Prognostic significance of NOTCH1 and FBXW7 mutations in pediatric T-ALL

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Abstract. The NOTCH signaling pathway plays an important role in the development of multicellular organisms, as it regulates cell proliferation, survival, and differentiation. In adults, it is essential for the T- or B-lymphocyte lineage commitment. NOTCH1 and FBXW7 mutations both lead to the activation of the NOTCH1 pathway and are found in the majority of T-ALL patients. In this study, the mutation analysis of NOTCH1 and FBXW7 genes was performed in 87 pediatric T-ALLs who were treated on the ALL-BFM protocols. In 19 patients (22%), activating NOTCH1 mutations were observed either in the heterodimerization domain or in the PEST domain and 7 cases (10%) demonstrated FBXW7 mutations (2 cases had both NOTCH1 and FBXW7 mutations). We also analyzed the relationship of the mutation data between the clinical and biological data of the patients. NOTCH1 and FBXW7, NOTCH1 alone were found correlated with lower initial leucocyte counts which was independent from the sex and T-cell immunophenotype. However, NOTCH1 and FBXW7 mutations were not predictive of outcome in the overall cohort of pediatric T-ALLs.

Keywords: T-ALL, NOTCH1, FBXW7, mutation, prognosis

1. Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy of thymocytes, generally characterized by very high circulating blast cell counts, mediastinal masses and central nervous system involvement [1,2]. It affects both children and adults and is rapidly fatal [3]. Although the outcome of pediatric ALL patients has improved in recent decades due to intensified therapies, less success has been achieved in adult T-ALL [1,4–7]. Malignant transformation of T-cell is caused by chromosomal translocation and other genetic, epigenetic abnormalities which lead to loss of cell-cycle control, unlimited self-renewal capacity, impaired differentiation and increased blastic cell proliferation [8].

Less than 1% of T-ALL shelter chromosomal translocation t(7;9) (q34;q34.3) involves NOTCH1 [9]. Weng et al. demonstrated activating NOTCH1 muta-
tions in more than 50% of T-ALL [12]. This observation gives an idea that NOTCH has an important role in T-ALL pathogenesis. It is reported that NOTCH signaling is also activated in cancers of other tissues with epithelial origin like breast, salivary gland, and colon [13–16].

The extracellular part of NOTCH1 comprises 36 epidermal growth factor (EGF)-like repeats, three Lin12/Notch repeats (LNRs), one N-terminal heterodimerization domain (HD-N) and one C-terminal heterodimerization domain (HD-C). The intracellular subunit consists of a RAM domain, a strong transcriptional activation domain, seven iterated ankyrin-like repeats and a C-terminal PEST domain [17–19]. By binding of NOTCH ligands, NOTCH receptor experiences a series of tightly reduced proteolytic cleavages followed by the translocation of the intracellular domain of NOTCH1 (ICN) into the nucleus where NOTCH interacts with other factors and regulates the transcription of many genes [20–22].

NOTCH1 mutations have been detected within HD-N, HD-C and PEST domains. These mutations cause up-regulation of NOTCH1-dependent signal transduction [12]. Mutations of HD-N and HD-C increase active ICN by destabilizing the intersubunit association [23,24]. Mutations of the PEST domain enhance ICN turn over [25]. Loss-of-function mutations of FBXW7 gene also lead to NOTCH1 activation by, inhibition of ubiquitin-mediated degradation of the activated form of NOTCH1 [26,27]. Several studies have reported mutations of FBXW7 gene in various tumors including endometrial cancers, T-ALL and inactivation of FBXW7 has been shown to lead to an increased genetic instability [28–30]. However, the clinical effect of NOTCH1 mutation in T-ALL still remains controversial and NOTCH1/FBXW7 mutations remain to be investigated. In this study, we analyzed the clinical and prognostic significance of NOTCH1/FBXW7 mutational status in 87 pediatric T-ALL patients.

2. Design and methods

2.1. Patients

Bone marrow/peripheral blood samples were obtained from 87 pediatric patients with T-ALL, who were diagnosed between 1997 and 2008 in Department of Pediatrics, Bakirkoy Maternity and Childrens Hospital; Pediatric Hematology Division of Istanbul Medical Faculty, Istanbul University; Unit of Hematology, Sisli Efthal Education and Research Hospital; Unit of Pediatric Hematology, Ministry of Health Gозtepe Teaching Hospital; Department of Internal Medicine, Haseki Education and Research Hospital, Istanbul, Turkey. At the time of diagnosis, bone marrow (n = 77) or peripheral blood samples (n = 10) from T-ALL patients (median age of 7, 6 years; min, 22 days- max, 16 years) were included in this study. Relapse material was available only from one of the patients and all samples were stored at −80°C after homogenization in RLT buffer (Qiagen, GmbH, Germany). Male, female ratio is 2.5:1. Immunophenotypic subgrouping of T-ALL is done according to the European Group for the Immunological Characterization of Leukemia (EGIL) guidelines [31]. All T-ALL patients received chemotherapy according to the ALL-Berlin-Frankfurt-Munster (BFM) 2000 protocol. The study was approved by the ethical committee of Istanbul Medical Faculty, Istanbul University and informed consents were obtained from all participating patients.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated by Qiagen RNeasy Protect kit (Qiagen, GmbH, Germany). RNA samples were treated by DNAse (1U/µg) for the possible DNA contaminations (MBI Fermentase, Germany). 1µg of total RNA was used for cDNA synthesis using random hexamers and MMLV reverse transcriptase according to the protocol provided by the manufacturer (MBI Fermentase, Germany) [32].

2.3. Detection of NOTCH1 mutations

N-terminal region of heterodimerization domain (HD-N) (exon 26), the C-terminal region of the heterodimerization domain (HD-C) (exon 27), the PEST domain (exon 34) of the NOTCH1, and exons 7,8,9,10 and 11 of WD40 domain of FBXW7 were amplified by using standard protocol [33]. Because of low sensitivity (20%) of direct sequencing, we used denaturing high-performance liquid chromatography (dHPLC) (WAVE DNA Fragment Analysis System, Transgenomics) technique, which have a sensitivity of 1–5% in mutation detection, to increase the sensitivity in addition to the sequencing [34–36]. All sequencing chromatograms analyzed by CLC combined Workbench software (V.3.6.1, Denmark).
Table 1

<table>
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<th>Patient no.</th>
<th>NucleotideΦ</th>
<th>Amino acid</th>
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<tr>
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<td>R479Q</td>
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<td>1393 C&gt;T</td>
<td>R465C</td>
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<td>-</td>
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<td></td>
<td>4796 G&gt;C</td>
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<td></td>
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<td>D400D</td>
<td>T28</td>
<td>4757 T&gt;C</td>
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<td>5064 G&gt;T, 5072 C&gt;T, 5082 ins 5083</td>
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Abbreviations: ins, insertion; dup, duplication. Φ Nucleotid number is according to the GenBank accession number NM_033632. Nucleotid number is according to the GenBank accession number NM_017617. Novel FBXW7 mutations are indicated by "♦" next to the sample identification number. Novel NOTCH1 mutations are indicated by "✸" next to the sample identification number. Nonsense mutations are indicated by an asterisk behind the amino acid number.

2.4. Statistical analyses

Proportional differences between groups were analyzed by X² test or Fisher’s exact tests. The median follow up was 11.8 months (min 1- max 133 months). Overall survival was defined by the interval from the date of diagnosis to the date of death or last follow-up. Relapse-free survival was the duration from the date of complete remission to the date of analysis or to the first event (failure to achieve remission (early death or resistant leukemia), relapse or death in complete remission). The Kaplan-Meier method was used to estimate survival rates. We compare the differences with the 2-sided log-rank test. Multivariate survival analysis was estimated according to the Cox regression model. All statistical analyses were done by SPSS 16.0 software and GraphPad Prism software.

3. Results

A total of 87 T-ALL patients were analyzed for NOTCH1 gene mutations. Heterozygous NOTCH1 mutations were found in 19 (22%) of 87 T-ALL samples. We have observed mutations in HD domain in 11 patients (12%), in the PEST domain in 6 patients (7%) and in both domains in 2 patients (3%). All the detected NOTCH1 mutations were listed in Table 1. Nine of the identified mutations were new mutations, whereas the remaining mutations had been reported previously [12, 37]. Most of the HD domain mutations were located at the HD-N region (n = 8) within the conserved amino acid residues between 1554 and 1610. All of the HD-N mutations were point mutations and 7 of the mutations were reported in previous studies [12,37]. 6 cases had L1586P/L1575P/R1599P missense mutations which increase the instability of the HD site [37]. One case had K1608N mutations which interrupt the conjunction of heterodimer. The initial and the relapse materials of one patient were available. That patient had the same point mutation (R1599P) of HD-N site both in the initial and the relapse samples. HD-C mutations were less common (n = 3) and clustered in amino acid residues 1681 to 1737. Within the HD-C domain, the patients had thymine insertions and one patient al-
so had point mutations. All variations created premature termination codons in exon 28/29. (Figs S1 and S2). Furthermore, a single nucleotide polymorphism C5097T was observed in the HD-C domain in 20 (23%) of 87 T-ALL patients [12].

The PEST mutations were observed in eight patients and clustered in amino acid residues 2375 to 2571. Two of the PEST domain mutations have been reported before [12]; on the other hand, seven of them were new mutations. Seven of nine PEST domain mutations induced premature stop codons. All of these premature termination codons were not expected to activate nonsense-mediated decay. Therefore, it is likely that these mutated mRNAs result in the production of C-terminally truncated proteins [38].

FBXW7 mutations were found in 7 (10%) of the 72 analyzed T-ALL samples (see Table 1). Two of the PEST domain mutations had co-existing NOTCH1 mutations, two in the HD domain (Tables 1 and 2).

The clinical and biological characteristics of the patients in this study are shown in Table 3. NOTCH1 and/or FBXW7 wild type and mutated cases (Fig. 1). Kaplan-Meier estimate of the probability of survival according to NOTCH1 mutations alone and FBXW7 mutations alone presence no significant difference (p = 0.38; p = 0.63).
Fig. 1. Outcome of pediatric patients with T-ALL treated on BFM protocol. (A) Event-free survival by NOTCH1 mutation. (B) Overall survival by NOTCH1 mutation. (C) Event-free survival by FBXW7 mutation. (D) Overall survival by FBXW7 mutation. (E) Event-free survival by FBXW7 and/or NOTCH1 mutation. (F) Overall survival by FBXW7 and/or NOTCH1 mutation. *P* value of 0.05 or less (two sided) was considered to indicate a statistically significant difference. None of the Kaplan-Meier analyses showed significant differences.

4. Discussion

In this study, we found 10% FBXW7 mutations and 22.2% NOTCH1 mutations in T-ALL patients. Mutations in NOTCH1 receptors domains are characterized with the up-regulation of NOTCH1-dependent signal transduction. HD domain mutations cause subunit dissociation or induce NOTCH1 to become susceptible to ligand-independent cleavage, resulting in increased ICN1 liberation and translocation to the nucleus [23,24,39]. Three of the patients showed L1575P mutations. This mutation, reduces the efficiency of furin cleavage and induce γ-secretase dependent NOTCH1 signaling and shows sensitivity to GSIs (γ-secretase inhibitors) (Table 1) [12,37]. The PEST domain is located in the C-terminal part of NOTCH1, which regulates protein turnover. Some deletions in this region increase the stability of the protein and up-regulation of NOTCH1 signaling [40–42]. Here, we define novel mutations as well as previously reported ones inside the PEST domain (Table 1) [12,38,43]. The novel mutations reside in the regions that control the protein stability, which
made us think that these mutations might cause the gain of function. The novel HD domain mutations are predicted to cause a substantial change of secondary structure by involving non-conservative amino acid substitution. Although this region is known to harbor most of the mutations, it can not be excluded that some of the newly identified mutations may represent polymorphism. The effect of novel mutations on ICN1 activity needs to be evaluated by functional analysis.

Mutations in the conserved protein-protein interaction domains of FBXW7 (R465C and R479Q) abrogate NOTCH1 binding and lead the NOTCH1 activation [33,44]. FBXW7 mutations have been associated with the HD domain which cause signal amplification, whereas FBXW7 mutations in PEST domain are less frequent [33,44,45]. Similar to other pediatric studies, we found no co-existing mutations in the PEST domain and the FBXW7 gene (Table 2). It has been proposed that mutations in the PEST domain relieve mutational pressure on FBXW7. Disruption of FBXW7 function may active in sustaining NOTCH signaling and also may prolong the half-life of substrates such as MYC [27,33,44]. Four novel mutations were found in FBXW7 and all of them were positioned in the evolutionary conserved side of the gene. Together the location and conservation statues suggest that the novel missense mutations may affect FBXW7 substrate targeting.

Currently, there are no genetic markers that reliably predict the treatment response or outcome for T-ALL [3,38]. Some genes (HOX11, TAL1, LYL etc.) have been reported to indicate a favorable or an unfavorable prognosis in a small number of patients and mRNA profiles might be used as a prognostic marker in the future [46–49]. The correlation between the NOTCH1 mutations and the clinical features and differences in disease outcome in T-ALL remains controversial [38,50]. Breit et al. [38] and Park et al. [51] revealed that NOTCH1 mutations are good prognostic markers for disease outcome where as Zhu et al. [52] showed that the NOTCH1 mutations are correlated with increased survival time. Furthermore van Grotel et al. [53], in spite of a high mutation rate (57%), did not show any correlation with outcome. In this study, we couldn’t find any significant correlation between the presence of activating NOTCH1 and/or FBXW7 mutations and long-term treatment outcome, perhaps because the short term follow up of the patients. It is compatible with study of Grotel et al. but much larger studies of pediatric patients and longer follow up time will be demonstrated the impact of NOTCH1/FBXW7 on survival and treatment outcome more clearly [53].

Overall, 22.2% of our T-ALL patients had NOTCH1 mutations and 10% had FBXW7 mutations. While FBXW7 mutation frequency is similar to previous studies, prevalence of NOTCH1 mutation was relatively lower in our group [12,43,44,54]. Although the European groups revealed a mutation rate of more than 50%, the frequency of Japanese population was determined as 30.8% [51,52]. Variable frequency of NOTCH1 mutations reported in other studies may be due to several factors like ethnicity, combination of susceptibility variants, and number of patients.

The characterization of the NOTCH1 mutations and certain targets on the NOTCH pathway are important for developing novel and specific therapeutic strategies. Inhibitors of γ-secretase have recently been tested in T-ALL cell lines and were shown to induce cell cycle arrest [55]. Importantly, FBXW7 mutations have been associated with resistance to γ-secretase inhibitor treatment. On the other hand, some HD domain mutations such as L1575P show sensitivity to the treatment [33]. Therefore, the identification of NOTCH1 and FBXW7 mutations needs to be taken into account when choosing target therapies in T-ALL patients. In our knowledge, this is the first NOTCH1/FBXW7 mutations study in Turkish pediatric T-ALL patients. It is known that NOTCH signaling members have important roles in the thymus and deregulated NOTCH activation is an independent oncogenic event involved in human T-ALL oncogenesis.

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Supplementary material

Supplementary data can be found on: http://www.istanbul.edu.tr/duyurular/2010/DMA0827.supporting.online.material.htm.

References


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