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Mutational status of EZH2 and CD79B hot spots in mature B-cell non-Hodgkin's lymphomas: novel CD79B variations have been revealed

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Abstract. – OBJECTIVE: We aimed to determine the hot spot mutational frequencies of Enhancer of Zeste homolog 2 (*EZH2*) and cluster of differentiation 79B (*CD79B*) genes in a cohort of mature B-cell non-Hodgkin's lymphomas.

PATIENTS AND METHODS: DNA samples from formalin-fixed and paraffin embedded (FFPE) tissues from a total of 37 patients with mature B-cell non-Hodgkin lymphomas were included in the study. Molecular genetic analysis was performed by direct sequencing of the DNA samples.

RESULTS: We analyzed formaldehyde fixed-paraffin embedded (FFPE) tumor tissue samples from 17 female and 20 male patients with a median age of 63.7 years at the time of diagnosis. None of the patients had previously reported hot spot mutations in *EZH2* and *CD79B*, but previously unreported single nucleotide variations of *CD79B* were present in nine patients. rs779833118 was the most frequent variation (7/37 patients, 18.9%). A non-synonymous variation rs757407417, which could have a potentially damaging outcome, was detected in two patients.

CONCLUSIONS: None of the patients had well-known hot spot mutations in *EZH2* and *CD79B*. However, we detected novel *CD79B* variations in mature B-cell non-Hodgkin's lymphoma patients.

Key Words:
EZH2, CD79B, Mature B-cell non-Hodgkin lymphoma, Mutation.

Introduction

Non-Hodgkin's lymphoma (NHL) is a heterogeneous group of tumors that arise from developing lymphocytes, and it is much more common than Hodgkin's lymphoma. NHL accounts for approximately 4% of all malignancies in the world[1]. In Turkey, NHL is the 8th and 7th most frequent cancer type in women and men respectively[2]. Nearly 80% of the lymphomas arise in B-cell lymphocytes. The most frequent B-cell lymphoma subtypes are diffuse large B-cell lymphoma (DLBCL) (37%), follicular lymphoma (29%), and chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) (12%)[3]. DLBCL is characterized by heterogeneous clinical outcome[1]. There are two major subtypes of the disease according to gene expression profiling: germinal center B-cell-like (GCB) and activated B-cell-like (ABC)[4,5]. These two subtypes have distinct clinical features, with the latter exhibiting poor prognosis[4].

Mature B-cell NHLs arise from differentiated subsets of mature B cells and comprise a heterogeneous group of lymphoproliferative malignancies. Chromosomal translocations, copy number alterations and gene mutations are involved in the pathogenesis of the lymphomas. It is also well known that the presence of molecular heterogeneity is typical for each lymphoma subtype[6,7]. Recently, several studies[8,9] have demonstrated that the mutations in enhancer of zeste homolog 2 (*EZH2*) and cluster of differentiation 79B (*CD79B*) genes play a role in lymphomagenesis. It has also been thought that these mutations might serve as potential candidates for targeted therapy in the future[10,11].

In this study, we wanted to demonstrate the hot-spot mutation frequencies of *EZH2* and *CD79B*, and to determine the association between the mutations and clinical characteristics in a group of Turkish mature B-cell NHL pa-

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tients. To the best of our knowledge, this is the first report regarding the presence of *EZH2* and *CD79B* mutations in a Turkish population.

Patients and Methods

Patients and Tumor Tissue Samples

A group of 37 patients pathologically diagnosed between January 2008 and July 2015 with mature B-cell NHL, as described in the World Health Organization's (WHO) classification, were enrolled in the study[1]. All of the samples were diagnosed and selected by a hematopathologist. Tumor tissues included 18 lymph nodes and 19 biopsy specimens of extranodal tissues from sites including bone marrow, brain, eye, liver, lung, stomach, and skin of the scalp.

All the patients were classified according to the Ann Arbor staging system and International Prognostic Index (IPI) score, following the criteria described in previous studies[12,13]. The classification of tumors as germinal center B-cell-like (GCB) and as the non-GCB subtype in DLBCL was performed according to the previously reported algorithm[14].

Two consecutive sections of formalin-fixed and paraffin embedded (FFPE) tissue were obtained for DNA isolation. DNA was isolated using a commercial kit and according to the manufacturer's instructions (Macherey-Nagel, Düren, Germany).

Tissue samples were collected from all of the patients before treatment. This study was approved by the Institutional Review Board of Pamukkale University and conducted in compliance with the Declaration of Helsinki.

Molecular Genetic Analysis

EZH2 exon 16, and *CD79B* exon 5 and 6 were amplified by polymerase chain reaction (PCR) using unique primers. The list of PCR primers and the length of the specific reaction products are given in Table I. Purified gene-specific amplicons from PCR were then sequenced by dye-terminated cycle sequencing using a commercial kit, according to the instructions of the manufacturer (GenomeLab™ Dye Terminator Cycle Sequencing Quick Start Kit, Beckman Coulter, Brea, CA, USA). Briefly, a DNA sequencing reaction was performed by using purified PCR products (as templates), forward or reverse sequencing primer, and the master mix supplied with the kit. The thermal profile of the reaction was: 96°C for 20 seconds, 50°C for 20 seconds and 60°C for 4 minutes, for 30 cycles. Purified products from the DNA sequencing reaction were then subjected to capillary electrophoresis in a Beckman Coulter CEQ™ 8000 Genetic Analysis System (Beckman Coulter, Brea, CA, USA).

Statistical Analysis

Statistical analysis was performed using the SPSS (version 11; SPSS Inc., Chicago, IL, USA) software. The χ^2 test was used to compare categorical variables between the two groups. Results were considered to be statistically significant when $p < 0.05$.

Results

Patients' Characteristics

The group of patients consisted of 17 females and 20 males whose median age at the time of diagnosis was 63.7 years with an age range of 28-80 years. The subtypes of mature B-cell NHL of the patients were DLBCL, mantle cell lymphoma (MCL), follicular lymphoma (FL), chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), and nodal marginal zone lymphoma (NMZL). Tumor stages I and II were classified as early, while stages III and IV were classified as advanced. Each patient was also categorized into either a low (IPI score 0-2) or high (IPI score 3-4) clinical risk group. Patients' tumor characteristics are summarized in Table II.

Table I. The list of PCR primers and the length of the reaction products.

Region	Forward primer	Reverse primer	Product length
EZH2 exon 16	5'-GCATCTATTGCTGGCACCATC	5'-CATTTCCAATCAAACCCACAGA	199 bp
CD79B exon 5	5'-AGAATGCACCTCACTCCTGACC	5'-CCGAGGACTCAGAGCTGCTGG	198 bp
CD79B exon 6	5'-GGCCACTATCTGCTGGTGTGG	5'-CATGAGCCAGGCAGCTCCGAAG	235 bp

bp: base pairs.

Table II. Tumor characteristics of the patients with mature B-cell NHL

Tumor characteristics	No. of patients (%)
DLBCL	26 (70.3)
Non-GCB subtype	12 (32.4)
GCB subtype	14 (37.8)
MCL	2 (5.4)
FL	2 (5.4)
CLL/SLL	4 (10.8)
NMZL	3 (8.1)
Stage (Ann Arbor)	
Early (I-II)	13 (35.1)
Advanced (III-IV)	24 (64.9)
IPI score	
Low (0-2)	22 (59.5)
High (3-4)	15 (40.5)
Tumor location	
Nodal	18 (48.6)
Extranodal	19 (51.4)

Molecular Genetic Analysis

All of the DNA samples from FFPE tissue specimens were analyzed for previously reported *EZH2* Y641 and *CD79B* ITAM domain (Exons 5 and 6) mutations. None of the patients had mutations in *EZH2* and *CD79B* exon 6, but several variations of *CD79B* exon 5 were detected in nine patients. Figure 1 shows the direct sequencing results of representative samples. Clinical characteristics and results of the cases with *CD79B* exon 5 variations are given in Table III.

Discussion

We have analyzed the mutation frequencies of *EZH2* exon 16 and *CD79B* exons 5 and 6 in 37 mature B-cell patients in this study and detected several variations in *CD79B* exon 5. Among the variations, rs779833118 was the most frequent (7/9), followed by rs199859520 (2/9) and rs757407417 (2/9), and a single case of rs752009987. However, no well-known mutations associated with mature B-cell NHL were detected in our patients, probably due to the low number of FFPE specimens or the genetic heterogeneous nature of the disease.

EZH2 (enhancer of zeste homolog 2) is the catalytic subunit of the polycomb repressive complex 2 (PRC2), which is a member of polycomb group proteins (PcG) and is responsible for gene silencing through methylation of Lys27 of histone H3 (H3K27)[15]. EZH2 adds one to three methyl groups to Lys 27. Methylation of Lys27 makes this amino acid an anchorage point for additional PcG proteins, which results in repressive chromatin state[16,17]. The interaction between EZH2 and various DNA methyltransferases in the establishment of gene silencing has also been shown[18]. EZH2 contributes to the cell fate decisions (self-renewal and differentiation) during the developmental processes in various tissues, including the hematopoietic system[9]. *EZH2* gain of function mutation Y641, which replaces a single tyrosine in the catalytic site of the SET domain, has been reported to occur in up to 24% of the GCB, and in a very little portion of ABC subtype DLBCL cases[8,19,20].

The B-cell receptor (BCR) signaling pathway is important for normal B-cell maturation as well as for development of B-cell malignancies[21-25]. Thus, members of this pathway are subject to several therapeutic approaches. The extracellular antigen recognition domain of the BCR is a tetramer consisting of two heavy and two light immunoglobulin chains. CD79B, together with CD79A, forms the cytoplasmic tail of the BCR that transmits the signal to the downstream cascade[26]. The highly conserved functional intracellular domains of CD79A and CD79B, immunoreceptor tyrosine-based activation motifs (ITAMs), become phosphorylated by the upstream kinases after binding of the antigen to the extracellular domain of BCR. The phosphorylated ITAMs then serve as docking sites for additional kinases and signal transduction molecules[27,28]. Mutations in the ITAM cause a chronic BCR signaling in approximately one-fifth of the ABC DLBCL patients, which may result in lymphomagenesis[8,10].

COSMIC (Catalogue of somatic mutations in cancer) (http://cancer.sanger.ac.uk/cosmic Access date: November 25, 2015) is a database that was designed to store and display somatic mutation information relating to human cancers[29]. There are plentiful mutations and variations of *CD79B* registered in the COSMIC database. Except for rs199859520, the SNPs we detected in our samples were lacking in the COSMIC database.

CD79B rs757407417 and rs752009987 are missense variants. rs757407417 is a C/T substitution, which results in encoded amino acid residue change from Asp to Asn (D193N). The clinical significance of this variation is unknown, but it had a SIFT score of 0 and PolyPhen score of 1, which predicts potential clinical significance. The amino acid position of this variation is in the ITAM domain of the protein and very close to the well-known Y196 mutation. One of the pa-

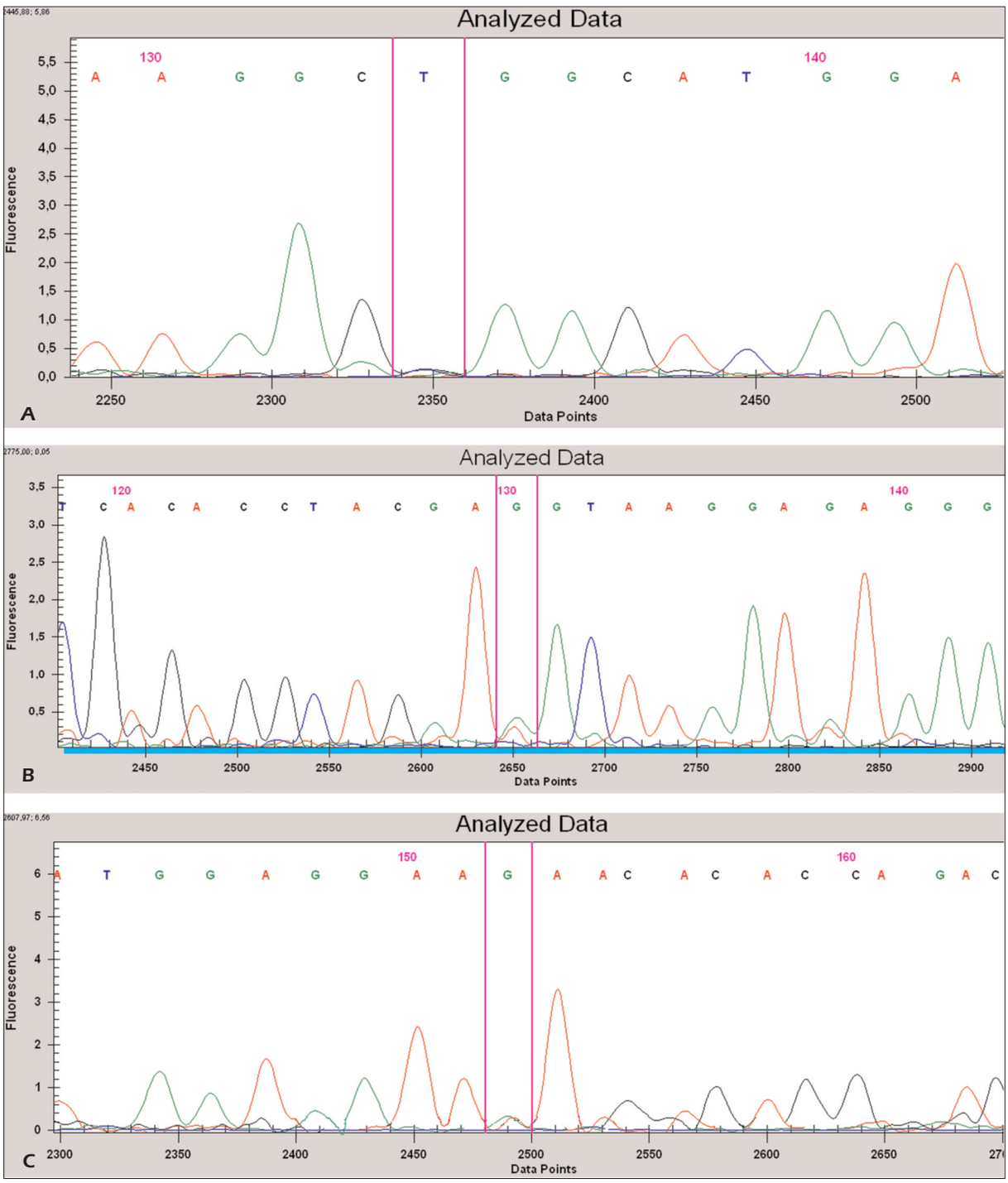

Figure 2. Direct sequencing results of the samples with ***A,*** rs779833118, ***B,*** rs199859520 and ***C,*** rs757407417 variations in CD79B gene. Box indicates the variation in nucleotide sequence.

tients who carried the SNP had grade II GCB type DLBCL with cervical localization and no extranodal involvement, and her IPI score was 2. The patient also had positive immunohistochemical staining (IHC) for the markers CD20, CD10, BCL-2, and BCL-6. Her Ki-67 proliferation index was 90%. However, another patient with rs757407417 was positive for CD20 and BCL-2 and her Ki-67 score was 40% with grade III disease. Although ITAM mutations frequently exist

Table III. Clinical characteristics of the patients with CD79B exon 5 variations.

Patient ID	Diagnosis	Sex	Age	GCB/ non-GCB	Tumor localization	Extranodal involvement (localization)	Ann Arbor stage	IPI score	CD79B exon 5 variation
1	MZL	M	80	N/A	Parailiac LN	+ (BM)	4	2	rs779833118 c.564T>G
2	DLBCL	M	49	GCB	Tonsilla	-	2	1	rs779833118 c.564T>G
3	DLBCL	M	65	Non-GCB	Inguinal LN	-	2	2	rs779833118 c.564T>G
4	DLBCL	F	78	Non-GCB	Cervical LN	+ (Lung)	4	3	rs779833118 c.564T>G
5	DLBCL	M	67	GCB	Inguinal LN	-	3	3	rs199859520 c.591G>A
6	DLBCL	M	76	GCB	Skin	+ (Brain)	4	3	rs779833118 c.564T>G rs199859520 c.591G>A
7	DLBCL	F	72	GCB	Cervical LN	-	2	2	rs757407417 c.577G>A
8	DLBCL	F	75	Non-GCB	Tonsilla	-	3	3	rs757407417 c.577G>A
9	DLBCL	F	53	Non-GCB	Cervical LN	-	3	2	rs779833118 c.564T>G rs752009987 c.563C>T

MZL: Mental zone lymphoma; LN: Lymph node; BM: Bone marrow; M: Male; F: Female; N/A: Does not apply.

in high-grade mature B-cell NHL, one of our patients with rs757407417 had grade II disease. Unfortunately, we did not have any DNA samples from other tissues of the patients to document rs757407417 in healthy, non-tumorous tissues.

rs752009987 is another missense variant with G/A/C substitution. Our sample had a C/A transition, which resulted in Ala/Val substitution in the encoded amino acid sequence. This substitution had a SIFT score of 0.16 and a PolyPhen score of 0.043, which predicts a possible mild clinical significance.

rs779833118 was the most frequent SNP in our samples. It is an A/C substitution, which results in a synonymous codon with no effect on the encoded amino acid chain. rs199859520 was found in two samples and in one of these was found together with rs779833118. This SNP is a synonymous and splice site variation. It is a C/T substitution with the ancestral allele being C. The clinical significance of this variation is unknown, but the patients carrying the SNP had advanced-stage diseases located in inguinal lymph nodes and skin, with the latter also involving the brain. The clinical importance of this substitution needs further clarification, including functional protein studies regarding the effect of splice site variation on gene product.

Mutations of *EZH2* and *CD79B* in NHLs, particularly in DLBCL have been studied widely in the literature. However, to the best of our knowledge, there are no reports regarding the status of mutation frequencies in our population. EZH2 Y641 mutation is one of the key points of DLBCL lymphomas with a frequency of about 10%[9]. However, we have not observed Y641 mutation in any of our GCB DLBCL samples.

CD79B ITAM domain mutations, particularly Y196, are also frequently reported in ABC type DLBCL patients. None of the well-known *CD79B* mutations were detected in our patients; however we found synonymous and non-synonymous single nucleotide variations in our samples. Bohers et al[8] analyzed activating mutations in DLBCL patients in BCR and NF-□B pathways, including CD79B and EZH2, in a cohort of 161 patients. The frequencies of *CD79B* and *EZH2* mutations were 5.6% and 24%, respectively. Except for various Y196 mutants, four novel mutations affecting the ITAM domain of CD79B were found, including Δ198-220, L199RfsX11,

K219R, and A205SfsX3. A205SfsX3, a frame shift mutation, was detected in a GCB type sample, while all other mutations were found in ABC type tumor specimens. K219R was found together with Y196S in one of the ABC type DLBCL patients.

Bruno et al[30] studied mutations of 37 genes, including *CD79B*, in primary central nervous system lymphomas by next generation sequencing. In three patients, three mutations were found in *CD79B* gene; Y196C, Y196H and T195S, all in the ITAM domain. However, variations found in our patients were not reported.

Conclusions

We have analyzed the hot-spot mutations of *EZH2* and *CD79B* in mature B-cell NHL patients from Turkey. In contrast with the literature, we did not detect any of the well-known hot-spot mutations in our cohort. This finding suggests that these tumors represent a heterogeneous group of neoplasms, which is a common phenomenon. Our findings also may show a population-based difference in DNA variations, although the sample size was relatively small. Moreover, direct DNA sequencing cannot detect DNA variations when tumor or mutation load is low, which may have affected our findings. One novel finding of our study was the single nucleotide variant in *CD79B*, rs757407417, which was found in one GCB type and one non-GCB type DLBCL patient. This variation should be remembered in further studies involving larger sample sizes.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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