

BRAF mutational analysis in ovarian tumors: recent perspectives

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Abstract: *BRAF* mutations are rare in ovarian cancer and mainly occur in indolent serous borderline tumors (SBTs), also known as serous tumors of low malignant potential or atypical proliferative serous tumors. The reported percentage of *BRAF* mutations in SBTs varies from 23% to 71%. Although a high percentage of stage II–IV SBTs with noninvasive implants have progressed to invasive low-grade serous carcinomas when patients were observed for 5 years or longer, *BRAF* mutations are rare in low-grade serous carcinomas as well as in invasive implants associated with SBTs. *BRAF* mutations in SBTs may prevent SBTs from progressing to invasive carcinomas. On the other hand, the reported percentage of *BRAF* mutations in mucinous carcinoma (20%) is much higher than that of mucinous borderline tumor (5%). Further investigation of the role of *BRAF* mutations in SBTs and mucinous tumor will shed light on the molecular mechanism underlying the role of *BRAF* mutations in tumor progression in different cellular context and the clinical utility of *BRAF* mutations in SBTs as a biomarker of favorable prognosis.

Keywords: *BRAF* V600E, ovarian cancer, COLD-PCR

Introduction

BRAF is a serine/threonine protein kinase of the RAF family that also includes ARAF and RAF1. *BRAF* has the highest basal level in the RAF family and is part of the mitogen-activated RAS/RAF/MEK/ERK protein kinase pathway, which acts as a signal transducer between the extracellular signals and the nucleus. Extracellular signals such as hormones, cytokines, and various growth factors interact with their receptors to activate the small G-proteins of the RAS family and subsequently activate *BRAF*. Active *BRAF* then activates MEK1/2 to phosphorylate ERK1/2, which leads to the expression of several downstream transcription factors that regulate cell growth, differentiation, and survival.

Mutations in the *BRAF* gene were first discovered by Davies et al¹ in 2002 through a systematic and genome-wide assessment of cancer-associated pathways in human cancer. The Catalogue of Somatic Mutations in Cancer (COSMIC, version 71)² database identified *BRAF* point mutations in over 40,000 cancer samples (Table 1). The frequency of *BRAF* mutations varies from over 40% in thyroid and skin tumors to 0%–12.5% in tumors in other organs. Coexisting mutations of *BRAF* and *KRAS* are very rare; however, such phenomena have been observed in a hyperplastic polyp of the colon³ and in ovarian mucinous carcinoma.⁴

The vast majority of missense mutations in *BRAF* involve a thymine to adenine substitution at nucleotide 1799 (c.1799T>A), which results in an amino acid change

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Table 1 Frequency of *BRAF* point mutations in tumors of different primary organ sites, according to the Catalogue of Somatic Mutations in Cancer (COSMIC) database

Primary organ site	Frequency of point mutation*	
	<i>KRAS</i>	<i>BRAF</i>
Thyroid	1.8% (141/7,717)	41.5% (19,297/46,463)
Skin	2.3% (86/3,729)	41.4% (8,134/19,667)
Large intestine	34.5% (18,551/53,826)	12.5% (9,253/74,074)
Eye	1.6% (4/258)	10.1% (84/828)
Bone	1.7% (11/643)	9.6% (53/552)
Hematopoietic and lymphoid	4.5% (532/11,956)	9.1% (786/8,636)
Pituitary	0% (0/315)	8.7% (20/230)
Central nervous system	0.9% (28/3,264)	7.0% (392/5,598)
Ovary	11.7% (660/5,653)	6.2% (270/4,386)
Biliary tract	23.3% (631/2,707)	5.8% (50/865)

Note: *Includes only primary organ sites for which more than 5% of the tumors had *BRAF* mutations.

from valine (V) to glutamate (E) at codon 600. This V600E mutation represents approximately 95% of all identified *BRAF* point mutations (Table 2). Mutated *BRAF* V600E activates ERK1/2 without the need for extracellular signals. Other relatively frequent *BRAF* missense mutations include mutations at codons 466, 594, and 601.

BRAF point mutation has been reported in approximately 6% (270/4,386) of ovarian tumor samples tested for *BRAF* mutations in the COSMIC database (Tables 2 and 3). The majority of these ovarian tumor samples (245/270) have a mutation in codon 600, with p.V600E being the predominant missense mutation. *BRAF* gene amplification and overexpression were found in approximately 12% and 7%, respectively, of high-grade ovarian serous carcinomas using the cBio Cancer Genomics Portal^{5,6} to interrogate the data generated from The Cancer Genome Atlas study.⁷

The available data indicate that *BRAF* mutations mainly occur in serous borderline tumors (SBTs),⁸ also known as serous tumors of low malignant potential or atypical proliferative serous tumors; SBTs also include micropapillary

Table 2 Frequency of common *BRAF* point mutations in all tumors and in ovarian tumors, according to the Catalogue of Somatic Mutations in Cancer (COSMIC) database

Point mutations in each codon	All tumors	Ovarian tumors
p.V600 (E, F, G, I, K, L, M, Q, R)	39,374	245
p.K601 (E, N, Q, R, T)	192	3
p.G466 (A, C, D, E, R, S, V, W)	158	2
p.D594 (A, E, G, H, K, N, V, Y)	157	2
Others	224	18
All point mutations	40,105	270

serous carcinoma (MPSC),⁹ a minor morphologically distinct subgroup that was first described by Burks et al¹⁰ in 1996. The reported frequency of *BRAF* mutations in SBTs ranges from 23% to 71%. We previously found that *BRAF* mutation is rare in advanced-stage ovarian low-grade serous carcinoma (LGSC).¹¹ *BRAF* mutation is also rare in primary clear cell ovarian carcinoma (1%),^{12,13} mucinous borderline tumors (2%),^{13–15} and endometrioid carcinoma (3%),¹⁶ but is relatively more frequent in mucinous carcinoma^{4,17} and serous adenoma.¹⁸

Detection of mutated *BRAF*

Most sequencing analyses of *BRAF* mutations in ovarian cancer have used direct Sanger sequencing. Standard polymerase chain reaction (PCR)-Sanger sequencing has a mutation detection sensitivity of approximately 10%.¹⁹ Since SBTs have a high component of stromal cells, the sensitivity for detecting the *BRAF* mutation may be compromised if DNA is extracted from bulk tissue for direct sequencing using regular PCR amplification of the targeted region (Figure 1A). To increase the mutation detection sensitivity, researchers can microdissect tumor cells from the paraffin section, but this strategy can be quite tedious. Several other methods, such as pyrosequencing,²⁰ matrix-assisted laser desorption/ionization time of flight mass spectrometry,²¹ and comparative allele-specific TaqMan PCR,²² can be used to screen for *BRAF* mutations in samples with low tumor cell purity. We have adopted the co-amplification at lower denaturation temperature (COLD)-PCR approach, described previously,²³ to enrich low-level mutant *BRAF* alleles in the DNA samples for detecting *BRAF* mutations by Sanger sequencing (Figure 1B).²⁴ As shown in Figure 1B, COLD-PCR amplifies the mutated allele (A) to have the same signal intensity as the wild-type allele (T).

Immunohistochemical staining for the *BRAF* V600E mutation-specific monoclonal antibody VE1²⁵ can also be used to detect *BRAF* mutation. The sensitivity of VE1 immunostaining has been validated for detecting the *BRAF* V600E mutation in formalin-fixed and paraffin-embedded ovarian SBT tissues.²⁶ Immunostaining was evaluable in most cases with sufficient tumor cells, but rare cases with scant cytoplasm and diffuse staining may be difficult to interpret.²⁷ The false-positive rate of VE1 immunostaining can be as high as 30%, so initial VE1 immunostaining should be validated with sequencing.²⁸ Figure 2 is an illustration of VE1 immunostaining on four ovarian tumor samples with confirmed *BRAF* mutation status by DNA sequencing. One of the SBT with wild-type *BRAF* gene (Figure 2B) (Figure 1A

Table 3 Frequency of *BRAF* point mutation detected in histologic subtypes of ovarian tumors, according to the Catalogue of Somatic Mutations in Cancer (COSMIC) database

Histology	Histologic subtype	Samples with point mutation n (%)	Samples tested
Adenoma	Adenofibroma	0	12
	Brenner tumor	0	43
	Cystadenofibroma	0	2
	Mixed	0	2
	Mucinous	0	40
	NS	0	6
	Serous	14 (10%)	142
Carcinoma	Adenocarcinoma	0	5
	Brenner tumor	0	1
	Carcinosarcoma (malignant mesodermal mixed tumor)	0	46
	Clear cell carcinoma	4 (1%)	352
	Endometrioid carcinoma	9 (3%)	262
	Mixed carcinoma	0	7
	Mucinous carcinoma	15 (10%)	144
	NS	11 (2%)	508
	Psamocarcinoma	1 (100%)	1
	Serous carcinoma	23 (1%)	1,663
	Serous micropapillary carcinoma	17 (40%)	43
	Small cell carcinoma	0	1
	Transitional cell carcinoma	0	5
	Undifferentiated carcinoma	0	10
Germ cell tumor	NS	0	1
	Teratoma	1 (4%)	28
	Yolk sac tumor	0	6
Tumor of low malignant potential (borderline)	Brenner tumor	0	20
	Endometrioid	0	3
	Mucinous	3 (2%)	125
	NS	4 (13%)	30
	Serous	162 (35%)	463
	Neoplasm	6 (2%)	298
Sex cord-stromal tumor	Fibroma-thecoma-fibrothecoma	0	37
	Granulosa cell tumor	0	80
	Total	270	4,386

Abbreviation: NS, not specified.

and 1B had wild-type *BRAF*; Figure 2C and 2D had mutated *BRAF* V600E) had faint false positive staining, which could be a result of an edge effect during staining and should be interpreted cautiously.

Role of *BRAF* mutation in the pathogenesis of ovarian cancer

There are four major histological subtypes of epithelial ovarian cancer (EOC), which is thought to arise from the surface epithelium of the ovaries but could also be from extra-ovarian origins.²⁹ An ovarian tumorigenesis model based on morphology and molecular genetics has been proposed.^{30,31} EOCs are classified as Type I or Type II. Type I tumors include low-grade MPSC, mucinous, endometrioid, and clear cell carcinomas and are characterized by high frequency of *KRAS*,

BRAF, *PTEN*, or beta-catenin mutations.^{30–34} Type II tumors include high-grade serous carcinoma, malignant mixed mesodermal tumors (carcinosarcomas) and undifferentiated carcinomas and are characterized by high genetic instability and high frequency of *TP53* mutation.^{31,32} Separating EOC into Type I and Type II based on genetic mutations is controversial. High frequency of *TP53* (57%, 8/14) and *KRAS* (57%, 8/14) mutations has also been found in Type I mucinous EOC.³⁵ Similarly, Type I endometrioid cancer also has a high frequency of *TP53* mutation (63%, 17/27).³⁶ Both endometriosis-related cancers (endometrioid and clear cell cancer) have high frequency of ARID1A mutations.^{37,38} SBT has high frequency of *BRAF* and *KRAS* mutations while LGSC has high frequency of *KRAS* mutation. Although the progression of LGSC to high-grade serous carcinoma is very

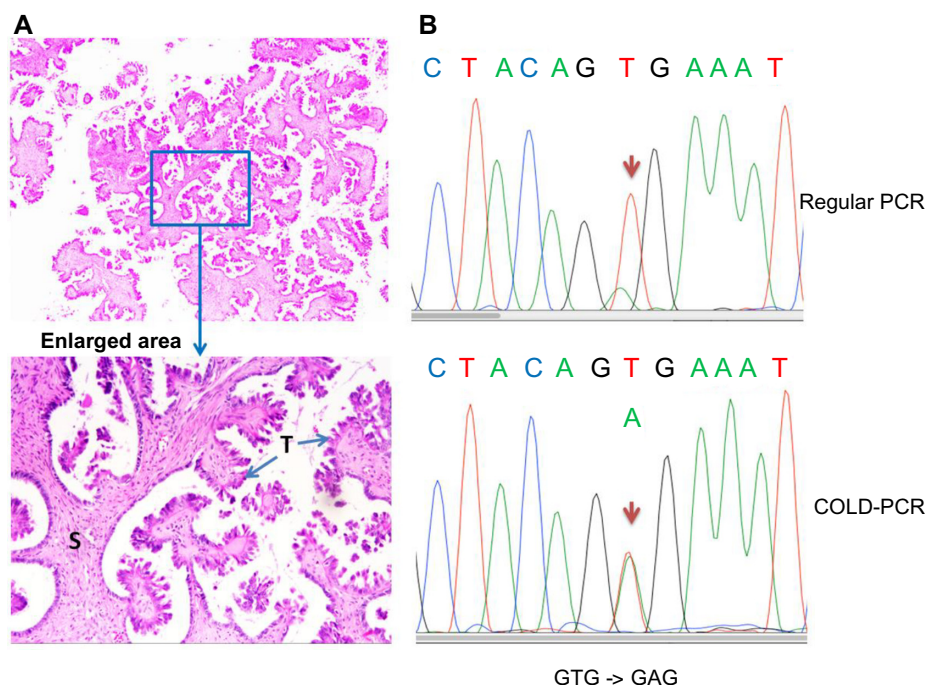


Figure 1 Co-amplification at lower denaturation temperature polymerase chain reaction (COLD-PCR) increases ability to detect *BRAF* mutations in ovarian serous borderline tumors (SBTs).

Notes: (A) Hematoxylin and eosin staining of a representative SBT showing the presence of a large component of stromal cells (S) in comparison to the tumor cells (T). The blue arrows indicate the tumor cells on the outside of the fibrous stroma. (B) Sequencing chromatogram showing the amplification of the mutant *BRAF* allele by COLD-PCR in comparison to regular PCR. The brown arrows indicate the mutated allele.

rare, several studies have reported the recurrence of high-grade serous carcinoma from SBT or LGSC.^{34,39,40} Thus, each ovarian carcinoma subtype should be treated as a different disease as suggested previously for future biomarker studies and clinical trials.⁴¹

No germline mutation of *BRAF* has been found, and thus the presence of *BRAF* mutation is probably not a genetic predisposition for the development of SBTs.¹⁶ Although a high percentage of stage II–IV SBTs with noninvasive implants have progressed to invasive LGSCs when patients were observed for 5 years or longer,⁴² *BRAF* mutations are rare in LGSCs as well as in invasive implants associated with SBTs.^{43,44} *BRAF* mutations are also rare in high-grade serous carcinomas. *BRAF* mutation is mainly found in ovarian serous adenoma, SBTs, invasive MPSC, and mucinous carcinomas.^{4,17,18,45,46}

It is generally believed that adenoma progresses to SBT and then to LGSC. The pathologic difference between SBTs and LGSC is the destructive stromal invasion in LGSC. One study proposed that MPSC is a step in the progression from SBTs to LGSC.⁴⁷ Destructive invasion of the ovarian stroma is rare in MPSC, but those with invasion are called invasive MPSC or are considered LGSC. MPSC represents approximately 6.5%–33% of all SBTs.^{10,16,48–51} However, there is a consensus that both typical SBTs and MPSC should be

classified as LGSC when areas of stromal invasion are greater than 5 mm. Patients with SBT with or without MPSC features have no difference in recurrence or disease-related mortality, although noninvasive SBTs with a micropapillary pattern may have invasive peritoneal implants more often than those without the micropapillary pattern.^{50–54}

Using sequencing analysis, Singer et al⁴⁵ found that ovarian SBTs and low-grade invasive MPSC shared similar frequencies of both *KRAS* and *BRAF* mutations, which are involved in the progression of SBTs to LGSC.⁴⁵ Another study found the same mutations in ovarian SBTs and the cystadenoma epithelium bordering the SBTs in six (86%) of seven samples.¹⁸ However, other studies suggest that ovarian SBTs with *BRAF* mutations may be less likely to spread or progress to LGSC. Most studies of ovarian LGSC reported a low frequency of *BRAF* mutations (Table 4).^{11,27,45,55–59} In one study of the implants that accompany ovarian SBTs, only six (13%) of 45 patients had *BRAF* mutations in SBTs with noninvasive peritoneal implants, and none of the patients had *BRAF* mutations in SBTs with invasive implants.⁴³ Heublein et al⁴⁴ detected *BRAF* mutations in only noninvasive implants associated with ovarian SBTs. Similarly, in our analysis of 36 patients with advanced-stage ovarian SBTs, we found that *BRAF* mutation was mainly associated with SBTs that did

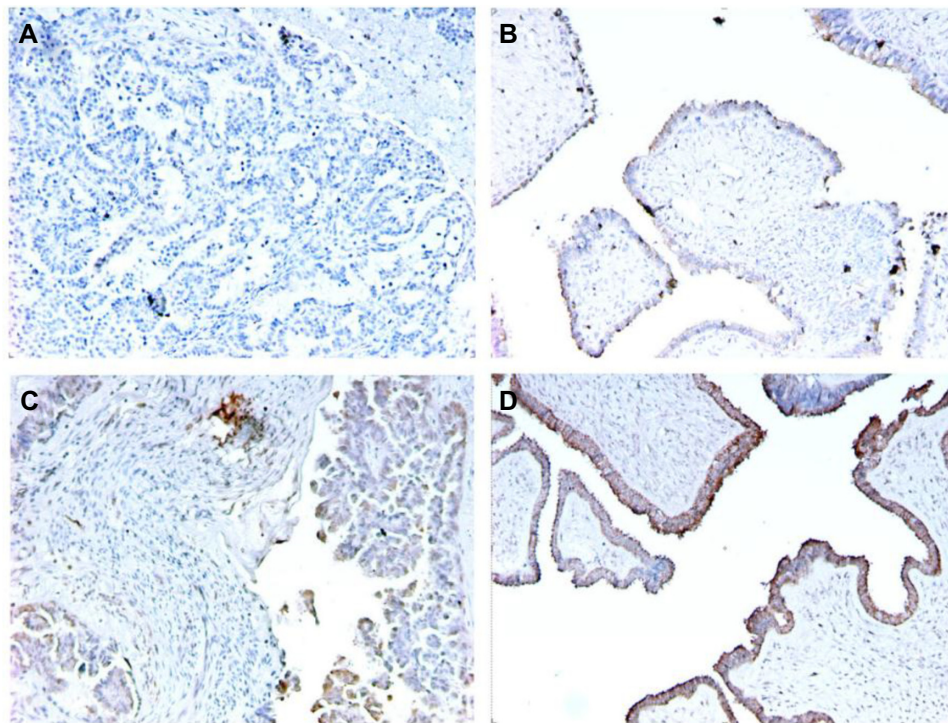


Figure 2 Examples of immunostains for *BRAF* V600E mutant protein with VE1 monoclonal antibody in ovarian tumor samples with *BRAF* mutation status confirmed by DNA sequencing.

Notes: (A) Low-grade serous carcinoma with wild-type *BRAF*; (B) serous borderline tumor with wild-type *BRAF*; (C) serous borderline tumor with *BRAF* V600E mutation; (D) serous borderline tumor with *BRAF* V600E mutation.

not progress to low-grade invasive tumors.²³ *BRAF* mutation is likely involved in the initiation and progression of ovarian adenoma to SBTs, but may not be involved in the progression of SBTs to carcinoma. By comparing the gene expression profiles between ovarian SBTs with wild-type *BRAF* and those with mutated *BRAF* V600E, we previously found that SBTs with *BRAF* V600E express high levels of genes associated with cell growth-inhibitory functions, including *CDC20B*, *PMEP1*, *PAEP*, *FOXCI*, and *SFN*.¹¹ Using immunohistochemical analysis, Heublein et al⁴⁴ also observed that ovarian SBTs with *BRAF*-mutated implants

tended to express high levels of p16. High expression of p16 may attenuate the mutated *BRAF*-induced MAPK signals that affect cell-cycle progression and thus may prevent further tumor progression.

On the other hand, the total reported percentage of *BRAF* mutations in mucinous carcinoma (20%) is much higher than that of mucinous borderline tumor (5%) (Table 5).^{4,13,15,17,46,60–62} Unlike the serous adenoma with similar *BRAF* mutations as SBTs, none of the mucinous adenomas (n=40) had a detectable *BRAF* mutation.¹⁵ Since it is believed that mucinous adenoma can progress as mucinous borderline tumor and then

Table 4 *BRAF* mutation frequency in serous borderline tumors (SBTs) and low-grade serous carcinomas (LGSCs)

SBT	LGSC	Reference
14/51 (28%)	7/22 (32%)	Singer et al (2003) ⁴⁵
NA	2/20 (10%)	Cho et al (2009) ⁵⁵
9/30 (30%)	1/39 (3%)	Wong et al (2010) ¹¹
10/43 (23%)	0/17 (0%)	Verezkey et al (2011) ⁵⁶
NA	0/11 (0%)	Sundov et al (2013) ⁵⁷
25/56 (45%)	1/19 (5%)	Grisham et al (2013) ⁵⁸
NA	2/34 (6%)	Farley et al (2013) ⁵⁹
22/31 (71%)	1/7 (14%)	Bosmuller et al (2013) ²⁷

Abbreviation: NA, not applicable.

Table 5 *BRAF* mutation frequency in mucinous borderline tumors (mBT) and mucinous carcinomas (MOC)

mBT	MOC	Reference
0/15 (0%)	1/10 (10%)	Mayr et al (2006) ¹³
NA	3/20 (15%)	Nakayama et al (2008) ⁴
0/17 (0%)	3/21 (14%)	Steffensen et al (2011) ¹⁷
0/3 (0%)	5/12 (42%)	Ryland et al (2013) ⁴⁶
NA	2/10 (20%)	Rahman et al (2013) ⁶⁰
2/38 (5%)	NA	Anglesio et al (2008) ⁶¹
3/22 (14%)	NA	Hunter et al (2012) ¹⁵
NA	1/2 (50%)	Gorringe et al (2008) ⁶²
Summary: 5% (5/95)	20% (15/75)	

Abbreviation: NA, not applicable.

mucinous carcinoma, a higher frequency of *BRAF* mutations in mucinous carcinoma may suggest a driver role of *BRAF* mutation in the pathogenesis of some mucinous carcinomas in contrast with that of SBTs.

Mutated *BRAF* as a potential prognostic marker for ovarian SBT or LGSC

Several studies have reported the association of *BRAF* mutation with a better clinical outcome for patients with ovarian cancer. Preusser et al²⁸ reported that patients with invasive ovarian carcinomas that stained positive for the *BRAF* V600E monoclonal antibody had a strong trend toward better survival. Similarly, in a study by Grisham et al,⁵⁸ *BRAF* V600E mutations were identified in 35% of 75 patients with ovarian SBTs or LGSC, and the *BRAF* V600E mutation in these tumors was associated with early-stage disease and improved prognosis. Grisham et al also concluded that patients with ovarian SBTs or LGSC who need systemic therapy are unlikely to have *BRAF* mutant tumors.⁵⁵ Pathologically, ovarian SBTs with *BRAF* mutation are associated with cellular features indicative of senescence, such as the expression of senescence-associated beta-galactosidase activity and abundant eosinophilic cells.⁶³ These data suggest that *BRAF* mutation is a biomarker of favorable prognosis and may prevent the progression of ovarian SBTs or early-stage LGSC to more aggressive disease, despite the fact that *BRAF* mutation is predictive of poor prognosis in other malignancies such as thyroid cancer and melanoma.

Future perspectives

SBT appears to be a unique disease with high frequency of *BRAF* mutations. Validation of *BRAF* mutations as a potential prognostic marker in a large cohort of patients with ovarian SBTs or LGSC is necessary to determine the clinical significance of *BRAF* status in the management of patients with this disease. Once the *BRAF* mutation has been confirmed as a protective factor against the progression of ovarian SBTs and early-stage LGSC into more aggressive disease,^{11,23,43,58} clinicians will be able to use the *BRAF* status of surgically resected ovarian SBTs to predict the risk of recurrence. Moreover, for progressive SBT/LGSC with *BRAF* V600E mutation, treatment with *BRAF* V600E specific inhibitor could be an alternative regimen.^{64,65} Furthermore, investigation of ovarian LGSC and mucinous carcinoma with *BRAF* mutation may unveil why *BRAF* mutations are associated with poor clinical outcomes in other cancers such as microsatellite-stable colon cancer,⁶⁶ melanoma,⁶⁷ and thyroid cancer.⁶⁸ In summary,

further study of *BRAF* mutation status will have a significant impact on the management of ovarian cancer.

Disclosure

The authors report no conflicts of interest in this work.

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