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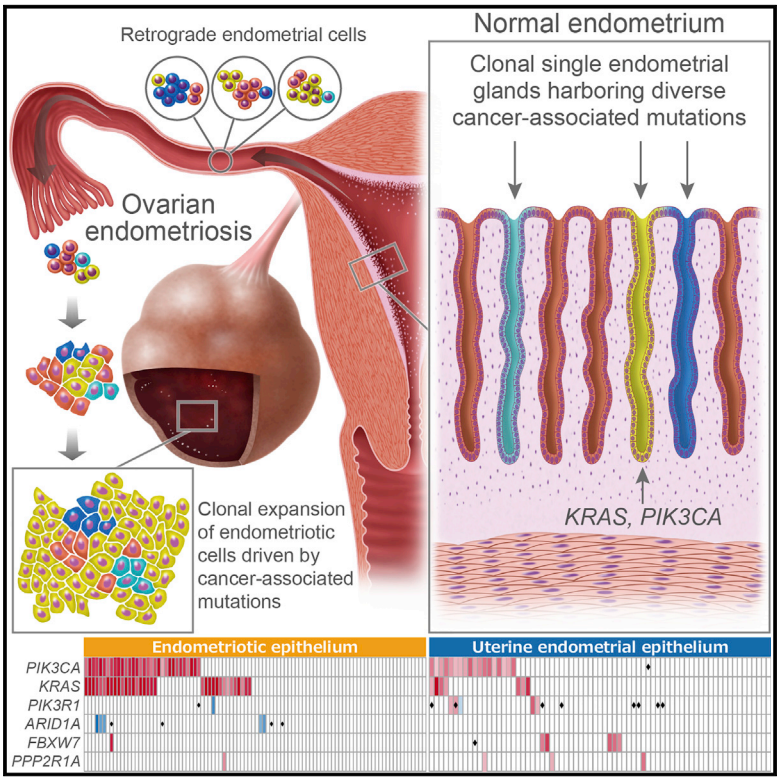
Clonal Expansion and Diversification of Cancer-Associated Mutations in Endometriosis and Normal Endometrium

Graphical Abstract



Authors

Kazuaki Suda, Hirofumi Nakaoka, Kosuke Yoshihara, ..., Teiichi Motoyama, Ituro Inoue, Takayuki Enomoto

Correspondence

yoshikou@med.niigata-u.ac.jp (K.Y.), itinoue@nig.ac.jp (I.I.), enomoto@med.niigata-u.ac.jp (T.E.)

In Brief

Suda et al. identify numerous cancer-associated mutations in epithelial cells from ovarian endometriosis and normal endometrium. They describe a heterogeneous and mosaic-like uterine endometrial epithelium, shaped by endometrial glands with distinct somatic mutations. They suggest clonal expansion of epithelial cells with cancer-associated mutations leads to the development of endometriosis.

Highlights

- Endometriosis and uterine endometrium exhibit cancer-associated somatic mutations
- Clonal expansion of epithelial cells with cancer-associated mutations in endometriosis
- Genomic architecture of epithelial cells in uterine endometrium is heterogeneous
- Single endometrial glands carry distinct mutations in cancer-associated genes

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Clonal Expansion and Diversification of Cancer-Associated Mutations in Endometriosis and Normal Endometrium

Kazuaki Suda,[1,8] Hirofumi Nakaoka,[2,8] Kosuke Yoshihara,[1,*] Tatsuya Ishiguro,[1] Ryo Tamura,[1] Yutaro Mori,[1] Kaoru Yamawaki,[1] Sosuke Adachi,[1] Tomoko Takahashi,[3] Hiroaki Kase,[4] Kenichi Tanaka,[5] Tadashi Yamamoto,[6] Teiichi Motoyama,[7] Ituro Inoue,[2,*] and Takayuki Enomoto[1,9,*]

[1]Department of Obstetrics and Gynecology, Niigata University Graduate School of Medical and Dental Sciences, Niigata 951-8510, Japan
[2]Division of Human Genetics, National Institute of Genetics, Mishima 411-8540, Japan
[3]Center for iPS Cell Research and Application (CiRA), Kyoto University, Kyoto 606-8507, Japan
[4]Department of Obstetrics and Gynecology, Nagaoka Chuo General Hospital, Nagaoka 940-8653, Japan
[5]Niigata Medical Center Hospital, Niigata 950-2022, Japan
[6]COI-s Biofluid Biomarker Center, Institute of Research Collaboration and Promotion, Niigata University, Niigata 950-2181, Japan
[7]Department of Molecular and Diagnostic Pathology, Niigata University Graduate School of Medical and Dental Sciences, Niigata 951-8510, Japan
[8]These authors contributed equally
[9]Lead Contact
*Correspondence: yoshikou@med.niigata-u.ac.jp (K.Y.), itinoue@nig.ac.jp (I.I.), enomoto@med.niigata-u.ac.jp (T.E.)
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SUMMARY

Endometriosis is characterized by ectopic endometrial-like epithelium and stroma, of which molecular characteristics remain to be fully elucidated. We sequenced 107 ovarian endometriotic and 82 normal uterine endometrial epithelium samples isolated by laser microdissection. In both endometriotic and normal epithelium samples, numerous somatic mutations were identified within genes frequently mutated in endometriosis-associated ovarian cancers. *KRAS* is frequently mutated in endometriotic epithelium, with a higher mutant allele frequency (MAF) accompanied by arm-level allelic imbalances. Analyses of MAF, combined with multiregional sequencing, illuminated spatiotemporal evolution of the endometriosis and uterine endometrium genomes. We sequenced 109 single endometrial glands and found that each gland carried distinct cancer-associated mutations, demonstrating the heterogeneity of the genomic architecture of endometrial epithelium. Remarkable increases in MAF of mutations in cancer-associated genes in endometriotic epithelium suggest retrograde flow of endometrial cells already harboring cancer-associated mutations, with selective advantages at ectopic sites, leading to the development of endometriosis.

INTRODUCTION

Five to fifteen percent of women of reproductive age suffer from endometriosis, which is histologically characterized by an extrauterine growth of endometrial-like epithelium and stroma and which causes pelvic pain, dysmenorrhea, and infertility (Giudice, 2010; Hickey et al., 2014). Endometriosis has been clinically recognized for nearly 150 years, yet the origin of the disease is still disputed. Several hypotheses, such as retrograde menstruation, coelomic metaplasia, and Müllerian remnants, have been proposed to account for the etiology and pathogenesis of endometriosis, but none have yet to be conclusively confirmed (Vercellini et al., 2014). Although endometriosis is thought of as a mostly benign gynecologic disease, several lines of evidence indicate that a history of endometriosis is associated with a significant risk for developing specific subtypes of ovarian carcinoma (Pearce et al., 2012). A more complete molecular characterization of the pathogenesis of endometriosis is required for the development of new treatments (Bulun, 2009).

Genome-wide association studies have identified several susceptibility loci for endometriosis (Adachi et al., 2010; Uno et al., 2010; Nyholt et al., 2012; Albertsen et al., 2013; Sapkota et al., 2017). Most of the identified variants were located in noncoding regions, suggesting expression levels of nearby genes were being altered (Maurano et al., 2012; Lappalainen et al., 2013). We previously demonstrated that allelic differences in a series of transcriptional regulation from transcription factor binding to gene expression mediated by chromatin interaction underlie the molecular mechanism of 9p21 endometriosis risk locus (Nakaoka et al., 2016). At the same time, studies exploring genomic alterations in endometriotic lesions have been intensively reported (Wiegand et al., 2010; Li et al., 2014). Focusing on the possibility that ovarian endometriosis could be the precursor of certain histological types of ovarian cancers, somatic mutations on genes frequently mutated in clear-cell and endometrioid ovarian cancers, such as *PTEN*, *PIK3CA*, and *ARID1A*, have been detected in ovarian endometriosis with or without concurrent ovarian cancers (Sato et al., 2000; Wiegand et al., 2010; Vestergaard et al., 2011; Yamamoto et al., 2011; Anglesio et al., 2015;

Er et al., 2016). The success of the Cancer Genome Atlas encouraged us to apply next-generation sequencing for the identification of somatic alterations to characterize the genomic features of endometriosis (Cancer Genome Atlas Research Network, 2011; Weinstein et al., 2013; Yoshihara et al., 2015). One of the key stumbling blocks to using next-generation sequencing for endometriosis has been that the lesion contains a relatively large proportion of non-epithelial stromal and inflammatory cells (McKinnon et al., 2016). To obtain a view of somatic mutations contributing to endometriosis not diluted by non-epithelial cells, we purified the endometriotic epithelial cells from the endometriosis tissue by laser microdissection (Matsuzaki et al., 2009).

In the current study, we performed whole-exome and target-gene sequencing for ovarian endometriotic epithelium samples obtained from subjects without concurrent gynecological cancers. Additionally, we analyzed histologically normal uterine endometrial epithelium samples obtained from subjects with benign gynecologic diseases. All the epithelium samples were isolated by laser microdissection from frozen sections. We exploited the distribution of mutant allele frequencies (MAFs) to infer the dynamic evolutionary process of a cell population driven by the accumulation of somatic mutations, based on the concept that the clonality of a subpopulation with a specific advantageous somatic mutation will be reflected as the MAF of the corresponding mutation (Nik-Zainal et al., 2012a; 2012b). Such analyses together with multiregional sampling from the same individuals will be useful to show how the genomes of endometriosis and normal uterine endometrium evolve through space and time (Russnes et al., 2011; Yates and Campbell, 2012). The identified mutations can be the genomic footprints tracing somatic cell lineage from ''normal'' uterine endometrial epithelium to endometriosis and on to certain subtypes of ovarian cancer. Furthermore, we conducted single endometrial gland sequencing to delineate the suspected heterogeneity in uterine endometrial epithelium. Our genomic study will provide further insights into the origins of endometriosis.

RESULTS

Whole-Exome Sequencing in a Discovery Cohort

We performed whole-exome sequencing for 13 endometriotic and 11 normal uterine endometrial epithelium samples, together with sequencing their matched normal blood samples (Figures 1A and 1B; Table S1). The average sequencing depth and the percentage of the exome that covered at least 20 reads were on average 97.4 and 84.0%, respectively. We detected a total of 4,192 somatic mutations in these 24 epithelium samples (median, 128; range, 72–536). The numbers of somatic mutations per Mb sequenced were not significantly different between ectopic endometriotic and normal uterine endometrial epithelium samples (Wilcoxon-Mann-Whitney test; $p = 0.61$; Figure S1A). In the five subjects (subjects 9–13) for which both endometriotic and uterine endometrial epithelium samples were sequenced, mutation profiles were discordant between their endometriosis and normal uterine endometrium (Figure 1B; Tables S2 and S3).

We identified 16 genes with non-silent mutations (i.e., missense, nonsense, or splice site) in three or more epithelium samples (Tables S2 and S3). Several cancer-associated genes, such as *KRAS*, *PIK3CA*, *FBXW7*, *PPP2R1A*, and *PIK3R1*, were recurrently mutated in both endometriotic and uterine endometrial epithelium samples, although these epithelia were histologically benign and normal. Somatic mutations on these genes have been detected in endometriosis-associated ovarian cancers, such as clear-cell and endometrioid histologic types (Jones et al., 2010; McConechy et al., 2014). In order to orthogonally confirm the presence of somatic mutations with low MAFs, we performed droplet digital PCR. A total of 11 mutation events at five single nucleotide variant (SNV) sites on cancer-associated genes (*KRAS*, *PIK3CA*, *FBXW7*, *PPP2R1A*, and *PIK3R1*) were selected for validation by droplet digital PCR as follows: two hotspot mutations for *KRAS* (p.G12V [c.G35T]) and *PIK3CA* (p.H1047R [c.A3140G]) and mutations with lowest MAF on *FBXW7* (p.R425C [c.C1273T]), *PPP2R1A* (p.D218V [c.A653T]), and *PIK3R1* (p.N564K [c.C1692G]). We successfully confirmed all of these 11 mutation events, indicating the reliability of our variant call procedure (Table S4).

KRAS and *PIK3CA* were the most frequently mutated genes in endometriotic and normal uterine endometrial epithelium samples, respectively (Figure 1B). All the *KRAS* mutations were located on the hotspots at amino acids 12, 13, or 61 (Figure 1C). It has been reported that these hotspot mutations impair GTPase-accelerating protein (GAP)-stimulated guanosine triphosphate (GTP) hydrolysis activity, lead to the persistence of active GTP-bound RAS, and in turn result in the constant activation of its downstream pathways (Scheffzek et al., 1997). The mutations in *PIK3CA* were widely distributed over functional domains of this gene, of which three were located on a hotspot at residue H1047 and the others also coincided with previously identified mutations in cancers based on the Catalogue of Somatic Mutations in Cancer (COSMIC) database (Forbes et al., 2017; Figure 1C). According to the finding that majority of *PIK3CA* mutations in cancers showed gain-of-function effects and growth advantages (Gymnopoulos et al., 2007), the mutations of *PIK3CA* in endometriotic and uterine endometrial epithelium samples at the same position as cancer mutations are likely to confer functional significance.

Although recurrently mutated genes were similar between two types of epithelium samples, the MAFs of the mutations were diverse. We classified the mutations into high and low MAF categories by setting dichotomizing threshold for MAF to be 15%. The number of high MAF mutations per Mb was larger in endometriotic epithelium than that in uterine endometrial epithelium (Wilcoxon-Mann-Whitney test; $p = 3.0 \times 10^{-4}$; Figures 1B and S1B). On the other hand, there was no statistically significant difference in the number of low MAF mutations between the two types of epithelium samples ($p = 0.19$). In order to assess whether the finding was robust to dichotomizing threshold for MAF, we conducted a sensitivity analysis in which different dichotomizing thresholds (0.10, 0.20, 0.25, 0.30, 0.35, and 0.40) were examined. In all the dichotomizing thresholds, we verified that the number of high MAF mutations was larger in endometriotic epithelium than that in uterine endometrial epithelium ($p = 0.015$, 5.3×10^{-4}, 2.1×10^{-4}, 5.3×10^{-4}, 8.1×10^{-4}, and 9.5×10^{-4}, respectively).

We examined the spectra of the identified mutations according to types of nucleotide substitutions (Lawrence et al., 2013; Alexandrov et al., 2013a, 2013b). The spectra of the somatic

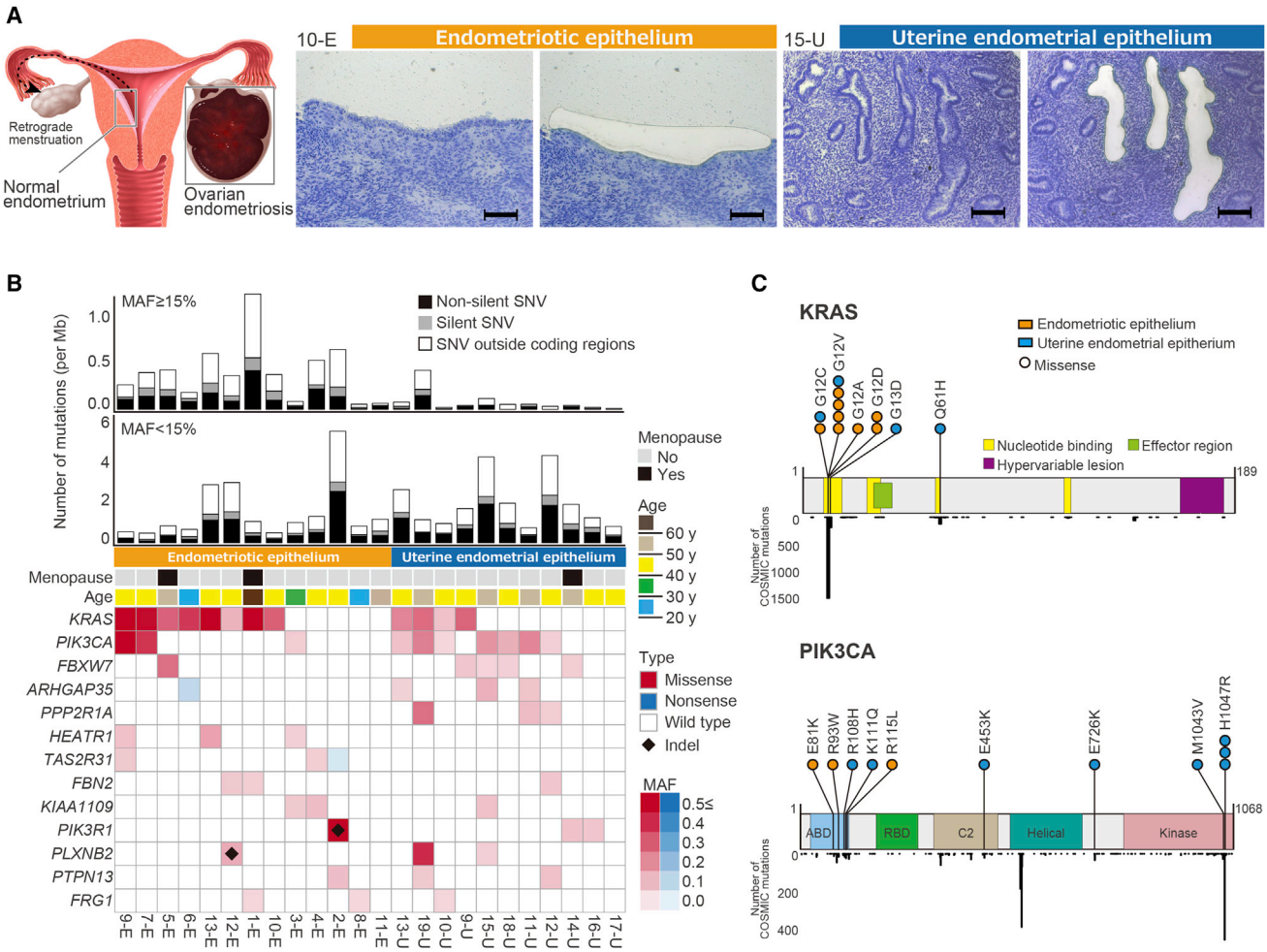

Figure 1. Whole-Exome Sequencing in Endometriotic and Uterine Endometrial Epithelium Samples Isolated by Laser Microdissection

(A) Scheme for tissue sampling and laser microdissection in ovarian endometriosis case. Scale bar, 100 μm.

(B) Summary of somatic recurrently mutated genes from whole-exome sequencing for 13 endometriotic epithelium and 11 uterine endometrial epithelium samples. Two bar graphs depict the numbers of somatic mutations with high or low MAF for each sample, respectively. A heatmap demonstrates distribution of recurrent mutations based on whole-exome sequencing across endometriotic and normal endometrial epithelium samples. Color and its density indicate type and MAF of each somatic mutation, respectively. Black diamond represents indel. *CSMD3*, *TTN*, and *MUC6* were recurrently mutated but not shown because these genes and their family genes have been reported as potentially spurious-positive genes (Lawrence et al., 2013; Tamborero et al., 2013).

(C) Schematic representation of locations of the identified somatic mutations in *KRAS* (upper) and *PIK3CA* (lower) along with known domain structures of the proteins. Numbers refer to amino acid residues. Each circle corresponds to an independent somatic mutation event. Black bar graph indicates the number of somatic mutations deposited in the COSMIC database.

mutations in the two types of epithelium samples were similar but clearly different from the germline variants of their matching normal blood samples (Figure S1C). The spectra of the somatic mutations were remarkably different regarding MAF (Figure S1D). Although the C > T transition at CpG dinucleotides, attributed to 5-methylcytosine, was common in high MAF mutations (Figure S1E), the C > A transversion, attributed to DNA damages by reactive oxygen species, was more abundant in low MAF mutations (Roberts and Gordenin, 2014).

We scrutinized the distribution of MAF of the somatic mutations in each sample. The distributions of MAF in endometriotic epithelium were higher than those in uterine endometrial epithelium (Figure 2A). Somatic mutations on representative cancer-associated genes (*KRAS*, *PIK3CA*, *FBXW7*, *PPP2R1A*, and *PIK3R1*) were located at or near the highest MAF in each sample (Figures 2A and S2). In 10 out of 24 epithelium samples, mutations on these cancer-associated genes ranked in the top 5% according to MAF. Particularly, five *KRAS* mutations showed highest MAFs in these samples. The average MAF rank of mutations on these cancer-associated genes was significantly higher than that of the other mutations (Wilcoxon-Mann-Whitney test; $p = 2.4 \times 10^{-5}$), suggesting these mutations were exerting a driving force for clonal expansion of their corresponding cell populations.

The majority of the normal uterine endometrial gland epithelium samples harbored multiple mutations of specific cancer-associated genes yet remained in a subclonal state

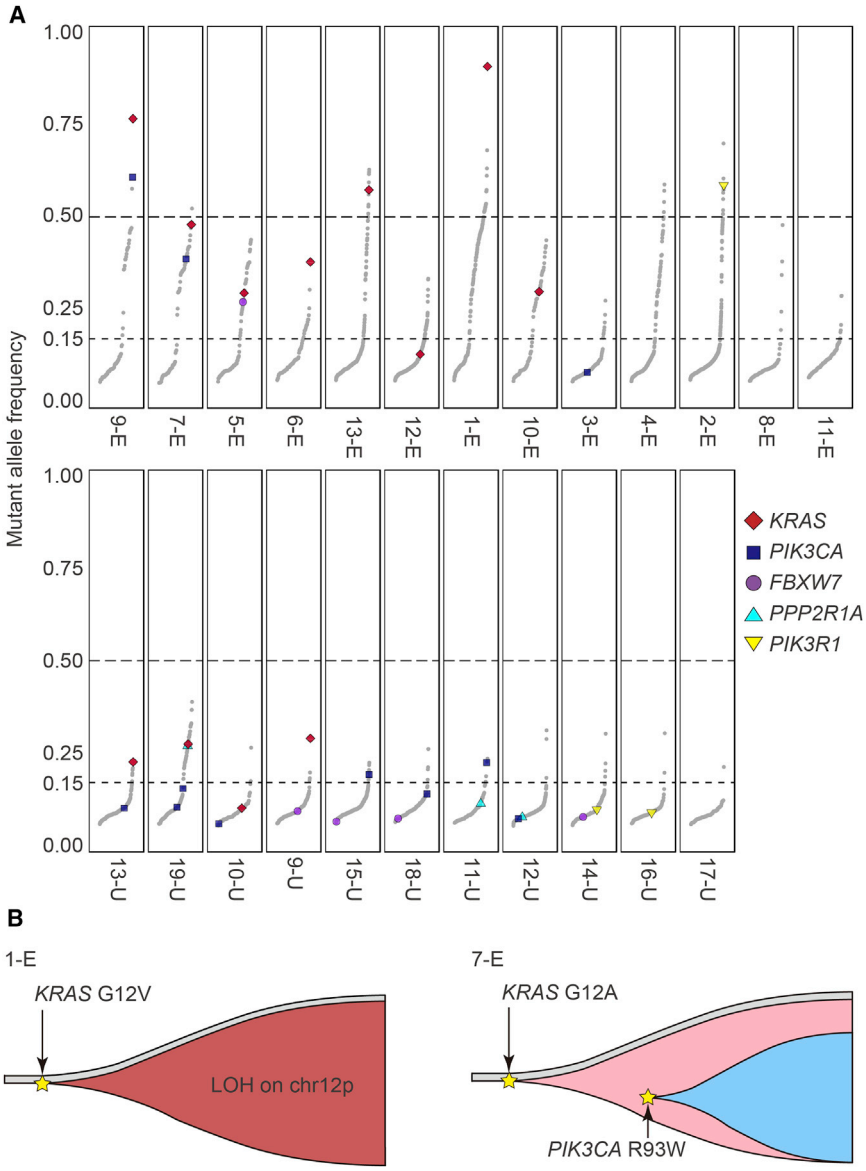

Figure 2. Clonal Expansion of Epithelial Cells with Cancer-Associated Mutations

(A) Distribution of MAFs of somatic mutations for each sample. Mutations are sorted according to their MAFs. Mutations on recurrently mutated cancer-associated genes (*KRAS*, *PIK3CA*, *FBXW7*, *PPP2R1A*, and *PIK3R1*) are color coded. The other mutations are represented by gray.

(B) Schematic representation for clonal expansion of *KRAS*-mutated epithelial cells in two representative endometriosis cases based on MAF.

(MAF << 0.5; Figure 2A). In contrast, mutations in these cancer-associated genes reached clonal status in the endometriotic epithelium samples (MAF of 0.5; Figure 2A). The MAFs for *KRAS* mutations in endometriotic epithelium were remarkably high (median of 0.425; range 0.10–0.93); in particular, the MAFs of a *KRAS* mutation (p.G12V) in samples 1-E and 9-E were 0.93 and 0.78, respectively (Figure S3A; Table S2). The dominance of mutant *KRAS* alleles in ectopic gland samples from 1-E and 9-E were confirmed by Sanger sequencing (Figure S3B) and droplet digital PCR (Table S4). Copy number analysis based on whole-exome sequencing demonstrated that the three endometriotic epithelium samples harboring high MAF mutations on *KRAS* had arm-level allelic imbalances, including loss of heterozygosity at chromosome 12p (Figure 3; Supplemental Experimental Procedures), leading to clonal expansion of epithelial cells carrying *KRAS* mutations (Figure 2B). By using Control-FREEC (FREE copy number and allelic content caller) (Boeva et al., 2011, 2012), we explored copy number alterations at the genome-wide scale. The result showed allelic imbalances at chromosome 12p in samples 1-E and 9-E were copy-neutral loss of heterozygosity. We could not detect the other recurrent copy number alterations at cancer-associated genes among multiple samples.

Target-Gene Sequencing in a Validation Cohort

We performed target-gene sequencing in a larger validation cohort, including 94 ovarian endometriotic epithelium samples from 45 subjects. We analyzed 71 histologically normal uterine endometrial epithelium samples from 29 subjects with benign gynecologic diseases (10, endometriosis; 18, myoma uteri; and 1, cervical intraepithelial neoplasia; Table S5). We selected 84 genes based on the results from our discovery phase and whole-exome sequence studies for ovarian clear-cell and uterine endometrial carcinomas (Table S6; Jones et al., 2010; Weinstein et al., 2013; Lawrence et al., 2014; Supplemental Experimental Procedures). By using a pre-capture pooling method followed by next-generation sequencing (Ahmadloo et al., 2017), we sequenced the targeted genes to the average depth of 78.7 reads and with coverage of at least 20 reads for 96.6%.

We confirmed that *KRAS* and *PIK3CA* were the most frequently mutated among the targeted genes (Figure 4). Additionally, the MAFs of these oncogenes were significantly higher in the endometriotic epithelium than in normal uterine endometrial epithelium (Wilcoxon-Mann-Whitney test; $p < 0.05$; Figure S5A). The MAFs of *KRAS* mutations in four endometriotic epithelium samples were significantly greater than 0.5 (range, 0.79–0.91; binomial test; $p < 0.05$), suggesting either the occurrence of loss of heterozygosity at chromosome 12p or regional amplification surrounding *KRAS*. These results further support the idea that clonal expansion of endometriotic epithelial cells was being driven by oncogenic somatic mutation events. The

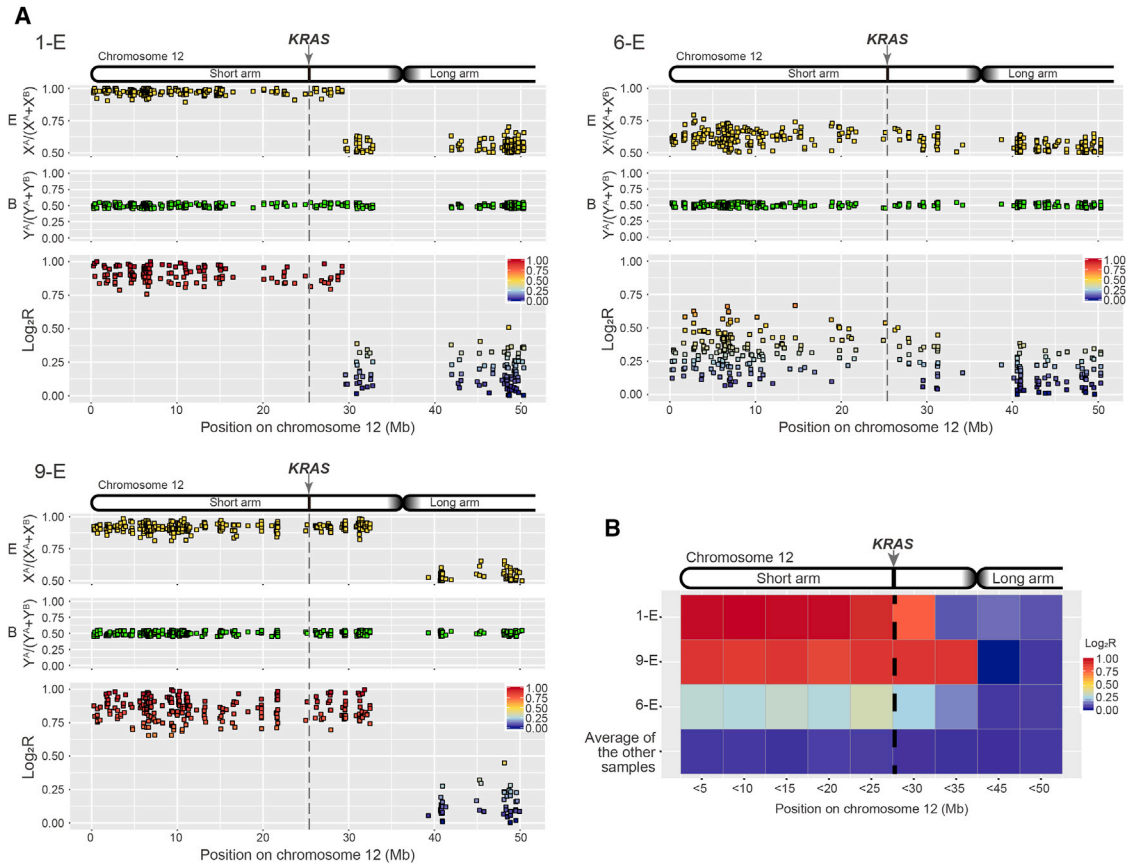

Figure 3. Arm-Level Allelic Imbalances at Chromosome 12p in Endometriotic Epithelium Samples Harboring High MAF Mutations on *KRAS*

(A) Signatures of allelic imbalances for three endometriotic epithelium samples at chromosome 12p are represented (1-E, 6-E, and 9-E). For each sample, top and middle panels depict ratios of sequence reads at germline heterozygote single-nucleotide variants in endometriotic epithelium and normal blood samples, respectively. Bottom panel shows $\log_2$R values calculated as relative ratios of the values shown in the top and middle panels. Deviation in $\log_2$R value from zero is a signature for allelic imbalance. Plots of $\log_2$R for almost the entire short arm of chromosome 12 in 1-E and 9-E locate near 1.0, suggesting the presence of arm-level deletion of wild-type allele (loss of heterozygosity) in these two samples (Supplemental Experimental Procedures).

(B) A heatmap showing somatic allelic imbalances on the short arm of chromosome 12. The color bar represents the averages of $\log_2$R values over germline heterozygote single-nucleotide variants located within 5 Mb bins. In two endometrial epithelium samples harboring *KRAS* mutations with high MAF (1-E and 9-E), $\log_2$R values were nearly 1.0 for the entire short arm of chromosome 12, indicating the presence of loss of heterozygosity. Another endometriotic sample harboring *KRAS* mutation with high MAF (6-E) showed statistically significant increases in $\log_2$R at the short arm of chromosome 12 compared to those of the other samples ($p < 2.2 \times 10^{-16}$), suggesting the subclonal loss of heterozygosity or allelic imbalance with relative increase of chromosome harboring mutant *KRAS* allele (Supplemental Experimental Procedures).

majority of mutations in the normal uterine endometrial epithelium remained in subclonal states (MAF < 0.5), corroborating the mosaic nature of their genomic compositions (Figure S6A).

We identified frequently mutated genes (mutated in >5% samples) in endometriotic (*PIK3CA*, *KRAS*, *ARID1A*, *TAF1*, *SPEG*, *ACRC*, and *FAT1*) and normal uterine endometrial (*ARHGAP35*, *PIK3CA*, *PIK3R1*, *HEATR7*, *KRAS*, *FBXW7*, *FGFR2*, *PLXNB2*, and *POLE*) epithelium samples, respectively (Table S6). It is notable that *ARID1A*, a gene that is thought to be involved in the progression of endometriosis to carcinoma (Wiegand et al., 2010; Yamamoto et al., 2012), was more frequently mutated in the endometriotic epithelium (Fisher's exact test; p = 0.038). Particularly, all the *ARID1A* mutations in the endometriotic epithelium were nonsense substitutions and frameshifting indels, suggesting their functional effects (Figure S4). On the other hand, mutations on *ARHGAP35*, *PIK3R1*, *FBXW7*, and *PLXNB* were more common in the uterine endometrial epithelium (Fisher's exact test; $p = 3.9 \times 10^{-5}$, 9.8×10^{-4}, 0.013, and 0.031, respectively). Well-established tumor suppressor genes and chromatin regulatory factors, such as *PTEN*, *ARID1A*, and *KMT2C*, were likely dysregulated by loss-of-function mutations (i.e., nonsense, splice-site, and frameshifting indels mutations; Figures 4 and S4). Similarly, we identified loss-of-function mutations in several other putative tumor suppressor genes (*PIK3R1*, *ARHGAP35*, and *FAT1*; Figures 4 and S4), supporting the antiproliferative functions of these genes (Sonoda et al., 2004; Taniguchi et al., 2010; Morris et al., 2013; Lawrence et al., 2014; Lin et al., 2014). Mutations in *TAF1* were mostly localized within the histone acetyltransferase and DNA binding domains (Figure S4; Louder et al., 2016).

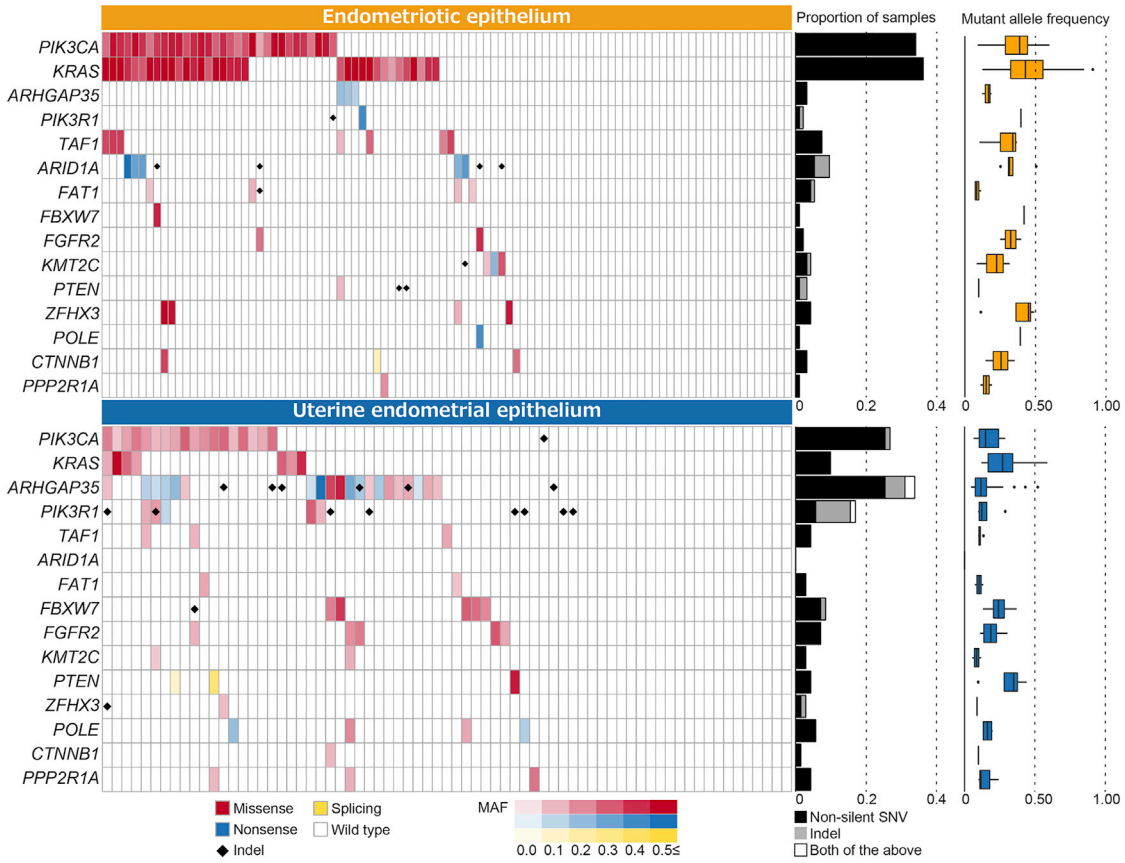

Figure 4. Mutation Landscapes of Endometriotic and Uterine Endometrial Epithelium Samples by Target-Gene Sequencing

Left panels show distribution of recurrent mutation based on target resequencing in endometriosis (top left) and uterine endometrium (bottom left). Color and its density indicate type and MAF of each somatic mutation, respectively. Black diamond represents indel. The middle panels (bar graphs) demonstrate the proportion of samples harboring mutation stratified by genes. The right panels (box-whisker plots) display variation of MAFs of somatic mutations stratified by genes.

Genomic Homogeneity and Heterogeneity in Multiregional Sequencing

We scrutinized the spatial distribution of somatic mutations in endometriotic and uterine endometrial epithelium samples by using multiregional sampling of several sites from the same individuals (Gerlinger et al., 2012; Yates et al., 2015).

We performed multiregional sampling for a total of 19 subjects with endometriosis, including whole-exome and target-gene sequencing. We were able to obtain 72 endometriotic epithelium samples, with a sampling depth range of 2–6 samples per subject. Similarly, we collected 77 normal uterine epithelium samples from 29 subjects (range, 2–4 samples per subject; Table S5).

Subject 30, a 40-year-old premenopausal woman, presented six spatially separated endometriotic epithelium samples (Figure 5A). All six endometriotic epithelium samples (E1–E6) harbored the same mutations on *PIK3CA* (p.C407W) and *SPEG* (p.R2055W) with high MAFs. E1 and E6 acquired distinct *FAT3* (p.S4515L) and *FAT1* (p.S404R) substitutions, indicating these later genomic events drove branching processes in the spatial evolution of the endometriotic epithelial cell population.

Subject 38, a 36-year-old premenopausal woman, presented with bilateral endometriomas. We collected spatially separated endometriotic epithelium samples from the right and left endometriomas, four and two samples, respectively (Figure 5B). Whereas all the endometriotic samples on the right ovary harbored a *KRAS* mutation (p.G12D), another *KRAS* mutation (p.G13D) and two frameshifting deletions on *PTEN* (p.F258fs and p.D331fs) were shared among the endometriotic samples on the left ovary. The *PTEN* mutation status distinguished all six samples as being either from the right and left endometrioma. Mutations of *PIK3CA* divided the four samples from the right endometrioma into two regional subgroups (p.H1047R for E1, E2, and E5 and p.P471L for E4). Furthermore, one of the endometriotic samples from the right endometrioma carried a *KRAS* mutation at low MAF level (0.12) that was eventually the same substitution with the one found in the left side ovary (p.G13D). These results suggest that the separate bilateral endometriomas of this subject originated from ancestral cell populations that went on to be characterized by distinct *KRAS* and *PTEN* mutations. The right-sided tumor then subsequently incurred further mutational events in *PIK3CA* and *KRAS* that rendered it spatially heterogeneous.

Subject 44, a 47-year-old premenopausal woman, presented with a unilateral endometrioma on her right ovary (Figure 5C).

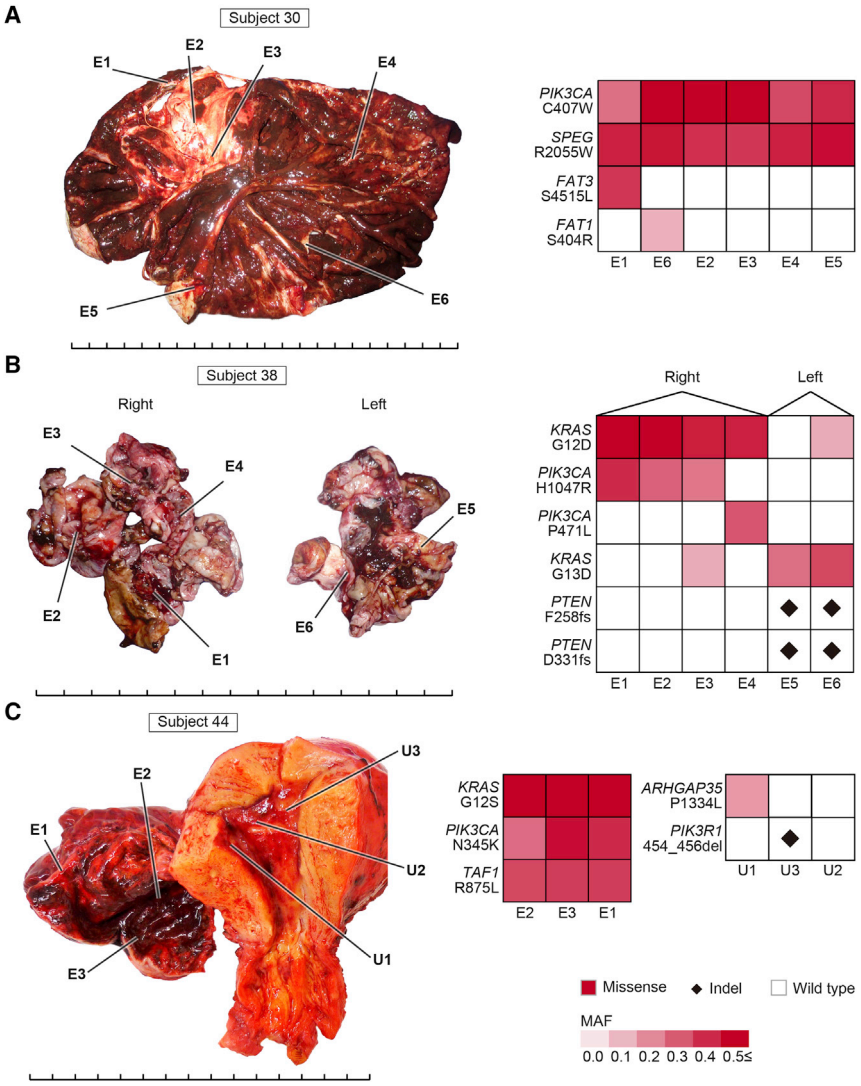

Figure 5. Genomic Homogeneity and Heterogeneity in Endometriosis and Uterine Endometrium

(A) Mutation sharing in a unilateral ovarian endometrioma from subject 30. Sampling sites are shown in the left panel. Color and its density indicate type and MAF of each somatic mutation, respectively. Each tick mark represents 1 cm.

(B) Difference in mutation-sharing pattern between right and left ovarian endometrioma in the same individual (subject 38). Color and its density indicate type and MAF of each somatic mutation, respectively. Black diamond represents indel. Each tick mark represents 1 cm.

(C) Commonality and diversity of cancer-associated mutations detected through multiregional sequencing of endometriosis and uterine endometrium from the same individual (subject 44). Color and its density indicate type and MAF of each somatic mutation, respectively. Black diamond represents indel. Each tick mark represents 1 cm.

Her endometrioma was histologically benign, and her uterine endometrium was normal. All three endometriotic epithelium samples (E1–E3) carried clonally expanded nonsynonymous mutations of *KRAS* (p.G12S; MAF 0.5). Her E1–E3 samples shared the same nonsynonymous substitutions in *PIK3CA* (p.N345K) and *TAF1* (p.R875L). The MAFs of the shared *PIK3CA* and *TAF1* mutations varied at an intermediate to high level (0.26–0.46). On the other hand, the mutation profiles of uterine endometrial epithelium samples (U1–U3) were divergent, as illustrated by the different mutations with low MAF level found in *PIK3R1* (0.16) and *ARHGAP35* (0.16).

We examined the patterns of sharing of mutations on cancer-associated genes (*KRAS*, *PIK3CA*, *FBXW7*, *PPP2R1A*, *PIK3R1*, and *ARID1A*) by comparing their carrier status between each pair of multiregional samples collected from the same individuals (Figure 6). We found that *KRAS* or *PIK3CA* mutations were completely shared across multiple regions of an individual subject with endometriosis (subjects 30, 35, 38, 41, 44, 46, and 52). Subject 46 had the same *KRAS* mutations (p.G12A) with high MAFs (0.66, 0.79, and 0.91), suggesting that all three lesions shared a common somatic progenitor cell and subsequently suffered a loss of heterozygosity or allelic imbalance at *KRAS*. Additionally, there were several cases harboring shared high MAF mutations together with unshared low MAF mutations (subjects 35, 38, and 41), proving that the unshared mutations in these endometriotic lesions were somatic events that occurred subsequent to the shared mutations. The MAFs of shared mutations were higher than those of unique mutations, presumably reflecting the clonal expansion of activated oncogene-carrying cells and to their wider distribution in subsequent endometriotic lesions (Figure S5B). To the contrary, sharing of these oncogene mutations was completely absent among multiple regions of the uterine endometrium of individuals. This result indicates that the normal uterine endometrium from individuals acquires spatially distinct patterns of somatic mutations that are precursors to endometriosis and potentially carcinoma.

Single Endometrial Gland Sequencing

To better delineate the genomic heterogeneity in endometrial epithelium cells, we focused on the endometrial glands, where each simple tubular gland is lined by columnar epithelium (Figure 7A; Tanaka et al., 2003).

We isolated 109 single endometrial glands from the uteri of three subjects (subjects 73–75; Supplemental Experimental Procedures; Table S7) and explored them for somatic mutations by applying target-gene sequencing. Remarkably, individual endometrial glands within the normal uterus of the same individual carried distinct somatic mutations (Figure 7B), aligning with their sporadic and spontaneous somatic origins during menstrual

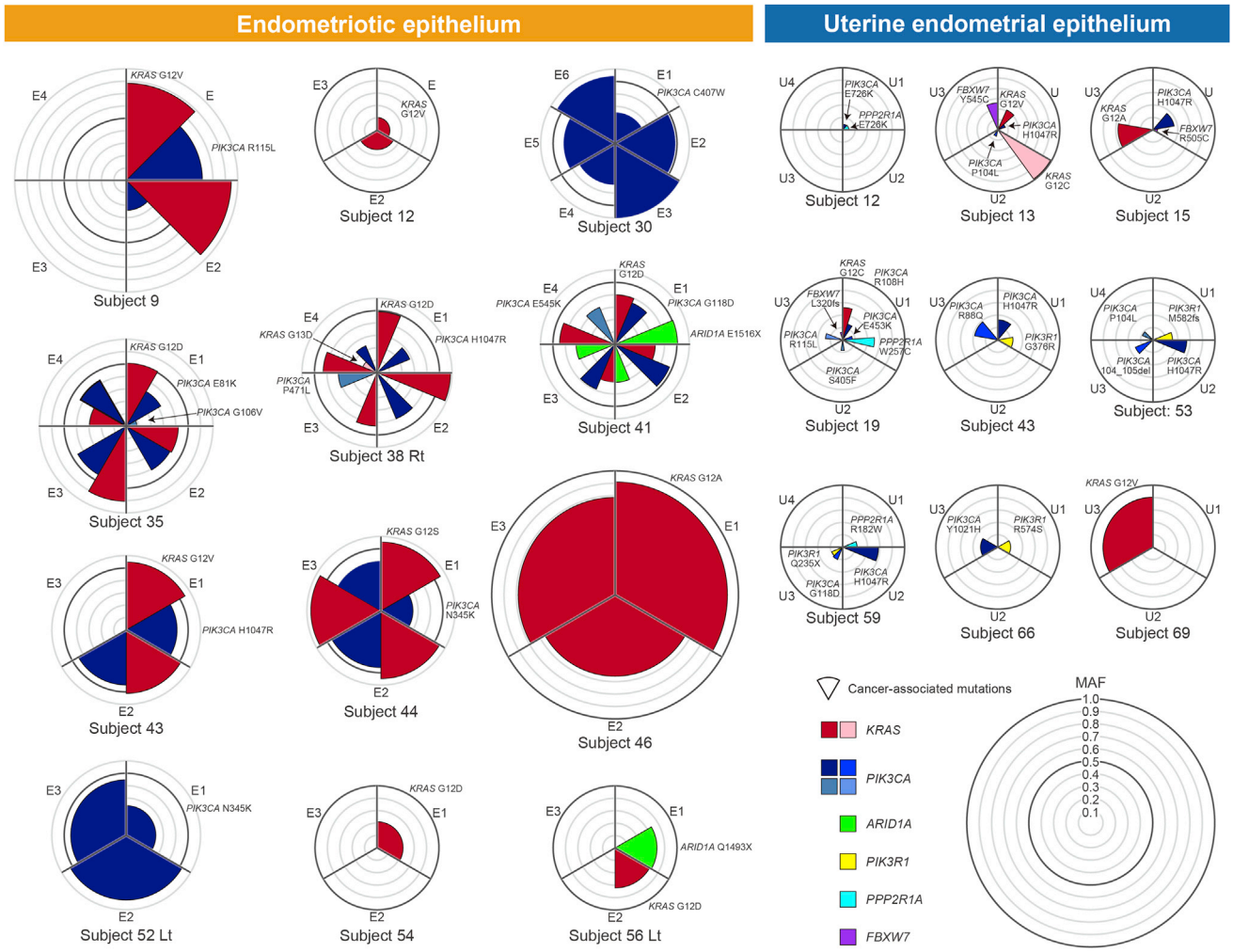

Figure 6. Sharing Patterns of Mutations on Cancer-Associated Genes among Multiregional Samples in Endometriotic and Uterine Endometrial Epithelium

Coxcomb plots show sharing patterns of mutations on representative cancer-associated genes (*KRAS*, *PIK3CA*, *FBXW7*, *PPP2R1A*, *PIK3R1*, and *ARID1A*) for subjects with at least three multiregional samples. For visualization, we focus on subjects carrying *KRAS* or *PIK3CA* mutation at any site. Multiregional samples are separated by radial lines. Each color represents a different mutation, and the same color indicates the same mutation in each subject. Concentric circles correspond with those of MAF scale.

glandular proliferation cycles. *PIK3CA* was the most frequently mutated gene across the single endometrial glands. We identified less frequent mutations within the other cancer-associated genes, including *KRAS* and *ARID1A* (Figure S7). The MAFs of these somatic mutations in endometrial glands were close to 0.5 (Figure 7C), indicating their clonal expansion within the gland from cells deep in the base of the tube. About one-third of the endometrial glands we sampled carried a *PIK3CA* mutation; however, each gland's mutation was different and each resulted in a unique amino acid substitution (Figure 7B). Only a very small number of the endometrial glands shared the same mutation. Two glands from subject 74 harbored the same *PIK3CA* mutation (p.K111N [c.G333T]) and the same *PPP2R1A* mutation (p.S256Y [c.C767A]; Figure 7B), implying that both glands were descended from a single common ancestral cell lineage carrying both these two mutations. The observed gland-to-gland genomic variation illuminates how heterogeneous and mosaic-like the nature of the uterine endometrial epithelium genome is shaped.

DISCUSSION

In this study, we identified numerous somatic mutations in cancer-associated genes in ovarian endometriotic epithelium cells isolated by laser microdissection from lesions of subjects without any detectable concurrent gynecologic cancers. While we were preparing to submit this manuscript, Anglesio et al. reported that 21% of the lesions in patients with deep-infiltrating endometriosis harbored somatic mutations in *ARID1A*, *PIK3CA*, *KRAS*, and *PPP2R1A* (Anglesio et al., 2017). Our results

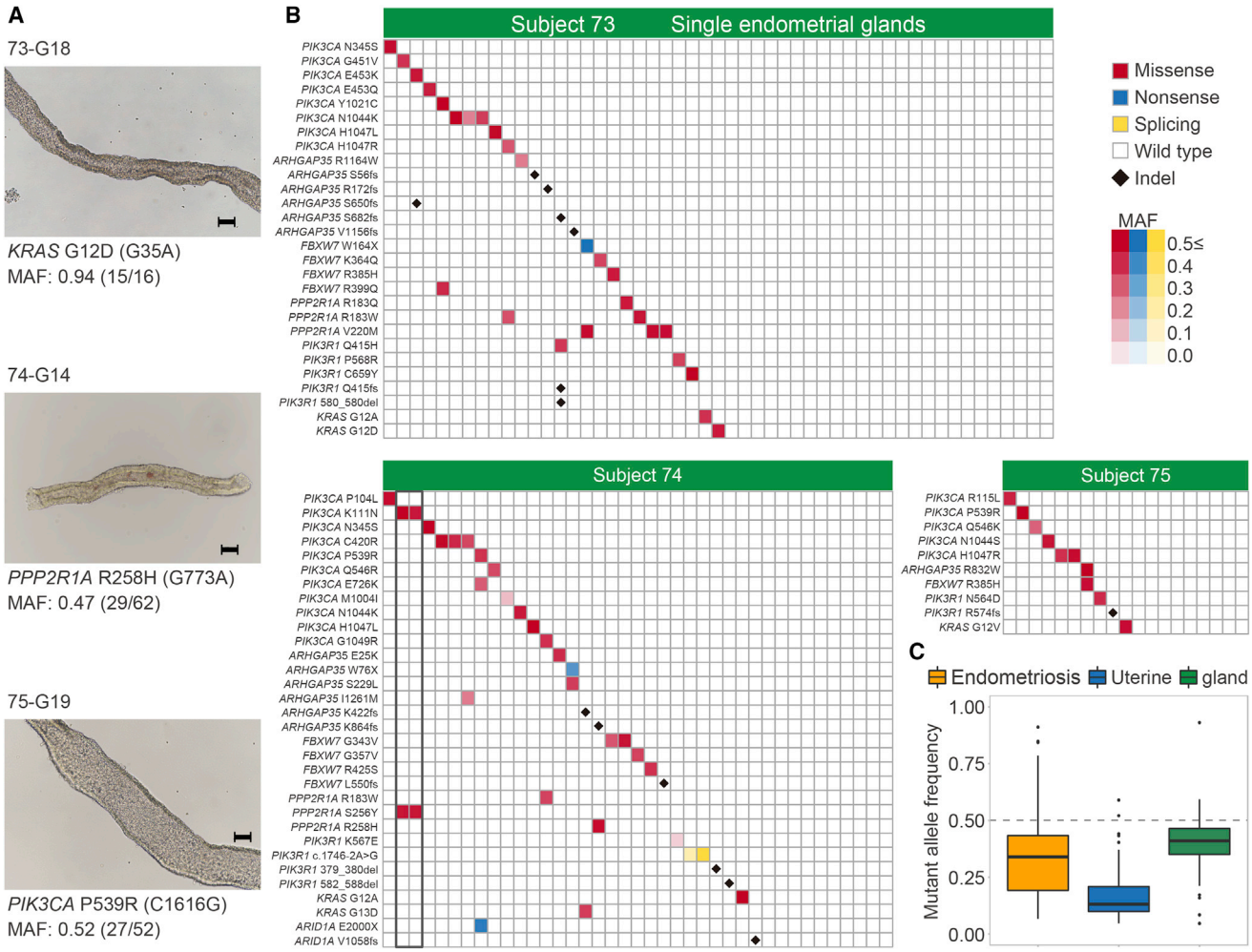

Figure 7. Diverse Mutations in Single Endometrial Glands

(A) Microscopic images of representative single endometrial glands (40× magnification; scale bars represent 100 μm).

(B) Each heatmap shows mutation profiles of single endometrial glands in each subject. Color and its density indicate type and MAF of each somatic mutation, respectively.

(C) High MAFs in endometrial glands support their clonal development.

corroborated their findings in a larger cohort of subjects with a more common type of endometriosis. At the same time, our work will provide several aspects that are not fully explored in Anglesio et al. First, the analyses of MAFs together with multiregional sequencing provided better understanding of spatiotemporal evolutions of the endometriosis and uterine endometrium genomes. Second, we showed that somatic mutations on cancer-associated genes were located at or near the highest MAF in each sample (Figures 2A and S2). Our results enabled us to infer evolutionary process of epithelial cells with cancer-associated mutations in ectopic sites, in which epithelial cells with cancer-associated mutations have selective growth advantages and therefore drive clonal expansion. Finally, most importantly, we provided a much broader perspective on the origin and pathogenesis of endometriosis by focusing on the genomic architecture of the normal uterine endometrium and its functional unit, the endometrial gland. The result showing the presence of somatic mutations on cancer-associated genes in histologically normal endometrial glands will provide important clues on the pathogenesis not only for endometriosis but also for a wide range of gynecologic diseases.

We demonstrated that the genes most recurrently mutated in endometriosis-associated ovarian cancers (Jones et al., 2010; Wiegand et al., 2010; McConechy et al., 2014) were also frequently mutated not only in endometriotic epithelium, considered the direct precursor to the carcinoma, but also in uterine endometrial epithelium samples, even though these epithelia were histologically benign and normal. The substantial overlap of recurrently mutated genes can be the genomic footprints linking somatic mutations, arising repeatedly and spontaneously throughout normal uterine endometrial epithelium, with subtypes of ovarian cancers via the intermediary lesion of endometriosis.

The analysis of MAF, combined with multiregional sequencing, revealed the unique mutational landscapes of endometriotic

epithelium and uterine endometrial epithelium samples. The distributions of MAF in endometriotic epithelium were higher than those in normal endometrial epithelium. It seems that the clonal expansion of the endometriotic lesions was driven by point mutations in the cancer-associated genes and by allelic imbalances, including loss of heterozygosity at *KRAS*. The multiregional sequencing of endometriotic epithelium samples showed that mutations on *KRAS* and *PIK3CA*, with high MAF, were frequently detected in 38% and 29% of subjects, respectively (Table S6), and that these were shared among spatially separated samples. The multiregional sequencing for subjects with bilateral endometriomas demonstrated that the shared mutations were specifically observed within the same endometrioma, but not between the left and right endometriomas, indicating that the left and right endometriomas arose independently. The steep increases in MAF of mutations on *KRAS* in endometriotic epithelium suggest that endometrial tissues harboring *KRAS* mutations were transported in a retrograde direction to the ovarian surface, where the specific *KRAS* mutations gave them selective advantages at this and other ectopic sites, leading to the development of endometriosis and widespread distribution of the clone across the endometriotic lesions (Figure S7). Although our results seem to be better fitted to retrograde menstruation hypothesis, we cannot exclude the possibilities that similar somatic mutations in cancer-associated genes occur in coelomic epithelium or Müllerian remnants, which might become precursor to endometriosis. Large-scale whole-genome or whole-exome sequencing efforts are required to create a comprehensive catalog of somatic mutations in heterogeneous epithelial cells in human female reproductive tract.

The mutational landscape of uterine endometrial epithelium represents the heterogeneous genomic compositions characterized by low MAF mutations and absence of shared mutations among spatially separated samples (Figure 6). The heterogeneous nature is clearly shown by the fact that 41% of subjects carried *PIK3CA* mutations but none of them had shared *PIK3CA* mutations across multiple regions collected from the same individuals.

To further decipher the heterogeneity in uterine endometrial epithelium, we focused on single endometrial glands, the minimum functional unit of uterine endometrial epithelium (Tanaka et al., 2003). The endometrial glands carried distinct somatic mutations in a nearly clonal state. Although 20%–33% of single endometrial glands from three subjects harbored *PIK3CA* mutations (Figure S6), the majority of the mutations resulted in different amino acid substitutions (Figure 7). The finding that multiple mutations were sometimes coordinately shared between two separated endometrial glands implies that both glands descended, post-mutations, from the same progenitor within the same general area of uterine endometrial epithelium. These results suggest that the heterogeneous and mosaic-like mutation landscape of uterine endometrial epithelium is shaped as a collection of endometrial glands with distinct genetic lineages (Figure S7).

Mutter and colleagues demonstrated PTEN-deficient glands were present in normal endometrium, and *PTEN* mutation status changed by contraceptive treatments and with the progression of endometrial metaplasia (Mutter et al., 2001, 2014; Lin et al., 2009). In our single endometrial gland sequencing, one out of 109 endometrial glands harbored a missense mutation of *PTEN* (<1%). Our results showed the other cancer-associated genes, such as *PIK3CA*, *KRAS*, *FBXW7*, *PPP2R1A*, *PIK3R1*, and *ARID1A*, were more frequently mutated. The single endometrial gland sequencing for women with or without contraceptive treatments will provide clues for molecular mechanisms underlying reduced risks for endometrial and ovarian cancers in users of contraceptives (Cibula et al., 2010).

Previous studies reported that the mutation status of *ARID1A* in samples from the same patients distinguished clear-cell carcinoma and adjacent atypical endometriosis from distant endometriotic lesions, suggesting that the *ARID1A* mutations were crucial for the malignant transformation from endometriosis to clear-cell and endometrioid types of ovarian cancers (Wiegand et al., 2010; Yamamoto et al., 2012). We identified five nonsense mutations and four frameshifting indels on *ARID1A* in endometriotic epithelium samples from patients without concurrent gynecological cancers. Moreover, one *ARID1A* nonsense mutation was detected in a normal endometrial gland. A mouse model study previously showed that an activating *Kras* mutation together with a *Pten* loss-of-function mutation developed an endometrioid type of ovarian cancer (Dinulescu et al., 2005). Similarly, coinciding *Arid1a* and *Pik3ca* mutations caused a clear-cell type of ovarian cancer in another mouse model (Chandler et al., 2015). In the same vein, we identified two endometriotic samples harboring activating *KRAS* mutations and frameshifting *PTEN* indels and five samples with both *ARID1A* and *PIK3CA* mutations. Considering that most *KRAS* and *PIK3CA* mutations achieved clonal status, substantial proportions of differentiated epithelial cells in the endometriotic lesions of these patients acquired combinations of mutations in oncogenes and tumor suppressors yet remained benign in appearance, suggesting that somatic mutations in "cancer-associated genes" are insufficient for malignant transformation.

Several lines of evidence demonstrate that mutations on cancer-associated genes are present in many "normal" tissues (Jaiswal et al., 2014; Xie et al., 2014; Martincorena et al., 2015; Martincorena and Campbell, 2015). Similar to these discoveries, benign endometriosis and normal uterine endometrium can be useful for exploring "resilience" factors, including genomic and environmental features that operate protective against malignant transformation or buffer the effects of such mutations on cancer-associated genes, which can provide clues for new therapies to prevent the developments of endometriosis and gynecologic cancers (Friend and Schadt, 2014).

The findings of this study reinforce John Sampson's century-old retrograde hypothesis of the origin of endometriosis at genomic level (Sampson, 1927). Further sequencing for endometrial epithelial cells from normal and retrograde menstruations will provide lineage-tracing genomic footprints from uterine endometrial epithelium to endometriosis.

EXPERIMENTAL PROCEDURES

Patient Samples

This study was approved by the institutional ethics review boards of Niigata University, Niigata Chuo General Hospital, and the National Institute of Genetics. We recruited study participants at the Niigata University Medical and Dental Hospital

and the Nagaoka Chuo General Hospital between September 2013 and March 2017. All subjects provided written informed consent for the collection of samples and subsequent analyses. The detailed description of the study participants and the method of laser microdissection to isolate epithelial cells can be seen in Supplemental Experimental Procedures.

Whole-Exome and Target-Gene Sequencing

We performed whole-exome and target-gene sequencing using genomic DNA derived from the 107 ovarian endometriotic and 82 normal uterine endometrial epithelium samples. Next, we conducted multiregional spatial target-gene sequencing for 19 endometriosis subjects and 27 subjects who provided normal uterine endometrium samples. Details of selection criteria for targeted genes and experimental methods for next-generation sequencing, orthogonal validations by Sanger sequencing and droplet digital PCR, and computational analyses are described in Supplemental Experimental Procedures.

Single Endometrial Gland Sequencing

We performed target-gene sequencing of DNA derived from individual endometrial glands. We collected a total of 109 endometrial glands from three subjects. Details of the single-gland isolation are provided in Supplemental Experimental Procedures.

Statistical Analysis

We conducted all standard statistical tests with the R program (http://www.r-project.org). We used Wilcoxon-Mann-Whitney test to compare (1) the number of mutations between endometriotic and normal endometrial epithelium samples, (2) the average MAF ranks between mutations on cancer-associated genes and the others, and (3) the MAFs of mutations between endometriotic and normal endometrial epithelium samples. The deviation of MAF of somatic mutation from 0.5 was assessed by binomial test. In the analysis of somatic allelic imbalances, we applied linear regression analyses to assess whether the $\log_2 R$ values for the endometriotic or uterine endometrial epithelium sample of interest was significantly deviated from those for baseline samples. We evaluated difference in the frequency of mutations on gene between endometriotic and normal endometrial epithelium by Fisher's exact test.

DATA AND SOFTWARE AVAILABILITY

The accession number for the sequencing data reported in this paper is EMBL-EBI: EGAS00001003095.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and seven tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.07.037.

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AUTHOR CONTRIBUTIONS

Conceptualization, K.S., H.N., K. Yoshihara, I.I., and T.E.; Methodology, K.S. and H.N.; Investigation, K.S. and H.N.; Validation, T.M.; Writing – Original Draft, K.S., H.N., and K. Yoshihara; Writing – Review & Editing, I.I. and T.E.; Funding Acquisition, H.N., K. Yoshihara, and I.I.; Resources, K.S., T.I., R.T., Y.M., K. Yamawaki, S.A., T.T., H.K., K.T., and T.Y.; Supervision, K. Yoshihara, I.I., and T.E.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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