*BRAF*p.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

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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.

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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.V600*K 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	—	3	3	Dead	1
14	V600R	M	61	Scalp	T2aN1M0	0	4	2	Partial	Nausea (II), headhache (I)	In response	2	Alive	0

F indicates female; M, male.

metastasis and death. Reports of melanoma patients with *BRAFp.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 *BRAFp.V600* mutations other than p.V600E, so that a higher number of patients might benefit from *BRAF* selective inhibitors. With this regard, *BRAFp.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.

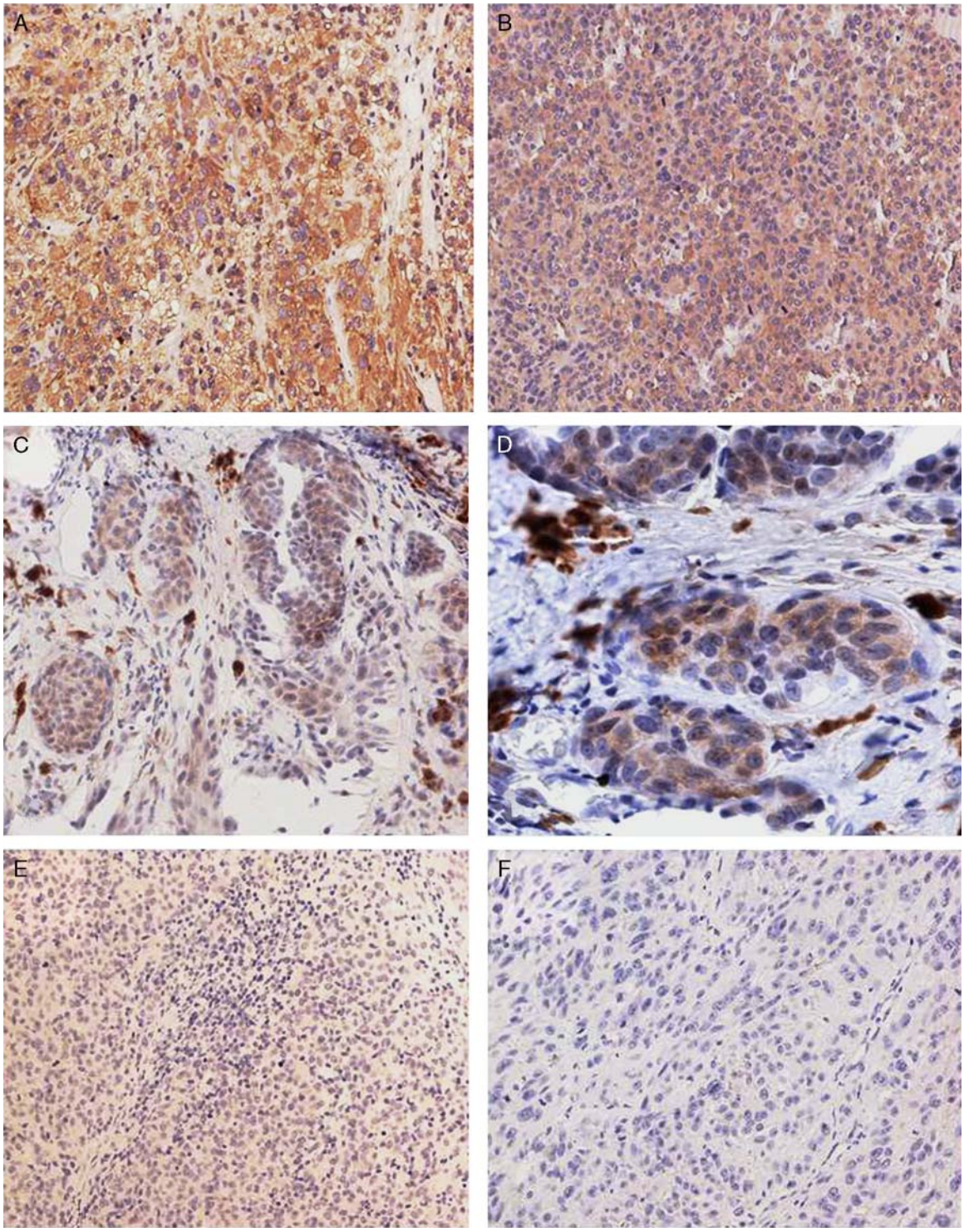

FIGURE 1. Immunohistochemistry with anti-*BRAF* p.V600E-specific VE1 antibody. p.V600E-mutated case with strong positive cytoplasmic staining of melanoma cells (A); p.v600E-mutated case with strong positive cytoplasmic staining of melanoma cells (B); p.V600R-mutated case with positive cytoplasmic staining 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).

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