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Original Article

Elucidation of genomic origin of synchronous endometrial and ovarian cancer (SEO) by genomic and microsatellite analysis

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ABSTRACT

Objective: Elucidation of clonal origin of synchronous endometrial and ovarian cancers (SEOs).
Methods: We reviewed 852 patients who diagnosed endometrial and/or ovarian cancer. Forty-five (5.3%) patients were diagnosed as SEOs. We evaluated blood and tissue samples from 17 patients. We analyzed the clonal origins of 41 samples from 17 patients by gene sequencing, mismatch microsatellite instability (MSI) polymerase chain reaction assay and immunohistochemical (IHC) staining of 4 repair genes.
Results: Sixteen of 17 patients had at least 2 or more trunk mutations shared between endometrial and ovarian cancer suggesting the identical clonal origins. The shared trunk mutation are frequently found in endometrial cancer of the uterus, suggesting the uterine primary. Four out of 17 (24%) SEOs had mismatch repair (MMR) protein deficiency and MSI-high (MSI-H) states. One case was an endometrial carcinoma with local loss of MSH6 protein expression by IHC staining, and the result of MSI analysis using the whole formalin-fixed, paraffin-embedded specimen was microsatellite stable. In contrast, ovarian tissue was deficient MMR and MSI-H in the whole specimen. This indicated that MMR protein deficiency could occur during the progression of disease.
Conclusion: Most SEOs are likely to be a single tumor with metastasis instead of double primaries, and their origin could be endometrium. In addition, SEOs have a high frequency of MMR gene abnormalities. These findings not only can support the notion of uterine primary, but also can help to expect the benefit for patients with SEOs by immuno-oncology treatment.

Keywords: Endometrial Carcinoma; Neoplasms; Epithelial Ovarian Cancer; Synchronous Multiple Primary; Molecular Genetics

Synopsis

Using gene sequencing, mismatch microsatellite instability polymerase chain reaction assay, and immunohistochemistry staining, we found that most synchronous endometrial and ovarian cancers have the same origin, which may be of uterine origin.

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Conflict of Interest
No potential conflict of interest relevant to this article was reported.

Author Contributions
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INTRODUCTION

Synchronous multiple cancers of the female genital tract, especially synchronous endometrial and ovarian cancers (SEOs), are reported as 1%–2% of all genital neoplasms [1-3]. Patients with disease of both the endometrium and the ovary can be classified into 3 groups: (1) synchronous and independent primary cancers of the endometrium and ovary, (2) endometrial cancer with metastasis to the adnexa, and (3) ovarian cancer with metastasis to the endometrium.

The distinction between independent primary tumors and metastasis from one site to the other (endometrium to the ovary or ovary to the endometrium) could be difficult on clinical and pathological features alone, but the distinction of the different groups on solid basis could be clinically significant in the management of SEOs.

In the past, the criteria for this distinction were based mostly on morphological features. Ulbright and Roth [4] proposed a set of histological criteria to distinguish the first 2 groups. Then Scully et al. [5] described a similar but more extensive list of clinical pathologic features. But more solid criteria for the distinction was needed for the benefit of patients' care.

Recent studies by genetic analysis on the clonal origins of SEOs suggested that most SEOs were single primary tumors with metastasis [6-8]. In 3 studies combined, 52 out of 55 (96%) SEOs were genetically similar or identical tumors. However, in these studies, it was not elucidated which is the primary, e.g., uterus or ovary.

In this study, we examined 17 SEOs by molecular and immunohistochemical (IHC) analyses and tried to elucidate their genomic origins.

MATERIALS AND METHODS

1. Patients

Of 852 patients who underwent surgical treatment for endometrial and/or ovarian cancer between January 2008 and December 2019 in our hospital, 45 (5.3%) patients had tumors in both endometrium and ovary (SEOs). Under the approval of the Ethics Committee of our hospital (Yamanashi Central Hospital, Ethics Review Committee for Clinical and Genomic Research, approval No. 180), of these 45, we obtained informed consent and performed genetic analysis on 17 (29%) patients.

All resected specimens were reviewed according to the criteria proposed by Scully et al [5]. The features indicative of double primaries (DPs) included (1) clear histological distinction of tumors of the endometrium and ovary; (2) no or minimal myometrial invasion; (3) absence of lympho-vascular space invasion; (4) unilateral ovarian tumor; and/or (5) ovarian endometriosis present. In individual cases, we scored how many met these criteria as "DP features." By these clinicopathological features, 10 out of 17 (59%) patients were considered as having DP and 7 (41%) as M (**Table 1**). We staged the cases according to the International Federation of Gynecology and Obstetrics guidelines [9].

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Table 1. Clinicopathological judgement and genetic clonality of 17 synchronous endometrial and ovarian cancers

Case	Uterine tumor					Ovarian tumor					DP features	Clinico-pathological judgement	Genetic clonality
	Histology	Stage	Size (mm)	Lymphovascular invasion	Myometrial invasion	Histology	Stage	Site	Size (mm)	Endometriosis			
1	EM	IA	3	–	-	EM	IIIC	Lt	90	+	4	DP	M
2	EM	IA	35	–	<1/2	EM	IIC	Lt	140	+	4	DP	M
3	EM	IA	5	–	-	S	II	Bilate	55/65	–	3	DP	M
4	EM	IIIC	20	+	<1/2	EM	IC	Lt	75	+	3	DP	M
5	EM	IA	10	–	<1/2	EM	IA	Rt	60	–	3	DP	M
6	EM	IA	30	+	<1/2	S	IV	Rt	30	–	3	DP	M
7	EM	II	40	–	<1/2	EM	IA	Rt	45	–	3	DP	M
8	EM	II	30	–	<1/2	EM	IIIC	Lt	70	–	3	DP	M
9	EM	IA	40	–	<1/2	EM	IC	Lt	170	–	3	DP	M
10	EM	IIIA	30	+	<1/2	EM	-	Rt	30	–	2	M	M
11	EM	IIIA	40	–	-	EM	-	Bilate	90/Micro	–	2	M	M
12	EM	IV	50	+	<1/2	EM	-	Lt	Micro	–	2	M	M
13	EM	IIIA	80	+	>1/2	EM	-	Lt	8	–	1	M	M
14	S	IIIA	60	+	>1/2	S	IC	Lt	80	–	1	M	DP
15	EM	IA	50	+	<1/2	EM	IVB	Bilate	80/60	–	1	DP	M
16	EM	IIIB	Micro	+	>1/2	EM	-	Bilate	Micro/ Micro	–	0	M	M
17	EM	IIIA	90	+	>1/2	EM	-	Bilate	20/30	–	0	M	M

DP, double primary; EM, endometrioid; Lt, light; M, metastasis; Rt, right; S, serous; micro, under 1 mm.

2. Blood and tissue sample preparation and DNA extraction

After centrifuging peripheral blood samples, buffy coats were isolated and stored at −80°C until DNA extraction [10,11]. Buffy coat DNA was extracted using the QIAamp DNA Blood Mini QIAcube Kit (Qiagen, Hilden, Germany) with QIAcube (Qiagen). DNA concentration was determined using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). We fixed tumor specimens with 10% buffered formalin to make formalin-fixed, paraffin-embedded (FFPE) tissues [12,13]. FFPE tissues were stained with hematoxylin and eosin and microdissected using the Arcturus LCM System (Thermo Fisher Scientific) to enrich tumor tissue [12]. Subsequently, the FFPE DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen).

3. FFPE DNA quality analysis

To determine the FFPE DNA quality, we performed real-time polymerase chain reaction (PCR) assay as previously described [13]. In brief, we used 2 types of primer sets including TaqMan RNase P Detection Reagents Kit and the FFPE DNA QC Assay v2 (Thermo Fisher Scientific). We diluted the human control genomic DNA to create a 5-point serial dilution for a standard curve analysis and determined absolute DNA concentrations.

4. Selection and sequencing of 52 significantly mutated genes (SMGs) associated with gynecologic cancers

We searched the literature and selected genes on the basis of the following criteria: recurrently mutated genes relative to the background mutation rates analyzed by the MutSigCV, genes involved in signaling pathways and potential therapeutic targets [14,15]; and known drivers of gynecologic carcinogenesis reported by The Cancer Genome Atlas (TCGA) projects [16,17] and other projects [18-21]. Furthermore, we included the hotspot mutation site of each gene from the Catalogue of Somatic Mutations in Cancer (COSMIC) database [22]. Ion AmpliSeq designer software (Thermo Fisher Scientific) was used to generate in-house panel. Targeted sequencing was conducted as previously described [23]. Briefly, multiplex PCR of was performed using the Ion AmpliSeq™ Library Kit Plus (Thermo

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Fisher Scientific). Primer sequences were digested with a FuPa reagent and adaptor ligation was conducted with Ion Xpress™ Barcode Adapters (Thermo Fisher Scientific). Ligated library was purified with Agencourt AMPure XP reagents (Beckman Coulter, Brea, CA, USA). The library concentration was determined using an Ion Library Quantitation Kit (Thermo Fisher Scientific). Emulsion PCR and chip loading were performed on the Ion Chef System with the Ion PI Hi-Q Chef Kit (Thermo Fisher Scientific), and sequencing were conducted on the Ion Proton (Thermo Fisher Scientific).

Sequence data were processed using standard Ion Torrent Suite Software (version 5.0.4; Thermo Fisher Scientific) running on the Torrent Server. Data processing pipeline comprised signal processing, base calling, quality score assignment, read alignment to the human genome 19 reference (hg19), quality control of mapping and coverage analysis. Following the data analysis, we performed the annotation of single-nucleotide variants, insertions and deletions by using the Ion Reporter Server System (Thermo Fisher Scientific) and used the buffy coat DNA as a control to detect variants in tumors (Tumor–Normal pairs). We used the following filtering parameters for detecting somatic mutations: the minimum number of variant allele reads was 5, the minimum coverage depth was 10, UCSC Common SNPs was 'not in' and confident somatic variants was 'in' [24].

Actionable mutations were referred to the OncoKB database (update: September 17, 2020) from the Memorial Sloan Kettering Cancer Center [25]. In this study, we defined "oncogenic mutations" as mutations annotated as oncogenic or likely oncogenic by OncoKB database.

5. Microsatellite instability PCR (MSI-PCR) assay

MSI analysis was performed on FFPE tumor DNA using the MSI Analysis System v1.2 (Promega, Fitchburg, WI, USA) or MSI (FALCO) Kit (FALCO Biosystems, Kyoto, Japan). Both assays examined 5 mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24, and MONO-27), as previously described [26]. Data were analyzed by GeneMapper Software 5 (Thermo Fisher Scientific). Tumors with more than 2 unstable microsatellite markers were defined as MSI-high (MSI-H). Tumors with less than one unstable marker were defined as microsatellite stable (MSS).

6. IHC

IHC of mismatch repair (MMR) proteins was performed as previously described [27]. Serial sections of FFPE tissue were deparaffinized and antigen activation was performed by heat treatment. We used the Dako EnVision FLEX kit, an automated staining instrument Autostainer Link 48 system (Dako, Copenhagen, Denmark), and primary monoclonal antibodies against anti-MLH1 (clone ES05; Dako), anti-MSH2 (clone FE11; Dako), anti-MSH6 (clone EP49; Dako), and anti-PMS2 (clone EP51; Dako). The tumors were classified into proficient MMR when all 4 MMR proteins were expressed in tumor nuclei and into deficient MMR (dMMR) when complete loss of the expression of at least one MMR protein.

7. Total number of samples analyzed

We analyzed the clonal origins of 41 samples (18 from uterus, 22 from ovaries and 1 from lymph node) from 17 patients by gene sequencing of 52 SMGs, mismatch MSI-PCR and IHC staining of 4 repair genes.

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RESULTS

1. DP features

Among DP features scored according to Scully's criteria, the highest score could be 5 and the lowest 0 (**Table 1**). We arbitrarily set DP as scores of 5 to 3, and M as scores of 2 to 0. By this scoring system, 10 (case 1 to 9, 15) were diagnosed as DP, and the rest as M (**Table 1**).

2. Genetic clonality

In order to see these clinical features are really reflecting genetic diagnosis, we analyzed the clonal origins of 41 samples (18 from uterus, 22 from ovaries and 1 from lymph node) from 17 patients by gene sequencing of 52 SMGs. Total of 235 mutations were detected from uterine and ovarian samples. Of the 235 mutations, 73 mutations (31%) were detected from uterine samples only and 121 (51%) from ovarian samples only. 41 mutations (17%) were shared between uterine and ovarian tissues.

Among oncogenic mutations defined by OncoKB, there were 65 mutations. Of 65, 19 (29%) were detected from uterine only, 20 (31%) from ovarian samples only, and 26 (40%) were shared. About half of the shared mutations between uterine and ovarian tumors were oncogenic mutations (**Fig. 1**).

Sixteen of the 17 SEOs had at least 2 or more common mutations (median, 3.0 mutations) and a higher frequency of oncogenic mutations in common, suggesting a clonal origin between endometrial and ovarian tumors (**Table 1**, **Fig. 2**). Of 10, all cases with clinicopathological DP had the common mutations, suggesting the possibility of metastasis of either cancer (**Fig. 2**). Only one case (case 14) shared no mutations, indicating DP (**Table 1**, **Fig. 2**)

The clinical classification showed 10 cases of DP and 7 cases of P, while the genetic classification showed 1 case of DP and 16 cases of M, with a concordance rate of 88%.

The most common oncogenic gene shared between endometrial and ovarian tumors was *PTEN* (6/17; 35.3%). This was followed by *ARID1A, KRAS* (5/17; 29.4%), *PIK3CA* (4/17; 23.5%), and *TP53* (3/17; 17.6%) in order of frequency (**Fig. 2**, **Fig. S1**).

We examined what signaling pathways the oncogenic mutations contributed to **Fig. S1**. The most frequent pathway common to both tumors was PIK3 pathway (12/17 71%). This result was similar to that of endometrial cancer (84%) in the TCGA data [17].

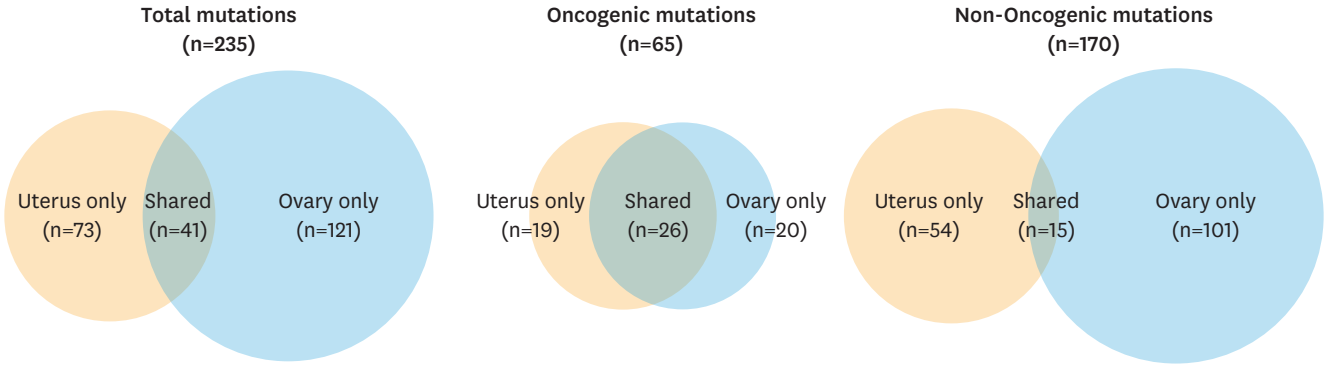

Fig. 1. Venn diagram showing the overlap of gene mutations.

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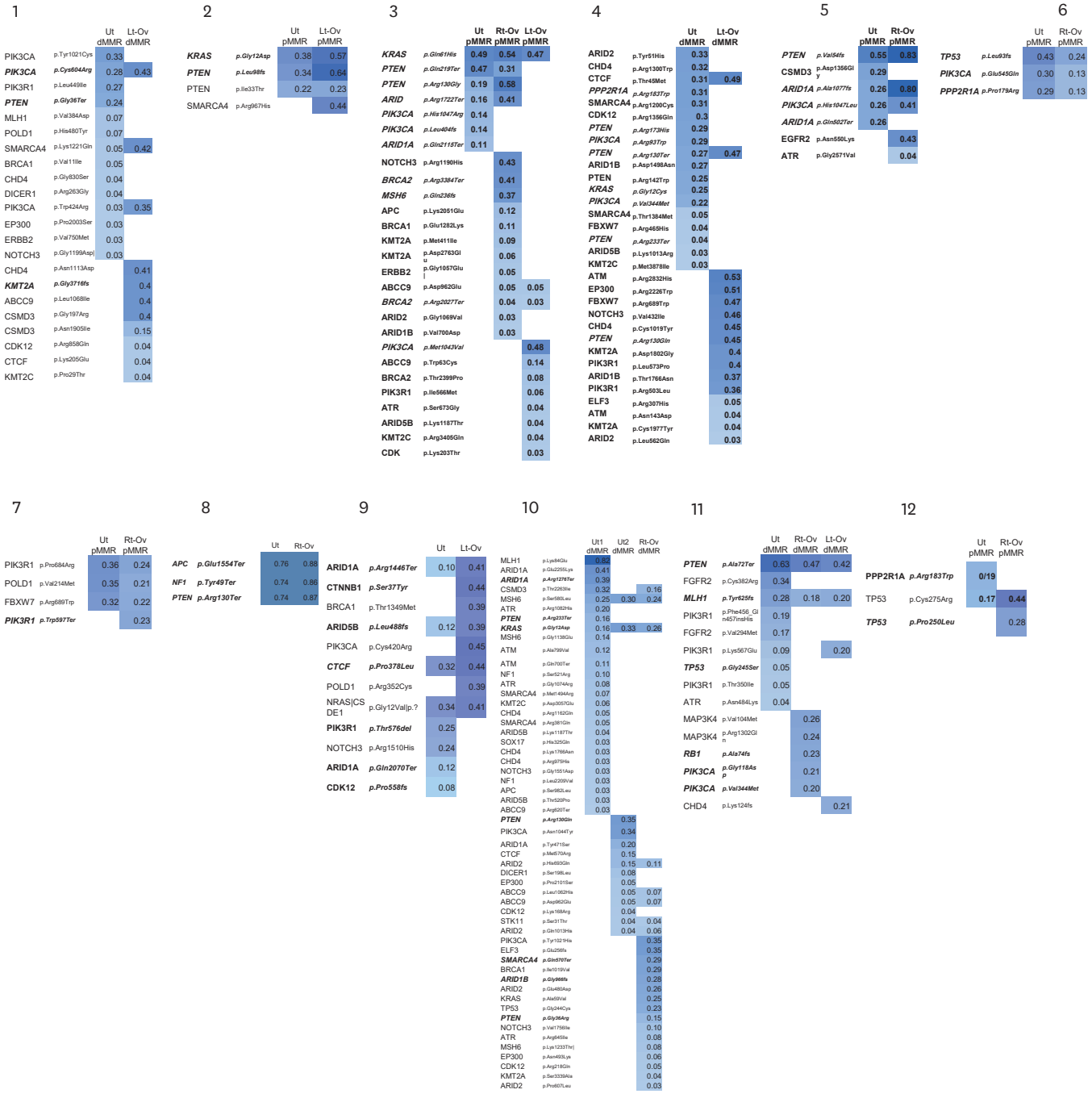

Fig. 2. Gene profiles of SEOs. Heatmap of gene mutations in patients with SEOs. These maps visualize the gene mutations in each cancer. Bold and italic gene mutations were oncogenic mutations defined by OncoKB.
AF, allele frequency; dMMR, deficient mismatch repair; LN, lymph node; Lt, left; Ov, ovarian tissue; pMMR, proficient mismatch repair; Rt, right; SEO, synchronous endometrial and ovarian cancer; Ut, uterine tissue.

(continued to the next page)

3. MSI-PCR and IHC

We then examined MSI using 2 different methods (MSI-PCR and IHC). All results of IHC and MSI analysis were consistent in uterus and ovary, and 4 of 17 (24%) SEOs had MMR defects and MSI-H (**Table 2**). While the frequency of MSI is reported to be low in ovarian cancer, it is reported to be 20-30% in endometrial cancer, suggesting that the uterus is also the primary site of this finding.

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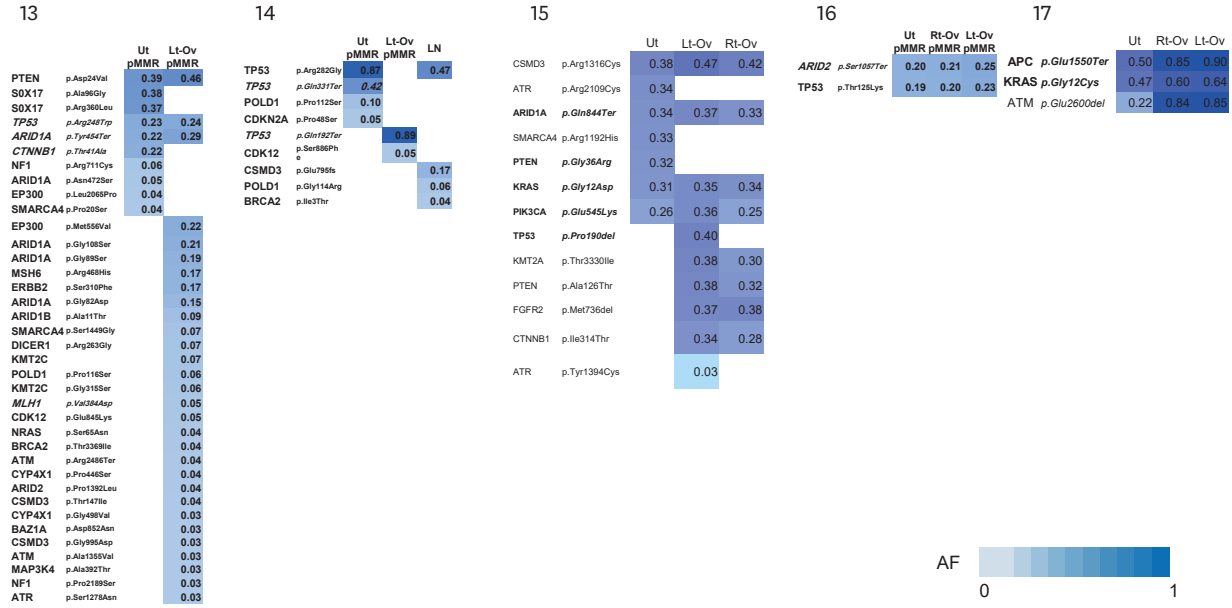

Fig. 2. (Continued) Gene profiles of SEOs. Heatmap of gene mutations in patients with SEOs. These maps visualize the gene mutations in each cancer. Bold and italic gene mutations were oncogenic mutations defined by OncoKB.
AF, allele frequency; dMMR, deficient mismatch repair; LN, lymph node; Lt, left; Ov, ovarian tissue; pMMR, proficient mismatch repair; Rt, right; SEO, synchronous endometrial and ovarian cancer; Ut, uterine tissue.

Table 2. The result of MSI assay and IHC staining

Case	Clinico-pathologic judgement	Genetic profile	MSI/IHC	
			Uterine	Ovary
1	DP	M	MSI-H/dMMR (MLH1&PMS2 loss)	MSI-H/dMMR (MLH1&PMS2 loss)
2	DP	M	MSS/pMMR	MSS/pMMR
3	DP	M	MSS/pMMR	MSS/pMMR
4	DP	M	MSS/p&dMMR (MSH6 loss)	MSI-H/pMMR (MSH6 loss)
5	DP	M	MSS/pMMR	MSS/pMMR
6	DP	M	MSS/pMMR	MSS/pMMR
7	DP	M	MSS/pMMR	MSS/pMMR
8	DP	M	MSS/pMMR	MSS/pMMR
9	DP	M	MSS/pMMR	MSS/pMMR
10	M	M	MSI-H/dMMR (MLH2-MSH6 loss)	MSI-H/dMMR (MLH2-MSH6 loss)
11	M	M	MSI-H/dMMR (MLH1-PMS2 loss)	MSI-H/dMMR (MLH1-PMS2 loss)
12	M	M	MSS/pMMR	MSS/pMMR
13	M	M	MSS/pMMR	MSS/pMMR
14	M	DP	MSS/pMMR	MSS/pMMR
15	DP	M	MSS/pMMR	MSS/pMMR
16	M	M	MSS/pMMR	MSS/pMMR
17	M	M	MSS/pMMR	MSS/pMMR

dMMR, deficient mismatch repair; DP, double primary; IHC, immunohistochemistry; M, metastasis; MSI, microsatellite instability; MSI-H, microsatellite instability-high; MSS, microsatellite instability stable; pMMR, proficient mismatch repair.

Among the 4 cases that showed MMR defects, case 4 was an endometrial carcinoma with local loss of MSH6 protein expression by IHC staining, and the result of MSI analysis using the whole FFPE specimen was MSS (**Fig. 3**). In contrast, ovarian tissue was dMMR and MSI-H in the whole specimen (**Fig. 3**). Therefore, we attempted to use laser capture microdissection to separate regions where protein expression was lost and regions where expression was maintained in uterine cancer. The results of the MSI test using microdissection were consistent with the IHC staining.

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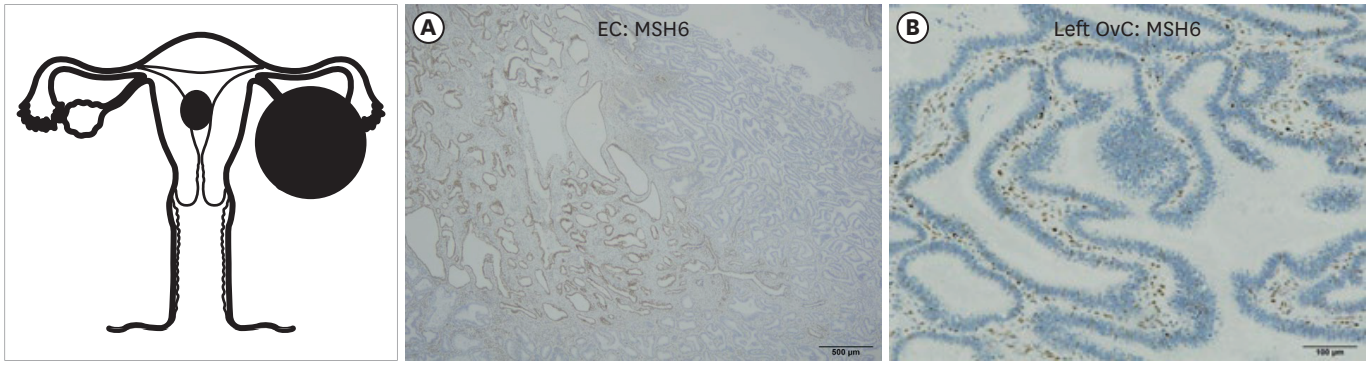

Fig. 3. MSH6 expression of case 4. EC with a discrete area with loss of MSH6. (A) ×40 OC all area with loss of MSH6. (B) ×100.
EC, endometrial cancer; OvC, ovarian cancer.

In addition, we identified germline mutations in these 4 cases of MMR defects. And found germline *MSH6* mutations only in case 4.

DISCUSSION

The difference between a single tumor with metastasis and dual-primary tumors in SEOs has been one of the most challenging clinical questions among gynecologists. Van Altena et al. [28] reported that after correcting for age, stage, histology and tumor grade, the survival rates of patients with SEOs and single ovarian cancers are similar; they highlighted that conventional early gynecological cancer treatment for patients with SEOs could be inadequate.

Although several clinicopathological features were considered useful, tumors did not fulfil all the differential criteria in several cases [4,5]. Thus, IHC, MSI, loss of heterozygosity and the mutational analyses of single or small sets of genes have been used as markers to separate synchronous primary tumors from metastatic disease [29-31]. Almost all these studies could not identify shared molecular alternations in most SEOs.

In recent studies used massively parallel sequencing to define the clonal relationship between SEO, most SEOs represented a single tumor with metastasis. [7,8]. Our mutation data substantially supported a clonal lineage. Anglesio et al. [6] investigated 2 cases with different histological types between the uterus and the ovary (endometrioid and clear cell) and deduced metastases. In this study, we compared the clinical classification with the genetic classification. The concordance rate was 88%, suggesting the limitation of clinical classification, which is mainly based on morphological diagnosis. Only case 11 was diagnosed as DP based on genetic profile. From these results, we considered that most of the SEOs were single tumors with metastasis.

However, previous studies have not clarified whether the uterus or the ovaries are the origin of the tumor. The most frequently mutated gene in endometrial cancers in previous reports was *PTEN* (60.6%). This was followed by *PIK3CA* (49.8%), *ARID1A* (38%), *TP53* (35.1%), and the majority of ovarian cancers were reported to be *TP53* (83.1%) [32]. In this study, our results showed that the most frequent oncogenic gene in endometrial cancers was *PTEN* (9/17; 52.3%), followed by *KRAS* (6/17; 35.3%) and *PIK3CA* (5/17; 29.4%). Ovarian cancers showed *PTEN* (7/17; 41.2%), *KRAS* (5/17; 29.4%), and *PIK3CA* (5/17; 29.4%), which differed from the mutation frequency in normal ovarian cancer, and most of them were found in endometrial cancer.

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Several prior reports suggest that *PTEN* mutations occur early in the neoplastic process of endometrial tumors and co-exist frequently with other mutations in the PI3K/AKT pathway [17,33]. Our results in this study also showed a high frequency of PI3K/AKT pathway abnormalities. There were striking similarities between the molecular profiles from the SEO subgroup and the TCGA 2013 endometrial carcinoma tumor set, implying the endometrium could be the primary origin for these cases rather than the ovary [17].

We added IHC and MSI-PCR. Several studies reported that the MMR deficiency was the most frequent in endometrial cancer (20%–30%). Whereas it was rare in ovarian cancer (0.5%–1%) [34-36]. In the current study, the frequency of MSI-H in SEO was 24%, which is comparable to the frequency of endometrial cancer. In other words, we thought that SEOs with MSI-H are likely to be ovarian metastasis of endometrial cancer, not DP cancer.

Furthermore, in case 4, the clinicopathological judgement was DP and the genetic classification was M. However, the MSI study showed areas of MSS and MSI-H in the endometrial tissue and only MSI-H in the ovarian tissue. Several studies on MSI have suggested that the loss of MSH6 protein expression may occur with tumor progression [37,38]. This indicated that MMR protein deficiency could occur during the progression of disease and it is highly probable that the cells in the MSI-H region of the endometrium metastasized to the ovary in the present case 4.

Regarding cases 3 and 6, which have different histopathologic diagnoses, we considered there were some limitations of morphological diagnosis. Anglesio et al. [6] concluded, as in the present case, that SEOs with some common mutations were not DP carcinoma but a single tumor with metastases, even though the histologic types are different.

In case 3, the histology of ovarian samples was diagnosed as “serous carcinoma,” but the genetic background was completely different from the characteristics of ovarian serous carcinoma (**Table 1**, **Fig. 2**). The uterine and ovarian samples shared a genetic mutation that is reported to be common in endometrial carcinomas [32]. Then we reexamined the histopathological sections. We found some areas that resembled serous carcinoma but were diagnostic of endometrial carcinoma. Several studies reported that cancers with the intratumor heterogeneity could change their morphology at the site of metastasis [39,40]. We considered that case 3 might fit that theory and that the ovarian lesions were most likely metastases of endometrial carcinoma. For case 6, among the gene mutations common to both uterine and ovarian samples, the *TP53* gene, which is considered to be common in serous carcinoma, was found. However, the remaining gene mutations belonged to the PIK3 pathway (**Fig. 2**, **Fig. S1**), and as in case 3, we suspected the ovarian lesions were metastases of endometrial cancer. There are several limitations to the current study. First, due to the rarity of SEOs, the number of patients included in this study was small. Secondly, only genetic variants were examined, and no epigenetic studies were performed. To validate the findings of this study, a larger cohort study should be conducted in the future.

In conclusion, most SEOs are likely to be a single tumor with metastasis and the origin might be endometrium especially they had endometrioid carcinoma. Furthermore, the frequency of MSI-H in SEOs could be higher than in endometrial cancers. The patients with SEOs may have more chances of immuno-oncology therapy than the patients with endometrial cancer alone.

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SUPPLEMENTARY MATERIAL

Fig. S1

Relationship between oncogenic mutations and pathways in each case.

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