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Original Article

TP53 variants in p53 signatures and the clonality of STICs in RRSO samples

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ABSTRACT

Objective: Precursor lesions may be identified in fallopian tube tissue after risk-reducing salpingo-oophorectomy (RRSO) in patients with pathogenic variants of *BRCA1/2*. Serous tubal intraepithelial carcinoma (STIC) is considered a precursor of high-grade serous carcinoma, whereas the significance of the p53 signature remains unclear. In this study, we investigated the relationship between the p53 signature and the risk of ovarian cancer.
Methods: We analyzed the clinicopathological findings and conducted DNA sequencing for *TP53* variants of p53 signatures and STIC lesions isolated using laser capture microdissection in 13 patients with pathogenic variants of *BRCA1/2* who underwent RRSO and 17 control patients with the benign gynecologic disease.
Results: *TP53* pathogenic variants were detected significantly higher in RRSO group than control (p<0.001). No difference in the frequency of p53 signatures were observed between groups (53.8% vs 29.4%; p=0.17). *TP53* sequencing and next-generation sequencing analysis in a patient with STIC and occult cancer revealed 2 *TP53* mutations causing different p53 staining for STICs and another *TP53* mutation shared between STIC and occult cancer.
Conclusion: The sequence analysis for *TP53* revealed 2 types of p53 signatures, one with a risk of progression to STIC and ovarian cancer with pathological variants in *TP53* and the other with a low risk of progression without pathological variants in *TP53* as seen in control.

Keywords: Prophylactic Surgical Procedures; Salpingo-Oophorectomy; Genes, p53; Genes, BRCA1; Genes, BRCA2; Carcinoma in Situ; Cystadenocarcinoma, Serous

Synopsis

We conducted a DNA sequencing for *TP53* variants in p53 signatures from patients with pathogenic variants of *BRCA1/2* who underwent risk-reducing salpingo-oophorectomy (RRSO) and control patients with the benign gynecologic disease. *TP53* pathogenic variants were detected significantly higher in RRSO group. The characteristics of p53 signatures might be different depending on BRCA status.

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Conflict of Interest
Hirasawa, a received lecture fee from Chugai pharmaceutical Co. Ltd.

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INTRODUCTION

TP53 mutation is one of the most common genetic alterations in cancer. Ovarian cancer, particularly high-grade serous carcinoma (HGSC), has the highest frequency of *TP53* mutations compared to all cancer types, with more than 90% of cases reported to have *TP53* mutations [1].

Serous tubal intraepithelial carcinoma (STIC), characterized by non-ciliated tubal epithelial cells that show marked nuclear atypia, mitotic figures, apoptotic bodies, loss of cellular polarization, abnormal p53 staining (a pattern compatible with either missense or deletion mutations), and an increased Ki-67 labeling index, has been recognized as a precancerous lesion of HGSC [2]. It is often found in fallopian tube tissue after risk-reducing salpingo-oophorectomy (RRSO) in hereditary breast and ovarian cancer (HBOC) patients with pathogenic variants of *BRCA1* and *BRCA2* and also found in fallopian tube tissue from patients with HGSC [3-6]. The coincidental finding of the *TP53* mutation in both HGSC and STIC has led to the recognition of STIC as an early lesion and the *TP53* mutation as an early driver mutation of HGSC [1,7-11].The similarity in morphology, immunophenotype, and gene expression patterns between fallopian tube epithelium and HGSC provides additional evidence that HGSC develops from the fallopian tube [12].

The p53 signature is another lesion that can be seen in epithelial cells in the fallopian tube and is characterized by 12 or more consecutive cells with abnormal p53 immunostaining in morphologically normal tubal epithelium. Often found in RRSO specimens, the p53 signature is also considered to be a precursor lesion for STIC. However, the p53 signature can also be detected in benign specimens without pathogenic *BRCA1/2* variants. Therefore, the association of the p53 signature and the risk of cancer development is unknown [13-15].

In this study, to investigate the risk of ovarian cancer in p53 signatures, we selectively analyzed *TP53* variants in p53 signatures on specimens from RRSO group and benign control group. We also analyzed the clonality of cancer and STIC by combining *TP53* variant analysis and panel sequencing for a case with different p53 staining STICs. This study clarified the significance of *TP53* variant analysis in p53 signatures and found new insights into the carcinogenesis of HGSC.

MATERIALS AND METHODS

1. Study groups

We investigated 13 HBOC patients with pathogenic germline *BRCA1* or *BRCA2* variants in whom RRSO was performed at Keio University Hospital between February 2013 and September 2019 (**Table S1**). All patients underwent genetic counseling, and their family history of cancer was obtained as previously described [16]. Seventeen patients with benign gynecologic disease in whom bilateral salpingo-oophorectomy was performed between February 2013 and September 2020 were also included as controls (**Table S2**). Clinical information was collected from the medical records. The study was approved by the ethics committee of Keio University School of Medicine (approval numbers: 20070081, 20130477, and 20160443). Informed consent was obtained from all patients prior to enrolment in the study.

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2. Pathologic analysis

All RRSO samples were analyzed using the Sectioning and Extensively Examining the FIMbriated end protocol [17]. In total, 313 embedded blocks were obtained from 13 patients; the mean number of blocks for each fallopian tube was 12 (range=6–22). The mean number of blocks per one fimbria was 3 (SD, ±1.5) in the RRSO group, no significant difference compared to the control group (2.5±1.0). The p53 signature was diagnosed as morphologically normal in tubal epithelial cells if there were at least 12 consecutive p53-positive secretory cells showing a low proliferative index (i.e., Ki67 <10%). STIC was defined as consecutive non-ciliated tubal epithelial cells showing marked nuclear atypia, mitotic figures, apoptotic bodies, loss of cellular polarization, an abnormal p53 staining pattern (compatible with either missense or deletion mutations), and an increased Ki-67 labeling index (Ki67 >10%). The samples were also evaluated for the presence or absence of occult cancer [13,18-21].

3. Immunohistochemistry

Expression levels of p53, Ki67/MIB1, c-Myc, Pax8, and WT-1 were analyzed in formalin-fixed, paraffin-embedded (FFPE) samples. Antigen retrieval was performed by autoclaving the samples in 10 mM sodium citrate buffer (pH 6.0) at 121°C for 1 minute. The following primary antibodies were used: anti-human p53 protein mouse monoclonal antibody (DO-7; Dako, Glostrup, Denmark), anti-human Ki67/MIB1 protein rabbit monoclonal antibody (SP6; Nichirei Bioscience, Tokyo, Japan), anti-human c-Myc protein rabbit monoclonal antibody (EP121; Nichirei Bioscience), anti-human Pax8 protein mouse monoclonal antibody (BC12; Nichirei Bioscience), and anti-human Wilms' tumor (WT-1) protein mouse monoclonal antibody (WT49; Leica Biosystems, South San Francisco, CA, USA). The secondary antibody was mouse and rabbit Histofine Simple Stain MAX-PO (Nichirei Bioscience). Protein expression was visualized by 3, 3′-diaminobenzidine and 3-amino-9-ethylcarbazole (codes 425011 and 415131, Nichirei Bioscience). The nuclei were counterstained with Mayer's hematoxylin. The stained slides were imaged using a Nano Zoomer-XR C12000 virtual slide scanner (Hamamatsu Photonics, Shizuoka, Japan).

4. Laser capture microdissection (LCM)

The FFPE tissue was cut into 3-μm sections and placed on a slide for LCM (Carl Zeiss Microscopy GmbH, Jena, Germany). The sections were deparaffinized and stained with hematoxylin. Approximately 20–50 epithelial cells from fallopian tube tissue were collected into 0.5-mL AdhesiveCap 200 under the microscope using the selective laser in the LCM system (PALM MB-IV; Carl Zeiss Microscopy). Genetic engineering research grade distilled water (Wako Pure Chemical Industries, Osaka, Japan) was then added to the micro tube cap and incubated overnight at room temperature.

5. I-PEP-PCR and direct sequencing of TP53

Exons 4–10 of the *TP53* gene were amplified from genomic DNA using the primer extension and I-PEP-PCR (improved primer extension and preamplification-polymerase chain reaction) method [22-24]. First-round PCR was performed using a multiplex method. The final volume of the reaction mixture was 30 μL and contained Ex Taq Hot Start Version and PCR reaction mix (Takara Bio Inc., Shiga, Japan). The second-round PCR was performed in a hemi-nested condition using AccuPrime Pfx DNA polymerase and Accu-Prime Pfx Reaction mix (Invitrogen, Carlsbad, CA, USA). The primer sequences are shown in **Table S3**. The PCR cycling conditions were as follows: preheating for 10 minutes at 95°C, 35 cycles at 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, 72°C for 10 minutes, and 4°C

thereafter. Direct sequencing was then performed using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA). The pathogenicity of the *TP53* mutations was assessed using the Catalog of Somatic Mutations in Cancer (COSMIC) database (https://cancer.sanger.ac.uk/cosmic) and the IARC TP53 database (https://p53.iarc.fr/TP53GeneVariations.aspx).

6. DNA analysis using next-generation sequencing

DNA was isolated from whole blood and HGSC tissue samples using the QIAamp Tissue and Blood Kit and the GeneRead DNA FFPE kit (Qiagen, Hilden, Germany). The quality of DNA was examined using the 4200 TapeStation (Agilent Technologies, Santa Clara, CA, USA) and Qubit 3.0 Fluorometer dsDNA BR assay kit (Thermo Fisher Scientific). A next-generation sequencing (NGS) library for amplicon sequencing was constructed from 40 ng (blood) and 250 ng (FFPE) of DNA using the QIAseq Targeted DNA Human Comprehensive Cancer Panel (Qiagen). which targets 275 cancer-related genes (qiaseq targeted dna panels - GeneGlobe (qiagen.com). The constructed library was quantified by quantitative PCR using the QIAseq Library Quant Assay kit (Qiagen). The library was denatured with 0.2 N NaOH (cat#72068; Sigma-Aldrich, St. Louis, MO, USA), diluted with hybridization buffer to a concentration of 20 pM pooled for multiplexed sequencing. High-throughput sequencing was then performed using an Illumina MiSeq instrument in 2×150 bp paired-end reads (Illumina, San Diego, CA, USA).

7. Bioinformatics analysis

Bioinformatics analysis of the QIAseq Targeted DNA Human Comprehensive Cancer Panel was performed using the GeneGlobe Data Analysis Center (https://geneglobe.qiagen.com/jp/analyze).

The read processing, including read trimming, read alignment, post-alignment read pair filtering, marking reads putatively from the same input molecule, and gene-specific primer alignment masking prior to variant calling, was performed, and variant calling was performed using the UMI-aware variant caller smCounter2. SnpEff was used to annotate variants (http://snpeff.sourceforge.net/index.html). Somatic variants were selected by the criteria that germline and somatic pair variants have allelic frequencies greater than 0.4% or somatic-only variants, that are registered as "pathogenic/likely pathogenic" in ClinVar or Cosmic database or the annotation impact of SnpEff was "HIGH". Copy number variant was analyzed by the published algorithm (https://github.com/reineckef/quandico).

8. Statistical analysis

Data were analyzed using a 2-tailed student's t-test under the assumption of normal distribution for biological parameters and the χ^2 test for the contingency table analysis by GraphPad Prism 8. The p-value of <0.05 was considered statistically significant.

RESULTS

1. p53 signatures in the RRSO and control specimens

Seven (53.8%) of the 13 patients with *BRCA1/2* pathogenic variants (BRCA-positive) harbored at least one p53 signature in their fallopian tube tissue (**Table S1**). One patient harbored both STIC lesions in the fallopian tube and occult cancer in the ovary. Seventeen lesions in 7 patients were diagnosed to contain p53 signatures. Twelve (70.5%) of these 17 lesions were identified in the fimbria, 2 (11.7%) in the infundibulum and ampulla, and 1 (5.8%) in the isthmus (**Table 1**, **Fig. 1**). There was no significant difference in age, body mass index,

Table 1. Location of p53 signatures in risk-reducing salpingo-oophorectomy samples

Right or left fallopian tube	Location				Total
	Isthmus	Ampulla	Infundibulum	Fimbria	
Right fallopian tube	0	2	1	6	9
Left fallopian tube	1	0	1	6	8
Right+left fallopian tube	1 (5.8)	2 (11.7)	2 (11.7)	12 (70.5)	17 (100.0)

Values are presented as number (%).

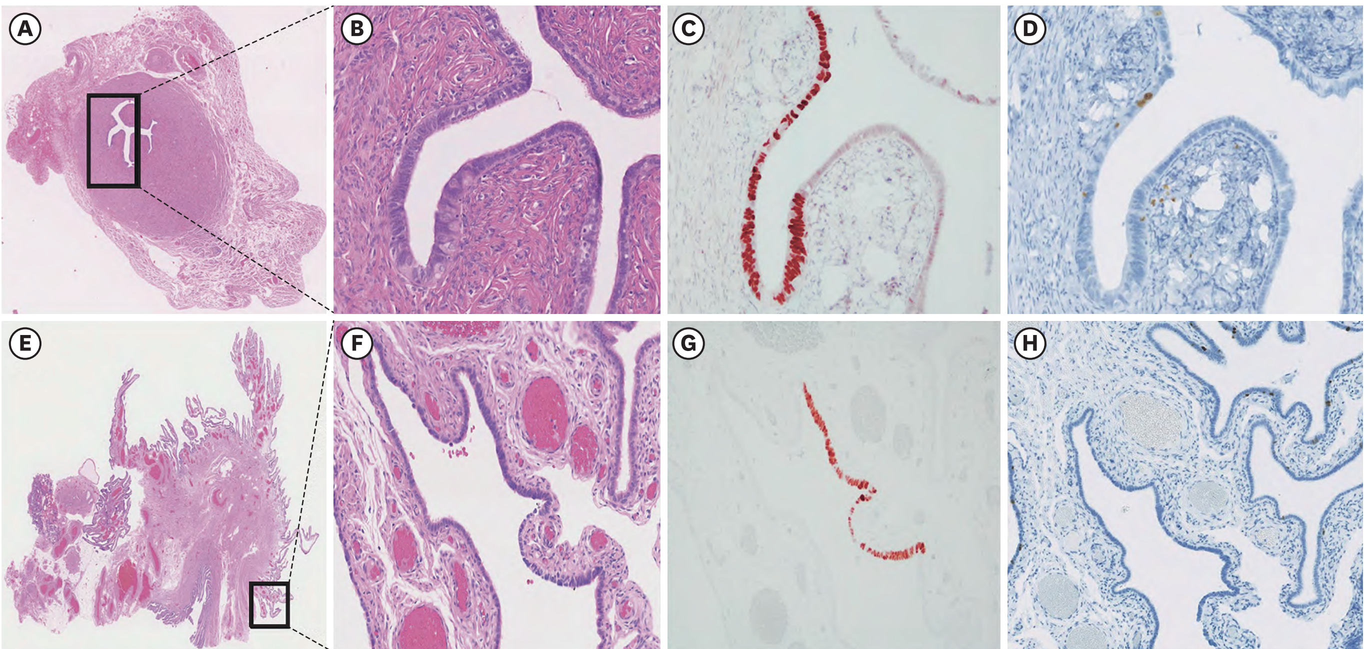

Fig. 1. Images of p53 signatures. (A, B) HE, (C) p53 IHC, (D) Ki67 IHC of the p53 signature in the isthmus of the fallopian tube in a specimen obtained during risk-reducing salpingo-oophorectomy. (E, F) HE, (G) p53, and (H) Ki67 IHC of the p53 signature in the fimbria of a control sample.
HE, hematoxylin and eosin; IHC, immunohistochemistry.

parity, frequency of hormonal therapy for breast cancer, or frequency of oral contraceptive use according to p53 signature status in the RRSO group, but there was a trend towards differences by pathogenic variants of *BRCA1* or *BRCA2* ($p=0.053$) (**Table 2**). Next, the frequency of the p53 signature was compared in the fimbria, where p53 signatures are usually found, between the 13 BRCA-positive women and the 17 control women with no family history of cancer (**Fig. 1**). There was no significant difference in the frequency of the p53 signature in the fimbria between the 13 BRCA-positive women (n=7, 53.8%) and the 17 control women (n=5, 29.4%, $p=0.17$, **Table 3**). Furthermore, there was no difference in background characteristics between the BRCA-positive group and the control group, except in parity (**Table S4**).

2. TP53 variants in lesions with a p53 signature

Next, we isolated the p53 signature lesions in fallopian tube epithelial cells by LCM and analyzed the *TP53* variants. In the RRSO group, 10 types of *TP53* variant were identified in 5 (71.4%) of 7 patients with the p53 signature. All 5 patients had germline *BRCA1* pathogenic variants. Nine (90%) of the 10 types of *TP53* variant were pathogenic and one was of unknown significance (**Tables S1** and **S5**). In the control group, 5 types of *TP53* variant were identified in 3 (50%) of 6 patients with the p53 signature. None of these 5 variants were pathogenic. More pathogenic variants were identified in the RRSO samples than in the control samples ($p<0.001$) (**Table S5**). We also sequenced normal fallopian tube epithelial cells (without the

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Table 2. Characteristics of patients with and without p53 signatures

Characteristics	RRSO patients (n=13)			Control patients (n=17)		
	p53 signature (n=7)	No p53 signature (n=6)	p-value	p53 signature (n=6)	No p53 signature (n=11)	p-value
Mean age at operation (yr)	49.4	47.3	0.57*	49	44.9	0.11*
BMI (mean)	20.95	21.03	0.95*	22.97	21.83	0.48*
Parity			0.9*			0.9†
Nulliparous	1	1		4	7	
Parous	6	5		2	4	
Hormone therapy for breast cancer			0.85†			
No	5	4				
Yes	2	2				
GnRH analog						0.11†
No				2	8	
Yes				4	3	
OC use			-			-
Never	7	6		6	11	
Ever	0	0		0	0	
Race			-			-
Asian	7	6		6	11	
BRCA1/2 pathogenic variant			0.053†			NA
BRCA1	6	2				
BRCA2	1	4				

NA, not available.
*t-test; †χ^2 test.

Table 3. Frequency of p53 signature in fimbria of RRSO and control samples

Group	RRSO (13)	Control (17)	Total	p-value
p53 signature in fimbriae				p=0.17 (χ^2)
Positive	7 (53.8)	5 (29.4)	12	
Negative	6 (46.2)	12 (70.6)	18	

Values are presented as number (%).
RRSO, risk-reducing salpingo-oophorectomy.

p53 signature) as a negative control in 6 patients in which the p53 signature was identified, but no variant was identified in any of these cases (**Table S2**).

3. The TP53 variant analysis suggested a relationship between STIC and the origin of the cancer

One of the 13 patients in the RRSO group had occult HGSC and 2 STIC lesions with morphologic changes. Ki67 stained diffusely in both STICs. One STIC lesion showed positive p53 staining while the other did not; therefore, these lesions were respectively termed "p53-positive STIC" and "p53-null type STIC" (**Fig. 2**). WT1 and PAX8, both of which are markers of HGSC, were positive in both STICs, and the pattern was comparable to that of occult cancer. Next, *TP53* variants were analyzed in both STICs using LCM (**Fig. 3**). In the p53-null type STIC, a nonsense mutation (c.617T>A) was detected, while in the p53-positive STIC, 2 mutations causing a frameshift of *TP53* were identified. One mutation (c.617delT) occurred at the same codon as the mutation (c.617T>A) detected in the p53-null type STIC, and the other mutation (c.983delT) was identical to that in the occult HGSC. The latter mutation in HGSC was confirmed by DNA analysis using NGS, which also revealed amplification of *MYC* and *CCND1*, the gene alteration commonly found in HGSC (**Fig. S1**). Immunohistochemistry confirmed that c-Myc protein expression was higher in the STIC and occult cancer than in normal fallopian epithelial cells (**Fig. 2**).

Fig. 2. Immunohistochemistry staining for p53, Ki67, c-Myc, PAX8, and WT1 in the normal fallopian tube epithelial cells without p53 signature (A, E, I, M, Q) and p53-null type STIC (B, F, J, N, R) and p53-positive STIC (C, G, K, O, S) and HGSC (D, H, L, P, T) in a patient who underwent risk-reducing salpingo-oophorectomy. HGSC, high-grade serous carcinoma; STIC, serous tubal intraepithelial carcinoma.

DISCUSSION

To our knowledge, this is the first report to reveal that the frequency of pathogenicity of *TP53* variants in p53 signatures was different between HBOC patients and control patients. Firstly, we found that the frequency of p53 signatures in the fimbria was similar between RRSO

Fig. 3. *TP53* variant analysis for a patient with both p53-negative/positive STICs and HGSC. A nonsense mutation (c.617T>A) was detected in the p53-null type STIC. In the p53-positive STIC, c.617del (G) at the same codon as the p53-null type STIC and the other mutation (c.983del) (I), which was not observed in the p53-null type STIC (H) but identical to that in the HGSC (**Table S6**), were detected.
HGSC, high-grade serous carcinoma; STIC, serous tubal intraepithelial carcinoma.

samples and benign control samples, which indicates that the likelihood of a p53 signature in the fimbria does not depend on whether patients have germline *BRCA1/2* pathogenic variants. The frequency of the p53 signature in RRSO samples was 53.8% in this study, which is within the range of 11%–71% in previous reports [13,15,21,25]. Secondly, in the sequence analysis, we identified 15 variants of *TP53* in the RRSO and control specimens, of which 9 (60%) variants were categorized as pathogenic while the others were of unknown pathogenic significance. In the RRSO samples, 9 (90%) of 10 variants identified were pathogenic, whereas none of the 5 variants identified in the control group were pathogenic. The proportions of pathogenic variants were significantly different between RRSO samples and controls ($p<0.001$). These results suggest that even if p53 signatures are identified at a similar frequency in RRSO samples and controls, its characteristics and the risk of carcinogenesis might be different. Another finding was that in RRSO samples, patients with

BRCA1 pathogenic variants tended to have a higher frequency of p53 signature than those with *BRCA2* pathogenic variants. In addition, all 5 patients with *TP53* pathogenic variants had *BRCA1* pathogenic variants, whereas none of the patients with *BRCA2* pathogenic variants had *TP53* pathogenic variants. This may be due to the higher risk of developing ovarian cancer in HBOC patients with *BRCA1* pathogenic variant than in those with *BRCA2* pathogenic variant [26]. These results suggest that there might be 2 types of p53 signatures, one with a low risk of progression to STIC as seen in the control group and the other with a risk of progression to STIC with pathological variants in *TP53*. These 2 types of p53 signatures could be classified by analyzing *TP53* variants.

Recent genomic analysis suggests that accumulation of *TP53* mutations leads to aging and development of cancer in various organs throughout the body, most notably in sun-exposed skin cells [27]. However, the impact of the p53 signature on carcinogenesis in the fallopian tube is unknown. Studies of p53 signatures in fallopian tube tissue from patients with HGSC have found that some p53 signatures show loss of heterozygosity in addition to *TP53* mutations, suggesting that some p53 signatures may already have a characteristics of cancer [28,29]. A basic study found that mouse fallopian tube-derived organoids with double-knockout of *Trp53* and *Brca1* formed tumors when implanted subcutaneously [30], which indicates that *TP53* mutations in cells with *BRCA* mutations may increase the risk of carcinogenesis compared to cells without *BRCA* mutations. In this study, however, the high prevalence of p53 signatures in normal controls, who are expected to have a low incidence of ovarian cancer, suggests that not all p53 signatures are associated with carcinogenesis. It has been reported that different types of *TP53* mutations have different impacts on the prognosis of HGSC [31]; thus, the pathogenicity of mutations may be important for the characteristics of the p53 signature. Therefore, to assess the risk of carcinogenesis for a p53 signature, it would be necessary to evaluate its characteristics, such as loss of heterozygosity and *BRCA* status in addition to *TP53* mutation. The type and pathogenicity of *TP53* mutations must also be considered. The concept of precursor escape has also been proposed [32], and how the p53 signature is involved in the development of peritoneal carcinoma will also require further exploration. The clinical significance of identifying p53 signature needs to be investigated in basic and clinical research in the future.

In this study, *TP53* variants were detected in most p53 signatures, but not in all. One potential explanation for why not all p53 signatures had *TP53* variants is that the section of the p53 signature from the tissue block was not the same as the section used for LCM, which could have prevented the p53 signature from being collected by LCM. In a previous study, the mutation detection rate for the p53 signature was found to be 57%, indicating the difficulty of analyzing the p53 signature in small lesions [13].

Two types of STIC, namely p53-positive STIC and p53-null type STIC, were identified in one patient who underwent RRSO. Both STICs were adjacent to each other, and both had mutations in the same codon of *TP53*, suggesting that they may have originated from the same cell. Furthermore, the p53-positive STIC had a mutation (*TP53* c.983delT) in common with the occult cancer in this patient, suggesting that p53-positive STIC was likely the origin of HGSC. These findings suggest a relationship between the 2 STICs and HGSC in terms of clonal evolution. Although only the p53-positive STIC had the same mutation as that in cancer, the patterns of PAX8, WT-1, and c-Myc protein expression and the Ki-67 index in both STICs were similar to those of HGSC, suggesting that both STICs possessed the characteristics of cancer. It has been reported that STICs could have a pattern of strong

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expression or loss of p53 protein [7,33]. Therefore, Ki-67 is important when screening for STIC in RRSO specimens, considering that p53-null type STIC might not be identified by p53 staining alone.

The main limitation of this research is that the analysis was based on a small number of cases from a single institution. To validate the results and obtain a more scientifically significant result, it would be needed to conduct a multicenter study with a large sample size. Further analysis of RRSO and control specimens will provide more detailed information on the characteristics of the p53 signature.

In summary, clinicopathologic analysis of the p53 signature and *TP53* variant analysis in RRSO and control samples found no difference in the frequency of p53 signatures but did reveal a difference in the frequency of *TP53* pathogenic variants in p53 signatures. Furthermore, *TP53* variant analysis allowed us to evaluate the clonality of STIC and occult cancer in an actual patient who had undergone RRSO.

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SUPPLEMENTARY MATERIALS

Table S1

RRSO patients' profile

Click here to view

Table S2

Normal control patients' profile

Click here to view

Table S3

Primer sequence

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Table S4

Characteristics of RRSO patients and control patients

Click here to view

Table S5

TP53 pathogenicity

Click here to view

Table S6

Gene list detected in high-grade serous carcinoma by next-generation sequencing

Click here to view

Fig. S1

Copy number variant analysis using DNA panel sequencing data of high-grade serous carcinoma lesion. Genes which copy number were elevated above 3.5 were *MYC* (3.87), *FOXL2* (3.74), and *CCND1/FGF4* (3.74).

Click here to view

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