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Mutations of BRAF and KRAS Precede the Development of Ovarian Serous Borderline Tumors

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ABSTRACT

Molecular genetic changes that are associated with the initiating stage of tumor development are important in tumorigenesis. Ovarian serous borderline tumors (SBTs), putative precursors of low-grade serous carcinomas, are among the few human neoplasms with a high frequency of activating mutations in *BRAF* and *KRAS* genes. However, it remains unclear as to how these mutations contribute to tumor progression. To address this issue, we compared the mutational status of *BRAF* and *KRAS* in both SBTs and the adjacent epithelium from cystadenomas, the presumed precursor of SBTs. We found that three of eight SBTs contained mutant *BRAF*, and four SBTs contained mutant *KRAS*. All specimens with mutant *BRAF* harbored wild-type *KRAS* and *vice versa*. Thus, seven (88%) of eight SBTs contained either *BRAF* or *KRAS* mutations. The same mutations detected in SBTs were also identified in the cystadenoma epithelium adjacent to the SBTs in six (86%) of seven informative cases. As compared to SBTs, the cystadenoma epithelium, like ovarian surface epithelium, lacks cytological atypia. Our findings provide cogent evidence that mutations of *BRAF* and *KRAS* occur in the epithelium of cystadenomas adjacent to SBTs and strongly suggest that they are very early events in tumorigenesis, preceding the development of SBT.

INTRODUCTION

It has been shown that tumors result from an accumulation of genetic alterations that result in uncontrolled cellular proliferation. Identification of the alterations that occur early in tumor development is critical to understanding carcinogenesis and can provide insight into potential markers for early detection (1–3). Ovarian cancer is one of the most lethal neoplasms in women, and serous carcinoma is the most common type (4), but the molecular events that underlie the development of ovarian serous carcinoma are largely unknown. Recent studies have shown that ovarian serous carcinoma develops along two distinct pathways, and we have proposed a model of ovarian carcinogenesis that reflects this concept (5–7). In one pathway, invasive low-grade serous carcinoma develops from a noninvasive (or *in situ*) tumor that has traditionally been termed "serous borderline tumor" (SBT; ref. 8). The progression of SBT to invasive low-grade carcinoma mimics the adenomacarcinoma sequence in colorectal carcinoma (1). Detailed analysis of SBTs shows that SBTs consist of two tumors at different stages of tumor progression, a benign tumor termed "atypical proliferative serous tumor," and an intraepithelial low-grade (noninvasive micropapillary serous) carcinoma, the immediate precursor of invasive low-grade serous carcinoma (5, 7). SBTs are frequently associated with serous cystadenomas that develop from ovarian surface epithelium through a hyperplastic process (9). Like ovarian surface epithelium, the epithelial cells of a cystadenoma do not show cytological atypia, and their proliferation index is extremely low (9). In the second pathway, high-grade serous carcinoma develops from ovarian surface epithelium or from surface inclusion cysts (10), but precursor lesions have not been well characterized. Accordingly, this process has been described as "*de novo*" (5).

Molecular genetic analysis has shown that SBTs and invasive low-grade serous carcinomas are characterized by mutations of *BRAF* and *KRAS* in 61 to 68% of cases (6, 7, 11, 12), but p53 mutations are rare (12–14). In contrast, high-grade serous carcinomas frequently contain p53 mutations (>50%) but rarely *BRAF* and *KRAS* mutations (6, 7, 13–17). These studies analyzed advanced stage tumors in which putative precursor lesions may have been obliterated by the tumor. In this study, we confined our analysis to small SBTs and associated cystadenomas to delineate the early molecular genetic events in their pathogenesis. Specifically, we compared the mutational status of *BRAF* and *KRAS* in SBTs and the adjacent nontransformed epithelium of serous cystadenomas.

MATERIALS AND METHODS

A total of eight small SBTs (corresponding to what has been classified as atypical proliferative tumor) and the associated cystadenomas were collected. The acquisition of tumor samples was approved by the Johns Hopkins institutional review board. The SBTs ranged from 8 to 20 mm (average 16 mm) in greatest dimension and associated cystadenomas ranged from 5 to 8 cm (average 6.8 cm). The SBTs occupied 5 to 15% of the total surface area of the cystadenomas. Only a small number of cases were studied because although cystadenomas and SBTs are not uncommon, cystadenomas containing synchronous small SBTs are relatively rare. Microscopically, the SBTs contained a hierarchical branching papillae lined by epithelial cells with mild to moderate cellular atypia (Fig. 1). The epithelium of the SBTs merged abruptly with the cystadenoma epithelium that was composed of a single layer of flat to columnar cells without atypia (Fig. 1). The Palm laser capture microdissection microscope (Zeiss) was used to separately collect the epithelium from the SBTs and adjacent cystadenoma. The PicoPure DNA extraction kit (Arcturus, Mountain View, CA) was used to prepare genomic DNA. PCR was then done, and an ABI 3100 sequencer (ABI, Foster City, CA) was used to do nucleotide sequencing. Exon 1 of *KRAS* and exon 15 of *BRAF* were both sequenced as each exon harbors almost all of the mutations in both genes (6, 7, 11, 18). The primers for PCR and sequencing were as follows: for *BRAF*, 5′-tgcttgctctgataggaaaatga-3′ (forward); 5′-ccacaaaatggatccagacaac-3′ (reverse); and 5′-gaaaatgagatctactgttttccttta-3′ (sequencing); for *KRAS*, 5′-taaggcctgctgaaaatgactg-3′ (forward); 5′-tggtcctgcaccagtaatatgc-3′ (reverse); and 5′-ctgcaccagtaatatgcatattaaaac-3′ (sequencing). The Lasergene program (DNASTAR, Madison, WI) was used to analyze the sequences.

RESULTS

The results of the mutational status correlated with the SBT or cystadenoma component of the tumors are shown in Table 1. We found that four SBTs (cases 1, 4, 5, and 8) contained activating *KRAS* mutations at codon 12 (three mutations of GGT to GAT and one mutation of GGT to GTT) and three SBTs (cases 3, 5, and 6) had *BRAF* mutations at codon 599 (all of T1796A mutation). As in our previous report (6), the presence of *KRAS* and *BRAF* mutations was mutually exclusive. Thus, seven (88%) of eight SBTs had either a *BRAF* or a *KRAS* mutation. Case 2 contained wild-type *KRAS* and *BRAF*. Analysis of the mutational status in the epithelium from the cystadenomas adjacent to the SBTs revealed that both the cystade-

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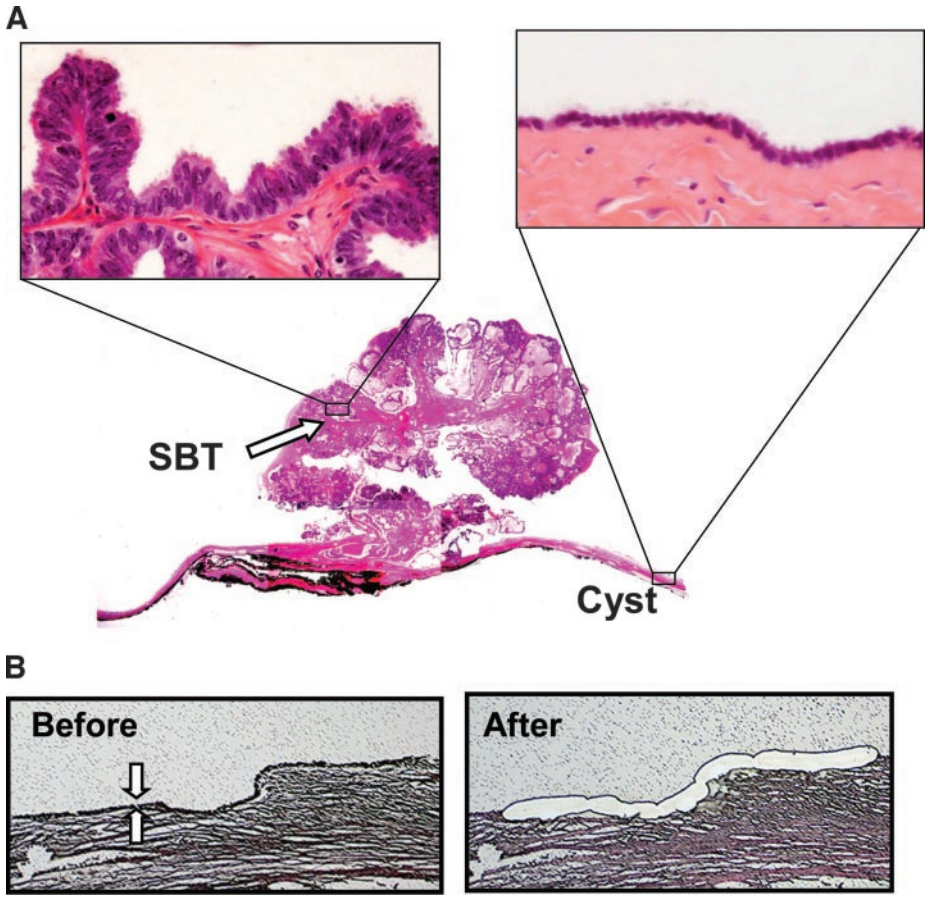


Fig. 1. *A*, small serous borderline tumor and associated serous cystadenoma (case E). The tumor measures 0.8 cm in greatest dimension and is composed of hierarchical branching papillae lined by cells with mild to moderate atypia (*left inset*). The adjacent cystadenoma epithelium is composed of a single layer of epithelium without cytological atypia (*right inset*). *B*, laser capture microdissection with minimal contamination from the underlying stromal cells were used to isolate epithelial cells lining the cystadenoma (between *arrows*).

noma and SBT components contained identical mutations in six of seven informative cases. Representative sequence analyses are shown in Fig. 2. The frequent mutations of *KRAS* and *BRAF* in small SBTs are consistent with previous reports showing mutations in either *BRAF* or *KRAS* in 66 to 68% of large SBTs (6, 11). The higher frequency of mutations (88%) in the current report is probably because of the use of purer tumor cell samples obtained by laser capture microdissection or may have resulted from the small sample size in the present analysis.

DISCUSSION

The findings in this study provide important insights into the molecular pathogenesis of low-grade ovarian serous tumors (Fig. 3). Because we only analyzed a single time point in the sequence of cystadenoma to SBTs, we can only infer that the findings truly describe the events in early tumor progression. However, the coexistence of a cystadenoma with a SBT strongly suggests that the latter arises from the former (Fig. 3). Accordingly, the presence of identical mutations in the cystadenoma epithelium that displayed no evidence of cytological atypia strongly suggests that mutations of *BRAF* and *KRAS* occur before the development of a SBT and indicates that cystadenomas are the precursors of SBTs. Our results support the view that mutations of *BRAF* and *KRAS* (or *NRAS*) are early events associated with tumor initiation as occurs in melanoma (19) and colorectal carcinoma (20).

We have recently studied 30 consecutive pure cystadenomas without SBTs and have shown an absence of *BRAF* and *KRAS* mutations in all of them (9). The frequency of mutations in *BRAF* and *KRAS* in cystadenomas associated with SBTs was significantly higher than those without SBTs ($P < 0.001$, Fisher's exact test; Table 2). This finding together with the fact that SBTs are relatively uncommon as compared to cystadenomas (21–25) suggests that only a small proportion of serous cystadenomas are neoplastic with the potential to progress to SBTs. Finally, our findings suggest a "gatekeeper" role of *BRAF* and *KRAS* genes in the development of low-grade serous carcinomas (26). This is supported by the observation that activating mutations in these genes are oncogenic in experimental cell culture systems (19, 27, 28) probably through a constitutive activation of mitogen-activated protein kinase (29, 30). Future experiments will determine whether mutations of *BRAF* and *KRAS* are sufficient to initiate the development of SBTs or additional genetic "hits" are required in tumorigenesis. Because mutations of *BRAF* and *KRAS* in serous cystadenomas are associated with the development of SBTs, detection of *BRAF* and *KRAS* mutations could facilitate the differen-

Table 1 *Mutational status of KRAS and BRAF genes in eight small serous borderline tumors and associated cystadenomas*

Gene	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8
KRAS								
SBT	G35A* G12D†	WT	WT	G35T G12V	G35A G12D	WT	WT	G35A G12D
Cyst	G35A G12D	WT	WT	WT	G35A G12D	WT	WT	G35A G12D
BRAF								
SBT	WT	WT	T1796A V599E	WT	WT	T1796A V599E	T1796A V599E	WT
Cyst	WT	WT	T1796A V599E	WT	WT	T1796A V599E	T1796A V599E	WT

Abbreviation: WT, wild-type.
* Alteration in nucleotide sequence.
† Alteration in amino acid sequence.

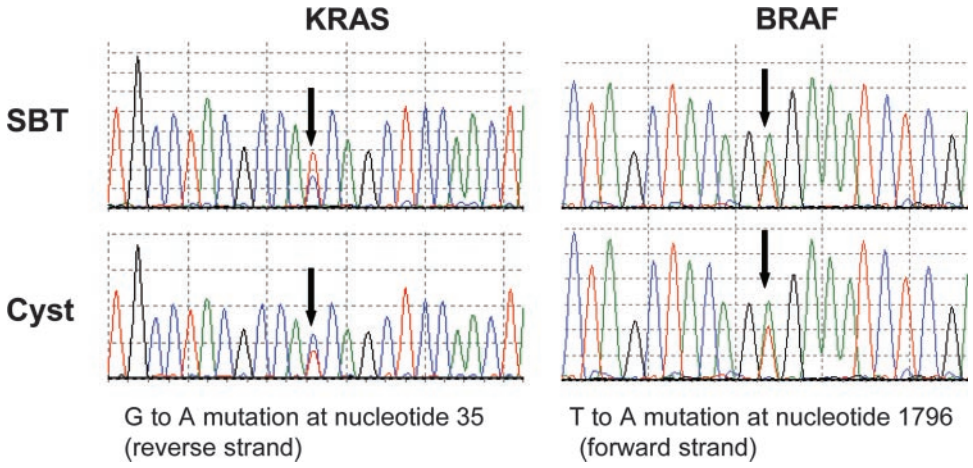


Fig. 2. Chromatograms of nucleotide sequences of *BRAF* and *KRAS* in two representative cases. *Left panel* (case 1) shows a point mutation in the *KRAS* gene in both SBT and the adjacent cystadenoma (Cyst) of the same specimen. *Right panel* (case 6) shows a point mutation in the *BRAF* gene in both the serous borderline tumor and the corresponding cystadenoma.

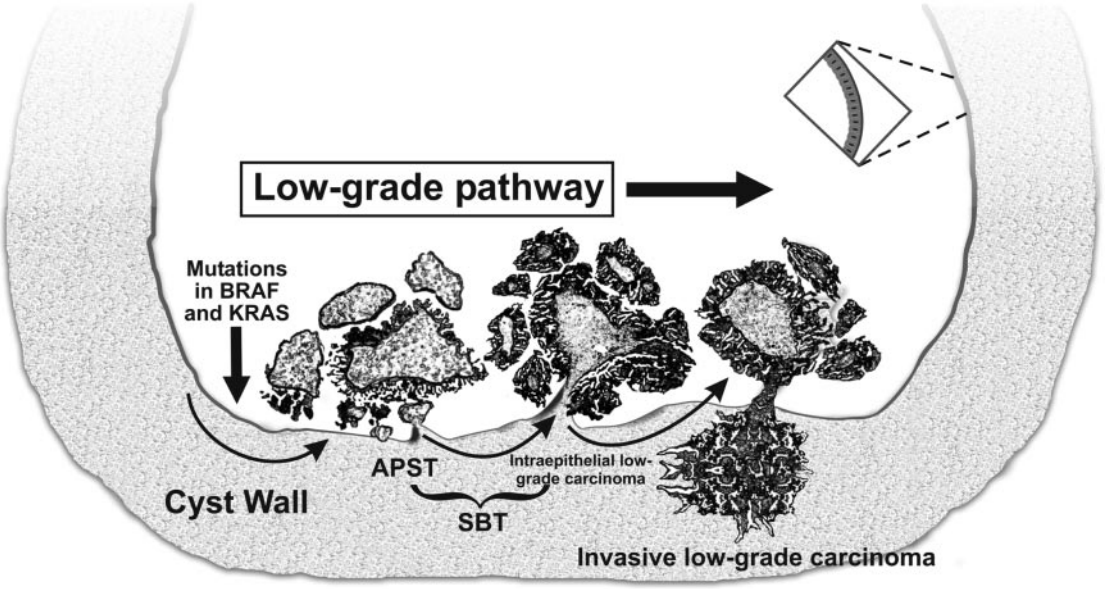
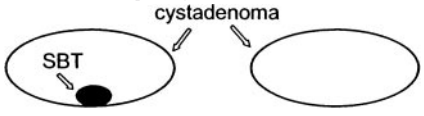


Fig. 3. Schematic representation of tumor progression in low-grade serous carcinoma. Mutations of *BRAF* and *KRAS* occur in a small proportion of cystadenomas that may contribute to the development of a SBT. Some serous borderline tumors progress further to intraepithelial and then to invasive low-grade serous carcinoma. *APST*, atypical proliferative serous tumor.

tiation of cystadenomas with a high risk of progression from the vast majority of cystadenomas that lack *BRAF* or *KRAS* mutations and have a very low risk of progression. Development of molecular assays (31, 32) that can detect such mutations (in cyst fluid, for example) could play an important role in the management of patients with ovarian cystadenomas, particularly young women who would prefer fertility-sparing treatment.

Table 2 *Frequency of mutations in BRAF and KRAS in cystadenomas with associated serous borderline tumor and pure cystadenomas*



	Cystadenoma associated with SBT	Pure cystadenoma
Mutations in *BRAF* or *KRAS*	6	0
Wild-type in *BRAF* and *KRAS*	2	30
Total cases	8	30

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