Chronic lymphocytic leukemia (CLL) is the most common leukemia of the adult population in Europe and the US. Several prognostic markers have been reported, which correlate with the clinical course and guide treatment decisions. Mutations in the NOTCH1 gene have recently been identified as new genetic alterations associated with shorter time-to-first-treatment and progression-free survival (PFS) [1–4]. Furthermore, clinical resistance to the anti-CD20 monoclonal antibodies in CLL patients with mutated NOTCH1 was found in some clinical trials, which manifested as a lack of benefit from the addition of rituximab to fludarabine-cyclophosphamide, or ofatumumab to chlorambucil [5–8].

The NOTCH1 gene encodes for a transmembrane receptor, which functions as a ligand-activated transcription factor. Upon binding with ligands of the Jagged or Delta families, a cascade of proteolytic cleavages take place that result in the release of NOTCH1’s intracellular domain from the membrane, translocation to the nucleus and subsequent activation or repression of target genes through interaction with other transcription factors such as CBF-1 [9]. The NOTCH1 signalling is involved in critical cellular processes such as proliferation, apoptosis and differentiation [10]. Alterations in NOTCH1 signalling have been reported in different diseases including several cancers [11].

In CLL NOTCH1 mutations are detected in 8–15% cases regardless of clinical phase of disease and are represented mostly by a recurrent 2-bp frameshift deletion (c.7541_7542delCT) localized in the exon 34. This deletion results in removal of the C-terminal PEST domain, which regulates protein turnover to the ubiquitin–proteasome complex for subsequent degradation, thereby leading to accumulation of constitutively active protein and deregulation of NOTCH1-dependent pathways [12]. Constitutive activation of NOTCH1 signalling is considered to contribute to apoptosis resistance and increased survival of CLL cells [13, 14]. For detection of NOTCH1 mutations conventional DNA sequencing techniques are generally used. Besides, Rossi et al. [1] have designed polymerase chain reaction (PCR)-based amplification refractory mutation system (ARMS) method for screening of the most common NOTCH1 c.7541_7542delCT mutation.

The aim of this paper was to evaluated real-time PCR assay system for detection of NOTCH1 c.7541_7542delCT mutation. We have not found any reports of the using real-time PCR for this purpose in the available literature.

**MATERIALS AND METHODS**

A total of 325 CLL patients referred to the State Institution “National Research Center for Radiation Medicine of the National Academy of Medical Sciences of Ukraine” (NRCRM) were included in study. CLL diagnosis was established according to standardized criteria of International Working Group on CLL [15]. Immunophenotyping of peripheral blood cells was performed in Laboratory of Immunocytochemistry, Department of Clinical Immunology, NRCRM. The staging was obtained according to both the Rai and Binet systems [16, 17]. The study was approved by the...
Ethical Committee of NRCRM and all patients gave informed consent.

Molecular studies were performed on DNA samples extracted from peripheral blood mononuclear cells with the QIAamp Blood Mini Kit (Qiagen, United Kingdom). In all observed CLL patients the presence of NOTCH1 c.7544_7545delCT mutation was investigated by ARMS using primers and PCR parameters developed by Fabbri et al. [14] with very little modifications. In brief, two forward primers (specific for the mutant allele, ForMUT: 5'-TCCTCACCCGGTCCCGA-3'; for both mutant and wild-type alleles, ForC: 5'-GTGACCGCAGCCCAATTGTT-3') and common reverse primer (Rev, 5'-AAGGCTTGGGAAAGGAAGC-3') were used. PCR reaction was performed with 50 ng of DNA in a 30 μL of final volume reaction mixture containing 15 ml 2X PCR Master Mix (Fermentas, Lithuania) and 0.1; 0.4 and 0.5 μM of ForC, ForMUT and Rev primers, respectively. PCR conditions were: 95 °C for 3 min followed by 30 cycles at 95 °C for 30 s, 59 °C for 40 s, 72 °C for 40 s with a final extension at 72 °C for 40 s in Bio-Rad C1000 thermal cycler. Amplified PCR products were separated on 2% agarose gel and visualized after staining with ethidium bromide. The 283 bp band indicated the wild-type allele was observed in all samples, whereas the additional band of 183 bp was found only in NOTCH1-mutated cases.

To confirm specificity of ARMS PCR for NOTCH1 c.7544_7545delCT detection, 5 DNA samples which were detected as positive and 5 negative samples were analysed by direct Sanger sequencing. Additionally, 7 cases which were doubtful with ARMS PCR also were sequenced. For this, PCR amplification was performed on 50 ng of DNA using abovementioned ForC and Rev primers (0.5 μM each) and 2X PCR Master Mix (Fermentas, Lithuania) in a total volume of 25 μL, and the same as for ARMS cycling conditions. Amplicons were spin column purified with PCR purification kit (Promega, USA) and sequenced directly using Rev primers and BigDye Terminator Cycle Sequencing Reaction Kit (Perkin Elmer, USA). Analysis was performed in an automated DNA sequencer ABI-310 (Applied Biosystem, USA) as reported earlier [18]. Data were compared with NOTCH1 germline sequence (RefSeq NM_017617.3).

Real-time PCR for NOTCH1 c.7544_7545delCT mutation was developed using the same ForMUT and Rev primers as for ARMS method and 2X Absolute Blue qPCR SYBR Green Mix (Thermo Scientific, USA) on the Bio-Rad IQ Real-time System. PCR reaction was performed in a final volume of 25 μL with 0.175 μL of each primer and 50 ng DNA. PCR conditions were: 95 °C for 15 min followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s. The program was ended with a dissociation curve analysis to verify the product and identify the presence of spurious PCR bands or primer dimmers. Each sample was assayed in duplicate. PCR standard curve was generated by a serial dilution of the NOTCH1-mutated DNA sample (50; 10; 2; 0.4; 0.08 ng). Amplification of the reference β-microglobulin (B2M) gene (forward primer 5'-CGGGCATTCCTGAAGCTGA-3' and reverse primer 5'-GGATGAGAACCAGACACATAG-3') was used as the internal control. The reaction mix without DNA template was used as the negative control. Levels of NOTCH1 c.7544_7545delCT in samples relative to the reference gene were evaluated based on the value of delta threshold cycles (∆Ct).

All 33 samples harboring c.7544_7545delCT and 10 cases lacking c.7544_7545delCT by ARMS were submitted to real-time PCR.

Statistics were performed using the SPSS 17.0 software package (SPSS, USA). Data shown are the means plus or minus standard deviations, and medians. The comparisons were analyzed with t-test. Correlations of ∆Ct and hematologic parameters of patients were assessed using Pearson’s correlation. Kaplan—Meier curves and log rank test were used to determine PFS for NOTCH1-mutated cases. p-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

NOTCH1 c.7544_7545delCT mutation was found by ARMS in 33 (10.1%) of 325 CLL patients (Fig. 1). This frequency was comparable with data from other CLL cohorts [19–21]. Results of ARMS reaction were apparent in 31 cases, but in 2 cases a weak mutational signals of a typical mobility were observed. Besides this, in 5 patients weak bands with mobility which was a little different from the 183 bp band of NOTCH1-mutated positive control were revealed. In repeated studies, these bands have been not identified, and cases were regarded as “presumably negative”.

![Fig. 1. Results of NOTCH1 c.7544_7545delCT mutation detection in CLL patients by ARMS method. The additional band of 183 bp identify of NOTCH1-mutated cases.](image-url)
85% of the blood leukocytes were CD19+/CD5+/CD23+ lymphocytes). Specific amplification of NOTCH1 deletion was found in all dilutions of DNA, including maximal (0.08 ng), which corresponds to approximately 10% of mutant alleles in the sample. The melting curve analysis revealed that the amplicon containing c.7544_7545delCT mutation melted at 91 °C in the form of a clear single peak. Correlation coefficient of the standard curve for NOTCH1 was 0.984 with a slope value of −3.38 (PCR efficiency — 97.6%) (Fig. 3). Correlation coefficient of the standard curve for B2M was 0.978 with a slope value of −3.44 (PCR efficiency — 95.0%). Thus, amplification efficiency of both genes was comparable, and B2M can be used as a referent for NOTCH1 detection. These obtained data regarding sensitivity of real-time PCR for detecting NOTCH1 mutations showed no significant difference from other methods (ARMS and direct DNA sequencing, which allow detecting mutations present in at least 10% of the alleles) [1, 22].

Amplification of the reference B2M gene using real-time PCR was similar in NOTCH1-mutated and NOTCH1-unmutated cases according ARMS. The mean ± SD of C_r values for B2M was 23.77 ± 0.84 and 24.51 ± 1.24, correspondingly (p = 0.234).

All 33 CLL cases with c.7544_7545delCT by ARMS were positive by real-time PCR also. The mean ± SD of C_r values for NOTCH1 was 28.17 ± 1.87, and median of ΔC_r was 3.9 (range 1.4–7.5 cycles), and the mean ± SD of ΔC_r was 3.9 ± 1.4. Specificity of reactions was confirmed by the melting curve analysis. In the most cases (26 of 33; 78.8%), ΔC_r values ranged from 1.4 to 4.9 cycles, and ΔC_r values more than 7 cycles (7.1 and 7.5 cycles) were observed only in 2 cases (weak mutational signal by ARMS reaction).

We did not observe a specific amplification in NOTCH1-unmutated cases, including "presumably negative" cases: in seven cases any amplification was found during the 40 cycles of the reaction, in other cases the mean ± SD of C_r values for NOTCH1 was 37.04 ± 2.6, median of ΔC_r was 10.9 (range 10.4–21.8 cycles), a clear single peaks in the melting curve were absent (Fig. 4). Thus, the application of real-time PCR allows to clearly identify CLL patients with c.7544_7545delCT NOTCH1 mutation even in cases of doubt by ARMS reaction. In such cases the use of real-time PCR is expedient as it has some advantages over DNA Sanger sequencing (time- and cost-effective).

Real-time PCR also can be used to determine the size of cell’s clone harboring mutations. Prognostic significance of quantitative assessment of some disease-associated genetic markers, which reflect tumor burden, was shown in CLL and other lymphoproliferative disorders [23–26]. However, as it was found by high-throughput sequencing and CD19+-selected cells, NOTCH1 mutations generally do not occur during...
In conclusion, we evaluated real-time PCR assay for detection of NOTCH1 mutations in comparison with ARMS method. Specificity and sensitivity of both techniques were comparable. NOTCH1 c.7544_7545delCT mutation was found in 10.1% of CLL patients, which is consistent with other reports [19–21, 27]. However, the results of ARMS in a minor number of cases (7 of 325 cases; 2.15%) were doubtful and required re-investigation. Sanger sequencing allows to precisely identify deletion, but it is quite laborious and more expensive than conventional PCR-based methods. In this context real-time PCR being time- and cost-effective, extends the opportunities for an objective assessment of the amplification’s specificity and thus might be used for fast screening for NOTCH1 c.7544_7545delCT mutation. Furthermore, under certain conditions it might allow a quantitative assessment of NOTCH1-mutated clone. CLL patients harboring NOTCH1 deletion showed a significantly shorter PFS in comparison with NOTCH1-unmutated cases sustaining reported adverse impact of this alteration on outcome [1, 20, 21]. Besides the prognostic implications, NOTCH1 mutations might guide treatment options in the context of advisability of anti-CD20 monoclonal antibodies addition [5–7], and also might provide a therapeutic target for NOTCH1 pathway inhibitors. The use of NOTCH1 inhibitors in combination with chemotherapy is considered as a promising approach for the treatment of CLL cases with NOTCH1-activating mutations [28].

REFERENCES


