Whole-exome sequencing of lobular endocervical glandular hyperplasia

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Abstract. Lobular endocervical glandular hyperplasia (LEGH) was first reported as a benign proliferative disorder of the uterine cervix. However, it currently remains unclear whether it has the biological characteristics of pyloric metaplasia or precursor of minimal deviation adenocarcinoma (MDA)/gastric-type mucinous cervical adenocarcinoma (GAS). Therefore, in the present study we performed whole-exome sequencing on three cases of LEGH collected by laser-microdissection from the frozen tissue sections of surgically removed uteri. Analysis of the results identified 50 somatic variants. After several filtering processes, we identified 13 functional variants, including 12 missense and one insertion-deletion variants. Of these mutations, keratinocyte proline-rich protein, olfactory receptor M4 and zinc finger protein 645 mutations were found in the Catalogue Of Somatic Mutations In Cancer but were not related to carcinogenic diseases. We did not detect any significant copy number alterations or signatures. Although this was a limited case series, we did not identify any variants relevant to the tumorigenesis of LEGH to MDA/GAS, suggesting a metaplastic aspect of LEGH.

Introduction

Lobular endocervical glandular hyperplasia (LEGH) was first described by Nucci *et al* (1) in 1999, as a benign lesion characterized by a prominent lobular architecture lined by tall, mucin-rich columnar cells located at the inner endocervix and surrounded by larger cysts. These glands mimic gastric pyloric glands and produce Periodic Acid-Schiff (PAS)-positive and HIK1083-positive intracytoplasmic neutral mucin (2). The incidence of LEGH was reported to be 0.7% in consecutive hysterectomy specimens in a single institution (8/1169) with a mean age range of 45-49 years (1,3). Clinically, patients present with symptoms such as an increased watery discharge and cervical mass (1). Gastric-type mucinous carcinoma (GAS) is defined as adenocarcinoma with a voluminous clear and pale eosinophilic cytoplasm and distinct cell borders (4). Minimal deviation adenocarcinoma (MDA), also known as adenoma malignum, is very rare and defined as the extremely well-differentiated form of GAS that often abundantly produces pyloric mucin (5). Since MDA/GAS exhibits an aggressive clinical behavior (6), its 5 year disease-specific survival rate is significantly worse than that of non-gastric-type adenocarcinoma (30% vs. 77%; P<0.0001) (5). Although LEGH was initially regarded as a benign lesion, it was subsequently reported as a potential precursor of MDA/GAS based on specific molecular findings as well as clinicopathological features (7,8). We previously demonstrated using a clonality analysis with the inactivation pattern of the human androgen receptor gene (HUMARA assay) that a subset of LEGH exhibited monoclonal growth and may be a precursor of MDA (9). Moreover, various findings on gene abnormalities common to MDA/GAS and co-existing LEGH, such as the gain/loss of chromosomes and GNAS, STK11, and KRAS mutations, have been reported (9-12). These findings support the nature of LEGH as a precursor of MDA/GAS. However, the molecular mechanisms involved in the carcinogenesis of LEGH as well as its comprehensive genetic profiles have not yet been elucidated. On the other hand, we often encounter clinical LEGH cases showing stable imaging and cytology findings for many years, which support a benign nature of LEGH. Therefore, to elucidate the molecular characteristics of LEGH is of importance.

In the present study, we performed whole-exome sequencing (WES) using LEGH and normal tissues precisely collected with laser microdissection (LM) to analyze the somatic variant profiles of LEGH.

Patients and methods

Patients and tissue samples. The three LEGH cases described herein were clinically diagnosed according to our management

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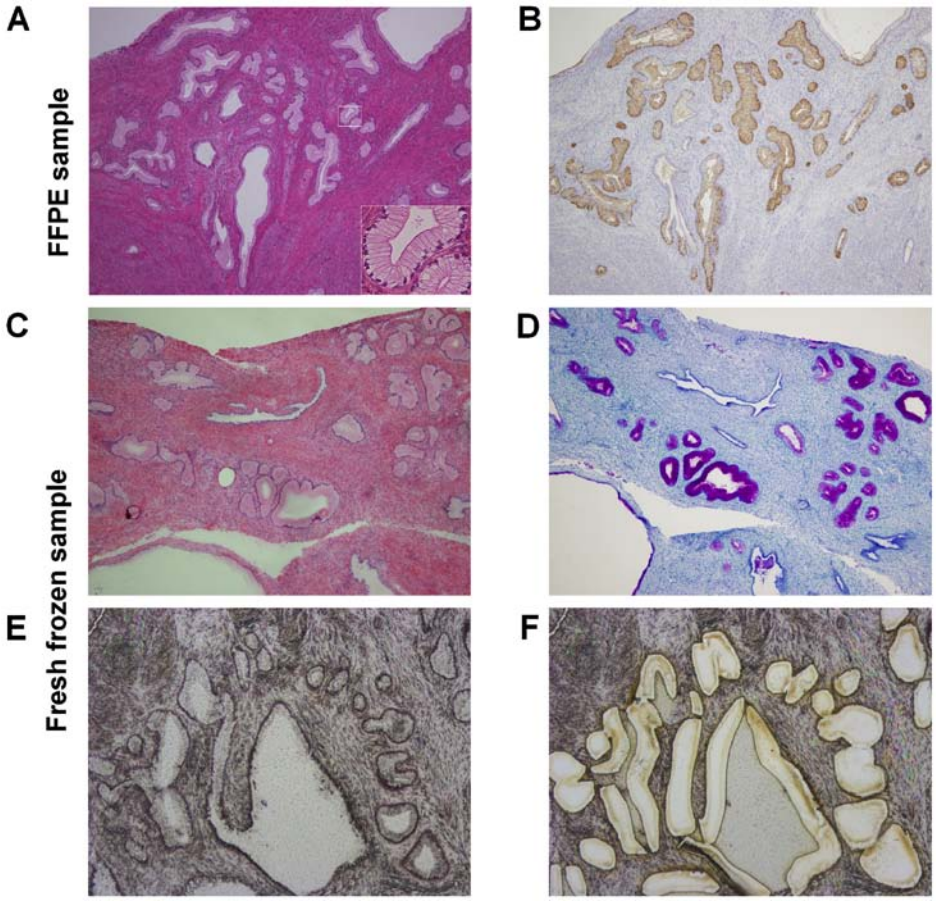

Figure 1. Staining of LEGH using the FFPE sample of case 3 and photomicrographs of LEGH using a fresh frozen sample of case 3. (A) H&E staining shows the explicit lobular proliferation of relatively small round glands surrounding larger cystic dilated glands. Insets show enlarged views of H&E staining. Magnification, x40; inset magnification, x200. (B) LEGH lesions of all cases show diffuse positivity for HIK1083. Magnification, x40. (C) Standard H&E staining and (D) Alcian Blue/Periodic Acid-Schiff staining at the same site are shown. Magnification, x40. Hematoxylin-stained sample (E) before and (F) after laser microdissection. H&E, hematoxylin and eosin; LEGH, lobular endocervical glandular hyperplasia; FFPE, formalin-fixed paraffin-embedded.

protocol (11,13). The present study was approved by the Ethics Committee of Shinshu University (approval no. 547) and patients provided written consent. The 3 LEGH patients underwent simple hysterectomy. Of the 3 cases, one had adenomyosis and 2 exhibited increases in the size of LEGH lesions. Several tissue specimens were collected from the removed uteri. They were placed in O.C.T Compound (Sakura Finetek, Tokyo, Japan), immediately frozen in liquid nitrogen, and stored at -80˚C until LM.

The remaining uterine tissues were fixed in 10% buffered formalin and embedded in paraffin wax for a pathological diagnosis. All three cases were histologically diagnosed with LEGH (Fig. 1A). In order to exclude intrinsic germline variants in WES, matched blood samples from all cases were collected and used as an internal reference. There were no patients with Peutz-Jeghers syndrome, which has been associated with LEGH (14). Table I shows the characteristics of the 3 patients.

Immunohistochemistry. Immunohistochemical staining for pyloric mucin was performed (Fig. 1B) using an anti-αGlcNAc antibody (clone HIK1083, 1:15 dilution, Kantokagaku, Tokyo, Japan) as the primary antibody. The staining procedure was performed according to a previous study (15). Negative controls were established by omitting primary antibodies from the procedure (15).

LM. Fifty to sixty serial sections (thickness of 10 μm for LM and 4 μm for H&E) were cut from fresh frozen samples using a cryostat (Leica CM1950; Leica BIOSYSTEMS, Nussloch, Germany).

One in every 10 sections was stained by H&E and Alcian Blue/Periodic Acid-Schiff (AB/PAS) to confirm LEGH, while the others were stained with hematoxylin only (Fig. 1C and D). In order to enrich LEGH cells more than 80%, LEGH lesions were precisely collected using the LM system (LMD6500; Leica MICROSYSTEMS, Germany) (Fig. 1E and F).

DNA extraction, WES. The genomic DNAs of LEGH and blood samples were purified using QIAamp DNA Micro kits and QIAamp DNA Blood Mini kits (Qiagen, Hilden, Germany) following the manufacturer's protocols. Double-stranded DNA concentrations were quantified using the Qubit dsDNA HS Assay and the quality of DNA was assessed by the 2200 TapeStation system (Agilent Technologies, Santa Clara, CA, USA). Three micrograms of genomic DNA, except for the sample from case 1 (0.3 μg), was used for WES. WES was performed using SureSelectXT Human All Exon V6 kits, except for case 1 for which we selected the low input protocol of the same kits according to the manufacturer's instructions (Agilent Technologies). Data were generated by 100-base paired-end reads on an HiSeq 2500 platform (Illumina Inc., San Diego, CA, USA). FASTQ sequences were aligned to the

Table I. Characteristics of three patients with LEGH.

Case	Age	WD	GM	Cytology	MRI findings	Surgical indication	Surgery	Period before surgery (months)
1	39	+	+	AGC-NOS	Cosmos	Lesion size-up	TAH	68
2	41	+	+	AGC-NOS	Cosmos	Lesion size-up	TLH	62
3	25	+	+	AGC-NOS	Cosmos	Adenomyosis	TLH	81

LEGH, lobular endocervical glandular hyperplasia; WD, watery discharge; GM, gastric-type mucin; Cosmos, Cosmos pattern; AGC-NOS, atypical glandular cells-not otherwise significant; TAH, total abdominal hysterectomy; TLH, total laparoscopic hysterectomy.

Table II. Number of somatic mutations and mutation rate in each case.

	Non-synonymous variant			
Case	Subtotal	Missense	Insertion-deletion (Indel)	Mutation rate Synonymous + non-synonymous (per Mb)
1	8	7	1	0.79
2	1	1	0	0.11
3	4	4	0	0.21
Total	13	12	1	

human reference genome (GCRh37/hg19) with BWA-MEM software version 0.7.11 (16) and Picard (http://broadinstitute.github.io/picard) was applied for post-alignment procedures as sorting, indexing, and marking duplicates.

Variant calling. Since our major objective was to detect somatic gene alterations in LEGH, germline alterations were not analyzed in the present study. We used SAMtools 1.2 (17) and VarScan 2.3.9 to call somatic mutations, copy number alterations (CNAs), and loss of heterozygosity (LOH) in tumor-normal pairs (18). Single nucleotide variants (SNVs) and indels (insertion-deletions) for somatic and germline sites were called using VarScan v2.3.9 with almost all default settings (19), except for a parameter of Fisher's exact test; somatic P-value 0.001. The somatic P-value threshold to call a somatic site was set as low as possible in order to avoid false positives due to the low coverage depth as recently reported (20). The effects of all non-synonymous somatic mutations on gene function were predicted using the annotation tool SnpEff v4.2 (21) to extract and select protein structure-altering variants showing the functional impact of Moderate or High. Furthermore, we browsed the Catalogue Of Somatic Mutations In Cancer (COSMIC) v78 (https://cancer.sanger.ac.uk/cosmic), the Cancer Gene Census (https://cancer.sanger.ac.uk/census), and ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) to clarify whether the mutations detected had previously been reported as being associated with diseases. We also detected CNAs with Samtools and VarScan in the exome data of LEGH and matched normal samples by comparing their log2 ratios of read depths within contiguous coverage regions separated by 100 base-pairs and by visually inspecting them for deviations from normal (thresholds in default settings: 0.20 for focal amplifications, -0.10 for deletions) (18).

Results

Patient characteristics. The mean age of the three patients was 35 years old and the mean follow-up period was 70 months (Table I). All three cases showed positive immunostaining for HIK1083 (Fig. 1B).

WES. WES was performed on DNA isolated from LEGH tissues and matched blood lymphocytes. The mean coverages of the target regions of LEGH and blood samples were 32.8x and 33.6x, respectively. Fifty somatic variants were identified. After the variants detected in blood samples were filtered out, we identified 13 functional variants, including 12 missense and 1 insertion-deletion variants (Table II). The mean mutation rate (± SD) of synonymous and non-synonymous mutations of the 3 patients was 0.37 (±0.37)/Mb (case 1; 0.79/Mb, case 2; 0.11/Mb, case 3; 0.21/Mb) (Table II).

Signature. The spectra of SNVs in the three cases were characterized by a predominance of C>T/G>A transitions, particularly at CpCpG nucleotides, and T>C/A>G transitions followed by C>G/C>G and C>A/G>T transversions (data not shown). These spectra did not match the seven validated mutational signatures of cervical cancer displayed in COSMIC (https://cancer.sanger.ac.uk/cosmic/signatures) (22).

Commonly mutated genes. We identified protein-altering somatic variants in 13 genes, including 12 non-synonymous mutations, and one frame-shifting deletion. However, we did not detect frequent mutations or mutations relevant to carcinogenesis in any of the 13 somatic variants (Table III). The mutations in KPRP, OR2M4, and ZNF645 were found in COSMIC but were not related to carcinogenic diseases. We also did not identify any functional mutations in ClinVar except

Table III. Somatic mutations identified by a whole-exome analysis of three patients with LEGH.

A, Patient 1

Gene	Mutation position (GRCh37/hg 19)		Variant allele frequency[a]	Mutation type	SnpEff		COSMIC ID	ClinVar allele ID
	Nucleotide (genomic)	Amino acid (protein)			Annotation	Impact		
KPRP	chr1:152733724C>T	p.R554W	0.39 (12:31)	Substitution	Missense	Moderate	COSM1334231	N/A
OR2M4	chr1:248402643C>T	p.P138L	0.30 (13:44)	Substitution	Missense	Moderate	COSM322280	N/A
TUBA3D	chr2:132237643C>T	p.A126V	0.24 (14:59)	Substitution	Missense & splice variant	Moderate	N/A	N/A
SST	chr3:187386990C>A	p.D72Y	0.35 (8:23)	Substitution	Missense	Moderate	N/A	N/A
CASC5	chr15:40915190A>G	p.R936G	0.61 (19:31)	Substitution	Missense	Moderate	N/A	N/A
UNK	chr17:7380988G>T	p.W266L	0.21 (6:29)	Substitution	Missense	Moderate	N/A	N/A
ZNF645	chrX:22292037G>A	p.R310H	0.43 (24:56)	Substitution	Missense	Moderate	COSM4589382	N/A
SHROOM2	chrX:9900287delC	p.P989fs	0.24 (21:88)	Deletion	Frameshift	High/LOF	N/A	N/A

B, Patient 2

Gene	Mutation position (GRCh37/hg 19)		Variant allele frequency[a]	Mutation type	SnpEff		COSMIC ID	ClinVar allele ID
	Nucleotide (genomic)	Amino acid (protein)			Annotation	Impact		
COL4A3	chr2:228111435T>C	p.L141P	0.66 (19:29)	Substitution	Missense	Moderate	N/A	N/A

C, Patient 3

Gene	Mutation position (GRCh37/hg 19)		Variant allele frequency[a]	Mutation type	SnpEff		COSMIC ID	ClinVar allele ID
	Nucleotide (genomic)	Amino acid (protein)			Annotation	Impact		
CAMTA1	chr1:7723593A>G	p.K329R	0.49 (34:69)	Substitution	Missense	Moderate	N/A	N/A
NBPF1	chr1:16901668T>C	p.K726E	0.30 (51:168)	Substitution	Missense	Moderate	N/A	N/A
VWA5B1	chr1:20662938A>G	p.K634R	0.65 (22:34)	Substitution	Missense	Moderate	N/A	N/A
ESRRA	chr11:64083320T>C	p.L385P	0.42 (13:31)	Substitution	Missense	Moderate	N/A	N/A

[a]Numbers in parentheses show variant reads:total reads. LEGH, lobular endocervical glandular hyperplasia; N/A, not applicable; COSMIC, Catalogue Of Somatic Mutations In Cancer.

p.L141P in COL4A3 indicating 'Benign.' Therefore, none of the mutations identified appeared to contribute functionally to the progression of LEGH.

CNAs in LEGH. We did not detect any copy number amplification or deletion in case 1 or 2. In case 3, one focal amplification, with an estimated copy number value of 2.9, was found between the range p35.3 to p36.31 in chromosome 1. However, cancer-related genes from the Cancer Gene Census in this range were all tumor suppressor genes, such as ARID1A and ID3, which suggested that functional copy number loss did not occur in this case. As a result, we did not find any significant CNAs related to the progression of LEGH.

Discussion

LEGH was regarded as a putative MDA/GAS precursor in the 2014 WHO classification, and an analysis of its genetic nature was expected to clarify the neoplastic nature of LEGH. Although the present study detected 13 variants in 3 LEGH cases, they did not appear to be oncogenic. There has been one report of aberrant COL4A3 expression being related to the pathogenesis of gastric carcinoma (23); however, the mutation of COL4A3 in the present results was 'Benign' in ClinVar. Therefore, the neoplastic or pre-cancerous nature of LEGH has yet to be elucidated in detail.

All three cases showed a low mutation rate, non-specific mutational signatures, and lower malignant potential missense or frame shift mutations. Of these mutations, KRPR, OR2M4, and ZNF645 were already reported in COSMIC. KPRP is an epidermal marker of differentiating keratinocytes, and its expression is elevated in psoriatic lesions (24). Regarding OR2M4, this olfactory receptor protein is one of the members of a large family of G-protein-coupled receptors, the signaling pathways of which are important for autism spectrum disorder (25). Furthermore, ZNF645, a human RING finger protein, possesses the domain of E3 ubiquitin ligase activity (26). These functions of all three COSMIC genes do not appear to be related to carcinogenesis.

The strengths of the present study are the LCM selection of samples to sequence, and comprehensive evaluation by WES analysis, which is the first report in this pathology. Also, limitations include the sample size, and the inherent shortcomings of current databases regarding this pathology, calling for future studies in the subject.

As previously reported, the GNAS, KRAS, and STK11 mutations were all exclusively detected in 11 (58%) LEGH cases (12). We also detected activating GNAS mutations in two 'LEGH with atypia' cases (14%) out of a total of 14 cases (9 LEGH and 5 'LEGH with atypia'), and a STK11 mutation was identified in only one MDA case (20%) (9,11). Collectively, the present results imply that somatic driver variants are not generated in the early stage of 'pure' or 'non-atypical' LEGH, but may occur in the late stage, i.e., LEGH already associated with atypia or MDA. Differences between the present results and previous findings may be attributed to their LEGH specimens having already acquired atypia.

In conclusion, although the present results indicate the metaplastic nature of LEGH, further research, including WES on atypical LEGH/LEGH with MDA, is needed to clarify the neoplastic characteristics of LEGH.

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Availability of data and materials

The datasets generated and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

KI designed the study, contributed to all experiments, data analysis and manuscript drafting. TM contributed to sample collection, data analysis, manuscript drafting and revising. AT and HA participated in the study design and data analysis of the whole-exome sequencing. RA, HT, SY and MO contributed to the recruitment of patients, collection of the LEGH tissues by laser microdissection, and DNA extraction used in the whole-exome sequencing. SA contributed to the pathological diagnosis and study design, and collected the LEGH tissues by laser microdissection. TS was the supervisor of this study, contributed to study design, data analysis, and drafting and revising the manuscript. All authors were given the opportunity to revise the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Shinshu University (approval no. 547) and patients provided written consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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