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Comprehensive genomic profiling reveals ubiquitous *KRAS* mutations and frequent *PIK3CA* mutations in ovarian seromucinous borderline tumor

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Abstract

The molecular underpinnings of seromucinous borderline tumor (SMBT) – an uncommon ovarian epithelial neoplasm characterized by association with endometriosis, frequent bilateral ovarian involvement, and occasional progression to invasive carcinoma – remain poorly understood. Here, we sought to comprehensively characterize the mutational landscape of SMBT and elucidate the clonal relationship between bilateral ovarian SMBTs. We also compared the mutational profiles between SMBTs and concurrent invasive carcinomas. Formalin-fixed, paraffin-embedded tissue specimens were retrieved from 28 patients diagnosed with SMBT. Massively parallel sequencing of 409 cancer-related genes was conducted to identify somatic mutations in 33 SMBT samples and four concurrent invasive carcinoma specimens. *TERT* promoter mutations were assessed by Sanger sequencing, whereas immunohistochemistry was used as a surrogate tool for detecting deletions or epigenetic silencing of relevant tumor suppressor genes. Twenty-six (92.9%) of the 28 patients were diagnosed with stage I SMBTs. Seven (25%) cases showed bilateral ovarian involvement and 13 (46%) had concomitant endometriosis. Concurrent ovarian carcinomas were identified in three patients, whereas one case had a synchronous endometrial carcinoma. Somatic mutations in the *KRAS*, *PIK3CA*, and *ARID1A* genes were identified in 100, 60.7, and 14.3% of SMBT samples, respectively. In contrast, *TERT* promoter mutations and DNA mismatch repair deficiencies were absent. Sequencing of paired specimens from patients with bilateral SMBT revealed the presence of at least two shared somatic mutations, suggestive of a clonal relationship. Similarly, we identified shared somatic mutations between SMBT samples and concurrent ovarian carcinoma specimens. Taken together, these findings demonstrated a distinct mutational landscape of SMBT in which (1) *KRAS* is invariably mutated, (2) *PIK3CA* is frequently mutated, and (3) *TERT* promoter mutations and DNA mismatch repair deficiencies are absent. Our findings represent the first extensive characterization of this rare ovarian neoplasm, with potential implications for disease classification and molecular diagnostics.

Introduction

Seromucinous borderline tumor (SMBT) – also known as atypical proliferative seromucinous tumor and endocervical-type (müllerian) mucinous borderline tumor – is an

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uncommon and still incompletely characterized ovarian epithelial neoplasm [1–3]. Although SMBT was formerly considered as a subtype of mucinous borderline tumor, the clinical presentation of SMBT more closely resembles that of serous borderline tumor – with frequent bilateral involvement of the ovary and sporadic extraovarian implantation [3–7]. Histologically, SMBT shows the hierarchical papillary architecture typical of serous borderline tumor. However, its papillae are generally lined by endocervical-like mucinous epithelial cells and frequently admixed with a variety of müllerian epithelial cells – including serous (ciliated) cells, endometrioid cells, clear cells, squamous cells, and "indifferent cells" with abundant eosinophilic cytoplasm [3–6]. Approximately one third of SMBTs are associated with endometriosis, which is seldom found in patients with serous or mucinous borderline tumors [3].

Although SMBT generally portends an excellent prognosis (with only a few cases of recurrence and a single death reported in the literature) [8], the neoplasm may display unfavorable histological features – including micropapillary growth pattern, intraepithelial carcinoma, and presence of microinvasion [3–6]. Furthermore, SMBT may progress toward a form of invasive carcinoma termed seromucinous carcinoma (SMC) by the 2014 World Health Organization (WHO) Classification of Tumors of Female Reproductive Organs [9]. Currently, SMC cannot be considered a histologically well-defined entity but rather shares significant morphological and immunophenotypical overlaps with other types of ovarian tumors – particularly endometrioid carcinoma and low-grade serous carcinoma with mucinous differentiation [10, 11]. Owing to its low morphologic reproducibility and lack of defining genetic characteristics, some pathologists have even proposed to discontinue the term SMC [10].

The molecular underpinnings of SMBT remains poorly understood as well. Mutational analysis of SMBT has only been performed in one study, which solely focused on *KRAS* and *PTEN* mutations in 16 samples [12]. *KRAS* mutations were found to be common (69% of cases), whereas no *PTEN* mutations were detected – resulting in a genetic profile similar to that of mucinous borderline tumor (of gastrointestinal type) [12]. Another report demonstrated the loss of immunohistochemical expression of ARID1A (a surrogate for *ARID1A* mutations) in 33% of SMBT samples, i.e., a frequency in line with that of other endometriosis-related ovarian neoplasms (clear cell carcinoma and endometrioid carcinoma) [13]. Here, we used massively parallel sequencing to comprehensively decipher the mutational landscape of SMBT – with a special focus on the clonal relationships between tumors showing a bilateral presentation. In an effort to shed more light on the controversial issue of SMC, we also investigated the genetic characteristics of invasive carcinomas that arose in association with SMBTs.

Materials and methods

Patient identification and retrieval of tissue specimens

We searched our departmental surgical pathology archives and identified 44 patients with ovarian tumors diagnosed as either seromucinous borderline (or borderline seromucinous) tumor ($n = 37$) or endocervical-type (or -like) mucinous borderline tumor ($n = 7$) between January 1, 2005 and December 31, 2016. Two pathologists (R.C.W. and S.M.J.) independently reviewed all hematoxylin and eosin-stained slides to identify SMBT specimens. Seven cases were excluded because the tumor histology is not typical for SMBTs. Additional 9 patients were excluded because the tumor sizes were deemed too small for further molecular analysis (atypical proliferative part <5 mm). The final study cohort consisted of 28 patients with a confirmed pathological diagnosis of SMBT (21 unilateral and seven bilateral). Concurrent invasive ovarian carcinoma adjacent to SMBT was evident in three patients, whereas one case had a synchronous uterine endometrioid carcinoma. For the purpose of the study, the following formalin-fixed, paraffin-embedded (FFPE) tissue specimens were retrieved: SMBT ($n = 33$, including five cases with bilateral disease), SMBT-concurrent carcinoma ($n = 4$), and matched benign control tissue from lymph nodes, fallopian tubes, or uterus ($n = 28$). Clinical characteristics included age at diagnosis, disease stage, type of surgery, and survival figures. Tissue specimens were drawn from the tissue bank of the Chang Gung Memorial Hospital (Taoyuan, Taiwan) after ethical approval was granted by the Institutional Review Board of Chang Gung Memorial Hospital (approval number: 201701220B0). Owing to the retrospective nature of the study, the need for informed consent was waived.

Sample processing and DNA extraction

A pathologist (R.C.W.) performed a careful selection of representative FFPE blocks and identified areas of SMBT or invasive carcinomas suitable for macrodissection. A thorough manual dissection of different tumor components was conducted on 10-μM-thick tissue sections to reduce contamination and – in case of invasive carcinoma samples – to rule out the presence of non-invasive tissue. Genomic DNA for mutation analysis was extracted from FFPE samples using a commercially available kit (Qiagen Inc., Valencia, CA, USA) as previously described [14, 15].

Massively parallel sequencing and data analysis

Paired tumor and normal samples were subjected to massively parallel sequencing targeting the coding regions of 409 cancer-related genes. The sequencing procedures and the approach used for data analysis have been previously described in detail [14, 15]. In brief, genomic DNA from each sample (80 ng) was amplified by polymerase chain reaction (PCR) to enrich the coding exons of targeted genes. To this aim, AmpliSeq Comprehensive Cancer Panel primer pools (Thermo Fisher Scientific, Waltham, MA, USA) were used. Amplicons were subsequently ligated with barcoded adaptors, conjugated with sequencing beads, and enriched using Ion Chef (Thermo Fisher Scientific) according to the Ion Torrent protocol. Sequencing was performed on an Ion Proton sequencer using the Ion PI chip (Thermo Fisher Scientific). Raw data from the sequencer were mapped to the hg19 reference genome using the Ion Torrent Suite (v. 4.2). Single nucleotide variants and short insertion/deletions were identified with the Torrent Variant Caller plug-in (v. 4.2). All variants were annotated using Variant Effect Predictor (VEP, release 78) and filtered out when their frequency was <5% or in presence of <50 reads. Variants not identified in matched normal samples were considered as somatic mutations. Further annotation was performed using COSMIC (v. 70), dbSNP (138), and 1000 Genomes (phase 1).

Mutation analysis of the *TERT* promoter

The promoter region of the *TERT* gene – which is known to contain two mutation hotspots (chr5: 1,295,228 and 1,295,250; hg19) – was amplified by PCR using the following primers: 5'-M13-CAGCGCTGCCTGAAACTC-3' and 5'-GTCCTGCCCCTTCACCTT-3', where M13 indicates a universal sequencing primer (5'-GTAAAAC GACGGCCAGT-3'). PCR conditions were as follows: 95 °C for 5 min, followed by 45 cycles at 98 °C for 20 s, 60 °C for 15 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. Amplified PCR products were purified and subjected to Sanger sequencing.

Immunohistochemistry

Immunohistochemical staining of 3-µm-thick paraffin sections was performed using the following antibodies: ARID1A (1:200 dilution; clone HPA005456, MilliporeSigma, St. Louis, MO, USA), ER (1:200; clone 6F11, Leica Biosystems), MLH1 (1:50 dilution; clone GM011, Genemed Biotechnologies, Torrance, CA, USA), Napsin A (1:200 dilution; clone IP64, Leica Biosystems), p53 (1:100 dilution; clone DO7, Leica Biosystems, Buffalo Grove, IL, USA), PAX8 (1:50 dilution; clone BC12, Biocare, Pacheco,

CA, USA), PR (1:400 dilution; clone 16, Leica Biosystems), PTEN (1:100 dilution; clone 138G6, Cell Signaling, Danvers, MA, USA), and WT1 (1:200 dilution; clone 6F-H2, Quartett, Berlin, Germany). Immunostaining was performed on a BOND-MAX automated stainer (Leica Biosystems). Heat-induced epitope retrieval was performed at 100 °C using citrate-based pH 6.0 buffer (BOND Epitope Retrieval Solution 1, Leica Biosystem) for Napsin A, and EDTA-based pH 9.0 buffer (BOND Epitope Retrieval Solution 2, Leica Biosystems) for the other antibodies. Immunoreactivity was assessed with a BOND Polymer Refine Detection system (Leica Biosystems). For ER, PR, Napsin A, and PAX8, reactions were interpreted as positive if at least 5% of tumor cells showed expression.

Results

Patient characteristics

The clinical characteristics of the 28 patients with SMBT (unilateral, $n = 21$; bilateral, $n = 7$) are summarized in Table 1. The median age at diagnosis was 37 years (range, 25–58 years). In general, patients presented with early-stage disease with 26 (92.9%) diagnosed at stage I and two at stage II, with a median tumor size of 7.7 cm (range, 2.7–15.2 cm). The median length of follow-up was 38.8 months (range, 14.6–240.6 months). Disease recurrence was observed in one case only (S20). The patient initially underwent surgical removal of an SMBT (stage II) involving the left ovary and the pelvic peritoneum. Twenty-seven months thereafter, a right ovarian SMBT was diagnosed and excised. A recurrence of SMBT at the right ovary was observed at 127 months of follow-up. The patient was successfully salvaged with hysterectomy and right salpingo-oophorectomy. At the time of last follow-up, all participants were alive without disease.

Histopathologic characteristics of seromucinous borderline tumors

All SMBTs were characterized by the presence of hierarchical papillary structures lined by endocervical-like mucinous epithelial cells and cilia-bearing serous epithelial cells (Fig. 1a, b). Moreover, variable amounts of endometrioid cells, squamous cells, piling-up clear cells, and "indifferent cells" with ample eosinophilic cytoplasm were observed (Fig. 1b–d). Nuclear atypia in SMBT cells was generally mild-to-moderate. All SMBTs showed, at least focally, prominent intraepithelial and mesenchymal neutrophilic infiltration (Fig. 1c, d). Five cases (17.9%) had evidence of focal intraepithelial carcinoma characterized by exuberant cribriform proliferation over papillary surfaces or

Table 1 Characteristics of patients with seromucinous borderline tumors.

Patient ID	Age (years)	Disease stage	Surgery type	Laterality	Tumor size (mm)	Follow-up (months)	Sample ID
S01	38	IB	LAVH+BSO+Om+Ap	B	L50/R19	14.6	S01L
S02[a]	40	IB	RSO+Lenu	B	L27/R99	17.4	S02L/R
S03	43	IA	LSO+Renu	L	60	18.2	S03L
S04	32	IC	RSO+Lenu (LS)	R	65	20.3	S04R
S05	39	IC	RSO+Lenu	R	80	23.6	S05R
S06	40	IA	ATH+BSO+Om+Ap+BPLN	L	78	24.9	S06L/EM[b]
S07	25	IC	Lenu (LS)	L	54	26.6	S07L
S08	47	IC	RSO	R	67	24.6	S08R/R_ca[c]
S09	29	IC	RSO (LS)	R	45	29.1	S09R
S10	32	IC	Renu (LS)	R	27	34	S10R
S11	30	IC	LSO+Renu+Om+Ap+LPLN	L	69	19.5	S11L
S12	26	IC	LSO (LS)	L	125	44.7	S12L
S13	31	IC	Renu+Lenu+BPLN (LS)	R	112	26.6	S13R
S14	49	IC	ATH+BSO+Om	R	76	43.6	S14R
S15	33	IC	RSO+RPLN+Om+Ap+Bx	R	152	25.4	S15R
S16[a]	32	IB	LSO+Renu	B	L92/R85	77.8	S16L/R
S19	30	IB	Renu+Lenu	B	L65/R105	73.9	S19R
S20	38	II	LSO+Om+LPLN ->Renu -> RSO+ATH	L ->R (recurrent)	100	240.6	S20R
S21[a]	51	IB	ATH+BSO+BPLN+Ap+Om	B	L35/R140	97.4	S21L/R/R_ca[d]
S23	41	IC	BSO (LS)	B	L70/R70	102.4	S23R
S24	35	IC	RSO+RPLN+Om+P Bx	R	70	115.5	S24R
S26	47	IC	ATH+BSO	R	105	128.6	S26R
S27	40	IC	RSO+Lenu+RPLN+Om+Ap	R	115	17.2	S27R
S28[a]	29	IB	LSO+Renu+BPLN+Om+Ap	B	L115/R118	58.3	S28L/R
S29	31	IC	ATH+RSO+Lenu+Om+Ap	R	109	120.5	S29R/R_ca[d]
S31	49	IC	ATH+BSO	L	90	133.7	S31L
S32[a]	58	IB	LSO+Renu	B	L51/R29	53.8	S32L/R
S33	30	II	LSO+Renu+CDS Bx	L	L85	61.9	S33L

ATH abdominal total hysterectomy, *LAVH* laparoscopically-assisted vaginal hysterectomy, *BSO* bilateral salpingo-oophorectomy, *BPLN* bilateral pelvic lymphadenectomy, *RSO* right salpingo-oophorectomy, *LSO* left salpingo-oophorectomy, *LS* laparoscopy, *Ap* appendectomy, *Om* omentectomy, *Lenu* left enucleation, *Renu* right enucleation, *LPLN* left pelvic lymphadenectomy, *CDS* cul-de-sac, *P* peritoneum, *Bx* biopsy.

[a]Bilateral seromucinous tumors were subjected to sequencing.

[b]Concurrent uterine endometrioid carcinoma.

[c]Concurrent ovarian clear cell carcinoma.

[d]Concurrent ovarian seromucinous carcinoma.

cystic linings (Fig. 1e–f). There was no evidence of micropapillary growth pattern or microinvasion in any of the study specimen. Three cases had foci of invasive growth adjacent to SMBT (all >5 mm in their greatest dimension) and their histological and genetic features will be subsequently described in detail. There were two cases in whom SMBT was associated with mature cystic teratoma ($n = 1$) and synchronous uterine endometrioid carcinoma ($n = 1$). A total of 13 (46%) patients had concomitant endometriosis (Fig. 2a). All endometriotic lesions were cystic involving ovary in 13 patients.

Mutational landscape of seromucinous borderline tumors

Massively parallel sequencing identified a median of three somatic mutations (range, 1–10) in each sequenced SMBT specimen. Six genes were found mutated in at least two cases (Fig. 2a; Supplementary Fig. 1). Strikingly, somatic *KRAS* mutations were invariably identified in all SMBT samples – all of them being hotspot mutations involving codons 12 or 13 (p.G12A, p.G12C, p.G12D, p.G12V, and p.G13D). Somatic mutations of the *PIK3CA*

Fig. 1 Representative photomicrographs of seromucinous borderline tumors (SMBTs). a Presence of hierarchical papillary structures. **b** Lined by an admixture of mucinous cells, cilia-bearing cells. **c** Endometrioid cells, piling-up clear cells. **d** Piling up squamous cells with mild-to moderate nuclear atypia, (**c** and **d**) prominent neutrophilic infiltration was evident. **e** An SMBT specimen with a region of intraepithelial carcinoma (lower portion of the figure). **f** Intraepithelial carcinoma characterized by an exuberant cribriform proliferation over papillary surfaces and cystic linings.
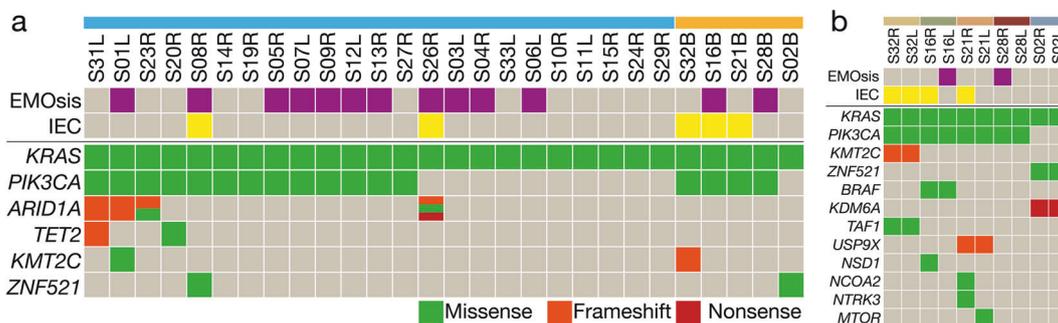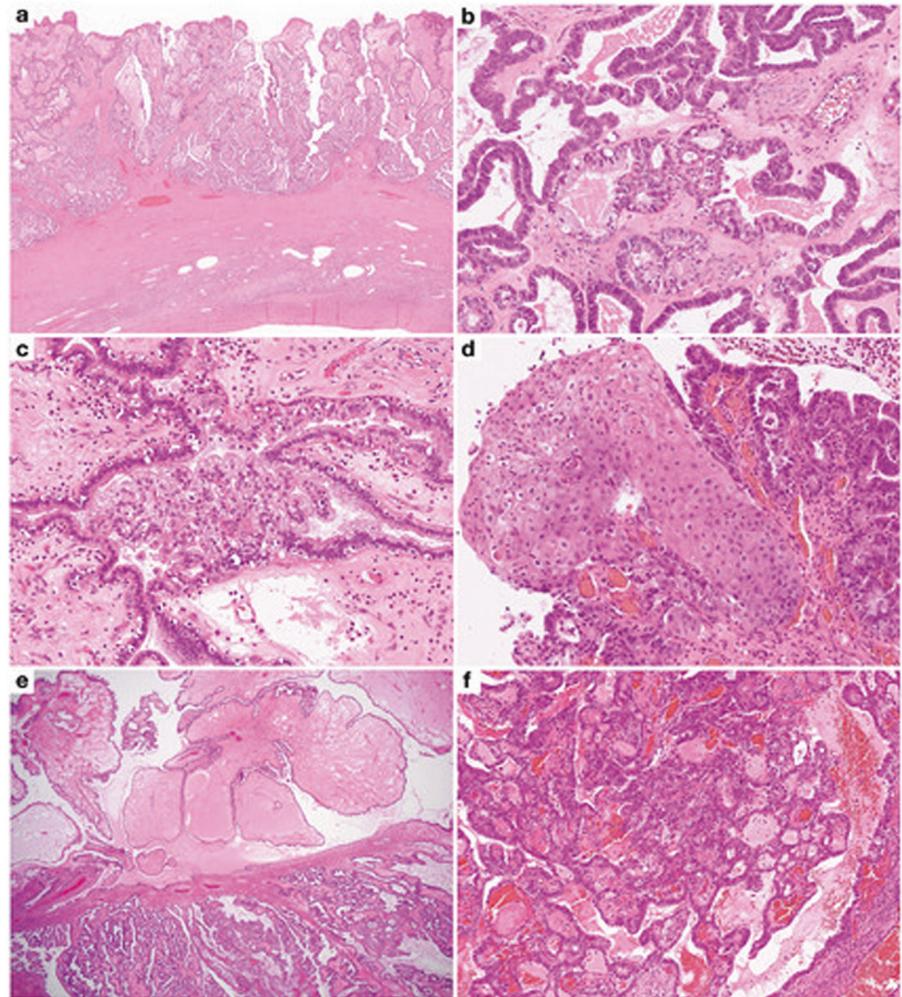

Fig. 2 Oncoplots summarizing nonsynonymous somatic mutations identified in seromucinous borderline tumor (SMBT) specimens. a List of the six most commonly mutated genes (in decreasing order from the top to the bottom). The five cases below the orange bar had bilateral disease (both lesions subjected to sequencing). **b** A clonal relationship between bilateral SMBTs was evident when the somatic mutations detected in paired samples were compared. The concomitant presence of endometriosis (EMOsis) and intraepithelial carcinoma (IEC) is reported in the first two rows.

and *PTEN* genes were identified in 60.7% (17/28) and 3.6% (1/28) of the SMBT specimens, respectively, in a mutually exclusive fashion. Four (14.3%) patients harbored *ARID1A* truncating mutations, whereas somatic mutations in the *KMT2C*, *TET2*, and *ZNF521* genes were identified in two (7.1%) cases. No mutations in genes coding for DNA mismatch repair proteins (i.e., *MLH1*, *MSH2*, *MSH6*, *PMS1*, and *PMS2*) were identified. Similarly, Sanger sequencing did not identify *TERT* promoter mutations. A complete list of somatic mutations detected

Fig. 3 Representative immunohistochemical staining of MLH1, p53, PTEN, and ARID1A in seromucinous borderline tumor (SMBT) specimens (20× objective lens). a An intact MLH1 nuclear expression was evident in all samples. **b** A heterogeneous, "wildtype" p53 staining pattern was detected in all specimens. **c** Loss of PTEN expression in an SMBT sample (S26R). **d** Loss of ARID1A nuclear expression in an SMBT sample (S31L).
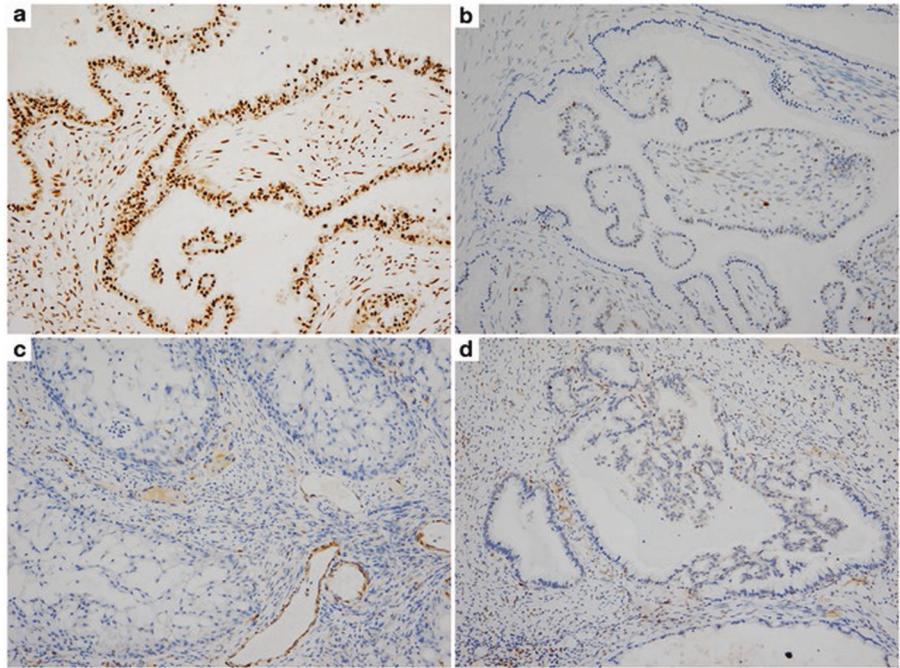
Fig. 4 Histological and genetic characteristics of carcinomas that arose concurrently with seromucinous borderline tumors (SMBTs). a A case (S29) of SMBT (left upper part of the image) with an adjacent invasive ovarian carcinoma (right lower part, 4× objective lens). **b** The invasive ovarian carcinoma diagnosed in case S29 – a seromucinous carcinoma (SMC) – consisted of confluent glandular structures lined by endocervical-like mucinous, endometrioid, and eosinophilic indifferent cells (20× objective lens). **c** The SMC diagnosed in case S21 was characterized by the presence of glandular structures lined by mucinous, endometrioid, and squamoid cells (20× objective lens). **d** The SMC diagnosed in case S21 showed focal neoplastic glands lined by cells with clear cytoplasm (20× objective lens). **e** A case (S08) of ovarian clear cell carcinoma that arose adjacent to SMBT; note the presence of tubulopapillary structures lined by clear cells (20× objective lens). **f** Oncoplot comparing the mutational landscapes of SMBT and concurrent ovarian carcinomas.
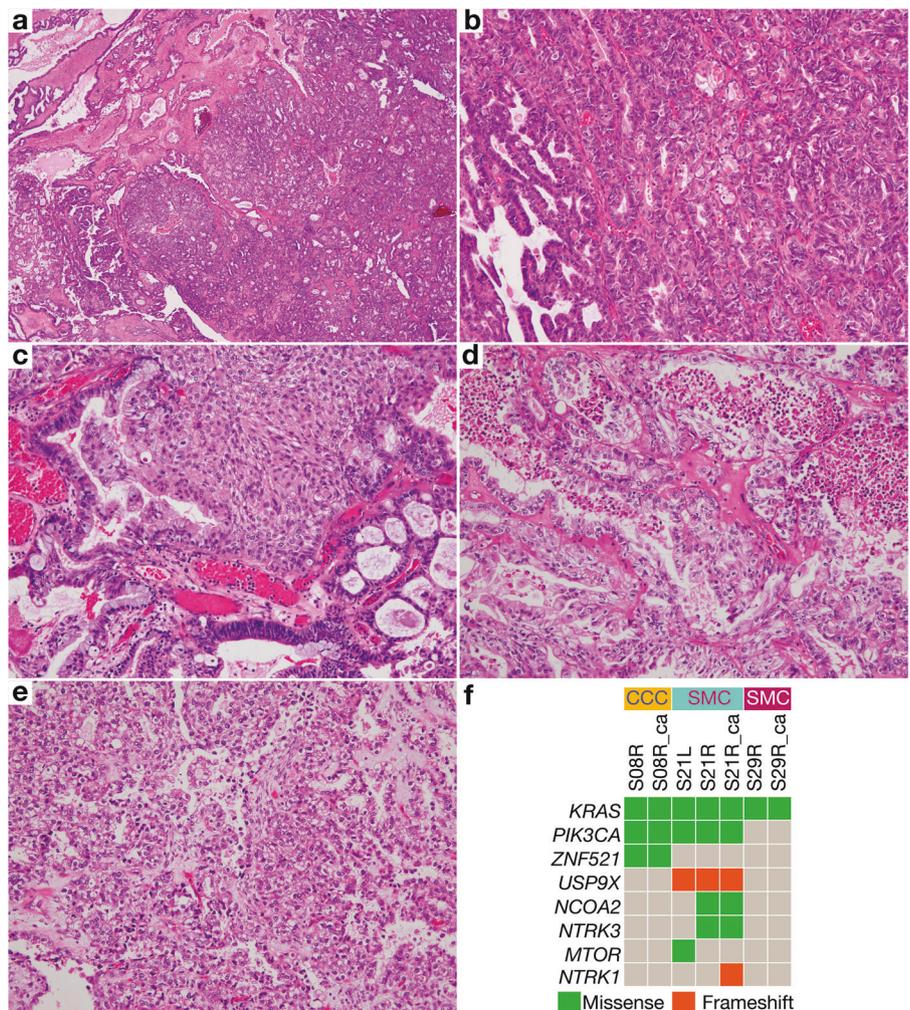
in SMBT specimens is provided in Supplementary Table 1.

Clonal relationship between bilateral seromucinous borderline tumors

We subsequently focused on cases with bilateral SMBT ($n = 7$). Of them, we were able to extract sufficient amounts of high-quality DNA from paired samples derived from five patients. Targeted massively parallel sequencing revealed that all paired specimens shared at least two somatic mutations (range: 2–4), suggesting that bilateral SMBTs were clonally related to each other (Fig. 2b). All of the five paired samples subjected to sequencing had an intact capsule without tumor cells over the ovarian surface – a finding that argued against the metastatic nature of the contralateral lesion.

Immunohistochemical analysis

All SMBTs were positive for PAX8 and negative for WT1. ER was expressed in all SMBTs and PR in most (91%) SMBTs. Napsin A was focally expressed (<25%) in two SMBT samples (S02R, S16R). The aforementioned results were in line with previous studies [16, 17], supporting the histological diagnosis of SMBT in our study cohort.

We analyzed the immunohistochemical expression of ARID1A, MLH1, p53, and PTEN in an effort to identify potential epigenetic silencing or large genomic deletions undetectable by targeted sequencing. All of the SMBT specimens were characterized by diffuse nuclear expression of MLH1 (Fig. 3a). These results indicate that epigenetic silencing of *MLH1* – which is commonly encountered in endometrioid carcinoma – is a rare event in SMBT. All of the samples showed heterogeneous p53 expression, suggesting that *TP53* genetic aberrations are invariably absent in SMBTs (Fig. 3b). Loss of PTEN expression was identified in a single specimen (sample S26R) known to harbor a somatic *PTEN* mutation (Fig. 3c). ARID1A expression was undetectable in three SMBT samples (S01L, S26R, and S31L), all of them carrying *ARID1A* mutations (Fig. 3d). Notably, the associated ipsilateral endometriosis also lost ARID1A expression (S01L and S26R). Taken together, the results of immunohistochemistry did not detect molecular aberrations other than those already identified by targeted sequencing.

Mutational analysis of concurrent gynecologic cancers in patients with seromucinous borderline tumor

Of the 28 study patients, three (S08, S21, and S29) had a concurrent invasive ovarian carcinoma adjacent to the SMBT (Fig. 4a). A fourth case (S06) was diagnosed with synchronous uterine endometrioid carcinoma. The invasive lesions identified in cases S21 and S29 were classified as SMC. In case S21, we observed an admixture of endocervical-like mucinous, endometrioid, and clear cells arranged predominantly in a confluent glandular pattern and focally in a solid pattern. In case S29, variable amounts of endocervical-like mucinous, endometrioid, and "indifferent cells" with abundant eosinophilic cytoplasm were structured in a confluent glandular pattern (Fig. 4b-d). The immunoprofiles of both SMCs were identical to those of the corresponding SMBTs. The invasive component of case S08 showed the typical histological findings of clear cell carcinoma – consisting of tumor cells with clear cytoplasm growing in either a tubulopapillary or solid pattern (Fig. 4e). Immunohistochemically, the clear cell carcinoma was positive for Napsin A and negative for ER and PR, whereas the adjacent SMBT was negative for Napsin A and positive for hormonal receptors.

Massively parallel sequencing revealed that all of the three concurrent invasive ovarian carcinomas shared certain somatic mutations with concomitant SMBTs – supporting the existence of a clonal relationship (Fig. 4f). The following three shared mutations were identified in patient S08: *KRAS* (c.35G>A), *PIK3CA* (c.1633G>A), and *ZNF521* (c.2836C>T). Five shared mutations were detected in patient S21, as follows: *KRAS* (c.35G>T), *PIK3CA* (c.3140A>G), *NCOA2* (c.1991C>A), *NTRK3* (c.446C>T), and *USP9X* (c.4548_4551delCAAA). A single shared mutation – *KRAS* (c.38G>A) – was identified in patient S29. The SMC identified in patient S21 also carried a mutation in *NTRK1* (c.1486_1487insC) – which was absent in the adjacent SMBT and may theoretically be involved in the progression from SMBT to SMC (Fig. 4f). In contrast, the synchronous uterine endometrioid carcinoma diagnosed in patient S06 did not share any somatic mutation with its coexisting SMBT (Supplementary Table 2).

Discussion

This study is the first comprehensive attempt to shed more light on the molecular underpinnings of SMBT through the application of next-generation sequencing on carefully dissected tumor specimens. Our main results can be summarized as follows. First, SMBT was found to have unique molecular features that set it apart from both other borderline tumors of the ovary and endometriosis-associated neoplasms. Specifically, the SMBT signature consisted of frequent somatic mutations in the *KRAS* (100%), *PIK3CA* (60.7%), and *ARID1A* (14.3%) genes, with *TERT* promoter mutations and DNA mismatch repair deficiencies being consistently absent. Second, we show that bilateral SMBTs

Table 2 Comparison of molecular aberrations among endometriosis-associated ovarian neoplasms.

Gene	SMBT	CCC	EMCA
KRAS	100%	4.7–7% [24, 27]	33.3% [22]
PIK3CA	60.70%	33–43% [21, 24, 27]	40.0% [22]
ARID1A	14.30%	46–57% [26, 27]	33.0% [26]
PTEN	3.6%[a&b]	5%[b] [24]	23.9%[a] [23]
CTNNB1	3.60%	1.0% [24]	53.3% [22]
TERT promoter	0%	15.9% [25]	0% [25]
dMMR	0%[a&b]	6%[a] [28, 29]	11.3%[a] [30]

SMBT seromucinous borderline tumor, *CCC* clear cell carcinoma, *EMCA* endometrioid carcinoma, *dMMR* deficient DNA mismatch repair.

[a]Detected by immunohistochemistry.

[b]By sequencing.

are frequent (25%) and clonally related to each other – as attested by the presence of shared somatic mutations. Third, a similar clonal relationship was identified between SMBT and concurrent ovarian carcinomas – including SMC and clear cell carcinoma. In light of these findings, the presence of *KRAS* mutations may serve as a genetic hallmark of SMBT – a finding that confirm and expand previous observations [12].

Formerly known as endocervical-type mucinous borderline tumor, SMBT was reclassified as a separate disease category in the latest 2014 WHO Classification of Tumors of Female Reproductive Organs [9]. Because the term "seromucinous" may cause confusion with serous and mucinous borderline tumors, Kurman and Shih have previously recommended the term "mixed müllerian borderline tumor" and emphasized the morphological and immunohistochemical differences between SMBT and serous/mucinous borderline tumor [18]. Our study provides further molecular genetic evidence that distinguishes SMBT from serous/mucinous borderline tumor. Differently from serous borderline tumor – which is characterized by mutually exclusive mutations in *KRAS* (17–39.5% of cases) and *BRAF* (23–48% of cases) [19] – our SMBT samples were found to invariably harbor *KRAS* mutations, with *BRAF* being rarely mutated (3.6%). Notably, a very high rate of *KRAS* mutations (92.3%) has been previously reported in mucinous borderline tumor [20] – potentially indicating a shared molecular basis with SMBT. However, *PIK3CA* mutations – which were identified in 60.7% of our SMBT samples – seem to occur much less frequently in MBT (15%) [20]. Moreover, we did not identify *CDKN2A* mutations – previously reported in 19.2% of MBT cases [20] – in our SMBT specimens.

Although SMBT is frequently associated with endometriosis (46% of cases in the current study), our data indicate that the mutational landscape of SMBT is distinct from that of other endometriosis-associated neoplasms — including ovarian endometrioid carcinoma and clear cell carcinoma (Table 2) [21–30]. Differently from ovarian endometrioid carcinoma, *CTNNB1* mutations, loss of PTEN expression, or DNA mismatch repair deficiencies were rarely identified in our SMBT specimens [22, 23, 30]. In addition, SMBT was distinct from ovarian clear cell carcinoma, as *TERT* promoter mutations were not found in SMBT whereas *KRAS* mutations were ubiquitous in SMBT [24, 25].

Not uncommonly, patients with SMBT show bilateral ovarian involvement at presentation [31]. Here, we demonstrated for the first time that bilateral ovarian SMBTs were clonally related to each other. Two potential explanations for this clonal relationship could be offered, including: (1) metastatic spread of a primary SMBT to the contralateral ovary, or (2) independent onset of contralateral SMBT from clonal endometriotic lesions affecting both ovaries. Notably, a similar clonal relationship has been previously reported for bilateral ovarian serous borderline tumor, which is likely attributable to contralateral ovarian metastasis owing to the frequent presence of ovarian surface involvement by these neoplasms [31]. However, all bilateral SMBTs identified in our study were characterized by an intact capsule and the absence of tumor cells over the ovarian surface. These observations argue against a metastatic origin and support the view that bilateral SMBTs arise independently of each other – most likely from clonally related bilateral ovarian endometriotic lesions harboring driver mutations in *KRAS*, *PIK3CA*, or other genes [32, 33].

SMC – a poorly characterized entity of ovarian epithelial cancer introduced in the 2014 WHO Classification of Tumors of Female Reproductive Organs [9] – can exhibit a wide variety of histopathological features consisting of an admixture of different cell types (including endocervical-like mucinous, endometrioid, eosinophilic "indifferent", hobnail, squamous, signet-ring, and clear cells) [11]. The question as to whether SMC should be regarded as a distinct category of ovarian cancer is still a matter of debate owing to its obvious morphological overlaps with low-grade serous, mucinous, and endometrioid carcinomas [10, 11]. From the perspective of multistep carcinogenesis, it is reasonable to regard as "true" SMC those arising in association with SMBT – which accounted for approximately 50% of cases previously reported in a large series [11]. SMBT-associated SMCs are likely to derive from SMBTs – as shown by the clonal relationship between SMBTs and SMCs identified in our study. They should be possibly regarded as a genetically distinct group of neoplasms in which *KRAS* and *PIK3CA* are commonly mutated. However, the differential diagnosis between SMCs without a coexisting SMBT component and other histotypes (especially endometrioid carcinoma with mucinous

differentiation) is challenging at best and frequently impossible [10]. Because *KRAS* is invariably mutated in SMBT, it can be hypothesized that the absence of *KRAS* somatic mutations may help identify at least certain SMC mimickers. Unfortunately, *KRAS* mutations are not uncommon in endometrioid carcinoma with mucinous differentiation – ultimately limiting the diagnostic utility of this molecular approach [10, 34].

In our study, we identified one patient with SMBT and concurrent ovarian clear cell carcinoma – with the two lesions being clonally related. To our knowledge, only another similar case has been reported in the literature [35]. While this observation seems to suggest that SMBT may act as a precursor to clear cell carcinoma, such lesions might as well be collision tumors. Indeed, an SMBT and a clear cell carcinoma within a collision tumor may appear clonally related to each other if they arise independently from the same endometriotic cyst that already harbors cancer driver mutations.

In conclusion, our current data represent the first extensive characterization of SMBT in terms of histology, immunohistochemistry, and molecular pathogenesis. If independently confirmed, our findings may have significant implications for disease classification and molecular diagnostics.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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