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BRAF mutation is associated with a specific cell-type with features suggestive of senescence in ovarian serous borderline (atypical proliferative) tumors

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Abstract

Serous borderline tumor (SBT) also known as atypical proliferative serous tumor (APST) is the precursor of ovarian low-grade serous carcinoma (LGSC). In this study, we correlated the morphologic and immunohistochemical phenotypes of 71 APSTs and 18 LGSCs with the mutational status of *KRAS* and *BRAF*, the most common molecular genetic changes in these neoplasms. A subset of cells characterized by abundant eosinophilic cytoplasm (EC), discrete cell borders and bland nuclei was identified in all (100%) 25 *BRAF* mutated APSTs but in only 5 (10%) of 46 APSTs without *BRAF* mutations ($p<0.0001$). Among the 18 LGSCs, EC cells were found in only 2 and both contained *BRAF* mutations. The EC cells were present admixed with cuboidal and columnar cells lining the papillae and appeared to be budding from the surface, resulting in individual cells and clusters of detached cells "floating" above the papillae. Immunohistochemistry showed that the EC cells always expressed p16, a senescence-associated marker, and had a significantly lower Ki-67 labeling index than adjacent cuboidal and columnar cells ($p=0.02$). *In vitro* studies supported the interpretation that these cells were undergoing senescence as the same morphologic features could be reproduced in cultured epithelial cells by ectopic expression of $BRAF^{V600E}$. Senescence was further established by markers such as SA-β-gal staining, expression of p16 and p21, and reduction in DNA synthesis. In conclusion, this study sheds light on the pathogenesis of this unique group of ovarian tumors by showing that *BRAF* mutation is associated with cellular senescence and the presence of a specific cell type

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characterized by abundant eosinophilic cytoplasm. This “oncogene-induced senescence” phenotype may represent a mechanism that prevents impedes progression of APSTs to LGSC.

Introduction

Serous carcinoma, the most common and lethal ovarian cancer, is composed of two types, low-grade serous carcinoma (LGSC) and high-grade serous carcinoma (HGSC), which are characterized by distinctly different clinicopathological and molecular features [1, 23–5]. It has been proposed that the immediate precursor of many HGSCs is an intraepithelial carcinoma in the fallopian tube, so-called “serous tubal intraepithelial carcinoma”, whereas the immediate precursor of most LGSCs is a noninvasive ovarian LGSC, also termed “serous borderline tumor, micropapillary variant”. The latter develops from a serous borderline tumor (SBT) also known as “atypical proliferative serous tumor (APST)”. While the majority of SBTs behave in a benign fashion, approximately 5% progress to LGSC which has a poor outcome for those with measurable disease after cytoreductive surgery [6]. At present, there are no markers that reliably predict progression to LGSC and some pathologists, therefore, prefer the designation SBT to draw attention to this possibility, whereas other pathologists prefer the designation APST to emphasize the benign nature of most of these tumors recognizing that some benign tumors have the potential to progress to malignant neoplasms. The recent WHO Classification of Tumors of the Female Reproductive Organs considers both terms synonymous [7]. In this manuscript the term APST is used.

In view of the uncertainty regarding the behavior of APSTs, patients and their physicians face a difficult dilemma in planning subsequent management, particularly for those women who present with advanced stage disease, as even the majority of these tumors do not progress to LGSC. The options are adjuvant chemotherapy, with its attendant potential complications, versus observation. In either case the anxiety associated with the uncertainty of the behavior of this tumor takes a significant emotional toll on the patient and her family. Accordingly, identification of a marker that reliably predicts outcome would be highly beneficial.

APST and LGSC are characterized by very low levels of DNA copy number changes as compared to other gynecologic tumors, reflecting relative genomic stability during tumor evolution[8, 9]. The most prominent molecular alterations so far described are somatic activating mutations of *KRAS* and *BRAF*, leading to their constitutive kinase activation. Approximately two thirds of APSTs contain either mutant *KRAS* or *BRAF* as the mutations are mutually exclusive[10–12]. Since *KRAS* and *BRAF* are involved in the MEK signaling pathway, it is believed that aberrant signaling activity of MEK, due to activating mutations in either *KRAS* or *BRAF*, plays a major role in the development of most APSTs[13].

We have previously identified a population of cells in APSTs with abundant eosinophilic cytoplasm (EC) that showed a significant decrease in steroid hormone receptors (ER and PR), WT 1 and Ki-67 proliferation index compared to neighboring cuboidal and columnar cells lacking abundant eosinophilic cytoplasm, suggesting that the EC cells were senescent[12,14]. The current study presents immunohistochemical and molecular genetic

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evidence showing that the EC cells occur preferentially in tumors harboring mutant *BRAF*. Moreover, *in vitro* studies demonstrated that ectopic expression of $BRAF^{V600E}$ in epithelial cells induces cellular senescence, thereby providing compelling evidence that APSTs with mutant *BRAF* are undergoing senescence and that EC cells are a useful morphologic marker.

Materials and Methods

Identification and selection of cases

The study group consists of 89 cases of APSTs (n=71) and LGSC (n=18) derived from two study sets. Most APSTs (n=49) were selected from the files of the nationwide Danish Pathology Data Bank as previously described [12]. The study was approved by the Danish Data Protection Agency and the Danish Scientific Ethical Committee. The remaining cases (22 cases of APST and 18 LGSC) were obtained from the pathology files of the Johns Hopkins Hospital. Acquisition of tissues specimen was approved by Institutional Review Board at the Johns Hopkins Hospital, Baltimore, Maryland. In addition to the primary ovarian tumor, sufficient tumor tissue was available from implants or metastatic lesions for 40 APSTs and 4 LGSCs. Bilateral APSTs were analyzed in 11 cases. This resulted in a total of 160 tumor lesions that were examined. Of the 71 APSTs 56 were advanced stage (FIGO II–IV), 6 were FIGO stage I and for 9 cases the information about FIGO stage was not accessible.

Morphologic evaluation and immunohistochemistry analysis

Sections of all 160 lesions were reviewed independently by two investigators (IMS, FZ) who were blinded to the clinical and mutational data. The number of cells with abundant eosinophilic cytoplasm (as previously described[12, 14]) was semi-quantitatively scored as a percentage of the total number of epithelial cells in a specimen as follows: not detectable (n.d.), <1 %, 1–25 %, and >25 % of epithelial tumor cells. Only cases with a sufficient amount of tumor cells (at least 400 tumor cells) were included. A subset of cases was selected for further immunohistochemical analysis. Sections were stained with anti-Ki-67 antibody (Ventana Medical Systems, Tucson, AZ; prediluted) and an anti-p16 antibody (Ventana Medical Systems, prediluted) and counterstained with either hematoxylin or eosin. The Ki-67 proliferation index was scored in a similar semi-quantitative manner by counting at least 400 cells and determining the percentage of positive cells. The EC and cuboidal/columnar cell populations were counted separately. p16 immunoreactivity in EC cells was reported as positive (both nuclear and cytoplasmic) or negative. Immunostaining with the mouse monoclonal VE1 antibody was used to detect mutant BRAF protein (V600E) as described previously (Spring Bioscience, Pleasanton, California, USA; 1:250) [12]. It has been shown that APSTs with BRAF V600E mutations were all positive for VE1 immunoreactivity while none of the wild-type BRAF specimens were positive for VE1[12]. VE1 immunohistochemistry was applied to determine the BRAF mutation status in six cases of which the mutational analysis was unsuccessful.

Mutational analysis

Mutational analysis was performed using the conventional Sanger sequencing technique in *KRAS* at exon 2 including codons 12–13 and *BRAF* at exon 15 including codon 600 as

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previously described[11]. PCR amplification was performed using genomic DNA from micro-dissected FFPE tissue with the following primers for exon 15 of *BRAF*: forward 5′-TGCTTGCTCTGATAGGAAAATGA-3′ and reverse 5′-CCACAAAATGGATCCAGACAAC-3′; for exon 2–3 of *KRAS*: forward 5′-TAAGGCCTGCTGAAAATGACTG-3′ and *KRAS* reverse 5′-TGGTCCTGCACCAGTAATATGC-3′. Amplified PCR products were sequenced at the Beckman Coulter, Inc., (Danvers, MA, USA) and analyzed with the Mutation Surveyor DNA Variant Analysis Software. Mutational analysis was successful in 83 cases. In additional six APSTs, that could not be analyzed for their mutational status, positive BRAF VE1 staining was used as a surrogate for $BRAF^{V600E}$ mutation.

Cell culture and Transduction—Primary human ovarian surface epithelial cells were prepared from ovaries obtained during prophylactic oophorectomies as described previously[15]. The cells were cultured in medium 199 and MCDB-105 (1:1) supplemented with 4% fetal bovine serum and 0.2 units/ml of insulin[16]. pBABE-puro and pBABE-puro-$BRAF^{V600E}$ were obtained from Addgene (Cambridge, MA). Retrovirus production and transduction were performed as described[17], using Phoenix cells to package the viruses (a gift from Dr. Gary Nolan, Stanford University). Cells infected with viruses encoding drug resistant gene to puromycin were selected in 1μg/ml. FG12-CMV-Lenti-GFP and FG12-CMV-Lenti-GFP-$BRAF^{V600E}$ were kindly provided by Dr. Daniel Peeper. Lentivirus was packaged using Virapower Kit from Invitrogen following manufacturer's instruction as described previously[18].

Immunofluorescence, BrdU labeling and SA-β-gal staining—The following antibodies were obtained from indicated suppliers: mouse anti-BrdU FITC (BD Bioscience), mouse anti-p21 (Santa Cruz), mouse anti-BRAF (Santa Cruz), mouse anti-β-actin (Abcam)[19], and mouse anti-p16 was a gift from Dr. Greg Enders. Immunofluorescence staining and BrdU labeling for cultured cells were performed as previously described using the antibodies listed above[1618]. SA-β-Gal staining was performed as previously described[20].

Statistical analysis

All calculations were done using the statistical software environment R, version 3.1.0 and Graph Pad Prism Version 5.0. Statistical significance was set at the level of 0.05.

Results

Morphologic examination of 71 APSTs including 25 with *BRAF* mutations, 29 with *KRAS* mutations and 17 with wild-type *BRAF* and *KRAS* revealed a population of epithelial cells containing abundant eosinophilic cytoplasm and discrete cell borders (EC) cells. The nuclei tended to be round with a smooth nuclear membrane and contained faintly staining chromatin. Mitotic figures were not observed in these cells. These cells were adjacent to cuboidal and columnar cells with round to oval nuclei in which the nuclear chromatin was more distinct and coarser and which were devoid of abundant eosinophilic cytoplasm. The EC cells appeared to bud from the overlying epithelial layer of the APST (Fig. 1). They were often detached from the epithelium, floating freely above the epithelial layer of the

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tumor and in one case in the underlying stroma of the tumor, so-called “microinvasion”. Using >1% EC cells as a cut point, it was found that they were present in all 25 APSTs harboring mutant *BRAF*, in 4 (14%) of 29 APSTs with *KRAS* mutation and in one (6%) of 17 APSTs with wild-type *KRAS* and *BRAF* (Table 1). The mutation status of APST according to clinical stages is summarized in Table 2. Because mutations of *KRAS* and *BRAF* are mutually exclusive, we combined the mutant *KRAS* and wild type *KRAS/BRAF* groups into one group, designated the “*BRAF* wild-type” group, and observed that EC cells were significantly more often present in the *BRAF* mutated than the wild-type group ($p< 0.0001$, Fisher’s exact test, two-tailed).

Immunohistochemical staining revealed that the EC cells in contrast to the adjacent cuboidal/columnar cells were intensely stained for both nuclear and cytoplasmic p16, a senescence-associated marker[21, 22] and were essentially negative for Ki-67, a proliferative marker (Fig. 2 and Fig. 3). EC cells had a significantly lower Ki-67 index than the cuboidal/columnar cells ($p=0.02$, paired two-tailed t-test). Occasional apoptotic cells adjacent to EC cells were also observed (Fig. 2B). They were positive for M30 staining, an apoptotic marker.

In our previous study, almost all primary APSTs and their concurrent implants were found to have the same mutation status of *KRAS* and *BRAF* [12]. In this study, there were 40 APSTs with at least one concurrent peritoneal implant that were available for study. Among them, 37 (92.5%) showed the same morphologic features in the primary tumor and implant in terms of the presence or absence of EC cells. Similar concordance of EC cells was observed in 10 (91%) of 11 bilateral APSTs. Two implants, one with many EC cells positive for VE1 and another without EC cells negative for VE1 are shown in Fig. 4. Interestingly, the cases in which there was discordance of EC cells between the primary tumors and implants occurred only in tumors and implants with mutant *KRAS* (3 of 23 cases). Those discordant cases include two with EC cells in the primary tumors but not in the implants and one case in which the primary tumor lacked EC cells, but they were present in the implants. All the cases with mutant *BRAF* and wild-type *KRAS* and *BRAF* showed concordant morphologic features in both the primary tumor and implants (17 of 17 cases), i.e. presence of EC cells in mutant *BRAF* cases and absent EC cells in both primary tumor and corresponding implant(s) in cases wild-type for *KRAS* and *BRAF*.

The above findings suggest that mutant BRAF protein may induce changes resulting in the development of EC cells that we interpreted on morphologic grounds as evidence of cellular senescence. To test this hypothesis, we ectopically expressed mutant $BRAF^{V600E}$ in human ovarian surface epithelial (HOSE) cells (Fig. 5). HOSE cells expressing $BRAF^{V600E}$ had a greater percentage of cells that were positive for SA-β-gal stain, a conventional marker of cellular senescence, than the control cells (27% vs. 3%). Immunofluorescent staining, also demonstrated that compared to control cells, HOSE cells expressing $BRAF^{V600E}$, had a much greater number of cells positive for p16 and p21, two proteins that are upregulated during cellular senescence. In contrast, in a BrdU incorporation study $BRAF^{V600E}$ transfected cells showed a decrease in DNA synthesis as compared to control cells (Fig. 5).

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Discussion

Cellular senescence is a fundamental biological phenomenon involved in embryogenesis, tissue development and various pathobiological conditions [23]. While it has been extensively studied in cell culture, microscopic characterization of senescence in tissue sections has not been well described. In this study, we identified a population of epithelial cells in APSTs characterized by abundant eosinophilic cytoplasm (EC), distinct cell borders, nuclei with faintly staining chromatin, strong expression of p16 and loss of proliferative activity as evidenced by essentially no Ki-67 immunoreactivity. These features are commonly seen in senescent cells *in vitro*. The EC cells, along with cuboidal and columnar cells, comprise the cellular population that lines the papillae of the APSTs. The latter two cell types have relatively scant cytoplasm, bland, round to oval nuclei and are frequently ciliated. The EC cells tend to be rounder, less frequently ciliated and are closely apposed to the cuboidal and columnar cells. When there is cellular stratification they tend to be at a slightly higher level than the cuboidal/columnar cells, approaching the surface of the papillae and then detaching, acquiring more abundant eosinophilic cytoplasm and losing their cilia in the process. As a consequence they appear to “float” above the papillae and occasionally are present in the underlying stroma, so-called “microinvasion”. Mitotic figures are present only in the cuboidal and columnar cells. Occasional apoptotic cells adjacent to the EC cells are also evident. The entire process appears to reflect senescence manifested by transformation of cuboidal and columnar cells to EC cells. This interpretation is supported by a previous study in which we demonstrated that compared to the cuboidal and columnar cells, the EC cells showed a significant decrease in ER, PR, WT1 and Ki-67 labeling index [14]. In the present study we found that the EC cells are specifically associated with *BRAF* mutations as they were detected in 100% of APSTs with *BRAF* mutations but in only 10% of APSTs with wild-type *BRAF* and *KRAS* or *KRAS* mutations, suggesting that they are a morphologic marker of *BRAF* mutation in these tumors. The above finding was confirmed by our *in vitro* cell culture studies showing that mutant *BRAF* leads to the development of senescent cells with similar morphologic features. Expression of p16 and absence of Ki-67 labeling has been previously reported in senescent cells in colorectal adenomatous polyps with *BRAF* mutations[22].

The findings in this study are reminiscent of “oncogene-induced senescence”, a well-established mechanism that impedes cancer development in humans[24, 25] in which oncogenic stress induced by an oncogenic protein such as mutant BRAF protein leads to DNA double strand breaks in non-transformed epithelial cells. This activates the DNA damage response that is responsible for growth inhibition and induction of senescence through activation of p53-p21 and p16 pathways[21, 26–29]. As *TP53* mutations are exceptionally rare in APSTs, the intact p53 protein may serve as the upstream checkpoint control that triggers the senescence pathway in *BRAF* mutated APSTs.

Our study raises a number of intriguing questions. First, if *BRAF* mutations result in cellular senescence in APSTs, why is this genotype clonally selected in many of them? We postulate that *BRAF* mutation, like *KRAS* mutation, is required for tumor initiation, because mutations in both genes have multiple tumor-promoting effects including upregulation of glucose transporter-1 (GLUT1), an essential surface protein that results in an increase in glucose

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metabolism required for tumor transformation [27]. Once an APST develops, the epithelial cells may initiate a mechanism to restrain tumor progression as occurs in many benign human neoplasms, including colorectal adenomatous polyps and melanocytic nevi[30–32]. This may account for why *BRAF* mutated advanced stage LGSCs are much less common than *BRAF* mutated advanced stage APSTs [33341235].

Second, how do tumors that progress to LGSC overcome the senescence induced by *BRAF* mutations? We suspect that as with the progression of precursor lesions to frankly malignant tumors, for example as occurs in melanoma and lung cancer[36, 37], additional molecular genetic alterations occur during progression from an APST to an LGSC, that abolish the checkpoint control by the p53-p21 and p16 pathways. We have previously analyzed the genome-wide copy number alterations in ovarian low-grade serous neoplasms and reported that hemizygous ch1p36 deletion and ch9p21 homozygous or hemizygous deletions are much more common in ovarian LGSCs than in APSTs[8]. The ch1p36 region contains several candidate tumor suppressors including miR-34a which is required for DNA damage response and is the direct p53 target that mediates its tumor suppressor functions[38, 39]. Likewise, the ch9p21 region corresponding to the *CDKN2A/B* locus encodes three well-known tumor suppressor proteins, p14 (Arf), p16 and p15 that inhibit cyclin-dependent kinase. Thus, deletions or silencing of miR-34a and *CDKN2A/B* loci may abrogate the p53 checkpoint on *BRAF* mutations and permit APSTs to escape senescence and progress to LGSC. In this regard, it has been reported that p16 expression levels are significantly decreased in LGSCs as compared to APSTs[40]. Finally, why are EC cells more commonly detected in *BRAF* mutated than *KRAS* mutated APSTs? It has been demonstrated that KRAS and BRAF kinases may form their own unique signaling networks in addition to the shared function in activating MEK signaling[41–43]. Therefore, in APSTs it is likely that although mutations of *KRAS* and *BRAF* contribute equally to tumor initiation, they may have different functions after the tumor has developed. Tumors with mutant *BRAF* undergo senescence, characterized by the presence of EC cells, whereas APSTs with mutant *KRAS* or wild type *BRAF* and *KRAS* are less likely to undergo senescence and therefore do not express the EC phenotype. Furthermore, after cytoreductive surgery occult implants harboring mutant *BRAF* are likely to undergo cellular senescence and have a reduced risk for recurrence and progression. In conclusion, this study provides new evidence that mutation of *BRAF* appears to have a paradoxical effect, acting as a driver mutation for initiation of an APST while also apparently inhibiting progression to LGSC. This finding sheds light on the pathogenesis of this unique group of ovarian tumors which has perplexed pathologists and gynecologists for over half a century. The study also has important clinical implications by correlating a specific morphologic phenotype, namely, EC cells with *BRAF* mutation, thereby providing pathologists with the ability to identify a group of APSTs that are likely to behave in a benign fashion from those that are more likely to progress to LGSC. This would allow gynecologists to target a group of patients for close surveillance and possibly further treatment while sparing another group of patients additional, and potentially harmful treatment. Although highly provocative, our conclusions must be validated by larger clinicopathologic studies.

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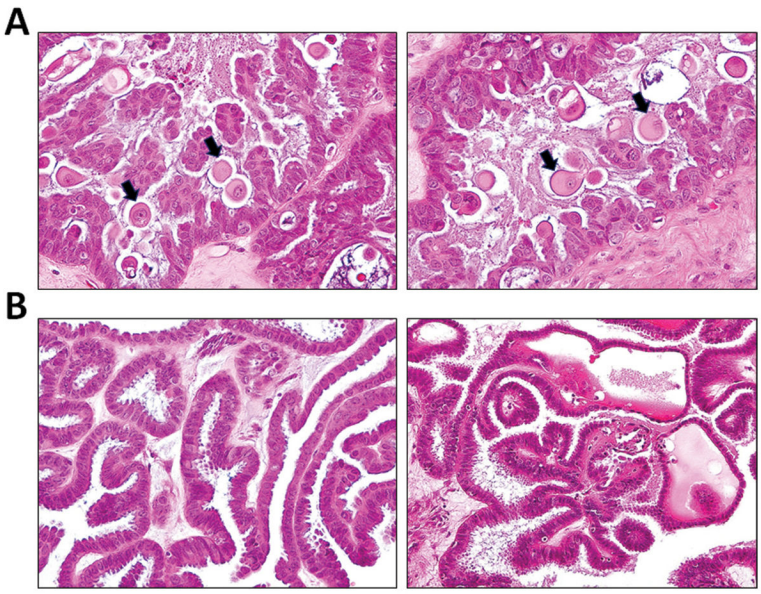

Fig. 1.

Fig. 1A. Eosinophilic cells (EC) in an APST with mutant *BRAF*^{V600E} are present on the surface of papillae along with cuboidal and columnar cells. Many EC cells are detached (arrows), floating above the surface of papillae. They are characterized by abundant eosinophilic cytoplasm and discrete cell borders. The nuclei are mostly round with a smooth nuclear membrane and contain faintly staining chromatin. EC cells appear to bud from cuboidal/columnar epithelial cells on the surface of the papillae. **1B.** Another APST without *BRAF* mutation does not demonstrate any EC cells.

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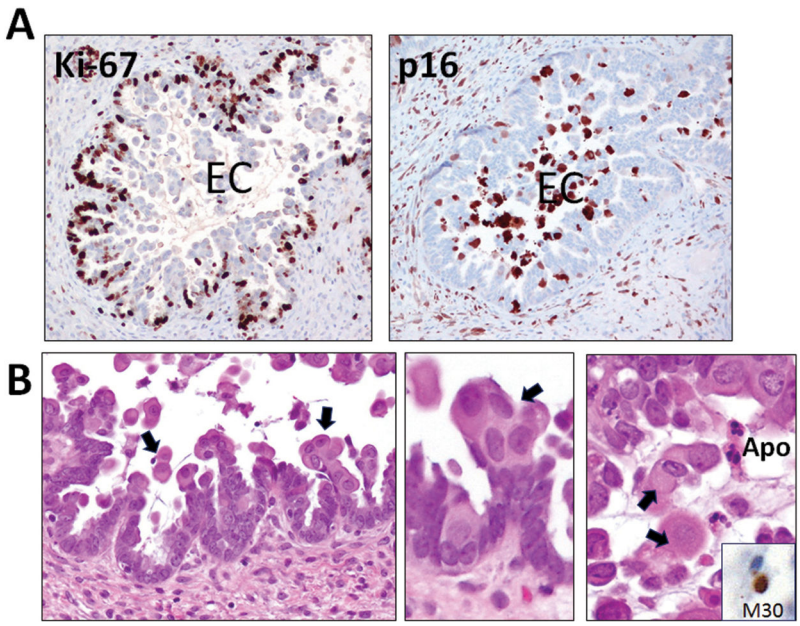

Fig. 2.

An APST harboring mutant *BRAF*^{V600} showing expression of Ki-67 and p16 **A.** EC cells tend to be Ki-67 negative and strongly and diffusely p16 positive. **B.** EC cells (arrows) showing senescent features characterized by nuclei with washed out indistinct chromatin (arrows). Apoptotic cells (Apo) are positive for an apoptotic marker, M30.

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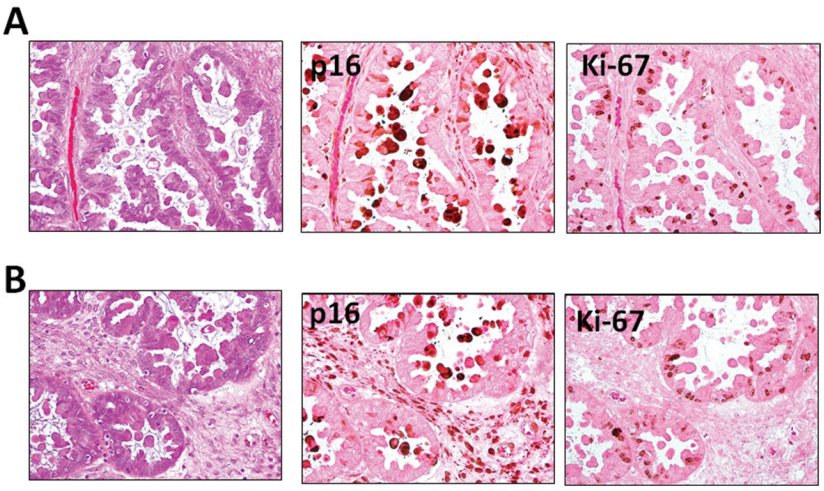

Fig. 3.

Immunostaining pattern of p16 and Ki-67 in an APST. EC cells that are positive for p16 and are devoid of Ki-67 immunoreactivity. A and B represents two different areas from the same case and the slides were counterstained with eosin only to reveal the abundant cytoplasm in EC cells.

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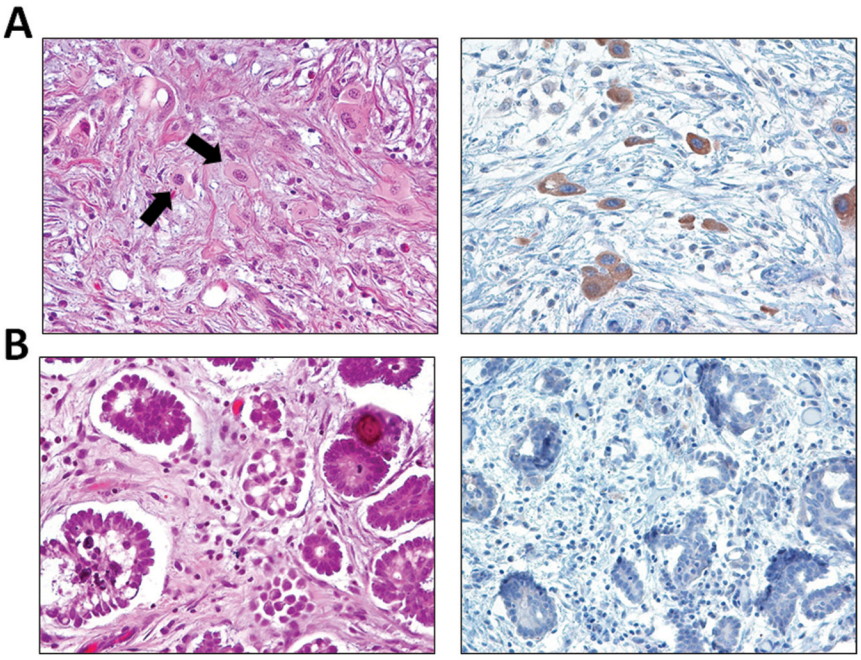

Fig. 4.

A. VE1 positive desmoplastic non-invasive implant contains many EC cells, identical to the EC cells in foci of “microinvasion” (arrows). **B.** VE1 negative implant does not contain EC cells.

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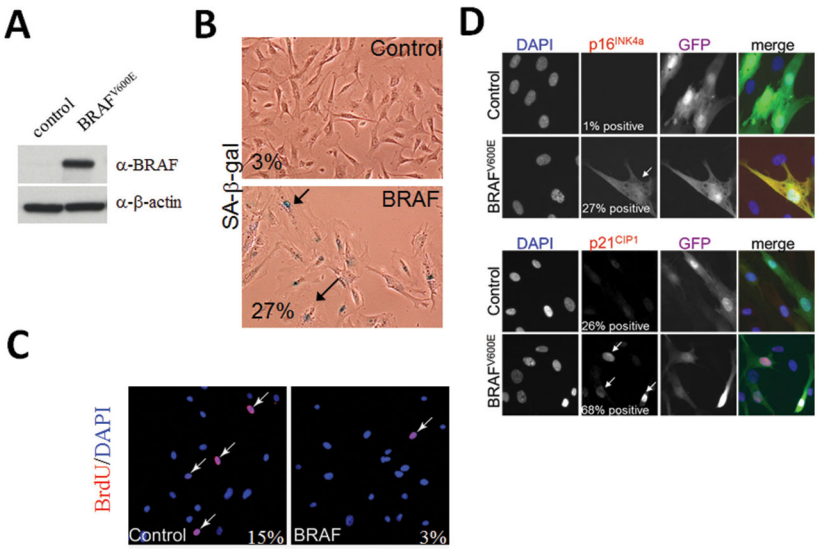

Fig. 5.

A. Western blot analysis shows expression of mutant BRAF protein in primary human ovarian surface epithelial (HOSE) cells transduced with the *BRAF*^{V600E} expressing construct. **B.** SA-β-gal activity in primary HOSE cells expressing *BRAF*^{V600E}. Arrows point to large, flat SA-β gal positive senescent cells. **C.** BrdU incorporation is decreased in primary HOSE cells expressing *BRAF*^{V600E} as compared to control non-transduced cells. Arrows point to BrdU positive cells (BrdU in red and cell nuclei counterstained with DAPI in blue). **D.** Expression of *BRAF*^{V600E} in HOSE cells in culture results in an increase in p16 and p21. Cells were engineered to co-express nuclear GFP when they were transfected with the expression plasmids. The fluorescence images were merged in the rightmost panel.

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Table 1

Summary of APST cases showing cells with abundant eosinophilic cytoplasm (EC).

Mutational Status		EC cells present	EC cells absent	Total
BRAF-mut[a]	*	25	0	25
BRAF-wt		5	41	46
KRAS-mut		(4)	(25)	(29)
BRAF/KRAS-wt		(1)	(16)	(17)

*P< 0.0001, Fisher's exact test, two-tailed

[a]for 6 APST, *BRAF* mutation was assumed after mutational analysis failed, but VE1 IHC stained positive

mut: mutant, wt: wild-type

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Table 2

Mutational status and FIGO stage of APST (n=71)

		BRAF-Mut	KRAS-Mut	WT
	n.a.	7	1	1
	I	6	-	-
FIGO STAGE	II	5	12	5
	III	6	16	11
	IV	1	-	-

Mut: somatic mutation; WT: wild-type KRAS and BRAF; n.a. not accessible.