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ORIGINAL ARTICLE ***Gynaecology***

Different mutation profiles between epithelium and stroma in endometriosis and normal endometrium

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STUDY QUESTION: Are there common mutation profiles between epithelial and stromal cells in ovarian endometriotic tissue and the normal endometrium?

SUMMARY ANSWER: Our study revealed no common mutations between epithelial and stromal cells in ovarian endometriotic tissue and the normal endometrium.

WHAT IS KNOWN ALREADY: Epithelial cells in both ovarian endometriotic tissue and the normal endometrium harbor somatic mutations in cancer-associated genes such as phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) and KRAS proto-oncogene, GTPase (*KRAS*).

STUDY DESIGN, SIZE, DURATION: We performed a retrospective study to identify the mutation profiles of stromal cells in endometriotic tissue and the normal endometrium. We collected 11 endometriotic stroma samples and 10 normal endometrial stroma samples between 2013 and 2017 at a tertiary care center.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The laser microdissection method was used to obtain stromal cells in ovarian endometriotic and normal endometrial tissues from patients with ovarian endometriosis and/or other non-invasive gynecological diseases. Target gene sequencing was performed to assess and compare the mutation profiles of stromal cells with those of epithelial cells obtained in our previous study. For target gene sequencing, 76 genes were selected based on previous genomic analyses for ovarian endometriosis, normal endometrium, endometriosis-related ovarian cancer and endometrial cancer.

MAIN RESULTS AND THE ROLE OF CHANCE: Stromal samples in ovarian endometrioma and normal endometrium harbor somatic mutations (18 mutations in 11 endometriosis samples and 16 mutations in 10 normal endometrial samples) but did not share any mutations with paired epithelial samples. The mutant allele frequency of stromal samples was significantly lower than that of epithelial samples in ovarian endometrioma ($P = 6.0 \times 10^{-11}$) and normal endometrium ($P = 1.4 \times 10^{-7}$).

LIMITATIONS, REASONS FOR CAUTION: The number of genes evaluated in the mutational analysis was limited. Additionally, the functional roles of somatic mutations in stromal cells remain unclear.

WIDER IMPLICATIONS OF THE FINDINGS: Different mutation profiles between paired epithelial and stromal cells in both ovarian endometrioma and normal endometrium suggest that origins of epithelial and stromal cells would be independent of each other in both normal endometrium and ovarian endometrioma; however, the theory of epithelial-mesenchymal transition is proposed in ovarian endometrioma.

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Key words: stroma / epithelium / endometriosis / uterine endometrium / somatic mutation / target gene sequencing

Introduction

Endometriosis, a benign gynecological disease affecting ~10% of reproductive-aged women, is histologically characterized by the growth and persistence of endometrial-like epithelium and stroma outside the uterus (Giudice, 2010; Vercellini *et al.*, 2014). The typical histological finding of endometriosis in women of reproductive age is one or more glands lined by endometrial-like epithelium and cuffs of endometriotic stroma, which is a mantle of densely packed small fusiform cells with scanty cytoplasm and bland cytology (Kurman *et al.*, 2011). Endometriotic stroma exhibits the unique feature of inflammatory responses, consisting predominantly of a diffuse infiltration of histiocytes at the time of menstruation. Detection of endometriotic stroma based on immunohistochemical staining with CD10 is used to diagnose endometriosis, particularly when the endometriotic epithelium is minimal or absent (Sumathi and McCluggage, 2002). Although numerous previous studies have used immortalized endometriotic stromal cell lines or clinical samples to evaluate the biological importance of stromal cells in endometriosis (Borghese *et al.*, 2017; Rekker *et al.*, 2018), the genomic alterations of endometriotic stromal cells are unclear.

Recently, we have focused on genomic alterations in the endometriotic epithelium to clarify the pathogenesis of ovarian endometriosis. In our latest report (Suda *et al.*, 2018), genomic analyses based on next-generation sequencing demonstrated that ovarian endometriotic epithelial cells harbor cancer-associated gene mutations with high mutant allele frequencies (MAFs), such as mutations in KRAS proto-oncogene, GTPase (*KRAS*) and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), suggesting clonal expansion of epithelial cells with cancer-associated mutations in endometriosis. We also clarified the genomic heterogeneity in the normal uterine endometrium, which is composed of innumerable endometrial glands with distinct somatic mutations. The commonality of cancer-associated mutations in endometriotic epithelium and uterine endometrial epithelium (gland) can provide molecular biological support for Sampson's theory, which states that retrograde menstruation through the fallopian tube leads to transfer of endometrial fragments into the peritoneal cavity, where they are implanted on the pelvic structures. However, the origin of endometriotic stromal cells remains unclear. The origin of endometriotic stroma is considered to be the stromal part of endometrial fragments in retrograde menstruation, endometrial stem cells (Gargett *et al.*, 2016; Pluchino and Taylor, 2016) or the epithelial-mesenchymal transition (Yang and Yang, 2017). Our previous study did not target stromal cells in ovarian endometriotic tissue and the normal endometrium, and the genomic relationship between epithelial and stromal cells was not clarified.

Therefore, in the present study we focused on stromal cells in ovarian endometriosis and normal endometrium to evaluate somatic mutations, with the aim of understanding the origin of stroma and epithelium in each tissue.

Materials and Methods

Ethical approval

This study was approved by the institutional ethics review boards of Niigata University, Niigata Chuo General Hospital and National Institute of Genetics.

Patient samples

Prior to the study, we obtained ethical committee approval and written informed consent from patients in the study. From the 94 samples from women with ovarian endometriosis samples analyzed in our previous study, we selected 11 that contained less stroma-infiltrating immune cells histologically. Of these 11 patients who underwent ovarian cystectomy or salpingo-oophorectomy, just one patient underwent hysterectomy at the same time: in addition to this normal endometrium sample, we randomly selected nine endometrium samples with no endometrial lesions from 70 endometrium samples analyzed in our previous study (Suda *et al.*, 2018). In total, 10 normal endometrium samples were used for the analyses, and only one case had both ovarian endometriosis and normal endometrium samples. The mean (± SD) ages of patients for ovarian endometriosis and normal endometrium analyses were 37.2 ± 6.6 and 45.9 ± 4.9 years, respectively. All samples were cut from surgical specimens, embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA, USA) in a Tissue-Tek Cryomold (Sakura Finetek) and quickly frozen in liquid nitrogen. To isolate the stroma cells from ovarian endometriotic and normal endometrial tissues, laser microdissections was performed on 10-μm-thick serial frozen sections fixed with 100% methanol and stained with toluidine blue with an LMD7 laser microdissection microscope (Leica, Wetzlar, Germany) (Fig. 1). As a guide for a stromal lesion, we performed immunostaining for CD10 with anti-CD10 antibody (ab208778, Abcam plc, Cambridge, UK) (Supplementary Fig. S1). We extracted DNA from isolated stromal cells using a QIAamp DNA Micro Kit (QIAGEN, Hilden, Germany). Prior to sampling of the stroma, we had isolated epithelial cells from ovarian endometriotic and normal uterine endometrial tissues in our previous study (Suda *et al.*, 2018).

Target-gene sequencing

Target-gene sequencing for endometriotic and uterine endometrial stroma samples for 76 genes was performed with the pre-capture pooling method described in our previous studies (Ahmadloo *et al.*, 2017; Suda *et al.*, 2018), with some modifications. Briefly, the 76 genes were selected (Supplementary Table SI) based on whole exome sequencing data for ovarian endometriosis and normal endometrium (Suda *et al.*, 2018), the mutation profiles in endometriosis-related ovarian cancer (Jones *et al.*, 2010) and in endometrial cancer (Lawrence *et al.*, 2014). The sequencing data for epithelium and blood samples from patients whose stroma samples were examined

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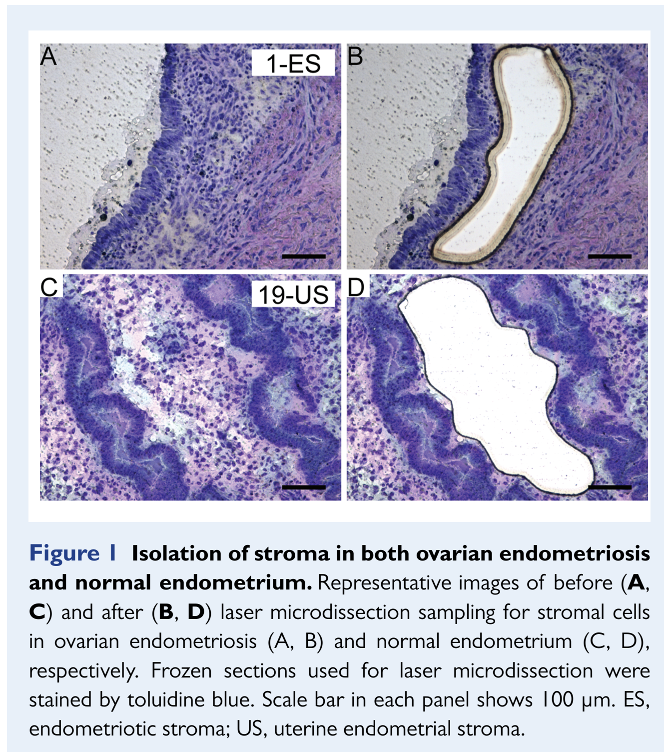

Figure 1 Isolation of stroma in both ovarian endometriosis and normal endometrium. Representative images of before (**A**, **C**) and after (**B**, **D**) laser microdissection sampling for stromal cells in ovarian endometriosis (A, B) and normal endometrium (C, D), respectively. Frozen sections used for laser microdissection were stained by toluidine blue. Scale bar in each panel shows 100 μm. ES, endometriotic stroma; US, uterine endometrial stroma.

here were retrieved from our previous study (Suda *et al.*, 2018). For sequencing library preparation, 25 ng of DNA derived from the laser microdissection procedure was repaired by using NEBNext FFPE DNA Repair Mix (New England Biolabs, Ipswich, MA, USA) followed by fragmentation with a KAPA Frag Kit (KAPA Biosystems, Wilmington, MA, USA). The sequencing libraries with distinct indices were generated with a NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs) and then pooled at equimolar amounts. Hybridization-based target enrichment was conducted with the SeqCap EZ Prime Choice System (Roche Diagnostics, Basel, Switzerland). The DNA probe set was selected by using NimbleDesign (http://design.nimblegen.com). The libraries were sequenced on a MiSeq platform with a 350- and 250-bp paired-end module (Illumina, San Diego, CA, USA). The bioinformatics pipeline to detect somatic mutations (single-nucleotide variants and short insertions and deletions) was implemented with the same method as in our previous study (Suda *et al.*, 2018). To avoid false positive variant calls, we excluded variants whose frequencies were >0.001 in any Exome Aggregation Consortium (ExAC) populations (Lek *et al.*, 2016). All somatic mutations detected by the pipeline are listed in Supplementary Table SII together with their frequencies in ExAC populations, COSMIC annotations (Tate *et al.*, 2019) and ClinVar classifications (Landrum *et al.*, 2018). For each somatic mutation detected in the epithelium sample, MAF of the same variant in the stroma sample derived from the same patient is listed in Supplementary Table SII, and vice versa.

Droplet digital PCR

Droplet digital PCR was performed using the Bio-Rad QX200 Droplet Digital PCR System (Bio-Rad Laboratories, Hercles, USA). Two mutations on *KRAS* (p.G12V [c.G35T] and p.G12A [c.G35C]) and one mutation on *PIK3CA* (p.H1047R [c.A3140G]) were selected for validation by droplet digital PCR. For each mutation, forward and reverse primers were designed to amplify the regions containing the corresponding mutations (Eurofins Genomics, Tokyo, Japan). Locked nucleic acid probes labeled with HEX and FAM, which specifically bind reference and mutant alleles of the mutations, respectively, were synthesized (Integrated DNA Technologies, Coralville, USA). Oligonucleotide sequences used in this study are shown in Supplementary Table SIII. As a positive control, we synthesized ~500-bp-long double-stranded DNA sequences with the corresponding mutations (gBlocks Gene Fragments, Integrated DNA Technologies). The synthesized DNA was serially diluted to a final concentration of 5 fg/μl. In order to minimize the absorption of the synthesized DNA to pipette tips and tubes, we used genomic DNA collected from peripheral blood sample of a healthy individual as carrier DNA. Therefore, the positive control samples show positive signals for both mutant and reference alleles (Supplementary Figs S2, S3 and S4). DNase/RNase-free distilled water was used as negative control. The reaction mixture and the thermal cycling conditions for droplet digital PCR are described in our previous study (Suda *et al.*, 2018).

Statistical analysis

Silent (synonymous) mutations and non-silent (missense, nonsense, splice site or frameshifting indels) mutations were used in the analyses of this study. The number of somatic mutations was presented as mean and SD. The Wilcoxon–Mann–Whitney test was used to evaluate the significance of differences between groups. $P < 0.05$ indicated statistical significance.

Results

No shared mutations between epithelium and stroma in ovarian endometriosis

We investigated somatic mutations of 76 endometriosis-associated genes in 11 paired endometriotic epithelial and stromal samples derived from 11 patients with endometriosis (Table I). The average sequencing depth and the percentage of the target region that covered at least 20 reads were on average 115 and 98.5% in endometriotic epithelial and stromal samples, respectively. The number of somatic mutations per sample was 1.8 ± 1.3 in the epithelium and 1.6 ± 1.5 in the stroma. There was no significant difference in the mutation frequency between endometriotic epithelial and stromal samples ($P = 0.71$). No shared mutations between endometriotic epithelial and stromal samples derived from the same patient were observed. Three missense mutations of AT-rich interaction domain 1A (*ARID1A*) were detected in two stromal samples. When cancer-associated genes were defined based on the Cancer Gene Census (Futreal *et al.*, 2004), the ratio of oncogene mutations per sample was significantly higher in the epithelium than in the stroma ($P = 0.025$), although there was no difference in the mutation frequency of cancer-associated genes between the two groups ($P = 0.32$). The MAFs of all mutations detected in the endometriotic stromal samples were less than 0.1 and were significantly lower than those in epithelial samples ($P < 0.0001$) (Fig. 2 and Supplementary Table SII).

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Table I Somatic mutations in stroma and epithelium for ovarian endometriosis.

Patient	Age (years)	Mutation in stroma	Mutation in epithelium*
		Gene (coding amino acid substitution)	Gene (coding amino acid substitution)
1	40	*CHD4* (G1204V)	*PIK3CA* (C407W), *SPEG* (R2055W)
2	36	-	*KRAS* (G12D), *KRAS* (G13D), *PIK3CA* (H1047R), *TAF1* (R825R)
3	47	*PLXNB2* (c.2817-2A>G)	*CTNNB1* (D32N), *CUX1* (E1418K), *KRAS* (G12V), *PIK3CA* (H1047R), *ZFHX3* (R1355T)
4	27	*FAM135B* (N947S), *KIAA1109* (T1412N), *SLC19A1* (G515G)	-
5	30	*FRG1* (S228I)	*PLXNB2* (K117E)
6	38	*ARID1A* (M793L), *LAMA2* (L2879Q), *PLXNB2* (E663fs),*ZFHX3* (L224L)	*ACRC* (D217N), *PIK3CA* (N345K)
7	33	*ARID1A* (T1514P), *ARID1A* (G1515C), *CAMTA1* (Q711Q), *CAMTA1* (A712A), *DISP2* (T1081A)	*KMT2C* (E1226X)
8	37	*ATM* (R189I), *DISP2* (R1146L), *PLXND1* (G203G)	-
9	32	-	-
10	46	-	*PIK3CA* (H1047R), *ZFHX4* (M1653I)
11	44	-	*ABCC1* (V926M), *ARHGAP35* (R783X), *KRAS* (G12A)

*Mutation data of epithelial cells was taken from Suda et al. (2018).

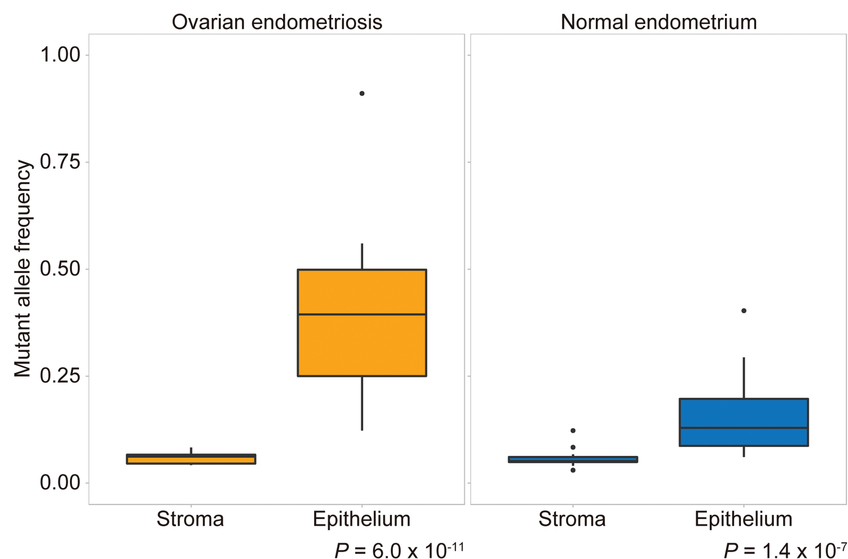

Figure 2 Comparison of MAF between stroma and epithelium. Box and whisker plots show mutant allele frequencies (MAFs) of all somatic mutations in ovarian endometriosis (left panel) and normal endometrium (right panel), in which MAFs were compared between stroma and epithelium samples. Wilcoxon–Mann–Whitney test showed MAFs were significantly lower in stroma than in epithelium for both ovarian endometriosis and normal endometrium. Error bars and black dots show SD and outliers, respectively.

No common mutations between epithelium and stroma in normal endometrium

Next, we examined the mutation profiles of 10 paired epithelial and stromal samples in the normal uterine endometrium through target-gene sequencing (Table II). The average sequencing depth and the percentage of the target region that covered at least 20 reads were on average 109 and 98.2% in normal endometrial epithelial and stromal samples, respectively. The number of somatic mutations per sample was 2.2 ± 1.8 in the epithelium and 1.6 ± 1.8 in the stroma. There was no significant difference in the mutation frequency between endometrial epithelial and stromal samples ($P = 0.36$). No shared mutations between endometrial epithelial and stromal samples derived from the same patient were observed. Although there was no difference in the mutation frequency of cancer-associated genes between epithelial

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Table II Somatic mutations in stroma and epithelium for normal endometrium.

Patient	Age (years)	Diagnosis	Mutation in stroma	Mutation in epithelium*
			Gene (coding amino acid substitution)	Gene (coding amino acid substitution)
11	44	Endometriosis	*DISP2* (L648P), *GPR50*, (V36V), *GPR50* (I37I),*KMT2C* (P2193A), *KMT2C* (P2193R), *PLXNB2* (P667fs)	*STT3A* (I569I)
12	52	Ov fibroma	*ARID1A* (T1514P), *TAF2* (S1188T)	*ARHGAP35* (K437fs), *KRAS* (G12A)
13	51	Uterine fibroid	*ANK3* (N366I), *XIRP2* (L2105M)	*PIK3CA* (S405F)
14	39	Uterine fibroid	*ZFHX3* (A222T), *ZFHX3* (L224L)	-
15	51	Endometriosis	-	*FBXW7* (H460Y), *FBXW7* (R609W)
16	44	CIN	*TAF1* (H1293N), *ZFHX3* (L224L)	*BCOR* (S209S), *FAT1* (V1952I), *PLXNB2* (Y1148C)
17	48	Uterine fibroid	-	*ARHGAP35* (W452G), *ARID5B* (D521fs), *KRAS* (G12S), *PIK3CA* (G1049R), *PIK3R1* (310_312del), *ZFHX3* (S2555fs)
18	41	Uterine fibroid	-	*KRAS* (G12V)
19	40	Uterine fibroid	*ARHGAP35* (Y1207X)	*ARHGAP35* (R610X), *ARHGAP35* (A613S), FAT3 (I1723I),*XIRP2* (D2602Y)
20	49	Uterine fibroid	*TAF2* (S1188T)	*FGFR2* (S252W), *MON2* (A721V)

*Mutation data of epithelial cells was taken from Suda *et al.* (2018).

Table III The results of droplet digital PCR.

Mutation	Stroma sample	Number of drop count		Proportion of mutation droplet	Corresponding epithelium	
		Mutation	Reference		Sample	MAF
PIK3CA pH1047R	3-ES	0	896	0	3-E	0.41
PIK3CA pH1047R	10-ES	0	1714	0	10-E	0.31
KRAS pG12V	3-ES	1	1623	0.0006158	3-E	0.55
KRAS pG12V	18-US	0	3256	0	18-U	0.40
KRAS pG12A	11-ES	7	1840	0.003790	11-E	0.91
KRAS pG12A	12-US	2	4261	0.0004692	12-U	0.28

In sample columns, each number represents each patient. ES, endometriotic stroma; US, uterine endometrial stroma; E, endometriotic epithelium; U, uterine endometrial epithelium; MAF, mutant allele frequency based on target gene sequencing.

and stromal samples (P = 0.24), the ratio of oncogene mutations per sample was significantly higher in the epithelium than in the stroma (P = 0.015). The stroma in the normal endometrium showed significantly lower MAFs compared to in the epithelium ($P < 0.0001$) (Fig. 2).

Validation experiment using droplet digital PCR

To validate the absence of epithelial mutations in stroma, we performed droplet digital PCR experiments for three endometriotic and two uterine endometrial stroma samples. For experimental assays, we selected somatic mutations in *PIK3CA* and *KRAS,* which were detected in corresponding epithelium samples with high MAFs. The result showed no, or very small proportions of, mutant signals in stromal samples (Table III and Supplementary Figs S2, S3 and S4), which was consistent with the target-gene sequencing data.

Discussion

Our targeted sequencing of 76 genes demonstrated that the epithelium and stroma did not share mutations in the ovarian endometriotic and normal uterine endometrial tissues. These findings suggest that the origin of stroma might differ from that of epithelium in both ovarian endometriosis and normal endometrium.

Recently, Noë *et al*. conducted droplet digital PCR to detect 6 synonymous and 13 missense passenger mutations in both endometriotic epithelial and stromal samples derived from six patients with non-superficial endometriosis (five deep infiltrating endometriosis and one ovarian endometriosis) (Noe, *et al*., 2018). Consistent with our results that non-silent mutations were not shared between epithelial and stromal cells in ovarian endometrioma, somatic passenger mutations detected in the endometriotic epithelium showed very low MAFs or were not detected in paired endometriotic stroma. More recently, Lac *et al*. performed droplet digital PCR to search for one driver

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mutation per case in both endometriotic epithelial and stromal samples derived from two incisional endometriosis cases and two deep infiltrating endometriosis cases (Lac, *et al.*, 2019). They demonstrated that four stromal samples contained driver mutations with very low MAFs (range: 0.0002–1.36%), which showed high MAFs in paired endometrial samples. Although these previous studies mainly used deep infiltrating endometriosis samples and different tissues from ours, as with these studies, we also observed very weak signals of epithelial driver mutations in three of five paired stromal samples by performing droplet digital PCR (range: 0.0–0.3%). We speculated these small proportions of mutant signals in stromal cells could result from the contamination by epithelial cells adjacent to stroma. The study by Lac *et al*. (2019) and the present study clarified that only the endometriotic epithelium harbors somatic mutations in cancer-associated genes with high MAFs, suggesting clonal expansion of endometriotic epithelial cells but not of stromal cells. The above two studies and ours did not support the hypothesis that some endometriotic epithelial cells turn into stromal cells through epithelial-mesenchymal transition to form the endometriotic stroma.

Although we sequenced only 76 genes, our study revealed different mutation profiles between the epithelium and stroma in ovarian endometriotic and normal endometrial tissues. Interestingly, compared to stromal cells, epithelial cells tended to harbor oncogene mutations in both the diseased and normal states (Tables I and II). On the contrary, a recent integrated genomic analyses of sarcoma, which is a representative malignant tumor derived from stromal cells, showed that many adult soft tissue sarcomas are characterized by high levels of copy number alterations and low levels of somatic mutations, unlike most epithelial malignancies (The Cancer Genome Atlas Research Network, 2017). Additionally, recurrent mutations across sarcomas are identified in only a few tumor suppressor genes, such as tumor protein P53 (*TP53*), ATRX chromatin remodeler (*ATRX*) and RB transcriptional corepressor 1 (*RB1*), but not in oncogenes. Although our study lacks copy number analysis, the occurrence of oncogene mutations may associate with the property of epithelial cell based on the results of The Cancer Genome Atlas study and ours.

Our previous study has reported that loss-of-function mutations in *ARID1A* are detected in ~10% of endometriotic epithelium samples and in a few single glands isolated from normal endometrium (Suda *et al.*, 2018). In the present study, we discovered three missense mutations of *ARID1A* in two endometriotic stromal samples. *ARID1A*, which is a subunit of the SWI/SNF chromatin remodeling complex, is a commonly mutated gene in cancer, and TumorPortal (Lawrence *et al.*, 2014) shows that *ARID1A* is highly significantly mutated in uterine endometrial cancer, bladder cancer and breast cancer. In addition, loss-of-function mutation in *ARID1A* is a key event in malignant transformation of ovarian endometriosis (Jones *et al.*, 2010; Wiegand *et al.*, 2010; Yamamoto *et al.*, 2012). However, the biological significance of *ARID1A* missense mutations with low MAF detected only in endometriotic stroma is still unknown.

The limitations of this study should be noted. The number of genes evaluated in this study was limited to 76. Genome-wide approaches, such as whole-exome and whole-genome sequencing, will provide further information including mutation signatures, which is useful to understand mutation-causing processes. Although we used laser microdissections to purify epithelial or stromal cells from surgical specimens, we could not exclude the possibility that small proportions of non-targeted cells were included. Indeed, we performed immunostaining for CD10 to confirm the area of stromal cells. However, we could not exclude the possibility that microdissected stroma would include a small population of CD10-positive immune cells because immune cells show CD10 positivity depending on the cycle phase (LeBien and McCormack, 1989). Additionally, we could not obtain a sufficient number of paired ovarian endometriosis and normal endometrium samples from the same cases, except for one woman who underwent salpingo-oophorectomy and hysterectomy. This is because endometriosis cases generally undergo only ovarian cystectomy or salpingo-oophorectomy, without hysterectomy. Further genomic analysis of paired ectopic and eutopic endometrium samples is needed to shed light on the pathogenesis of endometriosis.

In conclusion, our findings suggest that the origins of endometriotic epithelial and stromal cells might be different. Otherwise, if they do arise from common hypothetical precursor cells, they might have expanded independent of each other from the very early stage of divergence or differentiation, with distinct patterns of genome evolution.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

K.S., H.N., K.Y., I.I. and T.E. designed the study. K.S. collected specimens. H.N. performed sequencing experiments. K.S. and H.N. performed data analysis. K.S., H.N. and K.Y. drafted the article. All authors approved the submission of the manuscript.

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Conflict of interest

There are no conflicts of interest to declare.

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