Mutations of RAS genes in endometrial polyps

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Received May 31, 2019; Accepted August 27, 2019

DOI: 10.3892/or.2019.7353

Abstract. Endometrial polyps are common, yet the molecular mechanisms underlying their formation and progression remain unclear. We examined gene mutations possibly related to the pathogenesis of endometrial polyps, as well as to their clinical features. Four premenopausal patients with endometrial polyps, who were not under drug treatment, were recruited. Whole exomes of endometrial polyps and peripheral blood lymphocytes were analyzed by next-generation sequencing, and somatic mutations were derived by subtraction. Then, 35 samples of endometrial polyps and 12 samples of atypical polypoid adenomyoma were newly recruited to validate the identified mutations by polymerase chain reaction-reverse sequence specific oligonucleotide method. The mutations were also analyzed in separate stromal and glandular components of the polyps after laser-capture microdissection. Whole exome sequencing revealed that KRAS mutations were the only type of mutation detectable in multiple cases (2/4). Targeted mutation analysis revealed that 16 of 35 samples (45.7%) of endometrial polyps harbored RAS mutations. Mutation-positive cases exhibited a significantly higher number of endometrial polyps (3.25±2.70 vs. 1.74±0.87, P=0.045). Laser-capture microdissection in NRAS-mutated endometrial polyps revealed that both stromal and glandular components harbored RAS mutations. There was no RAS mutation in 12 samples of atypical polypoid adenomyoma. This is the first report demonstrating that pathogenic RAS mutations are frequent in non-treated endometrial polyps. RAS mutations may have an important role in tumorigenesis and in the formation of multiple endometrial polyps.

Introduction

An endometrial polyp is defined as a localized, disorganized proliferation of benign glandular and stromal elements protruding from the surface of the endometrium. Polyps are common and can occur at any age but are mostly observed in the perimenopausal period (1). Endometrial polyps are usually asymptomatic and found incidentally; however, they sometimes cause abnormal uterine bleeding and infertility. They arise as monoclonal overgrowths of genetically altered endometrial stromal cells with secondary induction of polyclonal benign glands (2). Tamoxifen and estrogen exposure are known to increase the frequency and number of endometrial polyps, and several studies have shown that body mass index, waist circumference, and insulin resistance are related to the presence of these polyps (3,4). The relationship of tamoxifen with endometrial polyps has been widely investigated, and it has been suggested that tamoxifen may induce KRAS mutations in endometrial cells and cause the recurrence of endometrial polyps and cancer (5,6). Several studies have also investigated the relationships of endometrial polyps with gene expression, mutation, and the immune system (7-10). However, the pathogenesis of endometrial polyps is still debated.

Next-generation sequencing has produced a breakthrough in genomic research and has facilitated mutational analysis by whole exome sequencing (11). To explore cancer genomic alterations, a large number of cancer genomes have been sequenced worldwide, resulting in the implementation of projects such as The Cancer Genome Atlas (TCGA) and The International Cancer Genome Consortium (ICGC). Whole exome sequencing has been the main platform for these sequencing initiatives, and data on the mutation of protein-coding regions have been accumulated in relation to all types of cancers (12,13).

In this study, we performed whole exome sequencing and targeted mutation analysis to verify the presence of somatic mutations in benign endometrial polyps and identify the relevant driver mutations. Our results indicated the importance of oncogenic mutation in benign endometrial polyps and may lead to a new discovery in the tumorigenesis of endometrial tumors.

Materials and methods

Patient selection and tissue samples for whole exome sequencing. After approval of the Ethics Committee of Keio University

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Key words: endometrial polyp, RAS mutation, tumorigenesis, atypical polypoid adenomyoma, benign tumors, endometrial cancer
School of Medicine (No. 2015-0032) and provision of written informed consent, four patients were recruited for whole exome analysis. These patients were scheduled for transcervical resection of endometrial polyps due to their symptoms (infertility, abnormal uterine bleeding). They were premenopausal and not taking any drugs that might affect the endometrium (e.g. oral contraceptives, tamoxifen). Endometrial polyps were removed by transcervical resection, and peripheral blood lymphocytes were collected as control samples. One polyp was randomly selected for analysis in patients with multiple polyps. These polyps were dissected for analysis and pathological assessment. Samples were stained with hematoxylin and eosin, and those containing about 20-50% of stromal components were selected. Two independent pathologists confirmed the diagnosis of endometrial polyp. Clinical data (age, body mass index, medical history) were obtained from the clinical records. All patients received an outpatient hysteroscopy prior to transcervical resection. The endometrial polyps were counted by two independent physicians who were blinded to information on gene analysis. Specifically, one physician counted the polyps at the time of outpatient hysteroscopy and another physician counted them by reviewing the recorded videos and photos of the hysteroscopy.

Whole exome sequencing. DNA was extracted from endometrial polyps and peripheral blood lymphocytes, and whole exome sequencing was performed using a next-generation sequencer HiSeq 2500 (Illumina, San Diego, CA, USA) according to the manufacturer's protocol and previous reports (14-16). In short, a sequencing library was build using a SureSelect XT Human All Exon kit and sequencing analysis was performed using a HiSeq 2500 system with HiSeq Rapid SBS Kit v2-HS (Illumina) according to the manufacturer's instructions. The sequencing quality was evaluated by a bio-analysis company (Takara Bio Inc., Shiga, Japan). Polyp-specific somatic mutations were derived by subtracting lymphocyte sequencing data from those of the polyps.

Confirmation of observed RAS mutation. We focused on one somatic mutation that was found in endometrial polyps. Validation was performed by targeted mutation analysis via the polymerase chain reaction-reverse sequence-specific oligonucleotide (PCR-rSSO) method. Specifically, 34 new patients were recruited by the same selection criteria that had been used for whole exome sequencing (endometrial polyp, premenopausal, no medication). Patient recruitment was approved by the Ethics Committee of Keio University School of Medicine (No. 2007-0081) and written informed consent was obtained from all participating patients. Tissue samples from endometrial polyps were formalin-fixed and paraffin-embedded (FFPE), and were diagnosed by two independent pathologists. A total of 35 samples were collected from the 34 patients, including one recurrence of endometrial polyps. DNA extraction and mutation analysis for RAS genes were conducted at LSI Medience Corp. (Tokyo, Japan). Genomic DNA was extracted from tissue and serial slices of 7 µm were prepared. After deparaffinization with xylene, the tissue sections were stained with hematoxylin and eosin, and the target lesions were dissected for analysis. PCR-rSSO with a Mebgen™ Rasket Kit (Medical and Biological Laboratories Co., Nagoya, Japan) was performed for RAS mutation analysis. This kit can detect 48 mutation hotspots of RAS (KRAS and NRAS) and is used clinically to detect RAS mutations in colorectal cancer as biomarkers of unfavorable response to the anti-EGFR antibody (17).

Laser-capture microdissection and RAS-targeted sequencing. We analyzed the RAS mutations in stromal and glandular components of endometrial polyps using laser-capture microdissection. An NRAS-mutated case was selected based on the presence of an adequate DNA quantity from each component. The RAS mutation was analyzed in separate glandular and stromal components. Serial slices of 7 µm were prepared from a block. After deparaffinization with xylene, the tissue sections were stained with hematoxylin and eosin, and the glandular and stromal lesions were dissected by a PALM-IV system (Carl Zeiss Microscopy, Oberkochen, Germany). All glandular lesions were microdissected and collected, and the remaining regions on the slide were regarded as stromal lesions. Each sample was placed in a 200-µl microtube and DNA was extracted using a NucleoSpin DNA FFPE XS kit (Takara Bio, Inc.). The NRAS exon 2 was amplified from the extracted DNA by Applied Biosystems AmpliTaq Gold DNA polymerase (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with the NRAS exon2 primer (Table S1). PCR products were purified using a NucleoSpin Gel and PCR Clean-Up Kit (Takara Bio) and then ligated to a pGEM-T easy vector (Promega, Fitchburg, WI, USA), followed by transformation of Sure2 competent cells (Agilent, Santa Clara, CA, USA). Competent cells were cultured on LB plates with ampicillin, and 30 colonies were chosen for each sample by color selection. The inserted DNA was amplified from each colony using SP6 and T7 primers (Table S1) and PCR products were analyzed by Sanger sequencing using the same NRAS exon2 primer pair.

Additional RAS mutation analysis for atypical polypoid adenomyoma. The RAS mutation was also investigated in atypical polypoid adenomyoma (APAM) by the PCR-rSSO method used in the analysis of endometrial polyps. Ten patients with APAM without premalignant or malignant components were recruited and provided written informed consent. In total, 12 samples from 10 patients were analyzed. APAM samples were obtained from two patients by diagnostic biopsy or resection and therapeutic hysterectomy. The pathological diagnosis was confirmed by two independent pathologists.

Statistical analysis. The clinical findings were compared by t-test and Pearson's Chi-squared test using Graph Pad Prism 7 (GraphPad Software, La Jolla, CA, USA), with the significance threshold set at P<0.05.

Results
Whole exome analysis of endometrial polyps. Whole exome sequencing was performed on samples from 4 patients using DNA isolated from endometrial polyps and peripheral blood lymphocytes. We identified 22 nonsynonymous somatic mutations, including 21 missense and 1 nonsense mutations in endometrial polyps. No indel, frameshift or synonymous mutations were detected. The number of mutations varied from 2 to 10 per patient. Two of 4 endometrial polyps harbored KRAS
mutations: c.37G>T (p.G12V) in patient 4. Additional mutations, including PPP2R1A (protein phosphatase 2 regulatory subunit A alpha) and ARHGAP35 (Rho GTPase activating protein 35), were found (Tables I and SII).

RAS mutation analysis by polymerase chain reaction-reverse sequence-specific oligonucleotide method. RAS mutations were analyzed in 35 endometrial polyp samples from 34 patients (including one recurrent case) and 12 APAM samples from 10 patients by PCR-rSSO for validation. This analysis revealed that 16 of 35 cases (45.7%) of endometrial polyps harbored RAS mutations (15 KRAS, one NRAS); 10 cases had a single RAS mutation, and 6 had multiple RAS mutations, varying from 2 to 3 per endometrial polyp. The RAS mutations were all found in exon 2 of KRAS and NRAS (KRAS G12V, G12D, G12C, G12A; NRAS G12D). Two metachronous endometrial polyp samples (cases 5 and 22) from a patient with recurrence of endometrial polyps harbored the same mutation (KRAS G12D). There were no mutations in the 12 atypical polypoid adenomyoma samples (Fig. 1). The comparison between 16 cases with RAS mutations and 19 cases without RAS mutations showed that the number of endometrial polyps was significantly higher in RAS-mutated cases, as assessed by outpatient hysteroscopy (3.25±2.70 vs. 1.74±0.87, P=0.045). There were no differences in age, body mass index, and parity between these two groups (Table II).

RAS mutations are present in both stromal and glandular components of endometrial polyps. We next used laser-capture microdissection to investigate whether the RAS mutations were harbored by glandular or stromal components of endometrial polyps. Case 14, carrying the NRAS mutation, was selected for this analysis because it contained an adequate quantity of DNA from each cellular component. The single endometrial polyp was 25 mm in size and had developed from the fundus of the endometrial cavity. Sanger sequencing of the glandular and stromal components showed that both harbored the same mutation (NRAS G12D). There were no mutations in the 12 atypical polypoid adenomyoma samples (Fig. 1).

Table I. Results of the whole exome sequencing for 4 cases of endometrial polyps.

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (years)</th>
<th>BMI (kg/m²)</th>
<th>Parity</th>
<th>Genes derived from NGS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>36</td>
<td>21.9</td>
<td>G0P0</td>
<td>PPP1R12B (c.2444A&gt;T, p.K815M) RAD21 (c.428T&gt;C, p.I143T)</td>
</tr>
</tbody>
</table>

NGS, next generation sequencing; BMI, body mass index.
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Discussion

In the present study, 45.7% of the examined endometrial polyps harbored RAS mutations. It is well known that endometrial cancers may contain KRAS mutations, with a reported rate of 10-30% (18-20). Furthermore, KRAS mutations were detected in 30-50% of POLE mutated (ultramutated) or microsatellite instability (hypermutated) endometrial cancers (21,22). We found a surprisingly high frequency of RAS mutations in endometrial polyps, a type of lesion generally regarded as benign. Endometrial polyps are monoclonal overgrowths of endometrial stromal cells with secondary induction of polyclonal benign glands (2). These non-atypical polyps usually do not develop into carcinoma, while colon polyps can sequentially

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>RAS mutation</th>
<th>Others</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>40.9 ±5.6</td>
<td>38.5 ±4.7</td>
<td>0.165&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>21.9 ±4.6</td>
<td>20.5 ±2.8</td>
<td>0.258&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Multigravida</td>
<td>4 (25.0%)</td>
<td>7 (36.8%)</td>
<td>0.493&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Numbers of polyps</td>
<td>3.25 ±2.70</td>
<td>1.74 ±0.87</td>
<td>0.045&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>t-test, <sup>b</sup>Fisher exact test. P-value in bold indicates statistical significance. All the data shown in this table with ± represented the mean ± standard deviation.
advance to colon carcinoma. *KRAS* mutations in colon polyps are correlated with the development of advanced polyps and adenomas. Moreover, *KRAS* mutations in colon polyps correlate with a larger lesion size and a higher number of polyps, and may be a useful marker for predicting the development of metachronous advanced neoplasia (23-25). In contrast, the pathogenesis of endometrial polyps is not well known, except for the fact that tamoxifen and estrogen exposure are risk factors. *KRAS* mutations in endometrial polyps have been found in elderly, tamoxifen-treated patients (5,6). We investigated endometrial polyps from premenopausal, drug-free patients to exclude age and artificial hormonal factors. In this study, several cases carried multiple *RAS* mutations, indicating that these events may favor the development of endometrial polyps.

The association between oncogenic mutations and benign tumors has been extensively investigated, and several oncogenes have been reported in benign conditions (26). Recent reports revealed that 26% of cases of deep infiltrating endometriosis harbor somatic cancer driver mutations (*KRAS, PIK3CA, ARID1A, PPP2R1A*) in glandular, but not stromal, compartments of deep endometriotic lesions (27,28). Furthermore, *KRAS, ARHGAP35*, and *PPP2R1A* mutations, which were detected by whole exome sequencing in this study, were recently found in uterine endometrial epithelium (29). Our result added a new information that *RAS* mutation in endometrial polyps has an important role in their multiple development.

Atypical polypoid adenomyoma (APAM) is defined as a mixture of polypoid lesion consisting of glands with cytological atypia and fibromuscular stroma (2). APAM is generally regarded as a benign lesion, however it frequently recurs and often coexists with atypical endometrial hyperplasia and endometrial adenocarcinoma (30). Therefore, we expected that APAM harbors *RAS* mutation more frequently than endometrial polyps. Previous small-scale studies have shown that APAM is associated with *MLH1* hypermethylation, and microdissected glandular components were shown to contain *CTNNB1* mutations (31,32). In addition, *KRAS* mutation was found in 4/16 cases (25%) without *BRAF* mutation (33), in contrast with the absence of *RAS* mutations in our APAM samples (0/12). Larger studies employing laser-microdissection followed by *RAS* mutation analysis in individual APAM components, will be necessary to draw final conclusions on this issue.

In conclusion, we found that *RAS* genes were frequently mutated in endometrial polyps, as assessed by whole exome sequencing and targeted mutation analysis. This is the first report showing a high frequency of pathogenic *RAS* mutations in non-treated endometrial polyps. *RAS* mutations may have an important role in tumorigenesis and in the formation of multiple endometrial polyps.

**Acknowledgements**

Not applicable.

**Funding**

This work was supported by the Japan Society for the Promotion of Science (JSPS) through a Grant-in-Aid for Scientific Research (C) (16K11154), and by the Keio Gijuku Academic Development Fund. The funders had no role in the study design, in the collection, analysis and interpretation of data, in the writing of the report, and in the decision to submit the manuscript for publication.

**Availability of data and materials**

The data analyzed during the current study are available from the corresponding author on reasonable request.

**Authors’ contributions**

The concept was designed by TT, KB and KK. draft preparation, writing, and editing of the manuscript were undertaken by TT and KB. Molecular analyses were performed by TT, KB, MA, and MY. Clinical procedures and sample collection were carried out by TT, KB, YK, MA, ET and DA. TT, KB and DA prepared the revised manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Ethics approval and consent to participate**

The Ethics Committees of Keio University School of Medicine approved the study. All the participants signed informed consent prior to participation.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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