

Mutational analysis of extranodal marginal zone lymphoma using next generation sequencing

SEOK JAE HUH¹, SUNG YONG OH¹, SUEE LEE¹, JI HYUN LEE¹,
SUNG HYUN KIM¹, MIN KYUNG PAK² and HYO-JIN KIM¹

Departments of ¹Internal Medicine and ²Pathology, Dong-A University College of Medicine,
Seo-gu, Busan 49201, Republic of Korea

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Abstract. Extranodal marginal zone lymphoma is a type of low-grade B-cell lymphoma that can be classified as a mucosal-associated lymphoid tissue (MALT) lymphoma. Recently, second-generation or next-generation sequencing (NGS), which allows simultaneous sequencing of hundreds to billions of DNA strands, has been a focus of attention and is rapidly being adopted in various fields. In the present study, paraffin-embedded tissue samples of gastric MALT lymphoma (n=1) and small intestine MALT lymphoma (n=4) were selected, and DNA was extracted from the tissue samples. After performing quality control, NGS was performed using HemaSCAN™, a custom panel of 426 genes, including essential blood cancer genes. NGS revealed single nucleotide variations (SNVs), short insertions and deletions (InDels) and copy number variations (CNVs). These genomic variants were reported as annotated, known or novel variants. An annotated variant, an erb-b2 receptor tyrosine kinase 2 gene amplification, was observed in one patient. Known and

novel variants, including SNVs of SET binding protein 6 (SETBP6), Runt-related transcription factor 1 and Kelch-like ECH-associated protein 1 genes, InDel of the marker of proliferation Ki-67 gene, and CNVs of the zinc finger protein 703 and NOTCH1 genes, were observed in ≥2 patients. Additionally, InDels with frameshift mutations were identified in the B-cell lymphoma/leukemia 10, DEAD-box helicase 3 X-linked, forkhead box O3 and mucin 2, oligomeric mucus/gel-forming genes in one patient. Since few NGS studies have been performed on MALT lymphoma, the current results were unable to determine if the different mutations that were identified are ‘actionable’ (that is, potentially responsive to a targeted therapy) Further studies are required to determine the associations between genetic mutations and the development of MALT lymphoma.

Introduction

Marginal zone lymphomas (MZLs) are a group of indolent B-cell lymphomas that arise from marginal zone B lymphocytes (1,2). MZLs comprises ~10% of all non-Hodgkin's lymphomas (NHLs) (1,3). They include splenic marginal zone lymphoma (SMZL) with or without villous lymphocytes, nodal marginal zone lymphoma (NMZL) and extranodal marginal-zone B-cell lymphoma of mucosal-associated lymphoid tissue (MALT) (4). MALT lymphoma is the most common subtype, comprising 7-8% of NHLs, with NMZL comprising <2% and SMZL <1% (3). MALT lymphoma arises from numerous epithelial tissues, including stomach (60-70%), lung (15%), ocular adnexa (10%) and other less frequent sites (thyroid, salivary glands, intestine, skin and liver) (5-7). These organs are usually almost devoid of lymphoid tissue; however, they accumulate B lymphocytes in response to persistent inflammation due to chronic infections, such as by *Helicobacter pylori* in the stomach (8). Anatomical sites may have prognostic relevance due to organ-specific clinical problems and different sites may have distinct natural history (9). In a previous study, long-term outcomes were investigated in patients with localized MALT lymphoma and the results identified a significantly improved outcome in gastric and thyroid lymphoma (10). In general, despite frequent relapses, MALT lymphoma most often maintains an indolent course (11).

Correspondence to: Dr Hyo-Jin Kim or Dr Sung Yong Oh, Department of Internal Medicine, Dong-A University College of Medicine, 26 Daesingonwon-ro, Seo-gu, Busan 49201, Republic of Korea
E-mail: kimhj@dau.ac.kr
E-mail: drosy@dau.ac.kr

Abbreviations: CNV, copy number variation; CLL, chronic lymphocytic leukemia; dbSNP, The Single Nucleotide Polymorphism database; DLBCL, diffuse large B cell lymphoma; EMZL, extranodal marginal zone lymphoma; FL, follicular lymphoma; InDels, short insertions and deletions; KEAP1, Kelch-like ECH-associated protein 1; MCL, mantle cell lymphoma; MALT, mucosal-associated lymphoid tissue; MZL, marginal zone lymphoma; NGS, next-generation sequencing; NMZL, nodal marginal zone lymphoma; PTCL, peripheral T-cell lymphoma; SETBP, SET binding protein; SMZL, splenic marginal zone lymphoma; SNP, single-nucleotide polymorphisms; SNV, single nucleotide variation; TNFAIP3, TNF- α inducible protein 3; ZNF703, zinc finger protein 703

Key words: mucosal-associated lymphoid lymphoma, next-generation sequencing, gene, mutation

Chronic inflammatory and immunological responses promote the acquisition of genetic abnormalities, malignant transformation and clonal expansion of transformed neoplastic cells (8,12). Acquired genetic abnormalities serve critical roles in the development of MALT lymphoma by modulating similar molecular processes (12). To the best of our knowledge, genetic abnormalities in MALT lymphoma have not yet been investigated using whole exome or genome sequencing. Furthermore, various repeat genetic abnormalities, including chromosomal translocations, somatic cell mutations and changes in the number of copies, have been reported to alter signaling pathways that modulate NF- κ B activity through standard pathways (13).

The etiology of MALT lymphoma involves various chromosomal translocations (14). The t(11;18)(q21;q21) chromosome translocation has been demonstrated to be the most common translocation that results in a functional chimeric fusion product between the N-terminal of baculoviral IAP repeat containing 3 (API2) and the C-terminal of mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) (15-17). The API2-MALT1 fusion product exhibits functional gain and activates both the canonical and non-canonical NF- κ B signaling pathways (13). The fusion product is often detected in stomach and lung MALT lymphoma (18). The t(1;14) translocation upregulates B-cell lymphoma/leukemia 10 (BCL10) proteins in 1-2% of MALT lymphoma cases, including in MALT lymphoma in the stomach, lungs and skin (12). The t(14;18) translocation results in the deregulation of MALT1 expression and it has been reported in 15-20% of MALT lymphoma cases (19). This translocation is most frequently detected in the MALT lymphoma of the liver, skin, ocular adnexa and salivary gland (19). The t(3;14) translocation results in forkhead box protein B1 upregulation (20). It is associated with MALT lymphoma of the thyroid, ocular adnexa and skin (20). Numerical chromosomal aberrations, including trisomy 3, 12 and/or 18 are present as sole abnormalities in 22% of cases (21).

In MALT lymphoma, TNF- α inducible protein 3 (TNFAIP3) deletions and mutations are found mainly in tumors of the ocular adnexa, salivary gland and thyroid (22-24). TNFAIP3 inactivation by deletion and/or mutation abolishes negative regulations of several signaling pathways, thus activating the canonical NF- κ B signaling pathway (25). The oncogenic activities of this inactivation depend on the presence of antigenic and inflammatory stimulations (12). The MYD88 innate immune signal transduction adaptor (MYD88) L265P somatic mutation can be observed in ocular adnexal MALT lymphoma (~5% of cases), although it appears to be infrequent at other anatomic sites (26). However, it has not yet been fully investigated (27-29). Somatic mutation in the MYD88 gene occurs at a single nucleotide (3p22.2) (26). This mutation leads to an amino acid change from leucine to proline (L265P), which activates MYD88-dependent signaling in Toll-like receptor signaling pathways, leading to NF- κ B activation (27,30).

Extranodal MZL (EMZL) usually has an indolent disease course (2,31). Patients with EMZL have a median survival time of >12 years (32). Gastric MALT lymphoma tends to be localized to primary tissue sites for a long time (33). The 10-year survival and disease-free survival rates for gastric MALT lymphoma are close to 90 and ~70%, respectively (33,34).

However, in rare instances, aggressive high-grade tumors can arise or be transformed from MALT lymphoma (35). Extranodal diffuse large B cell lymphoma (DLBCL) causes a drop in the 10-year survival rate to ~42% (33). EMZL is a clinically heterogeneous low-grade B cell lymphoma (1,3,5). Molecular pathways responsible for the pathogenesis, prognosis and drug resistance of EMZL have not been fully elucidated. Further research on genetic aberrations is required to identify the genetic abnormalities of MALT lymphoma.

Next-generation sequencing (NGS) provides a means of performing high-throughput, comprehensive analyses of landscapes of genetic aberrations, copy number alterations and transcript expression patterns, thus leading to improved understanding of various genetic abnormalities (29). The present study aimed to identify mutations that may be responsible for gastrointestinal MALT lymphoma, including 4 cases of MALT lymphoma in the small bowel, which is an uncommon anatomical site, by targeted sequencing using a panel (HemaScan™) of hematologic malignancy-related genes (Fig. S1). Genes that may be associated with the pathogenesis and progression of gastrointestinal MALT lymphoma were suggested.

Materials and methods

Patient characteristics. Patients who were diagnosed with gastrointestinal MALT lymphoma through endoscopy or surgery at Dong-A University Hospital were included in the analysis. Patient samples were excluded if they did not match the following criteria: Subjects should have enough FFPE samples for NGS and DNA preparation samples from the FFPE should qualify quality control (QC) criteria. A total of 5 patients (age, 49-72 years; 2 males and 3 females) underwent surgical biopsies. All patients were diagnosed histologically with MALT lymphoma. A total of 4 patients had small intestine lesions, and 1 patient had a stomach lesion. According to the Stage Lugano modification of the Ann Arbor staging system (36), 1 patient was in stage I, 3 patients were in stage III and 1 patient was in stage IV. None of the patients exhibited an increase in lactate dehydrogenase or B symptoms. All patients underwent bone marrow biopsies and no bone marrow involvement was noted. According to the International Prognostic Index (IPI) scoring system (37), 2 patients had an IPI score of 2 and 3 patients had a score of 0. *Helicobacter pylori* eradication therapy was performed for the patient with gastric MALT lymphoma.

All patients underwent surgery and 4 patients underwent chemotherapy with a regimen of cyclophosphamide, vincristine and prednisolone (CVP) for ≥ 4 -6 cycles. Each cycle of CVP chemotherapy regimens was administered intravenously with cyclophosphamide 750 mg/m², and vincristine 1.4 mg/m² (maximum dose of 2 mg) on first day, and orally with prednisone 100 mg on day 1 to 5. It was repeated every 21 days and treated up to six times. Additionally, the patient with gastric MALT lymphoma underwent radiotherapy prior to chemotherapy with the addition of intravenous Rituximab 375 mg/m² on day 1 to the CVP regimen. Intestinal MALT lymphoma recurred in one patient at 4 years and 6 months following the completion of the initial treatment. All patients were alive at the time of writing (68.3 months following initial treatment; Table I).

Table I. Patient characteristics.

Variable	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Sex	M	F	M	F	F
Age, years	59	72	53	55	49
Diagnosis	MALT	MALT	MALT	MALT	MALT
Site of lesion	Stomach	Small intestine	Small intestine	Small intestine	Small intestine
Stage	II	II	I	IV	II
B symptoms	-	-	-	-	-
Elevated LDH	-	-	-	-	-
BM involvement	-	-	-	-	-
ECOG PS	1	1	1	1	1
IPI score	0	1	0	1	0
<i>H. pylori</i> eradication	+	-	-	-	-
Chemotherapy	+	+	-	+	+
Radiotherapy	+	-	-	-	-
Surgery	+	+	+	+	+
Recurrence	-	+	-	-	-
Death	-	-	-	-	-

M, male; F, female; MALT, mucosal-associated lymphoid tissue; LDH, lactate dehydrogenase; BM, bone marrow; ECOG PS, Eastern Cooperative Oncology Group performance status; IPI, International Prognostic Factor; *H. pylori*, *Helicobacter pylori*.

The present study was approved by the Institutional Review Board of Dong-A University Hospital, Seo-gu, Busan, Republic of Korea (approval no. DAUHIRB 20-088).

Study design. All patients were diagnosed with gastrointestinal MALT lymphoma and underwent surgery to obtain tissue samples. Samples from the patients were obtained at Dong-A University Hospital (Busan, Korea) between January 2011 and December 2014. Immunohistochemistry for gastrointestinal MALT lymphoma diagnosis with LCA, UCHL-1, CD20, CD5, CD10, Bcl-2, Bcl-6, MUM-1, Cyclin D1 and CD23 was performed using formalin-fixed, paraffin-embedded (FFPE) tissues. The requirement for informed consent was waived since the tissues had been donated to the Dong-A University Hospital tissue bank.

DNA preparation. FFPE tissue samples were cut into 5- μ m thick sections, incubated at 60°C for 30 min, de-paraffinized in three containers of xylene baths at 60°C (3x5 mins), rehydrated with decreasing alcohols (100, 95 and 70%, two washes for 5 mins each) and washed with distilled water two times for 5 mins each. Subsequently, genomic DNA was extracted from the tissue samples using the Maxwell 16 CSC DNA FFPE kit (cat. no. AS1350, Promega Corporation) or the QIAamp DNA FFPE Tissue kit (cat. no. 56404, Qiagen, Inc.), which provides an automated purification of genomic DNA from FFPE tissue samples, thereby maximizing simplicity and convenience. DNA concentration and purity were verified using Nanodrop 8000 UV-Vis spectrometry (Thermo Fisher Scientific, Inc.) and Qubit 2.0 Fluorometry (Thermo Fisher Scientific, Inc.). Degrees of DNA degradation were measured using a 200 TapeStation Instrument (Agilent Technologies GmbH) and quantitative PCR (Agilent Technologies GmbH) (38).

Library preparation and sequencing. DNA was sheared using Covaris S220 (Covaris, Inc.). Target capture was performed using a SureSelect XT reagent kit, HSQ (Agilent Technologies GmbH) according to the manufacturer's protocol. A paired-end sequencing library was constructed using a barcode. Individual 'barcode' sequences are added to each DNA fragment during NGS library preparation so that each read can be identified and sorted before the final data analysis (39). After checking for library quality, sequencing was performed on a HiSeq 2500 (Illumina, Inc.) using 100 bp reads. SureSelect XT Human All Exon (version 5; Agilent Technologies, Inc.) was used for target capture for exome sequencing and a TruSeq Nano DNA Sample prep kit (Illumina, Inc.) was used for whole genome sequencing.

Panel design and sequencing. Samples were profiled on a HemaSCAN™, a targeted-sequencing platform designed by the LabGenomics in the Samsung Medical Center (81 Irwon-Ro Gangnam-gu, Seoul 06351, Korea). Samsung medical center participated in the panel design for the production of customized panels. The platform performs targeted NGS of cancer-related genes and allows for the detection of a variety of somatic mutations, including clinically actionable mutations. HemaSCAN™ analyzed 426 genes, including essential genes for different blood diseases, as follows: Plasma cell disease [NRAS proto-oncogene, GTPase (NRAS), KRAS and TP53], acute myeloid leukemia [CCAAT enhancer binding protein α , fms related receptor tyrosine kinase 3, Janus kinase 2 (JAK2), KIT proto-oncogene, receptor tyrosine kinase, nucleophosmin 1, disease resistance protein RUN1, TP53, isocitrate dehydrogenase (NADP(+)) 1 and isocitrate dehydrogenase (NADP(+)) 2], acute lymphoblastic leukemia (TP53, RB transcriptional corepressor 1, JAK2, NRAS and IKAROS family zinc finger 1) and malignant lymphoma

(MYD88, BRAF and TP53). Additionally, the platform analyzes single nucleotide variations (SNVs), short insertions and deletions (InDels), copy number variations (CNVs) and gene rearrangements in 426 target genes in clinical FFPE specimens. Furthermore, HemaSCAN™ allows researchers and clinicians to include target genes curated from literature by request (Fig. S1).

Purity estimation. Tumor purity is the proportion of cancer cells in a tumor sample and is a major confounding factor for analyzing cancer molecular and genomic data with NGS (40). The present study estimated tumor purity via the computational method that is derived from the distribution of variant allelic fractions (VAFs) of somatic single-nucleotide variants (SNVs) within copy-number neutral tumor segments. Tumor purity estimation using a panel sequencing is a more delicate process than using the whole genome due to the limited DNA region in the panel sequencing which could be insufficient for calculation of the genomic alterations (41). First, copy-neutral regions were identified by virtual karyotypes using SNP-based arrays. The minor allele frequencies at known single-nucleotide polymorphisms (SNPs) in the copy-neutral regions were near 0.5. Since only these SNPs were considered, their read densities corresponded to the most prominent peak in the distribution of read coverage at the SNPs, based on the hypothesis that pure polyploidy tumors with 4N in all chromosomes are extremely rare (42). The regions of copy number gain and loss were identified by their adjusted coverage relative to the copy number-neutral regions. Once the copy-neutral, gain and loss regions were delineated, the following formula was used to infer the proportion of each tumor clone: Alternative allele frequency = $[PxY + (1-P)] / [PxX + 2(1-P)]$ where, X and Y are the numbers of all and alternative alleles at each group of clustered SNPs in the tumor, respectively, and P is the proportion of tumor clones ranging between 0 and 1. Tumor purity was inferred from the maximum value among the Ps estimated at multiple positions, according to the hypothesis that the largest clone best represents tumor purity (43). Tumor purity <30% was less reliable and was not annotated.

Variant detection. Paired-end reads were aligned to the human reference genome (hg19 downloaded from <http://genome.ucsc.edu>) using BWA-MEM (ver.0.7.5) (<http://bio-bwa.sourceforge.net/>). SAMTOOLS (ver.0.1.18) (<http://samtools.sourceforge.net/>), GATK (ver.3.1-1) (<https://gatk.broadinstitute.org/hc/en-us>) and Picard (ver.1.93) (<http://picard.sourceforge.net/>) were used for file handling, local realignment and removal of duplicate reads, respectively. Base quality scores were recalibrated with GATK BaseRecalibrator, using known SNPs and InDels from SNP database 138 (dbSNP138).

To increase the sensitivity of SNV detection, two published methods, MuTect (version 1.1.4) (44) and LoFreq (ver.0.6.1) (45), were employed using default parameters. Unions of variants identified by the two callers (with high confidence set for MuTect) were used as the candidate set of variants. Small InDels were identified using Pindel (ver. 0.2.4) (46). To identify somatic CNVs, mean read depths at each exon were calculated and normalized to the coverage of target regions in each sample. Normalized read depth was further standardized by dividing the expected coverage for a

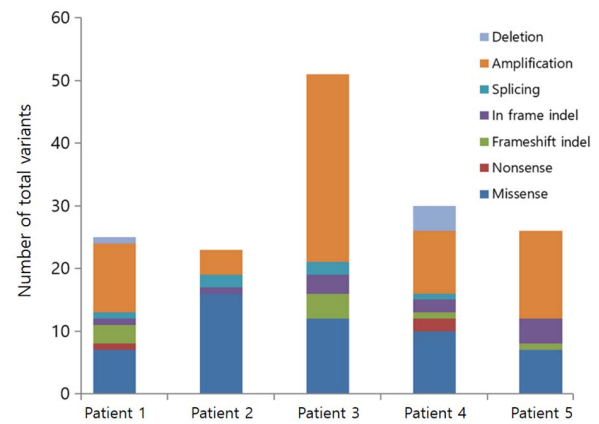


Figure 1. Numbers of total variants in the five patients. InDels, short insertions and deletions.

normal individual (the expected coverage at each exon was taken to be the median of the read depth at that exon across a set of normal individuals). These steps addressed variability in capture efficiencies and GC content at different exons. To infer the correct copy numbers, amplitudes of copy numbers were adjusted based on estimated purity. When the adjusted amplitude of a copy change was >1 or <1 on a log scale, the region concerned was defined as an amplification or a deletion, respectively. Furthermore, most fusions involve intronic breakpoints. To identify fusion using a gene panel, ‘hotspot’ introns that are known to contain most breakpoints were analyzed for a set of clinically relevant fusions.

Classification and interpretation of variants. Variants were classified into three tiers. Tier 1 variants included variants listed as therapeutic targets by the Korean MFDS or the US FDA, and variants reported to be candidates in clinical trials. HemaSCAN™ panel covered all positions of tier 1 variants. Tier 2 variants included any mutation reported in COSMIC (ver. 64) (<http://cancer.sanger.ac.uk>) and gene fusions involving a target gene and a known Tier 1 partner (in COSMIC) or a novel partner. Tier 3 variants included all other mutations. ‘Actionable’ (that is, potentially responsive to a targeted therapy) variants were defined as Tier 1 variants, while ‘known’ (but not actionable) variants were defined as Tier 2 variants.

Results

HemaSCAN™ panel. The HemaSCAN™ panel (Fig. S1) is a targeted NGS analysis panel that identifies clinically relevant genomic variants in tumor DNA and matches these variants with targeted therapies and/or clinical implications for diagnostic/prognostic purposes. NGS analysis revealed the following genomic variants in the five patients (Figs. 1 and S2): SNVs (Figs. 2 and 3), short InDels (Figs. 2 and 4) and CNVs (Fig. 5). These genomic variants were also reported as annotated, known and novel variants. Variant annotations were used to distinguish ‘real’ variants from sequencing artifacts and to distinguish potentially pathogenic variants from neutral variants (47). Estimated tumor purity in bioinformatics analysis was >90%. HemaSCAN™ analysis results revealed 25 variants

Table II. Sum and types of variation in the five patients.

Variation	Patient 1, n	Patient 2, n	Patient 3, n	Patient 4, n	Patient 5, n
SNV					
Missense	7	16	12	10	7
Nonsense	1	-	-	2	-
Splicing	1	2	2	1	-
InDel					
In-frame	1	1	3	2	4
Frameshift	3	-	4	1	1
CNV					
Amplification	11	4	30	10	14
Deletion	1	-	-	4	-
Total	25	23	51	30	26

SNV, single nucleotide variant; InDel, short insertions and deletions; CNV, copy number variation.

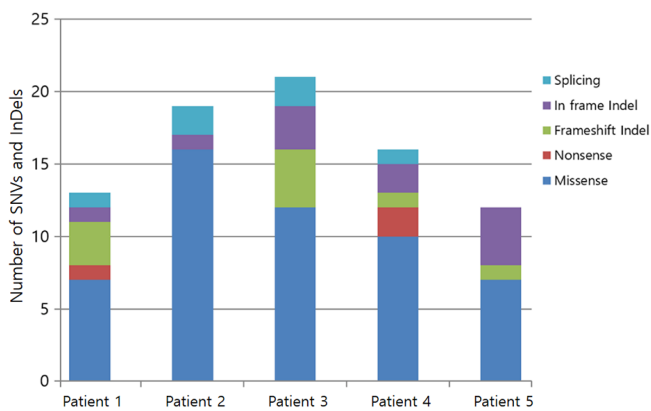


Figure 2. Numbers of SNVs and InDels. SNVs, single nucleotide variations; InDels, short insertions and deletions.

in patient 1, 23 variants in patient 2, 51 variants in patient 3, 30 variants in patient 4 and 26 variants in patient 5 (Table II).

SNVs. Different numbers of SNVs were identified in the five patients: Patient 1, 9; patient 2, 18; patient 3, 14; patient 4, 13; and patient 5, 7 SNVs. There were 6 SNVs [Kelch-like ECH-associated protein 1 (KEAP1), BLM RecQ like helicase, ETS variant transcription factor 6 (ETV6) and heat shock protein 90 α family class A member 1] at splicing sites, 3 SNVs [CREB binding protein (CREBBP), TNF receptor superfamily member 14 (TNFRSF14) and RAD50 double strand break repair protein (RAD50)] that caused stop codons and 52 nonsynonymous SNVs that altered protein amino acid sequences. No SNV was identified in the annotated variant. Among the known variants, the same nonsynonymous SNV was present in the SET binding protein 6 (SETBP6) gene in patients 2 and 3 (Fig. 3). The SETBP gene is located on chromosome 18 at 42,643,270 (<https://ghr.nlm.nih.gov/gene/SETBP1#conditions>). In exon 6 of the SETBP gene, the 4,398th cDNA nucleotide (guanine) was replaced by thymine and the amino acid at position 1,466 of the protein was altered from glutamic acid to aspartic acid (Fig. 3). Among known SNVs, three different SNVs in the ETV6 gene

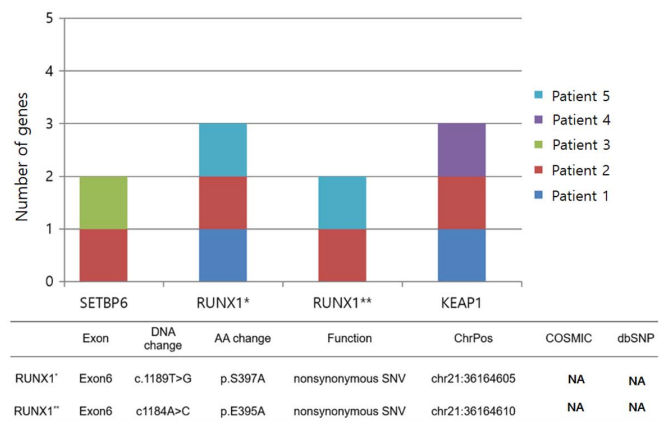


Figure 3. Genes for which SNVs were identified in ≥ 2 patients. SNVs, single nucleotide variations; RUNX1, runt-related transcription factor 1; KEAP1, Kelch-like ECH-associated protein 1; T, thymine; G, guanine; A, adenine; C, cytosine; AA, amino acid; ChrPos, chromosome position; SETBP6, SET binding protein 6; dbSNP, SNP data base; COSMIC, catalogue of somatic mutations in cancer; NA, not applicable.

were identified in patients 2 and 3 (Fig. S2). These SNVs were located in the same gene; however, they were at different positions in the 12th chromosome and different exons. Additionally, nucleotide substitutions and amino acid alterations also differed. Hence, they were deemed as different mutations.

Nonsynonymous SNVs were identified in two different Runt-related transcription factor 1 (RUNX1) genes (Fig. 3). One occurred in patients 1, 2 and 5 and the other in patients 2 and 5. Both were identified in exon 6, although nucleotide changes and the resulting amino acid changes differed. In the former, thymine at position 1,189 of cDNA was replaced by guanine, causing a change from serine to alanine at position 397. In the latter, adenine located at position 1,184 of the cDNA sequence was replaced by cytosine, causing a change from glutamic acid to alanine at position 395. SNVs in KEAP1 occurred at the splicing site and were identified in patients 1, 2 and 4. SNVs in CREBBP, TNFRSF1 and RAD50 caused stop codons (Fig. S2).

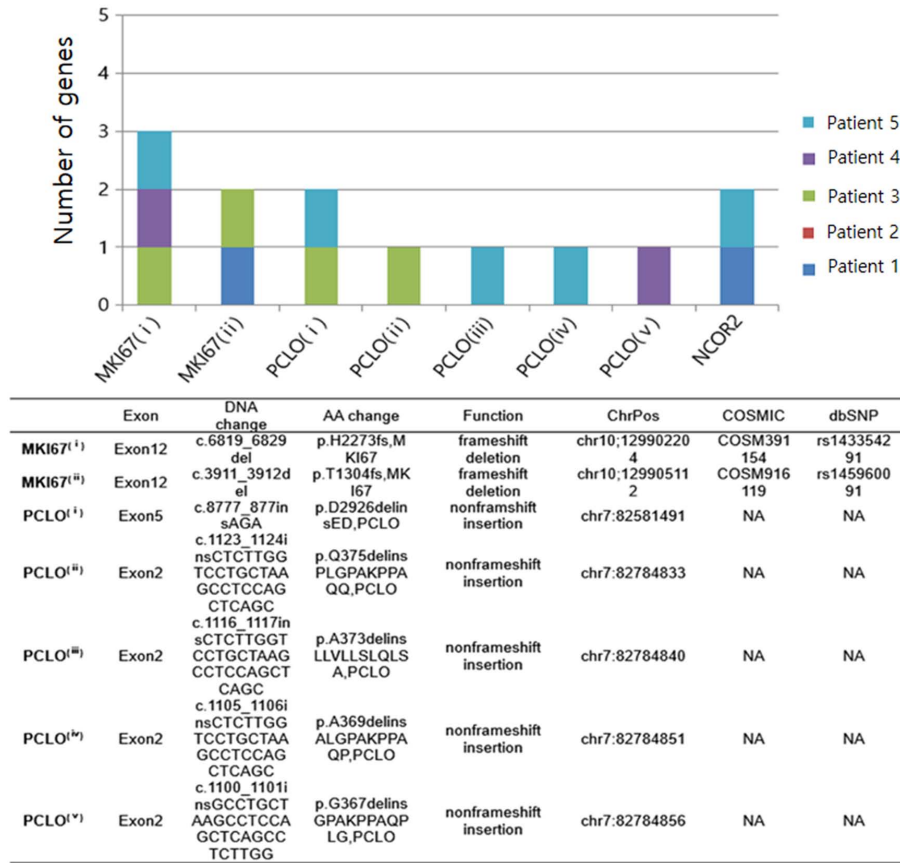


Figure 4. Genes for which short deletions and insertions were identified in ≥ 2 patients. MKI67, monoclonal antibody Ki-67; PCLO, piccolo presynaptic cytomatrix protein; NCOR2, nuclear receptor corepressor 2; AA, amino acid; ChrPos, chromosome position; dbSNP, SNP data base; COSMIC, catalogue of somatic mutations in cancer.

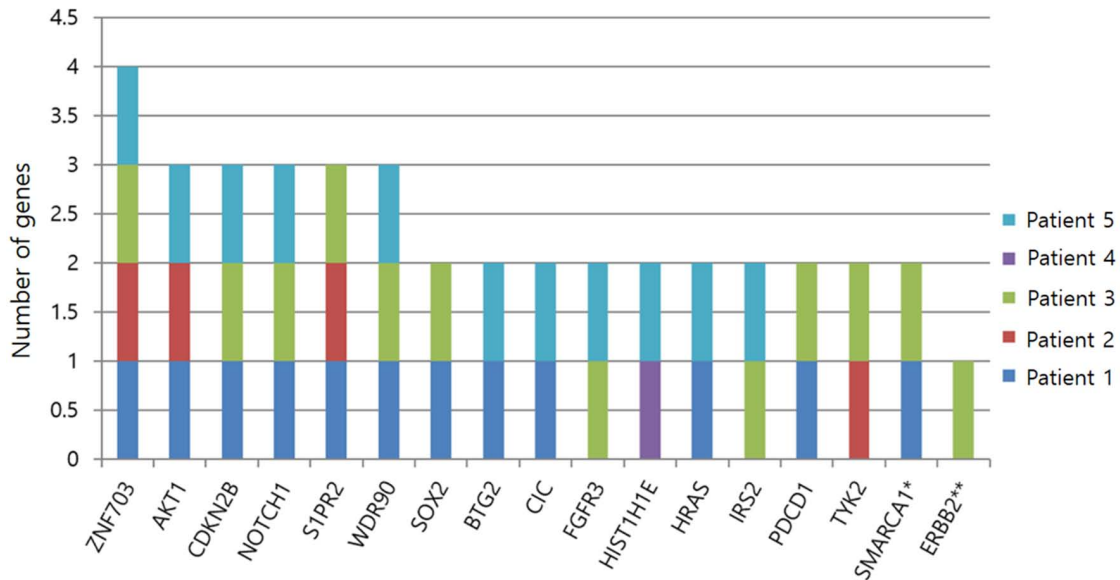


Figure 5. Genes in which copy number variations were identified in ≥ 2 patients. *Deletion; **annotated.

InDels. Among the three variant types, InDels exhibited the lowest frequency and were not identified in the annotated variant. Among the known variants, two single-base deletions were identified in the same marker of proliferation Ki-67

(MKI67) gene; however, these were at different chromosomal positions, resulting in a frameshift deletion (Fig. 4). This deletion occurred at amino acid 1,304 (threonine) of the protein in patients 1 and 3, and at position 2,273 (histidine) in patients 3, 4

and 5. Furthermore, additional frameshift insertions involving known variants were confirmed in the forkhead box O3, mucin 2, oligomeric mucus/gel-forming and BCL10 genes (Fig. S2). Among novel variants, five single-base insertions were identified in piccolo presynaptic cytomatrix protein (PCLO) at different chromosomal positions, resulting in non-frameshift insertions. Non-frameshift mutations occurred at five different positions of the protein: i) At position 2,926 (aspartic acid) in patients 3 and 5; ii) at 375 (glutamine) in patient 3; iii) at 373 (alanine) in patient 5; iv) at 369 (alanine) in patient 5; and v) at position 367 (glycin) in patient 4. Additionally, non-frameshift insertions in nuclear receptor corepressor 2 (NCOR2) were detected in patients 1 and 5.

CNVs. According to the results, CNVs were the most common variant. The largest number of CNVs was found in patient 3. Two types of CNV were identified: Amplification and deletion. Amplifications were encountered more frequently than deletions. Among the 74 CNVs identified, one was an annotated variant and six were known variants, while the others were novel variants.

Among the novel variants, a CNV in the zinc finger protein 703 (ZNF703) gene was the most common and was identified in patients 1, 2, 3 and 5. A total of 3 patients had CNVs in the AKT1, cyclin dependent kinase inhibitor 2B (CDKN2B), NOTCH1, sphingosine-1-phosphate receptor 2 and WD repeat domain 90 genes, two patients had CNVs in SRY-box transcription factor 2 (SOX2), BTG anti-proliferation factor 2, capicua transcriptional repressor, fibroblast growth factor receptor 3, H1.4 linker histone, cluster member (HIST1H1E), HRas proto-oncogene, GTPase (HRAS), insulin receptor substrate 2, programmed cell death 1, tyrosine kinase 2 and SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1 and one patient had a CNV in erb-b2 receptor tyrosine kinase 2 (ERBB2). CNVs were present in oncogenes (AKT1, ERBB2, SOX2 and ZNF703), a proto-oncogene (HRAS) and a tumor-suppressor gene (CDKN2B; Fig. 5).

Discussion

The present study performed a comprehensive analysis using an NGS customized panel for MALT lymphoma. NGS technology has been applied to lymphoid neoplasms to provide early insights into the mutational landscapes of several lymphoid cancers, including DLBCL (48), follicular lymphoma (FL) (48), chronic lymphocytic leukemia (CLL) (49), NMZL (50), SMZL (51), mantle cell lymphoma (MCL) (52) and peripheral T-cell lymphoma (PTCL) (53). Although the analyzed sample size was small, genetic mutations for rare gastrointestinal MALT lymphoma were identified. Various mutations or genetic alterations identified in previous studies using NGS of MALT lymphoma (54,55) were confirmed, and several variations affecting the development of MALT lymphoma for which the exact mechanisms have not yet been elucidated were identified. Hyeon *et al* (54) analyzed 19 cases of gastric MALT lymphoma using targeted sequencing and the majority of genes affected by genetic alterations were involved in NF- κ B pathway activation and included MALT1 rearrangement and somatic mutations of TNF receptor associated factor 3, TNFAIP3 and NOTCH1. Cascione *et al* (55) analyzed 72 cases of MALT lymphoma

derived from different anatomic sites using a large panel of previously known genes and reported genetic mutations or alterations (SNVs and CNVs) that code for proteins involved in chromatin remodeling and transcription regulation, the breakpoint cluster region protein/NF- κ B signaling pathway, immune escape and the NOTCH signaling pathway [trisomy 3, TNFAIP3, CREBBP, lysine methyltransferase 2C, tet methylcytosine dioxygenase 2, spen family transcriptional repressor (SPEN), trisomy 18, lysine methyltransferase 2D, LDL receptor related protein 1B, PR/SET domain 1, baculoviral IAP repeat containing 3-MALT, E1A binding protein p300 (EP300), TNFRSF14, NOTCH1/NOTCH2, β -2-microglobulin and 6p gains, in common order]. In the present study, genetic alteration was confirmed in NOTCH1 (80%). CNV was patients 1, 3 and 5 (Fig. 5). InDel/SNV was patient 1 and 2 (Fig. S2). And various other genetic alterations [caspase recruitment domain family member 11, SPEN, TNFRSF14, EP300, CREBBP, APC regulator of WNT signaling pathway, phosphoinositide-3-kinase regulatory subunit 2, SET binding protein 1 (SETBP1), HIST1HC, HIST1HD and HIST1H1E] (Fig. S2) were observed.

A total of three patients exhibited an SNV in the RUNX1 gene. This gene encodes a Runt-related transcription factor of the RUNX gene family. The protein encoded by RUNX1 constitutes the α subunit of core binding factor (56). RUNX1 exhibits nuclear localization and is widely expressed in hematopoietic cells (57). It serves an essential role in the development and maintenance of hematopoiesis (58). In adults, RUNX1 disruption by intragenic mutation leads to a pre-leukemic state that predisposes acute myeloid leukemia (59). In the present study, the RUNX1 mutation was observed at exon 6.

KEAP1 was identified in three patients at its splicing site. Oxidative stress is associated with cancer development (60). Therefore, oxidative stress rescuing mechanisms serve critical roles, as they can potentially cause chemo- and radio-resistance and affect outcomes (61). It has previously been reported that a genetic polymorphism in the KEAP1 gene is associated with breast cancer risk and survival outcomes (62).

In two patients, an SNV was identified in the SETBP6 gene. The SETBP1 gene provides instructions for making SET binding protein 1 (63). However, the function of SETBP1 protein and the effect of SET binding remain unclear. Further studies are required to determine if this genetic change influences disease progression.

InDels are the second most common type of human genetic variation (64). According to the Human Gene Mutation database (2008 update), most cases of severe disease are due to missense mutations (44%) and small coding InDels (23%), worldwide (65). In the present study, the most commonly detected gene mutation among frameshift InDels was detected for MKI67, and was a frameshift deletion. The MKI67 gene encodes the antigen pKi-67 in humans. The best-studied examples are carcinomas of the prostate (66), brain (67) and breast (68), as well as neuroblastoma (69) and neuroendocrine tumors (70). Furthermore, the role and significance of pKi-67 have been studied in NHL (71). Since pKi-67 appears to be crucial for cell proliferation, mutations in its gene may cause dysfunction in the cell cycle and cell division to influence the development of uterine cervix, colon and lung cancer (72). The present study did not clarify how frameshift deletions in the pKi-67 gene affect the development or progression of MALT lymphoma. MALT lymphoma

is an indolent disease for which a ‘watch and wait’ strategy is used (73,74). Therefore, low or no expression of pKi-67 may be associated with its indolent character.

Patient 1 exhibited a frameshift deletion in the DEAD-box helicase 3 X-linked (DDX3X) gene (Fig. S2). DDX3X is hypothesized to serve nuclear and cytoplasmic roles. Dysregulation of DDX3X has been associated with tumorigenesis caused by loss of function (75). Furthermore, DDX3X has been identified as a mutational cancer driver in medulloblastoma, cutaneous melanoma and chronic lymphocytic leukemia by PAN-cancer analysis (76-78). However, to the best of our knowledge, the role of DDX3X in MALT lymphoma has not been elucidated and the relationship between DDX3X mutations and disease occurrence has not been clarified.

Among the in-frame InDels detected, non-frameshift insertions were observed in PCLO and NCOR2. PCLO encodes the piccolo protein in humans, which functions as part of the presynaptic cytoskeletal matrix and is hypothesized to be involved in the regulation of neurotransmitter release (79). Mutations in the PCLO gene in DLBCL have been reported to be extremely common, although their role in cancer has not been clarified (80). NCOR2 encodes a nuclear receptor co-repressor that mediates transcriptional silencing of *CCND1*, *VDUP1* and *GADD45A* target genes (81). Aberrant expression of NCOR2 has been associated with acute promyelocytic leukemia, breast, bladder and prostate cancers (82). In the present study, mutations in PCLO and NCOR2 were found to alter their protein expression (Fig. S2).

Chromosomal aberrations are a hallmark of cancer cells. The term CNV indicates an intermediate-scale genetic change, defined as segments >1,000 base pairs in length; however, they are typically <5 megabases (83). ZNF703 is located on human chromosome 8 in the short arm region, commonly referred to as chromosome region 8p12 and is ubiquitously expressed in human tissues (84). It localizes to the nucleus under nonpathological conditions (85). However, it has been reported that this gene is amplified and/or upregulated in breast cancer, colorectal cancer, stomach cancer and pulmonary cancer (86-89). ZNF703 gene amplification was observed in four cases (80%). To the best of our knowledge, no previous studies have reported that ZNF703 acts as an oncogene in lymphoma. Further research is required to determine whether ZNF703 gene amplification is associated with the progression of diseases in primary gastrointestinal lymphoma, including MALT lymphoma.

NOTCH1 translocation-associated signaling is associated with direct cell-cell communication, thereby controlling cell differentiation, proliferation and apoptosis (90). Dysregulation of the NOTCH signaling pathway has been associated with several hematologic (ALL, CLL and MALT lymphoma) and solid malignancies (melanoma, cholangiocarcinoma, colorectal cancer, lung adenocarcinoma, renal cell carcinoma and prostate cancer) (91). Dysregulation of the NOTCH signaling pathway occurs by a variety of mechanisms, including mutational activation or inactivation, overexpression, post-translational modification and epigenetic regulation (91). Previous studies have indicated that NOTCH activation serves important oncogenic roles in various B-cell malignancies (92-94). In particular, NOTCH signaling is important for the generation of marginal zone B-cells (93,94), indicating that in MALT lymphoma, mutated NOTCH1 activation is not a common pathogenic mechanism (95). However,

since the NOTCH signaling pathway serves a crucial role in MALT lymphoma, further studies on other components of the NOTCH signaling pathway may elucidate the precise contribution to the pathogenesis of MALT made by NOTCH signaling. Further larger scale studies are required to confirm the effect of NOTCH1 mutations on MALT lymphoma.

The present study had several limitations. As some of the patient's clinical data had been analyzed, there may be other clinical correlations of genetic variation. In addition, the whole genome of each patient was not assessed, thus, there is a possibility that some genetic alterations, which could have clinical significance, remain unrevealed. Notably, the sample size was too small, thus a larger scale study is required to comprehensively examine the clinical relevance of genetic alternation and MALT lymphoma.

In conclusion, since few NGS studies have been conducted on MALT lymphoma, various mutations identified in the present study cannot be regarded as ‘actionable’ (that is, potentially responsive to a targeted therapy) and whether these mutations have therapeutic implications could not be confirmed. Further research is required for the development of treatments by genetic mutation observed in the present study.

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Availability of data and materials

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

Authors' contributions

SYO, JHL and HJK conceptualized and designed the current study and coordinated and supervised data collection. SJH, SL and SYO drafted and revised the manuscript and analyzed data. SJH, SHK and MKP conducted the pathological review and performed NGS data analysis. All authors agreed to be responsible for all aspects of the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of Dong-A University Hospital, Yeonje-gu, Republic of Korea (approval no. DAUHIRB 20-088). The requirement for informed consent was waived as the tissues had been donated to the Dong-A University Hospital tissue bank.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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