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

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**Abstract**. The NOTCH signaling pathway plays 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 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]. Malign 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]. The *NOTCH1* gene encodes regulatory single-pass transmembrane receptor that is evolutionarily conserved [10] and it is essential for T- or B- lymphocyte lineage commitment in hematopoietic progenitors [11]. Weng et al. demonstrated activating *NOTCH1* muta-

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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 *FBWX7* 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/FBWX7* mutations remain to be investigated. In this study, we analyzed the clinical and prognostic significance of *NOTCH1/FBWX7* 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 Etfal Education and Research Hospital; Unit of Pediatric Hematology, Ministry of Health Goztepe 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 ($1U/\mu g$) for the possible DNA contaminations (MBI Fermentase, Germany). $1\mu g$ 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
*NOTCH1* and *FBXW7* mutations in T-ALL patients

| Patient no. | FBXW7 Mutation | | NOTCH1 Mutation | |
| --- | --- | --- | --- | --- |
| | NucleotideΦ | Amino acid | Nucleotide¤ | Amino acid |
| T2 ◊ | - | - | 5082_5083 insT, 5093_5094 insT | 1694Q>1798*stop |
| T4 | 1436 G>A | R479Q | - | - |
| T7 | 1393 C>T | R465C | - | - |
| T13 | | | 4796 G>C | R1599P |
| T27 ♦ | 1200 C>T | D400D | 4796 G>C | R1599P |
| T28 | - | - | 4757 T>C | L1586P |
| T41 ◊ | - | - | 5082_5083 insT, 5093_5094 insT | 1694Q>1798*stop |
| T53 | - | - | 5064 G>T, 5072 C>T, 5082 ins 5083 | Q1688D, S1691L, 1694Q>1720*stop |
| T62 ◊ | - | - | 4681 G>A | D1561N |
| T70 ♦ | 1496G>A | G498A | 4724 T>C | L1575P |
| T73 | - | - | 4824 G>T, 7318 A>G, 7320_7321 insT,dup AGGGG, 7321_7322 ins TCCCTCTGA, 7329 C>A, 7359 G>A, 7364 C>A | K1608N, 2440S>2446*stop |
| T74 ◊ | - | - | 7553 C>T | P2518L |
| T77 | - | - | 4796 G>C | R1599P |
| T81 ◊ | - | - | 7331_7332 ins GAGCTTCG, 7357 G>C | 2444V>2561*stop |
| T84 ◊ | - | - | 7180 C>T | Q2394 *stop |
| T85 ♦ | 1948 dup gacttgaaaacggg | 650 dup DLKT | - | - |
| T87 | 1403 G>A | R465H | - | - |
| T89 | - | - | 7378 C>T | Q2460 *stop |
| T92 | - | - | 4724T>C, 7195 C>T | L1575P, Q2399 *stop |
| T95 ◊ | - | - | 7335_7336 insA, 7336_7337 insATG, 7338_7339 insGGCC, 7354 CC>TG 7355, 7378 C>A | 2445Q>2479*stop |
| T97 | - | - | 4724T>C | L1575P |
| T108 ◊ | - | - | 7207 C>T | Q2403*stop |
| T110 ◊ | - | - | 5094 C>T | D1698D |
| T115 ♦ | 1228A>C | T410P | - | - |

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 $X^2$ 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-

Table 2
Frequency of *NOTCH1* and *FBXW7* mutations in pediatric T-ALL

|  | *FBXW7* mutated ($n = 7$) | *FBXW7* wild-type ($n = 65$) | *FBXW7* not analyzed ($n = 15$) |
|---|---|---|---|
| *NOTCH1* wild type ($n = 68$) | 5 (71.4%) | 51 (85.0%) | 12 |
| *NOTCH1* mutated ($n = 19$) | 2 (28.6%) | 14 (15.0%) | 3 |

Table 3
Clinical and immunologic characteristics of 87 children with T-ALL according to either the presence or the absence of NOTCH1 and FBXW7 mutations

| Patients Characteristics | *NOTCH1* Mutation | | | *FBXW7* Mutation | | | *NOTCH1* and/or *FBXW7* mutation | | |
|---|---|---|---|---|---|---|---|---|---|
|  | Mutant, n (%) | Wild-type, n (%) | $P*$ | Mutant, n (%) | Wild-type, n (%) | $P*$ | Mutant, n (%) | Wild-type, n (%) | $P*$ |
| Sex |  |  | 0.77 |  |  | 0.37 |  |  | 0.78 |
| Female | 6 (31.6%) | 18 (26.5%) |  | 3 (42.9%) | 48 (75.0%) |  | 6 (30.0%) | 18 (26.9%) |  |
| Male | 13 (68.4%) | 50 (73.5%) |  | 4 (57.1%) | 16 (25.0%) |  | 14 (70.0%) | 49 (73.1%) |  |
| WBC count |  |  | **0.03** |  |  | 0.25 |  |  | **0.02** |
| Less than $50 \times 10^9$/L | 13 (68.4%) | 25 (36.8%) |  | 2 (28.6%) | 29 (45.3%) |  | 14 (70.0%) | 24 (35.8%) |  |
| $50 \times 10^9$/L or more | 4 (21.1%) | 35 (51.5%) |  | 5 (71.4%) | 26 (40.6%) |  | 4 (20.0%) | 35 (52.2%) |  |
| Unknown | 2 (10.5%) | 8 (11.8%) |  | 0 (0.0%) | 9 (14.1%) |  | 2 (10.0%) | 8 (11.9%) |  |
| T-cell Immunphenotype |  |  | 0.83 |  |  | 0.10 |  |  | 0.65 |
| Immature | 6 (31.6%) | 25 (36.3%) |  | 3 (42.9%) | 23 (35.9%) |  | 6 (30.0%) | 25 (37.3%) |  |
| Mature | 6 (31.6%) | 16 (23.5%) |  | 4 (57.1%) | 14 (21.9%) |  | 7 (35.0%) | 15 (22.4%) |  |
| Cortical | 3 (15.8%) | 15 (22.1%) |  | 0 (0.0%) | 15 (23.4%) |  | 3 (15.0%) | 15 (22.4%) |  |
| T not further classified | 4 (21.1%) | 12 (17.6%) |  | 0 (0.0%) | 12 (18.8%) |  | 4 (20.0%) | 12 (17.9%) |  |
| Lymph adenopathy ($n = 87$) |  |  | 0.29 |  |  | 0.69 |  |  | 0.44 |
| Yes | 32 (47.1%) | 6 (31.6%) |  | 4 (57.1%) | 29 (45.3%) |  | 7 (35.0%) | 31 (46.3%) |  |
| No | 36 (52.9%) | 13 (68.4%) |  | 3 (42.9%) | 35 (54.7%) |  | 13 (65.0%) | 36 (53.7%) |  |
| Central nervous system involvment |  |  | 0.21 |  |  | 0.89 |  |  | 0.25 |
| Yes | 4 (78.9%) | 7 (10.3%) |  | 1 (85.7%) | 8 (12.5%) |  | 4 (20.0%) | 7 (10.4%) |  |
| No | 15 (21.4%) | 61 (89.7%) |  | 6 (14.3%) | 56 (87.5%) |  | 16 (80.0%) | 60 (89.6%) |  |

WBC indicates white blood cell. $P*$ $X^2$ or Fisher's exact test. $P$ value of 0.05 or less (two sided) was considered to indicate a statistically significant difference and demonstrated bold font.

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 terminal 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). Three missense mutations were clustered in a 'hot spot' encoding arginine amino acid 465 and 479 residues. The mutations that altered conserved arginine residues in the WD40 domain are responsible for binding to the PEST domain of *NOTCH1* [30,33]. Of the seven iden-

tified *FBXW7* mutations, one duplication (T85), one synonymous mutation (T27), and two missense mutations (T70-T115) have not been previously described in T-ALL. Two (28,6%) of the 7 T-ALL patients with *FBXW7* 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 *FBXW7* and *NOTCH1* alone but not *FBXW7* alone, were found more frequently in T-ALL patients with low WBC count, $< 50X10^9$ /l, than in those with higher WBC count, $> 50X10^9$ /l ($p = 0.03$, $p = 0.02$ by $X^2$ test; Table 3).

Significant differences were not observed in the event-free and overall survival rates between *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 ($p = 0,38$; $p = 0,63$).
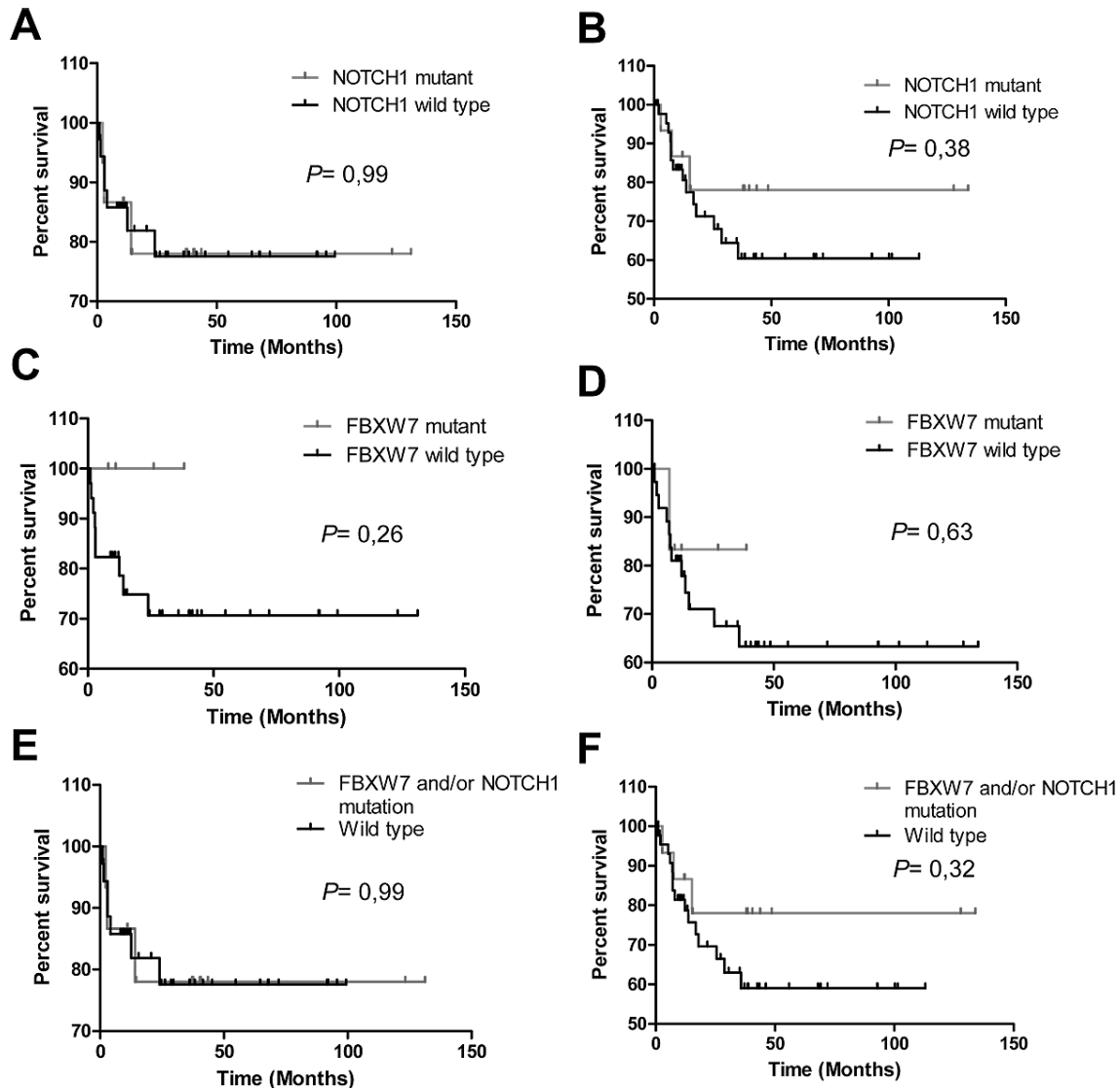
Fig. 1. Outcome of pediatric patients with T-ALL treated on BFM protocol. (A) Event-free survival by *NOTCH1* mutation. (B) Overall survival by *NOTCH1* mutation. (C) Event-free survival by *FBXW7* mutation. (D) Overall survival by *FBXW7* mutation. (E) Event-free survival by *FBXW7* and/or *NOTCH1* 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. Non 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 $\gamma$-secretase dependent NOTCH1 signaling and shows sensitivity to GSIs ($\gamma$-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 decreased 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 $\gamma$-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 $\gamma$-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.

## Acknowledgements

## Supplementary material

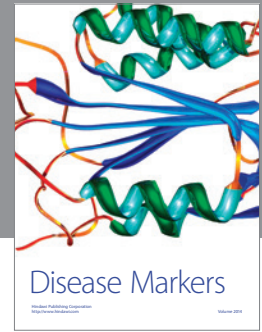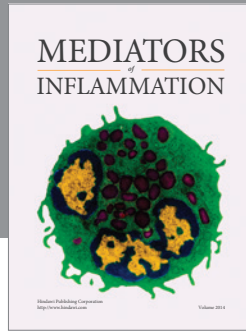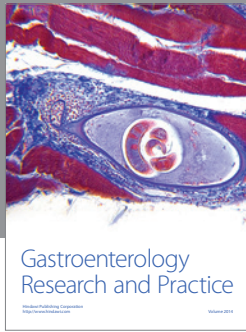Supplementary data can be found on: http://www.istanbul.edu.tr/duyurular/2010/DMA0827.supporting.online.material.htm.

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