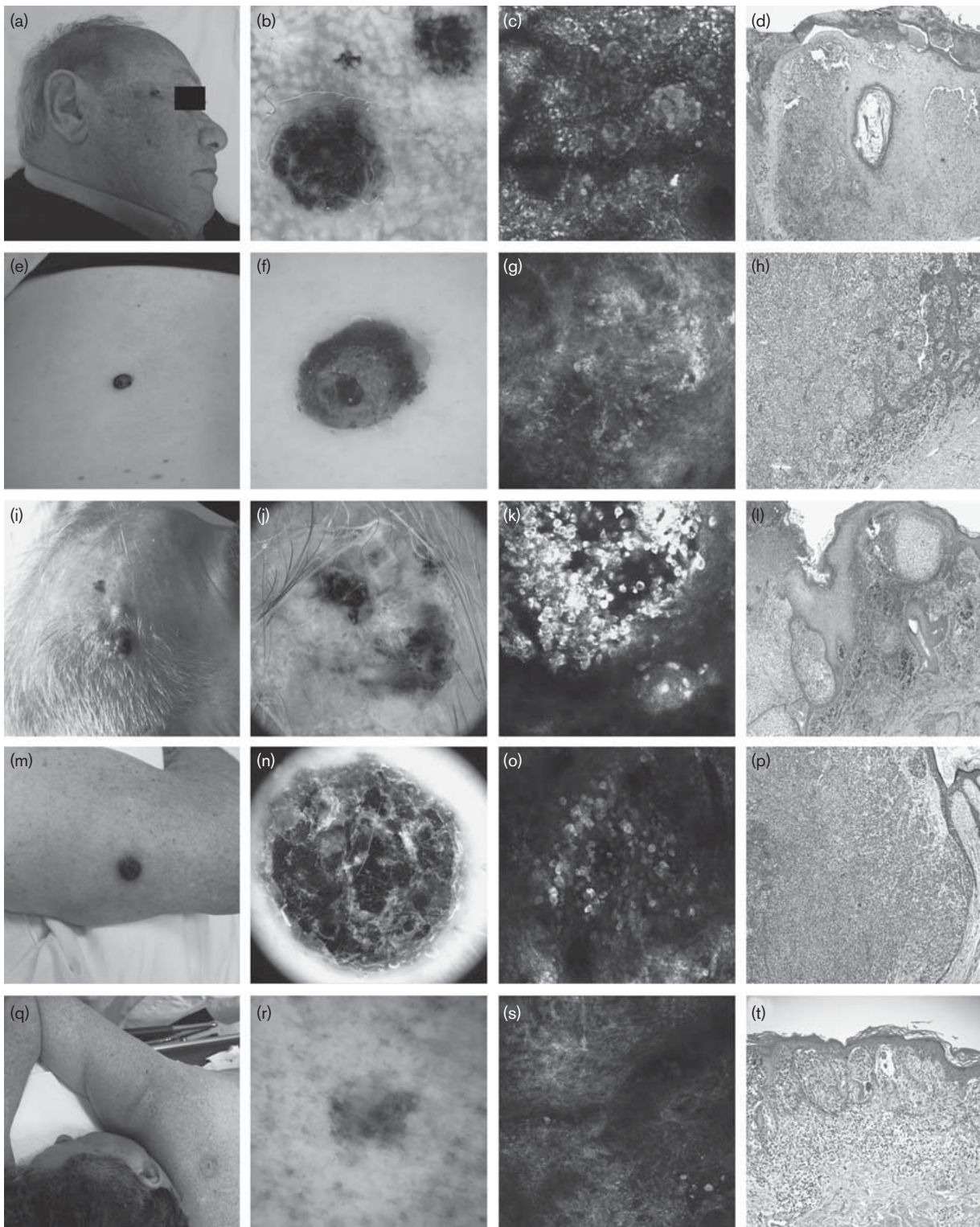
Clinical (a, e, i, m, q), dermoscopic (b, f, j, n, r), RCM (c, g, k, o, s), and histological (d, h, l, p, t) images of three melanomas with *BRAFpV600E* (a–l) and two wild-type melanomas (m–t). Globules, gray blotches, irregular vessels, white regression, irregularly distributed dots are present. RCM patterns are: pleomorphic pagetoid cells, disarrangement at the dermoepidermal junction, decohesed junctional nests, and bright particles, together with collagen bundles and plump bright cells. Histological sections confirm the diagnosis of melanoma showing the presence of atypical melanocytes, mainly aggregated to form dermal nodules, sometimes with regression areas (l). RCM, reflectance confocal microscopy.
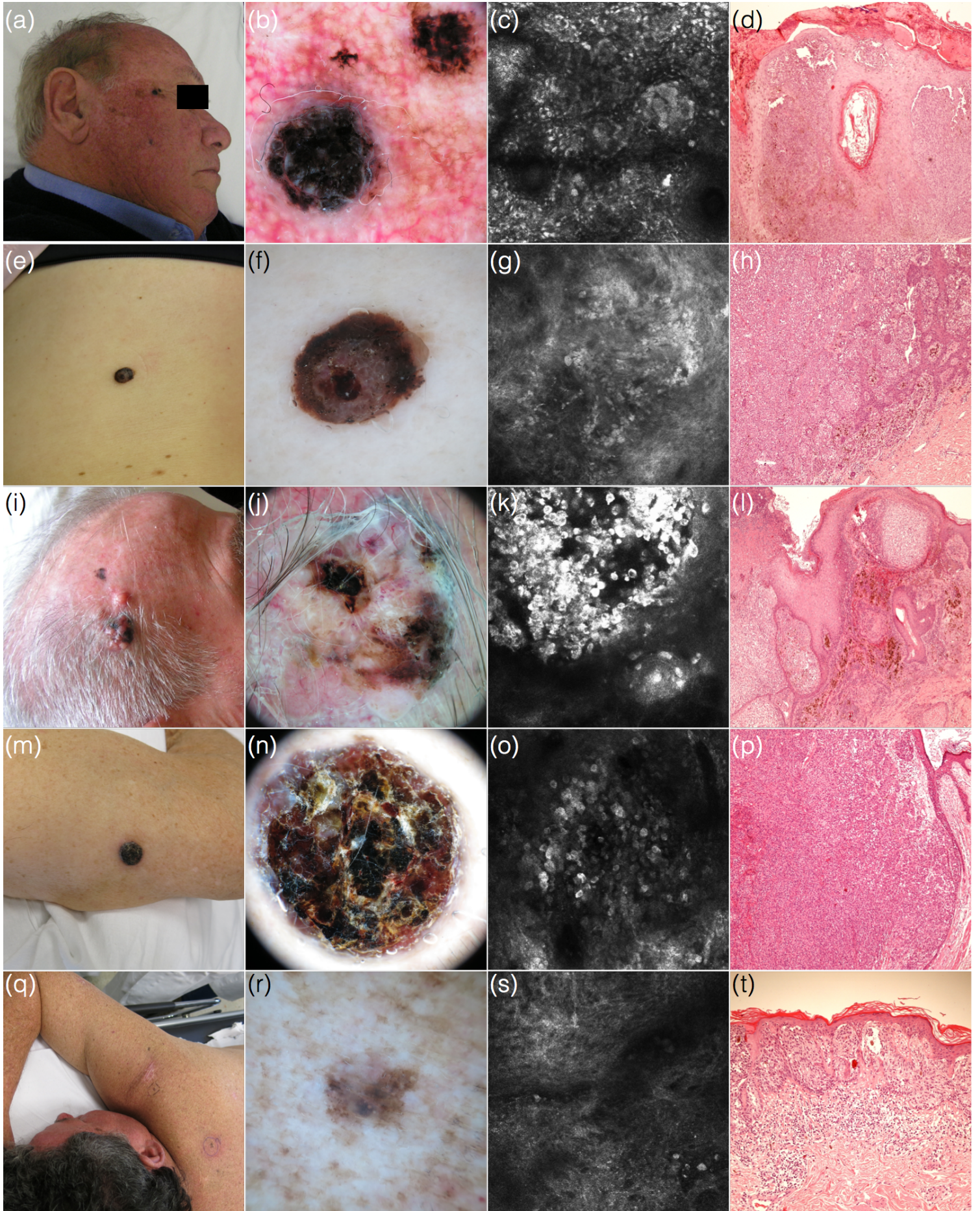
of cases; irregular vessels, white regression, and peppering were each present in 50% of cases. The RCM analysis shows that pleomorphic pagetoid cells, disarrangement at the dermoepidermal junction, decohesed junctional nests, and bright particles at the dermal–epidermal junction were each present in 75% of cases. Moreover, it was possible to correlate the detection of inflammation at the dermal–epidermal junction (collagen bundles and plump bright cells or bright particles) with the dermoscopic detection of peppering. The grading of the presence of plump bright cells on a semiquantitative scale (absent, few, abundant) showed that the abundant presence of these cells alone was correlated with the peppering phenomena. The detection of plump bright cells and of the inflammatory RCM parameters at the dermal–epidermal junction was more frequent than the detection of the peppering phenomena, being present in 67–75% compared with 50% of cases (Fig. 1). The frequency of dermoscopic descriptors found in wild-type melanomas marked the difference with BRAF mutated melanomas: globules were present in 75%, blue–gray blotches were found in 50%, irregularly distributed dots were present in 37%, and irregular vessels, white regression, and peppering were each found in 13% of cases. The RCM analysis shows that decohesed junctional nests were identified in 75%, disarrangement at the dermoepidermal junction was present in 62%, pleomorphic pagetoid cells were present in 50%, bright particles at the dermal–epidermal junction, and plump bright cells and collagen bundles were each found in 25–37% of cases.

The presence of peppering or the presence of plump bright cells was more frequently found in *BRAFp.V600E* mutated primary melanoma compared with wild-type *BRAF* primary melanoma, being present, respectively, in 63% of *BRAFp.V600E* mutated primary melanoma cases and in 37% of wild-type *BRAF* primary melanoma cases.

Discussion

Our study and its preliminary findings highlight for the first time the hypothesis that RCM can detect specific image patterns that can help distinguish different subtypes of mutated melanomas, with a focus on the most common *BRAFV600E* mutation. Histology is the gold standard for the diagnosis of melanocytic tumors. However, RCM is an important noninvasive diagnostic tool that has enabled satisfactory characterization of melanocytic and nonmelanocytic skin lesions. Numerous articles describe its utility in defining cell population and morphology in melanomas, which makes it a useful supplement to clinical and histologic findings, with which RCM shows precise correlates [10–14]. In addition, interestingly, it was used for the follow-up of changing moles in patients receiving treatment with *BRAF* inhibitors for metastatic melanomas [15]. Remarkable results

were also achieved in the search for distinctive RCM patterns in patients with multiple melanomas harboring *CDKN2A* and *MC1R* variants [16], pointing to the immense potential of in-vivo and noninvasive diagnostic tools, and in particular RCM, in the study of the cellular and architectural patterns of melanocytic lesions and their genetic signature.

Moreover, the study underlines the ability of RCM to evaluate the main morphologic patterns in melanomas with *BRAFV600E* mutations, and also in the cases where dermoscopy does not identify them. In particular, regression patterns were also detected in those melanomas that at the first dermoscopic evaluation showed an absence of dermoscopic peppering related to regression, which was found in histology. Regression patterns are represented by fibrosis (i.e. bright and thickened hyperrefractive collagen fibers) and diffuse melanophages in the dermis. Such cells are observed in RCM as irregularly shaped and ill-defined plump bright cells, larger than melanocytes, and with no visible nuclei. We assume that the increased number of melanophages and initial organization of collagen fibers become visible before regression is consistent enough to create dermoscopic peppering so that RCM provides an earlier and more precise characterization of melanoma features.

RCM can be an effective addition to dermoscopy, and can be used in further characterization of less common *BRAF* mutations such as *V600K* and *V600R*, which might share specific dermoscopic patterns compared with more frequent V600E1 [8,17,18]; in addition, *NRAS* and *C-KIT* mutated melanomas should be investigated for their imaging features.

Despite the small sample size, the authors believe that their first steps and preliminary findings may provide new insights into the characterization of malignant melanomas to promptly screen patients who require genetic testing. We confirm once again that RCM provides additional and more specific information on the cytoarchitectural structure of mutated melanomas compared with dermoscopy. For example, it is interesting that the semiquantitative evaluation of regression phenomenon can be performed by RCM through the numeric evaluation of plump bright cells and the assessment of fibrosis, and also in cases in which dermoscopic peppering is not clearly visible. The pathologist and the confocalist can therefore cooperate in a more precise overall assessment of tumor features. Of course, further studies are needed to clarify the role of RCM and other noninvasive diagnostic tools in the representation of morphologic and genetic patterns of melanoma, together with further histologic correlations.

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Acknowledgments