Short Communication

BRAF Mutation Is Rare in Advanced-Stage Low-Grade Ovarian Serous Carcinomas

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Low-grade ovarian serous carcinomas are believed to arise via an adenoma-serous borderline tumor-serous carcinoma sequence. In this study, we found that advanced-stage, low-grade ovarian serous carcinomas both with and without adjacent serous borderline tumor shared similar regions of loss of heterozygosity. We then analyzed 91 ovarian tumor samples for mutations in *TP53*, *BRAF*, and *KRAS*. *TP53* mutations were not detected in any serous borderline tumors ($n = 30$) or low-grade serous carcinomas ($n = 43$) but were found in 73% of high-grade serous carcinomas ($n = 18$). *BRAF* ($n = 9$) or *KRAS* ($n = 5$) mutation was detected in 47% of serous borderline tumors, but among the low-grade serous carcinomas (39 stage III, 2 stage II, and 2 stage I), only one (2%) had a *BRAF* mutation and eight (19%) had a *KRAS* mutation. The low frequency of *BRAF* mutations in advanced-stage, low-grade serous carcinomas, which contrasts with previous findings, suggests that aggressive, low-grade serous carcinomas are more likely derived from serous borderline tumors without *BRAF* mutation. In addition, advanced-stage, low-grade carcinoma patients with *BRAF* or *KRAS* mutation have a better apparent clinical outcome. However, further investigation is needed. (Am J Pathol 2010, 177:1611–1617; DOI: 10.2353/ajpath.2010.100212)

Serous carcinoma is the most common histological subtype of epithelial ovarian cancer. Recent evidence strongly suggests that a two-tier system stratifying serous carcinoma into low-grade and high-grade categories is biologically and clinically relevant.^{1–12} Low-grade serous carcinomas are believed to arise via an adenoma-serous borderline tumor-carcinoma sequence, whereas high-

grade serous carcinomas develop from an unknown precursor.^{6,7} While previous reports indicate that low-grade serous carcinomas may develop from either recurrence of a serous borderline tumor or *de novo*, the mechanism associated with the former type of development is not fully understood.^{13–15} Almost all serous borderline tumors that recur are associated with noninvasive or invasive peritoneal implants (stages II–IV) at initial diagnosis of the serous borderline tumor.^{13–17}

According to the World Health Organization, the pattern of serous borderline tumors may be classified as either typical or micropapillary.^{15,18–21} The micropapillary pattern was initially described in 1996; accounts for 8% to 18% of serous borderline tumors; and is associated with a greater frequency of bilaterality, a greater association with peritoneal implants (particularly invasive implants), and a higher risk of relapse than the typical pattern of serous borderline tumor.^{15,18–21} For all serous borderline tumors, regardless of the pattern, the risk of relapsing as low-grade ovarian serous carcinoma increases over time.²²

Mutation analysis could provide insight into how low-grade serous carcinoma develops. The initial report on *BRAF* and *KRAS* mutations in low-grade serous carcinomas, which were believed to be derived from serous borderline tumors with micropapillary pattern, indicated a rate of *BRAF* or *KRAS* mutation of 68%.²³ Similarly, in serous borderline tumors, a rate of *BRAF* or *KRAS* mutation of 61% was reported.²³ However, in invasive high-grade serous carcinomas, *BRAF* and *KRAS* mutations were not found.^{23–25} Conversely, *TP53* mutations are

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quite common in high-grade serous carcinomas but exceedingly rare in serous borderline tumors and low-grade serous carcinomas.²⁶ However, the correlation of *BRAF*/*KRAS* with patient outcome in low-grade ovarian serous carcinomas has not been explored. In this study, we investigated the *BRAF* and *KRAS* mutation status of advanced-stage low-grade serous carcinomas with and without a background of serous borderline tumor at initial diagnosis.

Materials and Methods

Patient Samples

Ninety-one ovarian tumor samples were obtained in accordance with human subject research protocols approved by our Institutional Review Board, and were retrieved from our Multidisciplinary Gynecologic Cancer Translational Research Tumor Bank. The 91 samples selected for this study included samples from 30 patients with serous borderline tumors, 43 patients with low-grade serous carcinoma, and 18 patients with high-grade serous carcinoma. All sections were reviewed by two gynecologic pathologists (M.T.D. and A.M.).

Microdissection and DNA Extraction

Optimal cutting temperature compound-embedded frozen tissues were sectioned and mounted on polyethylene terephthalate-membrane slides (Leica Microsystems, Wetzlar, Germany). These slides were immediately fixed in 70% alcohol for 30 seconds, hydrated in water, stained with methylene green, dehydrated in an ascending series of alcohol, and air dried. A Leica AS LMD Laser Capture Microdissection System (Leica Microsystems) with a 7.5- μ m diameter laser was used to microdissect and retrieve tumor cells from different regions of the sections. Two sets of approximately 5000 cells were dissected from each specimen. Using a microextraction kit, DNA was extracted as described by the manufacturer (Qiagen, Valencia, CA) and quantified using a NanoDrop spectrometer (NanoDrop, Rockland, DE).

Whole-Genome Amplification and DNA Purification

One microliter of 20 to 50 ng of DNA extracted from each microdissected sample was used as starting material for the amplification. The phi29-amplification was carried out with a GenomiPhi kit according to the manufacturer's instructions (GE Healthcare Biosciences, Pittsburgh, PA) using an incubation time of 16 hours. GenomiPhi reactions were checked on a 0.6% agarose gel. Amplification was considered acceptable when a smear of DNA fragments, ranging from 1 to 20 kb, was visible. Amplified DNA was ethanol precipitated and resuspended in molecular-grade water for subsequent single nucleotide polymorphism (SNP) array and mutation analyses.

SNP Array Analysis

SNP assays were performed on 32 samples: six adjacent normal tissues (2 were paired with samples from among the aforementioned 91 samples; 4 were from other patients), one serous borderline tumor, four early-stage low-grade serous carcinomas (2 stage I and 2 stage II), and 21 advanced-stage low-grade serous carcinomas (8 with adjacent serous borderline tumors, 13 without adjacent serous borderline tumors). Labeling, hybridization, washing, and staining of the 50K SNP mapping arrays were performed according to the standard Single Primer GeneChip Mapping Assay protocol (Affymetrix Inc., Santa Clara, CA) as described previously.^{27,28} Briefly, 250 ng of genomic DNA was digested with XbaI and then ligated to XbaI adaptor before subsequent PCR amplification using AmpliTaq Gold (Applied Biosystems, Foster City, CA). To obtain enough PCR products, four 100- μ l PCR reactions were set up for each XbaI adaptor-ligated DNA sample. The PCR products from the four reactions were then pooled and purified. A final 20 μ g of PCR product was fragmented with DNase I and visualized on 4% TBE agarose gel to confirm that the sizes ranged from 50 to 100 bp. Fragmented PCR products were then end-labeled with biotin. Hybridization and detection were performed with an Affymetrix Fluidics Station 450 and GeneChip Scanner 3000.

The cel files and corresponding SNP typing text files, exported from Affymetrix GTTYPE software,²⁹ were imported into dChip to identify regions of loss of heterozygosity (LOH) as described previously.³⁰ Chromosomal regions with LOH were inferred by HMM algorithm using CEPH 60 parents as a normal reference genotype file as described previously.³¹

Mutation Analysis

Mutation analysis for *BRAF*, *KRAS*, and *TP53* was performed on all 91 ovarian tumor samples. Analyses were conducted using the same primer sets described previously.²³

Gene Expression Analysis

Gene expression analysis was performed on five serous borderline tumors with *BRAF* mutation and five serous borderline tumors without *BRAF* mutation randomly. RNA was extracted from microdissected tumor cells. Expression profiling was carried out with Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays and data were analyzed as described previously.³²

Statistical Analysis

Statistical analyses were performed using SPSS 17.0 software (SPSS, Chicago, IL). Overall survival times were calculated from the date of diagnosis of low-grade serous carcinoma to the date of last contact or death and were estimated using the method of Kaplan and Meier. The log-rank test was used to compare differences between

Figure 1. Loss of heterozygosity (LOH) analysis of low-grade ovarian serous carcinoma with 50K SNP arrays. LOH regions were highlighted in blue color. Stars indicate Tumors with a *KRAS* mutation. SBOT, serous borderline ovarian tumor. LG/SBOT, low-grade serous tumor with adjacent serous borderline tumor. LG, low-grade serous tumor without adjacent serous borderline tumor.

survival curves. *P* values of $<.05$ were considered statistically significant.

Results

SNP Array Analysis

The LOH profiles indicated that tumor-adjacent normal tissues, serous borderline tumors, and early-stage low-

grade serous carcinomas did not have any major regions with LOH. On the other hand, advanced-stage low-grade serous carcinomas with and without adjacent serous borderline tumor had regions with recurrent LOH at 1p, 4, 6q, 8p, 9p, 17, 18q, and 22q (Figure 1).

Mutation Analysis

The samples from the 43 patients with low-grade serous carcinoma (median age, 47 years; range, 21 to 79 years; 39 stage III, 2 stage II, and 2 stage I) were initially analyzed for mutations at codon 600 of *BRAF* and codons 12 and 13 of *KRAS*. *KRAS* mutation was detected in eight samples (19%), and *BRAF* mutation was detected in one other sample (2%) (Table 1).

Subsequently, the samples from the 30 patients with serous borderline tumors (median age, 48 years; range, 21 to 73 years; 15 stage III, 2 stage II, and 13 stage I) were analyzed for both *BRAF* and *KRAS* mutations. *BRAF* mutation was detected in nine samples (30%), and *KRAS* mutation was detected in five other samples (17%), resulting in an overall mutation rate of 47% (Table 1). This frequency of *BRAF* and *KRAS* mutations in serous borderline tumors is similar to those previously reported.^{23–25} No *BRAF* and *KRAS* mutations were detected in any of the high-grade serous carcinomas.

No *TP53* mutations were detected in either serous borderline tumors or low-grade serous carcinomas; however, 73% of patients with high-grade serous carcinomas ($n = 18$; all stage III) were found to have a *TP53* mutation.

Gene Expression Analysis

Gene expression analysis of five serous borderline tumors with *BRAF* mutation and five serous borderline

Table 1. *BRAF*, *KRAS*, and *TP53* Mutations in Ovarian Serous Carcinoma Samples

Nucleotide	Amino acid	Serous borderline tumor ($n = 30$)	Low-grade serous tumor ($n = 43$)	High-grade serous tumor ($n = 18$)
<i>BRAF</i>				
c.T1799A	p.V600E	8	1	
c.A1803C	p.K601N	1		
Samples with mutation, n (%)		9 (30%)	1 (2%)	0 (0%)
<i>KRAS</i>				
c.G35A	p.G12D	4	5	
c.G35T	p.G12V	1	1	
c.G34C	p.G12R		1	
c.G35C	p.G12A		1	
Samples with mutation, n (%)		5 (17%)	8 (19%)	0 (0%)
<i>TP53</i>				
c.A395G	K132R			1
c.G524A	R175H			2
c.A536G	H179R			2
c.G733A	G245S			1
c.G772A	E258K			1
c.C574T	Q192X			1
c.C637T	R213X			1
c.C916T	A306X			2
c.455_456insC (insertion)	p.P153fsX28 (insertion-frame shift)			1
IVS6-1G>C (splice junction)	p.G187X (splice junction)			1
Samples with mutation, n (%)		0 (0%)	0 (0%)	13 (73%)

Figure 2. Genes differentially expressed between serous borderline tumors with *BRAF* mutation (Braf) and serous borderline tumors without *BRAF* mutation (wt).

tumors without *BRAF* mutation revealed 22 genes that were significantly differentially expressed between the two groups (>fourfold difference and $P < 0.0001$) (Figure 2).

Correlation of *KRAS*/*BRAF* Mutation Status with Patient Outcomes

Follow-up clinical data were available for 33 of the patients with stage III disease (Table 2). Of the eight patients with either *BRAF* or *KRAS* mutations, two (25%) have had no evidence of disease after optimal debulking surgery and platinum-taxane-based chemotherapy, three (38%) are alive with disease, two (25%) have died of disease, and one has died of other causes. On the other hand, 17 of the 25 patients (68%) with wild-type genotypes have died of disease, and eight patients (32%) are still alive with disease.

The median overall survival for patients in Table 2 was 53.9 months (95% CI, 28.4 to 79.5). The median follow-up time for patients who were alive at last contact ($n = 13$) was 62.3 months (range, 13.9 to 152.0). The median overall survival for patients whose tumors did not exhibit *BRAF*/*KRAS* mutations was 47.3 months (95% CI, 22.12 to 72.5), compared to 77.9 months for patients with *BRAF*/*KRAS* mutations ($P = 0.28$).

Discussion

The molecular events that give rise to low-grade ovarian serous carcinomas are not well understood, but molecular evidence has suggested that such tumors could be part of a continuum with serous borderline tumors of the ovary.^{7,8,33} In this study, we found that advanced-stage low-grade ovarian serous carcinomas with and without adjacent serous borderline tumors shared similar regions of LOH (Figure 1), which supports the hypothesis that such tumors could be part of a continuum with serous borderline tumors of the ovary. We also found a very low frequency of *BRAF* mutations in advanced-stage low-grade serous carcinomas, a finding that contrasts with previous findings²³ and suggests that aggressive low-grade serous carcinomas may be derived from serous borderline tumors without *BRAF* mutation.

Several regions of recurrent LOH were identified in the advanced-stage low-grade ovarian serous carcinomas in this study. However, which genes might be influenced by these LOH sites has not been studied and will need to be investigated. We speculate that 1p and 6q losses may be early events during tumor initiation or progression, since a previous study using comparative genomic hybridization identified 1p (3 of 8) and 6q (4 of 8) losses in both serous borderline and benign tumors.³⁴ In addition, comparative genomic hybridization analysis of serous borderline tumors with the typical pattern, serous borderline tumors with micropapillary pattern (noninvasive micropapillary serous carcinomas), and low-grade serous carcinomas (invasive micropapillary serous carcinomas) identified 1p loss as the most frequent event in serous borderline tumors with micropapillary pattern; the frequency of 1p loss in low-grade serous carcinomas was higher than that in serous borderline tumors with micropapillary pattern.³⁵ More recently, Kuo et al³⁶ have identified miR-34a, located at the 1p36 LOH site, as a potential tumor suppressor.

A *BRAF* mutation was only detected in 1 of 43 low-grade ovarian serous carcinomas. Together with the *KRAS* mutation, 8 patients with mutation appear to have a better clinical outcome. Although not definitive, the correlation of *BRAF*/*KRAS* mutation with a better clinical outcome suggests that the presence of mutations may be a favorable prognostic biomarker. However, our sample size is small and further investigation of a larger number of samples is warranted.

This interpretation was supported by the results of our gene expression profiling of serous borderline tumors with and without *BRAF* mutation. To our surprise, most of the genes overexpressed in serous borderline tumors with *BRAF* mutation (*CDC20B*, *PMEPA1*, *PAEP*, *FOXC1*, and *SFN*) have known cell growth inhibitory effects (Figure 2). *CDC20B*, regulated by microRNA449a/b, promotes apoptosis and suppresses proliferation of cancer cells.³⁷ *PMEPA1* exhibits a cell growth inhibitory function in prostate cancer.³⁸ *PAEP* can induce cell differentiation, and ovarian cancers with *PAEP* expression have more favorable outcome.³⁹ *FOXC1* is involved in negative regulation of cell growth.⁴⁰ *SFN* (14-3-3 σ) is a tumor sup-

Table 2. Mutation Status and Outcomes in 33 Patients with Stage III, Low-Grade Ovarian Serous Carcinoma

Sample	Mutation	Patient age at diagnosis, years	Time to last follow-up, months	Status at last follow-up
LGS49	BRAF	53	30	Alive with disease
LGS46	KRAS	36	47	Alive, NED
LGS50	KRAS	51	27	Alive, NED
LGS6	KRAS	33	97	Alive with disease
LGS22	KRAS	40	153	Alive with disease
LGS5	KRAS	49	78	Deceased (NED but died of other cause)
LGS23	KRAS	59	8	Died of disease
LGS24	KRAS	40	27	Died of disease
LGS1	None	70	132	Alive with disease
LGS11	None	64	123	Alive with disease
LGS13	None	41	82	Alive with disease
LGS17	None	31	62	Alive with disease
LGS47	None	49	41	Alive with disease
LGS48	None	59	32	Alive with disease
LGS51	None	38	86	Alive with disease
LGS52	None	22	14	Alive with disease
LGS2	None	56	16	Died of disease
LGS3	None	59	55	Died of disease
LGS7	None	59	3	Died of disease
LGS8	None	21	26	Died of disease
LGS10	None	47	36	Died of disease
LGS15	None	23	168	Died of disease
LGS18	None	52	55	Died of disease
LGS21	None	54	23	Died of disease
LGS32	None	70	36	Died of disease
LGS33	None	37	24	Died of disease
LGS34	None	39	84	Died of disease
LGS37	None	75	12	Died of disease
LGS38	None	28	60	Died of disease
LGS39	None	34	48	Died of disease
LGS40	None	29	24	Died of disease
LGS41	None	45	24	Died of disease
LGS43	None	79	74	Died of disease

NED, no evidence of disease.

pressor gene and is involved in cellular senescence of melanoma cells.⁴¹ These findings suggest that low-grade ovarian serous tumors are more likely to derive from serous borderline tumors without *BRAF* mutations than from serous borderline tumors with *BRAF* mutations. This hypothesis is further supported by the fact that two of the patients with serous borderline tumors without *BRAF* or *KRAS* mutations developed recurrent low-grade serous carcinoma 17 and 60 months after the initial diagnosis of a serous borderline tumor.

In pathological studies, 2%–3% of high-grade serous carcinomas have been observed to have low-grade serous carcinoma components.^{1,42} Vang et al found no *BRAF* or *TP53* mutations in a series of six such carcinomas with both high-grade and low-grade components.⁴² It is tempting to speculate that some low-grade serous carcinomas without *BRAF* mutation could progress to high-grade serous carcinomas.

In summary, we found a low frequency of *BRAF* and *KRAS* mutations in low-grade serous carcinomas, a finding that contrasts with findings of a previous report.²³ This difference is possibly due to the fact that most of our samples of low-grade serous carcinoma were of advanced stage whereas most of the low-grade serous carcinomas previously reported in the 2003 study by Singer et al²³ were exclusively obtained

from the primary micropapillary serous carcinomas adjacent to well-established serous borderline tumors of the ovary rather than from primary low-grade serous carcinomas with disseminated or metastatic disease (Dr. Ie-Ming Shih at Johns Hopkins, personal communication). Recent studies suggest that an activating *BRAF* mutation could induce senescence.^{43,44} Furthermore, our expression profiling data indicated that serous borderline tumors with *BRAF* mutation have a high level of expression of genes involved in cell growth inhibition. Thus, *BRAF* mutation could be a favorable prognostic marker in patients with advanced-stage serous borderline ovarian tumors or recurrent low-grade serous carcinoma unless a second mutational event occurs, such as the loss of *IGFBP7*, critical in the pathogenesis of melanoma with *BRAF* mutation.⁴⁴ Alternatively, the low frequency of *BRAF* mutations could suggest that some low-grade serous carcinomas develop *de novo* rather than arising from a pre-existing serous borderline tumor.

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