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ANALYSIS OF THE 3′UTR REGION OF THE *NOTCH1* GENE IN CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS

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Deregulation of *NOTCH1*-signalling pathway is common in chronic lymphocytic leukemia (CLL). The most of studies are focused on detection of the hotspot c.7541_7542delCT *NOTCH1* mutations in exon 34, while studies of mutations in the 3′UTR region are rare. *The aims* of work were to evaluate the frequencies of mutations in the 3′UTR region of the *NOTCH1* gene (9:136,495553-136,495994) in Ukrainian CLL patients, the distribution of rs3124591 genotypes located in that area, and association of *NOTCH1* mutations with structure of B-cell receptor. *Materials and Methods:* Detection of mutations in the 3′UTR region of the *NOTCH1* was performed by direct sequencing in 87 previously untreated CLL patients (from the total group of 237 CLL patients) with unmutated immunoglobulin heavy-chain variable (UM *IGHV*) genes and without mutations in hotspot regions of *TP53*, *SF3B1,* and exon 34 of *NOTCH1* genes. *Results:* Mutations in the 3′UTR region of the *NOTCH1* were revealed in three of 87 CLL patients (3.4%). Two cases with non-coding mutations were related to subset #1 of stereotyped B-cell receptors, and one case belonged to stereotyped subset #28a. Analysis with inclusion of 30 UM *IGHV* cases with previously detected c.7544_7545delCT mutations revealed that the frequency of UM *IGHV* genes of I phylogenetic clan (except *IGHV1-69)* was significantly increased, and the frequency of UM *IGHV3* and *IGHV4* genes, on the contrary, was reduced in *NOTCH1*-mutated cases comparing with *NOTCH1*-unmutated cases ($p = 0.002$) and the general group ($p = 0.013$). SNP rs3124591 did not affect the risk of CLL and survival parameters of the patients. At the same time, differences were found in the frequency of *IGHV* gene usage and in the structure of HCDR3 in carriers of individual genotypes. *Conclusion:* The frequency of *NOTCH1* mutations in 3′UTR region was low. Our findings confirmed current data on the association between the structure of the B-cell receptor and the appearance of *NOTCH1* mutations. Some features of HCDR3 structure were identified in carriers of TT and CC genotypes of rs3124591.
Key Words: **NOTCH1 mutations, 3′UTR region of the *NOTCH1,* rs3124591, *IGHV* genes.**

Deregulation of *NOTCH1*-signalling pathway is common in chronic lymphocytic leukemia (CLL). This gene encodes transmembrane receptor, and active intracellular fragment of NOTCH1 (ICN1) releasing after ligand binding, conformation changes and proteolytic cleavages translocates to the nucleus, where it mediates the transcription of target genes involved in cell differentiation, proliferation and apoptosis [1, 2]. Expression of ICN1 was found in 50.5% of CLL cases lacking *NOTCH1* gene mutations regardless of mutation status of immunoglobulin heavy-chain variable (*IGHV*) genes [3]. Prognostic significance of such constitutional ICN1 expression in CLL is not yet known.

Activated *NOTCH1* mutations in CLL were first found through whole-genome sequencing by two independent groups in 2011 [4, 5]. They were commonly represented by a single 2-bp deletion (c.7544_7545delCT, P2514fs) in exon 34 and resulted in the removal of C-terminal PEST [proline (P), glutamic acid (E), serine (S), and threonine (T) rich] domain involved in proteasomal degradation of ICN1. *NOTCH1* c.7544_7545delCT mutations occur in 8–11% in newly diagnosed CLL, 10–15% at the time of first treatment and 15–20% at chemorefractoriness and, according to the data of several authors, are associated with short progressive-free (PFS) and overall survival (OS) [6, 7]. Later, in the minority of CLL cases point mutations in the 3′UTR region of *NOTCH1* were found that resulted in the removal of C-terminal PEST domain [8]. We revealed c.7544_7545delCT mutations of *NOTCH1* in 13.4% of untreated CLL patients. Associations between the presence of *NOTCH1* mutations and unmutated (UM) *IGHV* genes, more advanced stage of the disease, higher initial WBC count, bulky disease, short time-to-treatment (TTT) period and PFS were found [9]. The aims of present work were to evaluate the frequencies of mutations in the 3′UTR region of the *NOTCH1* gene (9:136,495553–136,495994) in Ukrainian CLL patients, the distribution of rs3124591 genotypes located in that area, and association of *NOTCH1* mutations with structure of B-cell receptor.

MATERIALS AND METHODS

Samples of 87 previously untreated CLL patients with UM *IGHV* genes and without mutations in hotspot regions of *TP53*, *SF3B1,* and *NOTCH1* (exon 34) genes were selected for the study from the total group of 237 patients. Such choice of patients was based on the current data on a rare association of the *NOTCH1* mutations with mutations of *TP53* and *SF3B1* genes, and their prevalence in cases with UM *IGHV* genes. All patients (69 males and 18 females) were referred to the National Research Center for Radiation Medicine, Kyiv, during the period of 2002–2016. The study was approved by the local Ethics Review

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Abbreviations used: CLL – chronic lymphocytic leukemia; HCDR3 – heavy chain complementarity-determining region 3; ICN1 – intracellular fragment of NOTCH1; IGHV – immunoglobulin heavy-chain variable (genes); OS – overall survival; PFS – progression-free survival; TTT – time-to-treatment; UM – unmutated.

Committee, and all patients gave informed consent prior to participation in it. The diagnosis of CLL was based on clinical history, lymphocyte morphology, and immunophenotypic criteria.

Genomic DNA for molecular analysis was extracted from peripheral blood mononuclear cells with the QIAamp Blood Mini Kit (Qiagen, Crawley, United Kingdom) according to the manufacturer's protocol. Screening for presence of *TP53*, *SF3B1* mutations and *NOTCH1* mutations in exon 34 as well as mutational status of *IGHV* genes was performed in all 237 patients.

IGHV-D-J rearrangements were amplified according to the BIOMED-2 consortium rules [10] as described previously [11]. *IGHV* rearrangements were analyzed by IMGT/V-QUEST [12]. Mutational status of *TP53* gene was performed for exons 3 to 10 as described previously [13]. *SF3B1* mutations were analyzed in exons 14, 15 and 16 by PCR amplification followed by direct sequencing with the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) according to Rossi *et al.* [14]. It should be noted that sequencing was carried out in those regions of *TP53* and *SF3B1* genes where the vast majority of mutations is located (> 95%) [15, 16].

NOTCH1 mutations were analyzed in the hotspot c.7282_7680 region in exon 34 of *NOTCH1* gene by PCR amplification followed by direct sequencing as described previously [9]. Detection of mutations in the 3′UTR region of *NOTCH1* gene (9:136,495553–136,495994) was performed by direct sequencing according to Puente *et al.* [8] using original primers. PCR conditions and primers are described in: dx.doi.org/10.17504/protocols.io.qfhdtj6.

Analysis of rs3124591 genotypes was performed using SNPstats tool (http://bioinfo.iconcologia.net/snpstats/start.htm).

TTT period, PFS and OS were estimated by the method of Kaplan and Meier and assessed by the log-rank test.

RESULTS

***The frequency* of *NOTCH1* mutations and association with HCDR3 structure.** Mutations in the 3′UTR region of the *NOTCH1* were revealed in three of 87 CLL patients (3.4%). Non-coding mutations were represented by 139390152T>C *(*two cases*)* and 139390145T>C (one case).

Two cases with non-coding mutations were related to subset #1 of stereotyped B-cell receptors, and one case belonged to stereotyped subset #28a (according to classifications of Stamatopoulos *et al.* [17], Murray *et al.* [18], Bomben *et al.* [19]).

To study the associations of *NOTCH1* mutations and the stereotyped B-cell receptors, we have added to the studied group 30 UM *IGHV* cases with previously revealed c.7544_7545delCT mutations from the total group of 237 CLL patients. Thus, combined group of 117 UM-*IGHV* cases included 33 cases with *NOTCH1* mutations and 84 cases without *NOTCH1* mutations in exon 34 and in the 3′UTR region. It was divided into three subgroups according to the principle used by Sutton *et al.* [20].

The frequency of *NOTCH1* mutations in subgroup of 22 CLL cases with UM *IGHV* genes of I phylogenetic clan (comprising *IGHV1, IGHV5* and *IGHV7* genes), except *IGHV1-69*, was the highest (13 of 22 cases; 59.1%). This subgroup included 9 cases of subset #1 (five with *NOTCH1* mutations), 2 cases of subset #28a (both *NOTCH1*-mutated), one case of subsets #59, #95, #UA/ref4 [21] (all *NOTCH1*-mutated), six cases showing homology with CLL sequences represented in the databases, but did not include in the stereotyped subsets (three with *NOTCH1* mutations), and two *NOTCH1*-UM heterogeneous cases. Lengths of HCDR3 in *NOTCH1*-mutated cases varied from 12 a.a. to 21 a.a. and did not differ from *NOTCH1*-UM cases (15.66 ± 0.98 a.a. *vs* 18.55 ± 1.93 a.a.; $p = 0.165$).

Additional *NOTCH1*-mutated CLL case, not included in this subgroup, in its structure of *IGHV-D-J* rearrangement was close to cases of subsets #1 (structures of HCDR3 in CLL cases with *NOTCH1* mutations are available by request).

In subgroup of 37 CLL cases with UM *IGHV1-69* gene, *NOTCH1* mutations were revealed in 12 cases (32.4%). *NOTCH1*-mutated cases belonged to subsets #6 (1 of 3), #9 (1 of 3), #UA/ref2 (1 of 2), #UA/ref11 (one case). Additional one *NOTCH1*-mutated case was revealed among six cases homologous with CLL sequences represented in the databases, but did not include in the stereotyped subtypes, and seven *NOTCH1* mutations were found among fourteen CLL cases with heterogeneous HCDR3s. Without *NOTCH1* mutations there were cases of subsets #7 (n = 5), #UA4 (n = 1), #UA6 (n = 1), and #UA7 (n = 1). Lengths of HCDR3 in *NOTCH1*-mutated cases varied from 17 a.a. to 25 a.a. and did not differ from *NOTCH1*-UM cases (21.08 ± 0.75 a.a. *vs* 21.64 ± 0.71 a.a.; $p = 0.635$).

In subgroup of 57 CLL cases with other UM genes (*IGHV3*, n = 39; *IGHV4*, n = 16; *IGHV2*, n = 2) *NOTCH1* mutations were present in seven cases (12.3%). Two *NOTCH1*-mutated cases belonged to subsets #22 (1 of 3) and #25 (one case), two had homology with CLL sequences represented in the databases, but not included in the stereotyped subtypes (total n = 15), the three remaining cases had heterogeneous HCDR3s (total n = 25). CLL cases from subsets #7 (n = 2), #9 (n = 1), #31 (n = 1), #41 (n = 2), #44 (n = 1), #50 (n = 1), #109 (n = 1), #UA5 (n = 2), and #UA9 (n = 2) were *NOTCH1*-UM. Mean lengths of HCDR3 did not differ in *NOTCH1*-mutated (21.14 ± 0.98 a.a.) and *NOTCH1*-UM cases (20.60 ± 0.64 a.a.; $p = 0.760$).

Differences in the frequency of *NOTCH1* mutations in subgroups of cases expressed UM *IGHV* genes of I clan (except *IGHV1-69*), *IGHV1-69* gene and other UM *IGHV* genes were significant ($p = 0.001$). The distribution of UM *IGHV* genes of the selected subgroups among *NOTCH1*-mutated cases was significantly different comparing with the distribution in *NOTCH1*-UM cases, in the analyzed group

of 117 CLL cases as well as in total group of 237 CLL cases (Table 1).

Table 1. The distribution of UM *IGHV* genes in studied CLL patients

Groups of CLL patients	Subgroups of UM *IGHV* genes			*p* value in comparison with *NOTCH1*-mutated cases
	Clan I except *IGHV1-69*	*IGHV1-69*	Other *IGHV* genes, including *IGHV6-1* case	
NOTCH1-mutated, n = 33	13 (39.4)	12 (36.4)	8 (24.2)	–
NOTCH1-UM, n = 84	9 (10.8)	25 (27.7)	50 (59.5)	0.002
Whole group of 117 patients	22 (18.8)	37 (31.6)	58 (49.6)	0.013
161 cases with UM *IGHV* genes from total group of 237 patients	32 (19.9)	50 (31.0)	79 (49.1)	0.013

Analysis of rs3124591 SNP distribution and its association with survival parameters and IGHV gene usage. The distribution of rs3124591 genotypes was as follows: CC genotype — 20 cases (21.5%), CT genotype — 41 cases (44.1%), and TT genotype — 32 cases (34.4%) and did not deviate from the Hardy — Weinberg equilibrium ($p = 0.04$). In comparison with healthy individuals of European ancestry retrieved from the 1000 Genomes Project dataset (CC genotype 26.2%; CT genotype 47.4%; TT genotype 26.0%; http://www.1000genomes.org/) no significant differences were found. All three cases with non-coding *NOTCH1* mutations were represented in carriers of TT genotype.

The impact of rs3124591 on duration of TTT period, PFS and OS was insignificant ($p = 0.425$, $p = 0.380$, and $p = 0.722$, correspondingly).

The spectrum of used *IGHV* genes tended to be narrower in CC homozygotes than in carriers of CT and TT genotypes (correspondingly 22, 20, and 8 *IGHV* genes, $p = 0.125$; $p = 0.043$ in comparison TT *vs* CC genotype carriers) (Table 2).

Table 2. The distribution of UM *IGHV* genes in carriers of different rs3124591 genotypes

IGHV genes	Genotypes of rs3124591		
	CC, n = 19	CT, n = 37	TT, n = 31
IGHV1 family	9 (47.4)	17 (45.9)	9 (29.0)
IGHV1-69	8 (42.1)	13 (35.1)	4 (12.9)
IGHV2 family	0	0	1 (3.2)
IGHV3 family	7 (36.8)	13 (35.1)	14 (45.2)
IGHV3-11	4 (21.1)	1 (2.7)	2 (6.5)
IGHV4 family	3 (15.0)	7 (18.9)	4 (12.9)
IGHV4-39	2 (10.5)	2 (5.4)	0
IGHV5 family	0	0	3 (9.7)

Expression of only two *IGHV* genes was detected in more than half of CC homozygotes (8 cases with *IGHV1-69* and 4 cases with *IGHV3-11* gene).We found a reduced *IGHV1*-69 gene usage in carriers of TT genotype compared to carriers of CT and CC genotypes (12.9%, 35.1%, and 42.1%, correspondingly; $p = 0.038$). The distributions of *IGHD* and *IGHJ* genes were comparable in carriers of different genotypes. It should be noted that the frequencies of *IGHV* and *IGHJ* genes usage and different stereotyped subsets in this studied group did not differ from those in the previously studied large group cohort.

The HCDR3 length did not differ in carriers of different genotypes with UM *IGHV* genes (CC genotype 21.43 ± 1.14 a.a.; CT genotype 21.05 ± 0.73 a.a.; TT genotype 19.16 ± 0.76 a.a.; $p = 0.140$). However, the number of N nucleotides inserted in the V_HD junctions was significantly less in carriers of TT genotype than in carriers of CT and CC genotypes (Table 3).

The comparison of CLL sequences with non-CLL sequences available from public databases showed that most cases that had HCDR3 homology with antibacterial or antiviral Ig clones were present in TT homozygotes (22.6% *vs* 2.7% in carriers of CT genotype and 0% in carriers of CC genotype, $p = 0.006$; Table 4). All CLL sequences homologous with autoreactive clones were revealed in carriers of TT (9.7%) and CT (10.8%) genotypes. A number of CLL cases that were similar to Ig sequences expressed by normal B-cells (elderly, neonate, cord blood, tonsils) and to Ig sequences from patients with X-linked hyperIgM did not differ in carriers of different rs3124591 genotypes (Table 5, 6).

Table 4. HCDR3 homology in carriers of different rs3124591 genotypes

HCDR3 homology with:	Genotypes			*p value*
	CC	CT	TT	
Normal B cells	4 (21.1)	10 (27.0)	5 (16.1)	0.515
Ig from X-HIgM syndrome	0	1 (2.7)	3 (9.7)	0.226
Antibacterial or antiviral Ig clones	0	1 (2.7)	7 (22.6)	0.006
Autoreactive clones	0	4 (10.8)	3 (9.7)	0.332

DISCUSSION

To investigate non-coding mutations in the 3′UTR region of the *NOTCH1*, previously untreated CLL cases with no mutations in *TP53*, *SF3B1,* and *NOTCH1* (exon 34) genes were selected. All

Table 3. N-nucleotide additions and exonuclease activities in the V_HD and DJ_H junctions in carriers of different rs3124591 genotypes

Parameters	Genotypes			*p* value
	CC	CT	TT	
V_HD N-nucleotide addition (bp)	8.68 ± 1.05	9.33 ± 1.01	5.56 ± 0.83	0.014
V_HD with no addition (% of sequences)	0	8.1	16.1	0.144
V_HD N-nucleotide addition (bp) except cases with no addition	8.68 ± 1.05	10.18 ± 0.96	6.68 ± 0.83	0.032
DJ_H N-nucleotide addition (bp)	10.21 ± 2.19	7.72 ± 0.95	8.56 ± 1.10	0.443
DJ_H with no addition (% of sequences)	0	10.8	3.22	0.190
DJ_H N-nucleotide addition (bp) except cases with no addition	10.21 ± 2.19	8.68 ± 0.94	8.86 ± 1.10	0.716
V_H 3' coding end excision (bp)	1.56 ± 0.19	1.63 ± 0.25	1.73 ± 0.43	0.542
V_H 3' without excision (% of sequences)	36.8	27.03	38.71	0.415
V_H 3' coding end excision (bp) except cases without excision	1.83 ± 0.29	2.26 ± 0.27	3.05 ± 0.60	0.157
D 5' coding end excision (bp)	5.11 ± 1.10	4.33 ± 0.61	4.23 ± 0.85	0.762
D 5' without excision (% of sequences)	0	16.2	25.8	0.049
D 5' coding end excision (bp) except cases without excision	5.11 ± 1.10	5.20 ± 0.62	5.77 ± 0.96	0.848
D 3' coding end excision (bp)	5.05 ± 0.77	5.00 ± 0.71	3.73 ± 0.72	0.365
D 3' without excision (% of sequences)	15.8	18.9	22.6	0.808
D 3' coding end excision (bp) except cases without excision	6.00 ± 0.68	6.21 ± 0.72	4.86 ± 0.79	0.395
J_H 5' coding end excision (bp)	3.00 ± 0.87	3.66 ± 0.59	4.10 ± 0.64	0.581
J_H 5' without excision (% of sequences)	26.3	27.03	22.58	0.918
J_H 5' coding end excision (bp) except cases without excision	4.07 ± 1.04	5.07 ± 0.62	5.34 ± 0.62	0.510

Table 5. Structure of HCDR3 region in CLL cases with *NOTCH1* mutations (MAS – mean alignment score)

CLL cases	HCDR3 amino acid sequence	Subset
1-03*01_6-19*01_4*02_UM_EF407847	AREQWLGPSYFDY	1
1-02*02_6-19*01_4*02_UM_EU350413	ARAQWLVQLSLDY	1
1-02*02_6-19*01_4*02_UM_S42	ARLQWLWPRKLDY	1
5a*01_3-22*-1_4*02_UM_EF441753	ARIQWLLLPHFDY	1
5a*03_3-16*-1_4*02_UM_KC802107	ARLQFLGISDPFDY	1
1-02*02_1-26*01_6*02_UM_EF091912	ARPYSGSYPWYYYGMDV	28a
1-02*02_3-10*01_6*02_UM_EU667602	ARLYSGSYFYYYYGMDV	28a
1-58*01_3-3*01_6*02_UM_EF091913	AAGYDFWSGMDV	59
1-02*02_3-22*01_4*02_UM_GU358667	ERSYDSSGYYCHFDY	95
1-58*01_6-13*01_3*02_UM_EF175413	ALASSWIFDAFDI	UA/ref4
1-02*04_2-8*01_6*02_UM_EU433868	AKPSFYCTNGVCYTDYYYGMDV	Homology with CLL cases DQ100687 (MAS 68.2), EF177969 (MAS 68.2)
1-46*01_3-9*01_5*02_UM_GU358664	ARDRGYFDWLLRNGWFDP	Homology with CLL case EF441746 (MAS 72.2)
5-51*01_3-3*01_4*02_UM_JQ928948	ARHGMYYDFWSGYYLAAYFDY	Homology with CLL cases EU099117 (MAS 66.6); DQ100922 (MAS 61.9)
6-1*01_6-19*01_4*02_UM_JF810281	ARDEYWGSGWDY	
1-69*06_3-16*02_3*02_UM_GU358670	ARGGDYDYIWGSYRSNDAFDI	6
1-69*01_3-3*01_6*02_UM_EF407834	ASKSLPITIFGVVISDYYYYGMDV	9
1-69*01_6-13*01_6*02_UM_EF175412	ARVQGGSAAAYENYYYYYGMDV	UA/ref2
1-69*01_2-2*02_5*02_UM_EU350410	AREFSDIVVVPAAIIRNWFDP	UA/ref11
1-69*01_3-3*01_6*02_UM_EU667595	ARAPDFWSGYYFRGGGMDV	Homology with CLL case DQ100846 (MAS 68.4)
1-69*01_2-2*02_6*01_UM_EU433873	AREGGDIVVVPAAIISWSRYYGMDV	
1-69*01_3-22*01_4*02_UM_S9	ARWGGGAYYYDSSGYYYGFDYYFDY	
1-69*01_3-22*01_5*02_UM_KC02101	ARRNSGYYYYKEYNWFDP	
1-69*01_3-9*01_4*02_UM_EF441755	ARDSRELRYFDWLSQEGYFDY	
1-69*13_5-24*01_6*02_UM_GU358657	AREGDGYNYGYYYYYGMDV	
1-69*01_6-19*01_6*03_UM_JX462742	ARVGGYSSGWYQNYYYYYMDV	
1-69*01_3-9*01_4*02_UM_HM173329	VRMHFDWLRPAFYSFDY	
3-11*01_3-3*01_6*02_UM_EF175391	ARTYYDFWSGYDGHYGMDV	22
3-30*03_3-3*01_6*02_UM_EU814961	AKDGLGIRFLEWLSTSYYYGMDV	25
4-04*02_2-2*01_5*02_UM_EU350427	ARVPIVVVPAAVSLMRVNWFDP	Homology with CLL case EU099080 (MAS 77.2)
4-34*01_6-6*01_6*03_UM_GU358661	ARGLTYSSSSYYYYYYMDV	Homology with CLL case EF407849 (MAS 68.4)
3-53*01_3-3*01_6*02_UM_EF175410	ARDASPSLYYDFYPYGMDV	
3-64*05_6_19*01_2*01_UM_HM173330	VKDSTPGIAVAGTWGYWYFDL	
2-70*01_3-3*01_4*02_UM_S10	ARTTKLSVYDFWSGYYTGSGLGYFDY	

of them expressed UM *IGHV* genes. Such choice was due to the literature data on a rare association of the *NOTCH1* mutations with mutations of the other genes. In addition, *NOTCH1* mutations are mainly detected in CLL patients with UM *IGHV* genes. For example, in the study of Rossi *et al.* 76.5% of CLL cases with *NOTCH1* mutations in exon 34 expressed UM *IGHV* genes, and more than 90% of them did not have mutations of *TP53* gene [6]. In the study of Schnaiter *et al.*, in none of 97 fludarabine-refractory CLL patients concurrent *NOTCH1* and *SF3B1* were found, but 23.1% of *NOTCH1*-mutated cases had simultaneously *TP53* gene mutations. All revealed *NOTCH1* mutations in this study were in patients with UM *IGHV* genes [22]. In our group of 237 CLL patients, mutations of *TP53*, *SF3B1*, and c.7544_7545delCT of *NOTCH1* were found in 12.1%, 10.8%, and 13.1% of cases, correspondingly. Only single case harbored mutations in both *NOTCH1* and *TP53* genes and two cases — in *NOTCH1* and *SF3B1* gene. All except one *NOTCH1*-mutated cases had UM *IGHV* genes. Therefore, we investigated a group of CLL patients with an increased chance to identify mutations in the 3′UTR region of *NOTCH1* gene.

Non-coding mutations in the 3′UTR region of *NOTCH1* gene were first identified by Puente *et al.* [8]. Authors revealed 12 *NOTCH1* mutated cases of 176 CLL patients with UM *IGHV* genes (6.7%). The most frequent recurrent non-coding mutation was 139390152T>C, and two cases had 139390145 and 139390143 point mutations. Nadeu *et al.* detected 3′UTR mutations in 22 of 391 CLL patients (5.6%) [23]. We have identified two cases with 139390152T>C mutation and one case with 139390145T>C mutation among 87 *IGHV*-UM CLL cases (3.4%). The frequency of non-coding mutations in our group and their localization were comparable with previously published data.

Our data confirmed current data on the association between the structure of the B-cell receptor and appearance of *NOTCH1* mutations. In one of the largest series of studied stereotyped subset CLL cases (565 cases assigned to one of 10 major stereotyped subsets) the skewed distribution of *NOTCH1* mutations within exon 34 was found. Enriched mutations were cases belonged to subsets #1, #59, #99, #6, and #8. *NOTCH1* mutations were relatively infrequent in cases from subsets #3, #5, #7 and, especially, from subsets #2 and #4 [20]. In our group, five of the nine cases from stereotyped subset #1 were *NOTCH1*-mutated: three had mutations within exon 34 and two had non-coding mutations in the 3′UTR region. On the contrary, *NOTCH1* mutations were not found among nine cases of subset #7. The number of cases belonged to other subsets was too low for comparison. However, the observed differences in the frequency of *NOTCH1* mutations in subgroups of cases that expressed UM *IGHV* genes of I clan (except *IGHV1-69)*, UM *IGHV1-69* gene and other UM *IGHV* genes (59.1%, 32.4%, 12.3%, correspondingly) cannot be explained solely by the presence in the first subgroup of the cases belonged to subset #1. Excluding cases of subset #1, *NOTCH1* mutations were detected in eight of 17 remaining cases of the first subgroup (47.0%). Both cases of subset #28a were *NOTCH1*-

Table 6. CLL cases showing HCDR3 homology with immunoglobulins of known specificity (MAS – mean alignment score)

IGHV gene	HCDR3 amino acid sequence	MAS	Subset	Antigen specificity
CC genotype				
3-11*01_3-3*01_6*02_UM_LM647765	ARA--YDFWSGY-YFERYGMDV			Peripheral blood B lymphocyte
3-11*01_3-3*02_6*02_UM_JQ928963	AR**WGP**YDFWSGY**S**Y**YYY**YGMDV	68.1	22	
1-69*01_2-2*01_6*02_UM_AB204177	AR_GGYCSSTSCTILSYYYYGMDV			Umbilical cord blood CD19$^+$IgD$^+$CD27$^-$CD38$^+$ B lymphocyte
1-69*01_2-2*02_6*04_UM_JX462747	AR**WS**GYCSSTSC**MGAD**YYYYGMDV	75.0		
4-39*01_3-10*01_5*02_UM_AY607328	ARRLSYYYGSGSYYNWFDP			Term cord blood
3-11*01_3-10*01_5*02_UM_JQ928962	AR**DNVL**YYGSGSY**F**NWFDP	73.0		
4-34*01_3-3*01_6*03_UM_LM647851	ARGRVGYDFWSGS-PYYYYYYMDV			Peripheral blood B lymphocyte
1-69*01_3-3*01_6*03_UM_KC802110	ARGR**N-**YDFWSG**PTWG**YYYYYMDV	73.0	7	
TC genotype KC802096				
4-34*01_2-2*02_4*02_UM_AY607318	ARTIIVVVPAAIRWFDP			Term cord blood
4-34*01_2-2*02_4*02_UM_KC802096	AR**ED**IVVVPAAL**YY**FD**Y**	70.6		
3-11*04_3-10*01_4*02_M_AB202917	ARDSLWFGEFMY--FDY			Tonsillar B cell
3-11*01_3-10*01_4*02_UM_EU433870	ARD**T**LWFGEF**HAYY**FDY	70.6	UA/ITA_13	
1-8*01_-0*01_3*02_UM_EU571887	ASVLGTYYYGSGSYYDAFDI			IgM$^+$ B lymphocyte 18 week gestation fetus spleen
4-30-4*01_3-0*01_3*02_UM_GU358680	ARK--TYYYGSGSYYDAFDI	77.7		
6-1*01_6-6*03_6*03_UM_LM648697	AREGSSWSGNYYYYYYMDV			Peripheral blood B cell
3-49*03_6-13*01_6*-3_UM_EF175429	**TSHS**SSW**DYY**YYYYYYMDV	63.1		
3-33*01_6-19*01_6*02_UM_AF174112	ARDRLAVAGTVYYYYGMDV			Elderly B cell
3-33*01_4-11*01_6*02_UM_EF407833	ARD**LH**AV**TTRN**YYYYGMDV	68.4	44	
3-07*1_3-22*01_6*01_UM_DQ454347	ARV-GDYYDSSGYYYYYYGMDV			Neonate peripheral blood
4-59*_3-22*01_6*02_UM_GU358662	AR**GL**GDYYDSSGY**LH**YYYGMDV	81.8	50	
3-33*01_3-3*01_6*02_UM_AY607518	ARDTPY-DFWSGYYYYYYGMDV			Term cord blood
3-33*01_3-3*01_6*02_UM_JX462745	ARDT**RVD**DFWSGY**FV**YYYGMDV	77.0	22	
3-30*03_3-22*01_6*02_UM_LM648585	ARDWASRDSSGYYYL-GYYYYMDV			Peripheral blood B cell
3-07*1_3-22*01_6*02_UM_EU814965	ARD**TYYY**DSSGYYY**PYY**YYY**G**MDV	65.0		
1-69*01_2-2*01_6*02_UM_LM648973	ARDS--DIVVVPAARGPYYYGMDV			Peripheral blood B cell
1-69*01_2-2*01_6*02_UM_EF091906	AS**PGPQ**DIVVVPAA**Y**YYYYYGMDV	79.0		
1-8*01_2-2*01_6*02_UM_AJ414783	AR--VTGYCSSTSCTKVYYYYYGMDV			B cell
3-21*-1_2-2*01_6*02_UM_EF091902	AR**NRY**T**E**YCSSTSC**HPS**YYYYYGMDV	73.0	41	
3-33*01_3-3*01_6*02_UM_EF541600	ARDISTDFWSGYYT--GSYYYGMDVW			Ig in X-HIgM Syndrome patient
1-2*01_3-3*01_6*02_UM_EU667601	AR**GV**S**YD**FWSGYY**IRE**G**D**YYYGMDV	72.0	7D	
5-10-1*01_3-10*01_3*02_M_JF274048	ARRATYYYGSGSYFDAFDI			Anti-Influenza H5N1 Viruses
4-30-4*01_3-0*01_3*02_UM_GU358680	AR-**K**TYYYGSGSYYDAFDI	89.7		
1-69*13_2-2*01_6*02_UM_U86795	AGT-IVVVPAAGGIFFYYYGMDV			Heterohybridoma derived from CD5$^+$ CLL B lymphocytes with rheumatoid factor activity
1-69*01_2-2*01_6*02_UM_HM173323	A**RT**D**I**VVVPAA**MI**I-**Y**YYYGMDV	72.7		
1-69*06_3-16*02_3*02_UM_AF460965	ARGGNYDYIWGSYRSNDAFDI			Antiphospholipid antibodies
1-69*01_3-16*02_3*02_UM_EF091909	ARGG**D**YDYIWGSYR**P**NDAFDI	90.5	6	
1-69*01_3-16*02_3*02_UM_GU358676	ARGGNYDYIWGSYR**T**NDAFDI	95.0	6	
PH0955	ARVSIFGVVQHYYYYYYMDV			Rheumatoid factor
1-69*01_3-3*01_6*02_UM_KC802106	ARV**QV**FGVV**NT**YYYYYYMDV	80.0		
TT genotype				
4-34*01_3-3*01_6*03_UM_LM647851	ARGRVGYDFWSGS-PYYYYYYMDV			Peripheral blood B cell
4-34*01_3-3*01_6*03_UM_EF091922	ARG**FG**YYDFWSG**TH**P**PN**YYY**Y**MDV	70.8		
3-21*01_6-13*01_6*02_UM_LM647465	ARDQGSSSSWFDYYYYGMDV			Peripheral blood B cell
3-21*01_6-13*01_6*02_UM_EF091900	ARD**R**G**V**SSSW**YLS**YYY**Y**MDV	70.0	26	
1-8*01_6-13*01_4*01_UM_LM647894	AR-GARYSSSWYPFDY			Peripheral blood B cell
3-30*01_6-13*01_4*01_UM_HM173331	AR**V**G**TG**YSSSWYPFDY	81.0		
3-74*02_3-3*01_6*02_UM_LM647144	AAIYDFWSGYWSYYYYYGMDV			Peripheral blood B cell
3-48*03_3-3*01_6*02_UM_JQ928950	A**RD**YDFWSGY**YA**YYYYYGMDV	31	80.9	
3-7*01_3-3*01_6*02_UM_LM648392	ARGLYDFWSGYYPHYYYYGMDV			Peripheral blood B cell
1-69*01_3-3*01_6*02_UM_S17	A**AQ--**DFWSGYYPHYYYYGMDV		60.0	
3-64*01_2-2*03_6*02_UM_EF541666	ARDGYCSSTSCYLDGGLYYY-GMDV			Ig from X-HIgM patient
1-2*04_2-15*01_6*02_UM_JF810257	AR**E**GYCSG**G**SCY**PPPGN**YYYYY**Y**GMDV	69.0	UA/ITA_17	
3-48*02_3-3*01_6*02_UM_AF077457	ARDSTIFGVVII-DYYYGMDV			Ig from X-HIgM patient
3-30*03_3-3*01_6*02_UM_EU433876	**RAQM-**IFGVVII**E**DYYYGMDV	70.0	9	
3-30*03_-3*01_6*02_UM_EF542369	ASHYDFWSGHYEPYYYYGMDV			Ig from X-HIgM patient
3-48*03_3-3*01_6*02_UM_JQ928950	A**RD**YDFWSGY**YA**YYYYYGMDV	31	80.9	
1-2*04_3_3*01_6*02_UM_AY686915	ARES--YYDFWSGKRN-YYYYGMDV			Anti-rotavirus Ig
1-2*04_3_3*01_6*02_UM_EU433869	AR**DGLQ**YYDFWSG**SDLA**YYYYGMDV	68.0	7D	
3_11*05_3-3*01_5*02_UM_DQ322854	ARDTRPYDFWSGYYP-NWFDP			Anti-pneumococcal polysaccharides Ig
4-61*01_3-3*01_5*02_UM_EF091926	ARH**RGD**YDFWSGYYP**Y**NWFDP	80.9	UA_5	
4-59*01_3-3*01_6*03_UM_FJ385336	ARDLTYYDFWSGYYPDYYYY-MDV			Anti-respiratory syncytial virus Ig
4-34*01_3-3*01_6*03_UM_EF091922	AR**GFG**YYDFWSG**TH**P**PN**YYY**Y**MDV	66.6		
1-2*02_1-26*01_6*02_UM_AY944713	ARVYSGSYPYYYYGMDV			Anti-rotavirus Ig
1-2*02_1-26*01_6*02_UM_EF091912	AR**P**YSGSYP**W**YYYGMDV	88.0	28	
3-21*01_6-13*01_6*02_UM_KJ409179	ARDKEYSSSWYLYPYYYYMDV			Anti-HCV Ig
3-21*01_6-13*01_6*02_UM_EF091900	ARD**RGV**SSSWYL-**S**YYYYMDV	75.0	26	
1-18*01_1-26*01_4*02_M_JX213637	ARDVQYSGSYLGAYYFDY			Anti-influenza B viruses Ig
4-59*01_1-26*01_4*02_UM_JX462751	AR**HDP**YSGSYL**V-**YYFDY	70.5		
3-11*01_3-3*01_5*02_UM_DQ322847	ARDTRPYDFWSGYYP-NWFDP			Anti-pneumococcal polysaccharides Ig
3-11*01_3-3*01_5*02_UM_EU667604	ARD-R-YDFWSGY**IGY**NWFDP	73.6	UA_5	
4-34*01_3-3*01_4*02_UM_X54441	ARGGSVLRFLEWLLYPAFDY			Rheumatoid factor
1-69*01_3-3*01_4*02_UM_EU667599	AR**S**G**E-**LRFLEWLL**SAD**FDY	68.4	UA_7	
3-33*01_4-17*01_3*02_UM_AJ305181	AKGDYGDYSFAFDI			Anti-SLE Ig
3-33*01_4-17*01_3*02_UM_EF407846	A**R**GV**P**GDY**VM**AFDI	71.0	UA_9	
3-30-3*01_4-17*01_3*02_UM_EF407832	A**R**G**PR**GDY**VSS**FD**L**	57.0	UA_9	

mutated (mutations within exon 34 and non-coding region). The occurrence of UM *IGHV* genes of I clan (except *IGHV1-69)* among *NOTCH1*-mutated cases was twice as high as in the general group. Therefore, it can be suggested that expression of UM *IGHV* genes of I clan (except *IGHV1-69)* is a risk factor for the presence of *NOTCH1* mutations. On the contrary, the probability of detecting *NOTCH1* mutations was lower in the carriers of other UM genes (it should be noted that subset #8 was absent in our cohort).

When searching for non-coding mutations in the 3´UTR region of the *NOTCH1*, we paid attention to rs3124591, which is localized in the amplified region. Preliminary data suggested its functional significance. Quan *et al.* found association of CC genotype of rs3124591 (16.4% among cases and 11.1% in the control group) with the risk of lung cancer in northeast Chinese non-smoking females [24]. In study of Gao *et al.*, also performed in Chinese population, CC genotype of rs3124591 was absent in the control group (n = 100) and among patients with invasive ductal carcinoma (IDC, n = 100) and ductal carcinoma *in situ* (DCIS, n = 50) [25]. The C allele was significantly associated with high risk of DCIS, but not IDC; the TC genotype was significantly associated with an increased risk of IDC and DCIS and poorly differentiated IDC. In addition, Notch1 protein expression was significantly higher in DCIS patients with the TC genotype. Although Notch1 protein expression was higher in IDC patients with the TC genotype, this association did not reach significance. Authors concluded that the impact of the rs3124591 variant on Notch1 protein expression mainly occurs early in IDC development.

In our study, rs3124591 did not affect the risk of CLL and survival parameters of patients. At the same time, differences were found in the frequency of *IGHV* gene usage and in the structure of HCDR3 in carriers of individual genotypes. Leukemic cells of CC homozygotes expressed the most limited spectrum of UM *IGHV* genes (mostly *IGHV1-69* and *IGHV3-11*), and their HCDR3 sequences were homologous only with the sequences of normal B-cells. Conversely, leukemic cells of TT homozygotes used the largest number of UM *IGHV* genes, more frequently had HCDR3 homology with antibacterial or antiviral Ig clones, had less N nucleotide additions in DJ_H junctions, and the number of sequences that lacked N additions at DJ_H junctions had a tendency to be higher. By two last parameters, they were a bit like a memory B cells, characterized by Tian *et al*. [24]. It is known that Notch1 signaling regulates B and T lymphocyte development [27–30]. Taking into account data of Cao *et al.* [25] on the functional significance of rs3124591, we hypothesized that rs3124591 could influence on the selection of B-cell clones during early stages of CLL development. This assumption are in an agreement with reports regarding the role and possible mechanisms of NOTCH signaling in regulation of the normal B-cell repertoire, summarized by Cruickshank and Ulgiati [31].

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