Expression of BRAF V600E Mutant Protein in Epithelial Ovarian Tumors

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Background: Genetic analyses have identified BRAF V600E mutations in a subset of ovarian carcinomas. The aim of this study was to investigate the expression of BRAF V600E aberrant protein using a novel mutation-specific antibody in epithelial ovarian tumors.

Methods: We immunohistochemically analyzed expression of V600E-mutant BRAF protein in archival formalin-fixed, paraffin-embedded tissue specimens of 142 epithelial ovarian tumors [98 invasive carcinomas and 44 tumors of low malignant potential (LMP)] using monoclonal antibody VE1. *BRAF* mutation status was validated in all immunopositive cases and in 6 immunonegative control cases by gene sequencing.

Results: We found anti-BRAF V600E immunolabeling in 4 serous carcinomas and 5 serous LMP. Presence of a *BRAF* V600E mutation was confirmed by sequencing analysis in 6 of the 9 cases (3 LMP tumors, 3 low-grade carcinomas). In 2 partially VE1-positive tumors deriving from 1 patient (1 LMP and 1 contralateral invasive high-grade serous carcinoma), genetic analysis repeatedly resulted in *BRAF* wild-type, arguing for false-positive immunostaining results. One immunopositive case was repeatedly inconclusive in genetic analysis. In all 6 genetically confirmed cases, BRAF V600E mutant protein expression was homogenous throughout the tumor tissue.

Conclusions: We found *BRAF* V600E mutations in 13% (4/31) of serous LMP and 5% (3/62) of invasive serous carcinomas. No *BRAF* V600E mutations were detected in nonserous epithelial ovarian tumors. For reliable assessment of the *BRAF* V600E status in ovarian epithelial tumor samples, an integrated approach using immunohistochemistry and genetic analysis seems advisable, as both methods lead to incorrect results in some cases.

Key Words: ovarian carcinoma, BRAF, BRAF V600E, BRAF mutation, immunohistochemistry

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V-RAF murine sarcoma viral oncogene homolog B1 (BRAF) is a serine/threonine kinase involved in the mitogen-activated protein kinase (MAPK) pathway. Activating mutations of *BRAF*, most commonly (> 95%) of the V600E type, are found in a wide range of human tumors including melanoma, papillary thyroid cancer, hairy cell leukemia, and others.[1] The *BRAF* V600E mutation causes a substitution of valine by glutamic acid in the activating segment of the kinase domain of BRAF, thus leading to constitutive kinase activity.[2] Activation of downstream targets leads to increased proliferative and metastatic tumor activity, and several specific inhibitors of BRAF V600E-mutated protein have been developed. Most notably, the oral BRAF inhibitor vemurafenib showed remarkable increases of overall and progression-free survival in BRAF V600E-mutated metastatic melanoma and recently received approval by the US Food and Drug Administration.[3,4]

In epithelial ovarian tumors, genetic studies have identified *BRAF* mutations including V600E in up to 30% of serous tumors with low malignant potential (LMP) and low-grade serous carcinomas, whereas they are rare in high-grade serous carcinomas.[5–8] The Catalogue of Somatic Mutations in Cancer database specifies *BRAF* mutation rates of 4% for ovarian carcinomas (56 mutations in 1253 samples) and 34% for LMP tumors (172 mutations in 508 samples). Among all listed *BRAF* alterations, mutations of the V600E type constitute 39/53 (74%) in ovarian carcinoma and 169/172 (94%) in ovarian tumors of LMP.

We have recently introduced the mutation-specific monoclonal antibody VE1, which allows immunohistochemical detection of V600E-mutated BRAF protein with high sensitivity and specificity in formalin-fixed and paraffin-embedded (FFPE) tumor tissue samples.[9,10] Furthermore, we showed that BRAF V600E mutant protein is

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A.v.D. and D.C. declare shared inventorship of BRAF antibody clone VE1. A patent for diagnostic application of VE1 has been applied for. All terms are being managed by the German Cancer Research Center in accordance with its conflict of interest policies. The remaining authors declare no conflict of interest.
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generally expressed homogenously throughout the tumor tissue, although to date most analyzed samples represented metastatic lesions. Here, we utilized antibody VE1 to investigate BRAF V600E protein expression in a series of human epithelial ovarian tumors.

MATERIALS AND METHODS

Patients and Materials

Ninety-eight FFPE cases of epithelial ovarian carcinoma, FIGO stages I to IV were retrieved from our archive. Before therapy, patients were evaluated by clinical and ultrasound examination, chest x-ray, and computerized tomography or magnetic resonance imaging. Extensive surgery with total abdominal hysterectomy, bilateral salpingo-oopherectomy, pelvic lymph node dissection, and omentectomy, was performed in all patients. Surgery was followed by 6 cycles of a platinum/taxol containing adjuvant chemotherapy, except in patients with grade 1, stage IA tumors.

Response to chemotherapy was rated as described previously.[11] In brief: "No evidence of disease" was defined as the complete disappearance of all evidence of disease for at least 4 weeks, confirmed by physical examination, computed tomographic scan, and ultrasound. "Partial response" was defined as a $\geq 50\%$ decrease in the sum of the products of the diameters of measurable lesions for at last 4 weeks. "Stable disease" was defined as a steady state of response less than a partial response or progression $<25\%$ of at least 4 weeks duration. "Progressive disease" was defined as an increase of $\geq 25\%$ in the size of the measurable lesion or the appearance of a new lesion within 2 months after beginning of chemotherapy. Patients were followed at 3-month intervals.

In addition, 44 ovarian tumors of LMP (so called "borderline tumors") stage IA (limited to 1 ovary without rupture of the ovarian capsule) were investigated. In 1 patient an LMP tumor in 1 ovary and a serous carcinoma in the other was investigated (included in the above numbers).

Methods

Immunohistochemistry

We performed anti-BRAF V600E immunostaining using the novel monoclonal mouse antibody VE1 (antibody kindly supplied by A.v.D.) as described previously on 3-μm-thick tissue sections from FFPE tumor tissue blocks.[9,10] Scoring of immunostaining results was performed by 2 observers (M.P. and P.B.) blinded to all clinical, histopathologic and genetic data. Immunoreaction was scored positive, when viable tumor cells showed a nonambiguous cytoplasmic staining for VE1. A faint diffuse staining, any type of isolated nuclear staining, weak staining of single interspersed cells, or staining of monocytes/macrophages was scored negative.

Data on HIF-1α, p21, p53, p21, and bcl-2 expression and microvessel density (MVD) were available from a previous study for all cases.[11] Expression of HIF-1α, p21, and bcl-2 was scored as "negative," "weak," "moderate," and "strong," expression of p53 as "positive" or "negative" as described previously.[11] MVD was assessed by counting the field of view with the highest numbers of microvessels using a magnification of $\times 200$.

Polymerase Chain Reaction Amplification and Direct Sequencing

In 15 selected cases (all immunopositive or equivocal tumors and 6 negative control cases), we performed direct sequencing of *BRAF* exon 15 mutations for validation of immunostaining results. In all 15 cases, gene sequencing was performed at the Department of Pathology (Medical University of Vienna) as described previously using capillary sequencing and mutations were identified using SeqScape (Applied Biosystems).[12] In 2 LMP cases with low tumor cell content macrodissection was applied to separate tumor cells from non-neoplastic tissue parts and a second round of gene sequencing using the Quiagen V600 kit (QIAGEN GmbH, Düsseldorf, Germany) was performed. Two tissue blocks derived from a single patient (1 LMP and 1 contralateral serous carcinoma) showed heterogenous VE1 immunostaining. These 2 blocks were subjected to a second round of *BRAF* exon 15 sequencing after macrodissection using the Qiagen V600 kit in Vienna and a third round of sequencing (in one of the blocks with prior macrodissection of immunostained vs. nonimmunostained tissue parts) at the Department of Neuropathology (University of Heidelberg) as described previously.[9]

Statistical Analysis

χ^2 test and Mann-Whitney test were used when appropriate. Survival analysis was performed only in patients with invasive carcinoma. For statistical analyses, *BRAF* V600E mutation status was defined as follows: Cases with unequivocal V600E mutant protein expression were regarded as "BRAF V600E positive," also in cases in which genetic analysis was inconclusive. Cases with no or equivocal VE1 immunostaining result and without gene mutation were considered as "BRAF V600E negative." Overall survival (OS) was defined from the day of primary surgery until cancer death of the patient. Deaths from other cause than ovarian carcinoma or survival until the end of the observation period were considered as censored observations. Disease-free survival (DFS) was defined from the end of primary therapy until first evidence of progression of disease. Patients with progressive disease under primary chemotherapy were excluded from analysis of DFS.

Log-rank test was used for univariate analysis of survival. A 2-tailed P value of ≤ 0.05 was considered as significant

RESULTS

BRAF V600E Status

We found cytoplasmic anti-BRAF V600E immunolabeling of tumor cells in 4 serous carcinomas (3 low

grade, 1 high grade) and 5 serous LMP (Fig. 1). No staining of ovarian superficial epithelial cells or cells of the epithelium of the fallopian tube (if present) was seen in any case. In 4 of these cases, genetic analysis confirmed the presence of a *BRAF* V600E mutation and in 1 no evaluable DNA sequence could be generated. In 2 LMP cases with low tumor cell content, genetic analysis initially showed *BRAF* wild-type, but a second genetic analysis of macrodissected tumor cells lead to the identification of *BRAF* V600E mutations in both cases (Fig. 1C and D). Two tumors deriving from the same patient (1 LMP and 1 contralateral invasive high-grade serous carcinoma) showed heterogenous VE1 immunostaining of weak to moderate intensity (Fig. 1E). In both of these paraffin blocks genetic analysis showed *BRAF* wild-type in 3 independent runs (2 were done in Vienna and 1 in Heidelberg), arguing for false-positive immunostaining results. In all genetically confirmed cases, BRAF V600E mutant protein expression was homogenous throughout the tumor tissue. In 6 cases of serous invasive ovarian carcinoma with negative VE1 immunostaining result, genetic analysis confirmed *BRAF* wild-type. Table 1 shows the correlation of immunohistochemical and gene sequencing results and the correlation of BRAF V600E status with clinical parameters is shown in Table 2.

Cell Cycle–related and Hypoxia-related Proteins and Vascularization

The frequency of expression of bcl-2, HIF-1α, p21, p53 in invasive carcinomas and LMP is shown in Table 3. Mean MVD was 24 ± 17 (SD) microvessels/field.

Statistical Analyses

Although V600E mutations were only found in serous tumors, significance was missed when comparing frequency of V600E positivity in serous versus all other histologic subtypes ($P = 0.096$, Fisher exact test). No significant difference in V600E status was seen when comparing LMP versus invasive tumors, also when analyzing only serous tumors ($P > 0.05$, respectively, χ^2 tests).

In the subpopulation of invasive carcinoma patients, we observed a significant difference in FIGO stage between VE1 positive and negative cases ($P = 0.035$, Mann-Whitney test): whereas in VE1-negative cases the median FIGO stage was IIIB, it was IA in positive cases. We found no association of V600E status with patients' age, MVD, HIF-1α or p53 expression in this cohort ($P > 0.05$, Mann-Whitney test or χ^2 test, respectively). In serous ovarian carcinomas, V600E positivity was significantly more common in low grade compared with high-grade tumors ($P = 0.022$, Fisher exact test, Table 2).

There was a significant positive correlation of BRAF V600E expression with p21 expression in the entire cohort and in the subpopulation of LMP tumors, respectively. In both groups, median p21 expression score was "absent" in V600E-negative and "moderate" in V600E-positive tumors ($P = 0.008$ and 0.019, respectively, Mann-Whitney test).

When analyzing LMP and invasive carcinomas together, we observed a negative correlation of BRAF V600E status with bcl-2 expression ($P = 0.048$, Mann-Whitney test): Median BCL-2 score was "weak" in V600E-negative and "absent" in V600E-positive cases.

Survival Analysis

In patients with invasive carcinoma, the mean observation time was 37.4 ± 9.1 months. During this time period, 25 developed recurrent disease and 39 patients (39.8%) died from their carcinoma. A strong trend toward better DFS and OS in carcinomas with BRAF wild-type status was seen, which did not reach statistical significance (DFS: 0.304; OS: $P = 0.218$, log rank test; Fig. 2).

DISCUSSION

According to the dualistic model of ovarian carcinogenesis, ovarian carcinomas are divided into 2 broad categories (type I and II neoplasms) that form through distinct pathogenic pathways.[14] Type I tumors include low-grade serous carcinomas, oftentimes develop from LMP, characteristically show genetic alterations of the MAPK pathway and have a less aggressive clinical course.[15] In agreement with this model and with previous genetic studies *BRAF* V600E mutation was exclusive to serous LMP and serous carcinoma and we observed a significant association of *BRAF* V600E status with low FIGO stage in invasive carcinomas.[5–8]

BRAF inhibitors are emerging as novel and effective anticancer drugs. Vemurafenib was approved for the treatment of metastatic melanoma with *BRAF* V600E mutations and clinical trials with other BRAF inhibitors and in other patient populations are underway.[3,4] BRAF inhibitors may also be effective in some ovarian tumors, as drug-induced inactivation of the MAPK has been shown to induce growth inhibition and apoptosis in serous ovarian tumors harboring *BRAF* V600E mutations.[16] We show in the present study that BRAF V600E mutant protein is generally expressed homogenously throughout the tumor tissue in mutated cases, thus making it an attractive target for systemic targeted drugs. Such a therapeutic approach could be particularly interesting for low-grade serous carcinomas that have been shown to have lower response rates to conventional chemotherapy than high-grade serous carcinomas.[17] Of note, we found the *BRAF* V600E mutation in one of 15 cases of brain-metastatic serous ovarian carcinoma in a previous study.[10]

Our findings have general implications for clinical *BRAF* V600E testing. Obviously, correct identification of mutated and nonmutated cases is of paramount importance to avoid patient mistreatment with or without BRAF inhibitors. So far, testing of the BRAF status is performed using various DNA-based methods and a real-time polymerase chain reaction based assay to detect *BRAF* V600E mutations was FDA-approved for patient selection for vemurafenib therapy. Our data suggest that an integrated approach using anti-BRAF V600E

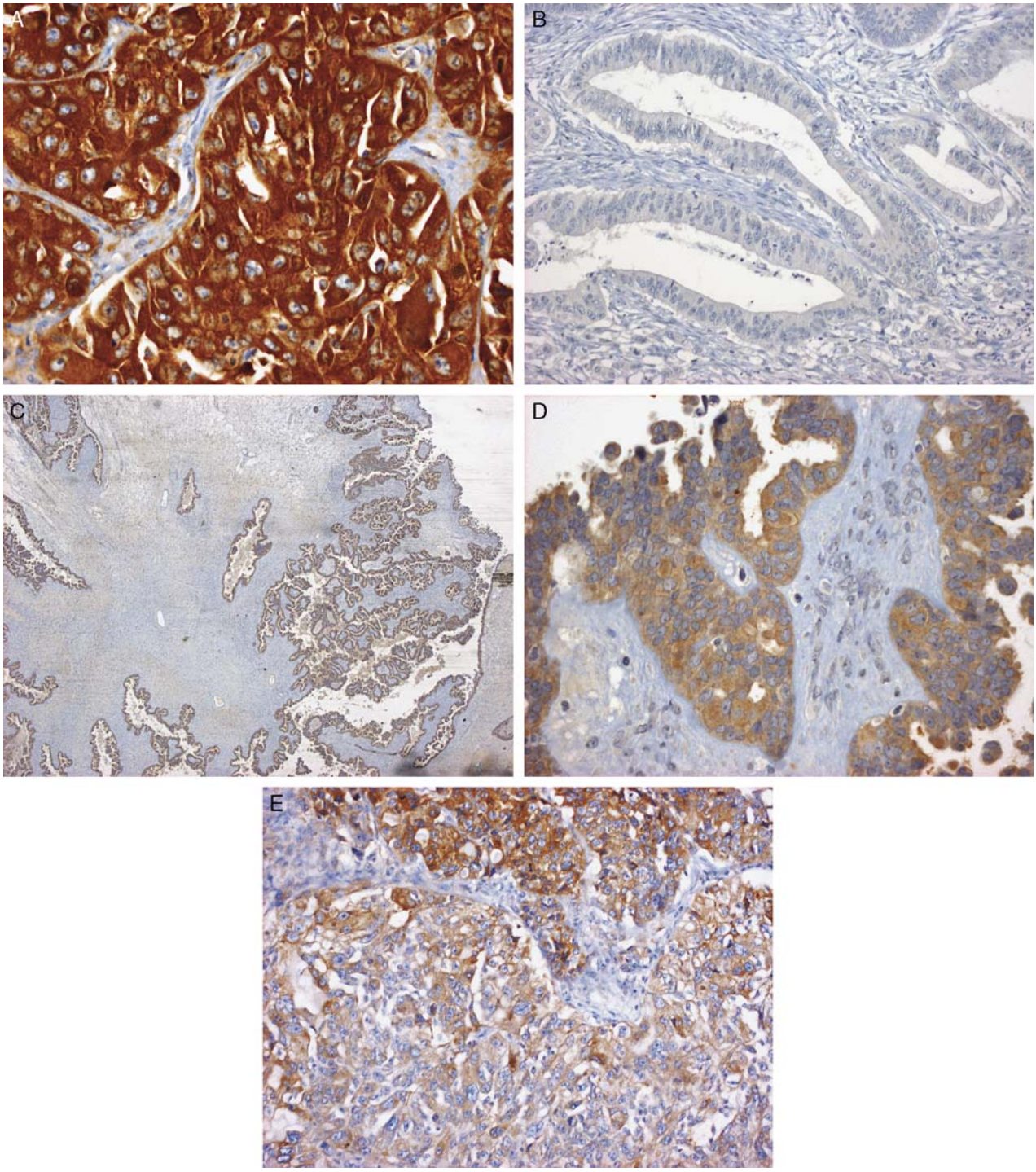

FIGURE 1. A, VE1 immunostaining showing strong and homogenous cytoplasmic labeling of tumor cells in a case of serous ovarian carcinoma with *BRAF* V600E mutation (original magnification ×400). B, Negative VE1 immunostaining result in a case of serous ovarian carcinoma with *BRAF* wild-type (original magnification ×200). C and D, In this case of LMP, VE1 immunostaining labeled tumor cells embedded in large areas of non-neoplastic tissue (C, original magnification ×25). Genetic analysis initially showed *BRAF* wild-type, but a second genetic analysis of macrodissected tumor (D, original magnification ×400) lead to the identification of a *BRAF* V600E mutation. E, This serous ovarian carcinoma showed heterogenous VE1 immunostaining of weak to moderate intensity (original magnification ×200). Genetic analysis repeatedly showed BRAF wild-type.

TABLE 1. Correlation of VE1 Immunostaining and *BRAF* Gene Sequencing Results

	BRAF V600E Mutation	*BRAF* Wild-Type	*BRAF* Sequencing Inconclusive
VE1 immunostaining negative		6	
VE1 immunostaining positive	6*	2†	1

*All these cases showed homogenous VE1 immunostaining throughout the tumor tissue. In 2 of these 6 cases, genetic analysis initially showed *BRAF* wild-type, but a second genetic analysis of macrodissected tumor cells lead to the identification of *BRAF* V600E mutations in both cases.
†These cases showed inhomogenous VE1 immunostaining and repeated gene sequencing showed *BRAF* wild-type.

immunostaining in addition to a DNA-based method may increase the diagnostic accuracy, as none of the techniques has perfect analytical performance. DNA-based analysis may incorrectly indicate *BRAF* wild-type (false-negative results) in samples with high content of non-neoplastic DNA such as cases with small tumor cell formations embedded in surrounding tissue parts. Separation of tumor cells from surrounding tissues by macrodissection or microdissection may increase the accuracy of DNA-based testing, but this is currently not feasible in the routine clinical setting. Another problem is that impaired DNA quality due to tissue processing (FFPE) or mixture with necrotic tissue may lead to noninterpretable results of DNA-based analysis. We show that in at least a proportion of cases with false-negative or inconclusive gene sequencing results, VE1 immunostaining is able to label BRAF V600E-expressing tumor cells. Therefore, anti-BRAF V600E immunohistochemistry may allow identification of patients eligible for BRAF-inhibiting therapies that would have been missed based on DNA-based analysis alone. In contrast, interpretation of VE1 immunostaining has also some caveats. First, VE1 immunostaining is not able to identify rare non-V600E mutations including the *BRAF* V600K mutation, which seems to also confer sensitivity to BRAF-inhibiting drugs. In our present study we show that in single cases VE1 may produce an unspecific immunostaining reaction, possibly leading to false-positive results. In contrast to the cases with confirmed *BRAF* V600E mutation at the genetic level, the immuno-positive cases with wild-type *BRAF* showed an inhomogenous staining reaction, which may help to identify false-positive cases. In addition, as discussed previously focal weak or absent VE1 immunostaining in otherwise immunoreactive tumor tissue samples may be observed in areas of beginning necrosis (prenecrosis) and in relation to artificial tissue damage.[9] It is important to note that sections have to be freshly cut from FFPE blocks for optimal VE1 immunostaining results, as we observed marked decrease of VE1 immunostaining intensity in tissue sections stored for longer than a few weeks.[9,10]

Interestingly, we found a significant positive correlation of BRAF V600E expression with expression of p21 and an inverse correlation of BRAF V600E expression with bcl-2 expression. It must be noted, however, that the

TABLE 2. Clinical Characteristics of 142 Patients With Epithelial Ovarian Tumors

Factor	No. Cases	BRAF V600E Positive (Unequivoval Protein Expression)
Invasive carcinoma (n = 98)		
FIGO stage		
IA	14 (14.3%)	2 (14.3%)
IC	14 (14.3%)	0
IIA	10 (10.2%)	1 (10%)
IIB	4 (4.1%)	0
IIC	2 (2%)	0
IIIA	2 (2%)	0
IIIB	12 (12.2%)	0
IIIC	33 (33.7%)	0
IV	7 (7.1%)	0
Histologic grading serous carcinomas (n = 62)[13]		
Low grade	15 (25.4%)	3 (16.7%)
High grade	44 (74.6%)	0
Histologic grading nonserous carcinomas (n = 36)		
G1	12 (33.3%)	0
G2	9 (25%)	0
G3	15 (41.7%)	0
Histologic type		
Serous	62 (63.3%)	3 (4.8%)
Mucinous	13 (13.3%)	0
Endometroid	16 (16.3%)	0
Clear cell	7 (7.1%)	0
Residual tumor after primary surgery		
None	44 (44.9%)	2 (4.5%)
$< 2\,cm^3$	17 (17.3%)	1 (5.9%)
$\geq 2\,cm^3$	37 (37.8%)	0
Tumors of low malignant potential (n = 44)		
Serous	31 (70.5%)	4 (12.9%)
Mucinous	13 (29.5%)	0

TABLE 3. BRAF V600E Status in Relation to Expression of Cell Cycle–associated and Hypoxia-associated Proteins

Protein	Invasive Carcinoma V600E + /Cases	LMP (Borderline) V600E + /Cases
Bcl-2		
Absent	2/47 (4.3%)	4/26 (15.4%)
Low	1/20 (5%)	0/5 (11.4%)
Moderate	0/18	0/8 (18.2%)1
Strong	0/13	0/5 (11.4%)
HIF-1α		
Absent	1/31 (3.2%)	0/5
Low	1/20 (5%)	2/12 (16.7%)
Moderate	1/23 (4.3%)	2/14 (14.3%)
Strong	0/24	07 13
P21		
Absent	2/72 (2.8%)	0/24
Low	0/9	0/5
Moderate	1/16 (6.3%)	4/14 (28.6%)
Strong	0/1	0/1
P53		
Negative	3/57 (7.5%)	3/40 (7.5%)
Positive	0/41	1/4 (25%)

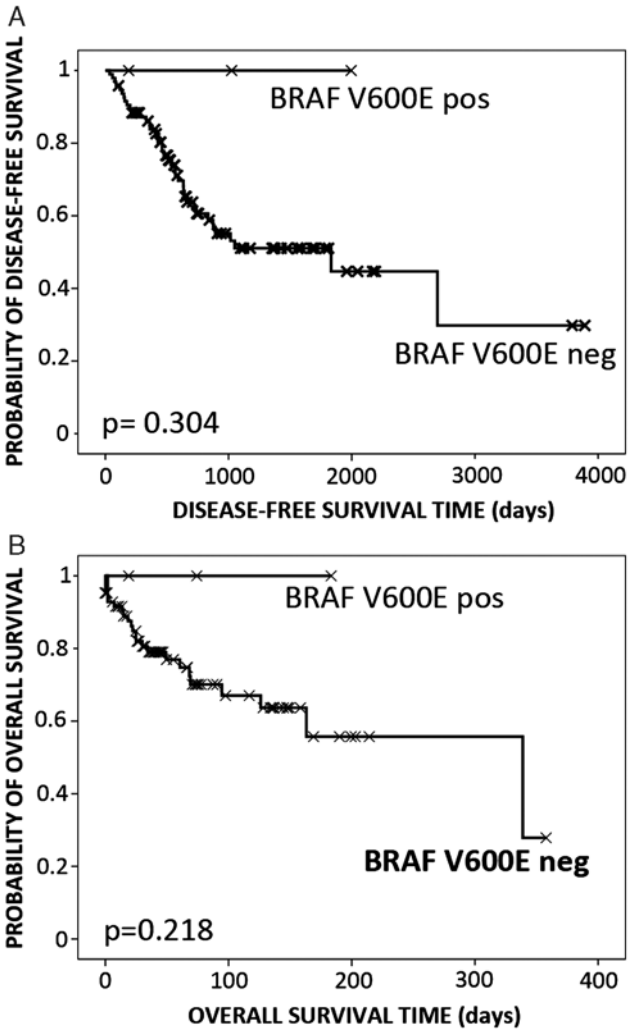

FIGURE 2. Kaplan-Meier curves showing the correlation of BRAF V600E status with disease-free survival (A) and overall survival (B) in our cohort of patients with invasive ovarian carcinoma.

power of our statistical correlations is very limited by the small number of V600E mutated ovarian tumors. Still, in line with our findings high p21 promoter activity and expression in response to BRAF V600E signaling by MEK and ROS-dependent phosphorylation of FOXO4 has been identified in melanoma cells.[18] This cascade has been proposed to participate in the regulation of oncogene-induced senescence response. Our data may suggest a potential role of this mechanism in the pathogenesis of *BRAF* V600E-mutated ovarian tumors. Further studies are needed to clarify the role of the cell cycle–associated protein p21 and bcl-2 in their interaction with BRAF in ovarian tumors.

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