Evaluation of PAX5 gene in the early stages of leukemic B cells in the childhood B cell acute lymphoblastic leukemia


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ABSTRACT

B-lineage acute lymphoblastic leukemia (B-ALL) is a common subtype of acute leukemia in children. PAX5 plays a central role in B-cell development and differentiation. In this study, we analyzed PAX5 expression levels, transactivation domain mutations/deletions in B-ALL patients (n = 115) and healthy controls (n = 10). Relative PAX5 mRNA levels were significantly increased in B-ALL patients (p < 0.0001). PAX5 expression was also evaluated in three different B-ALL subgroups (pro B, Common B and Pre B ALL) and showed stage specific expression levels. Pro B (p = 0.04) and pre B (p = 0.04) patients showed significantly high PAX5 mRNA levels compared to stage specific controls. At least one deletion of exons 7–8 or 9 has been identified in the 41% of the patients. CD34 positivity in patients and presence of large deletions (Δ7/8/9) showed a significant correlation (p = 0.05). None of our patients showed PAX5 point mutations, but two previously identified SNPs (rs3780135 and rs35469494) were detected. Our results support that PAX5 is a critical factor in B-ALL development and aberrant PAX5 expression especially at early stages may lead to leukemic transformation.

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1. Introduction

B lymphocytes develop from hematopoietic stem cells (HSCs) through intermediate lymphoid progenitors and developmental progression from early pro-B cells to immunoglobulin-secreting plasma cells is regulated by a multitude of extracellular signals and transcription factors [1,2]. PAX5 (B-cell-specific activator protein, BSAP) is a member of the paired box domain gene family that encodes nuclear transcription factors important in development, differentiation, cell migration and proliferation [3]. The B cell transcription factor PAX5, plays an important role in B lineage commitment by controlling B cell development from the pro B to the mature B cell stage [4]. The earliest B cell progenitors (pre-pro-B cells), initiate expression of B-cell specific transcripts and undergo the first step in immunoglobulin gene rearrangement. These cells first mature pro-B and then pre-B cells and PAX5 expression starts at the differentiating point from pro B cells to pre B cells [5,6]. PAX5 is also important for maintaining the identity and function of mature B cells in late B lymphopoiesis but its expression is downregulated during the plasma cell differentiation [7]. Pax5 down-regulates the expression of many cell surface receptors and intracellular transducers to mediate signaling in early progenitors and activates B cell-specific genes in including BLNK, CD19, LEF-1 and MB-1 genes [8,9].

PAX5 expression in normal adult tissue is limited to the hematopoietic system whereas it is aberrantly expressed in a number of solid cancers as well as B-cell malignancies where it functions as an oncogene [1,10,11]. Loss of PAX5 expression, arrests B cell development at an early pro-B-cell stage and reverts committed B-cell precursors (BCPs) to progenitors [12,13]. Conversely, ectopic
Table 1
Clinical parameters of B-ALL patients.

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>Pro B-ALL (n=11)</th>
<th>Common B-ALL (n=88)</th>
<th>Pre B-ALL (n=16)</th>
<th>Total B-ALL (n=115)</th>
</tr>
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<tbody>
<tr>
<td>Sex</td>
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<td>1</td>
<td>4</td>
</tr>
<tr>
<td>&lt;10</td>
<td>4</td>
<td>73</td>
<td>10</td>
<td>89</td>
</tr>
<tr>
<td>≥10</td>
<td>6</td>
<td>13</td>
<td>5</td>
<td>24</td>
</tr>
<tr>
<td>WBC count (g/l)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
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<td>42.450</td>
<td>58.000</td>
<td>48.110</td>
</tr>
<tr>
<td>Min-max</td>
<td>4800–400.000</td>
<td>1700–702.000</td>
<td>3600–395.000</td>
<td>1700–702.000</td>
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<tr>
<td>Hb (g/dl)</td>
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</tr>
<tr>
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<td>8.25</td>
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<tr>
<td>Min-max</td>
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<td>1.4–11.9</td>
<td>1.4–12</td>
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<td>Plt values (g/l)</td>
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<td>Median</td>
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<td>79.000</td>
<td>76.600</td>
<td>76.500</td>
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<tr>
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<td>3000–490.000</td>
<td>3000–348.000</td>
<td>3000–490.000</td>
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<td>Bone marrow blast (%)</td>
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<td></td>
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<td></td>
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<tr>
<td>Median</td>
<td>84</td>
<td>92</td>
<td>84</td>
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</tr>
<tr>
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<td>65–100</td>
<td>16–100</td>
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<td>4</td>
</tr>
<tr>
<td>t(12;21)</td>
<td>2</td>
<td>8</td>
<td>2</td>
<td>12</td>
</tr>
</tbody>
</table>

PAX5 expression in hematopoietic stem cells and precursor cells inhibit T cell proliferation and leads to uncontrolled B cell proliferation [14].

PAX5 has frequently been targeted by mutations and chromosomal translocations in childhood acute lymphoblastic leukemia (ALL) [15–19]. Five human Pax-5 isoforms, which occur through the alternative splicing of exons that encode for the C-terminal transactivator domain, identified and characterized. These isoforms mostly arise from exon 7, exon 8, and/or exon 9 and expressed in peripheral mononuclear cells, cancerous and noncancerous B cell lines, as well as primary B cell lymphoma tissue [20,21].

Here, we investigated the PAX5 expression in childhood B cell acute lymphoblastic leukemia (B-ALL) patients (n=115), with different immunophenotypic stages and healthy controls. Transactivator domain mutations and gene deletions were also investigated to understand the incidence and clinical relevance of the PAX5 gene.

2. Materials and methods

2.1. Patient samples and control selection

One hundred and fifty (50 girls and 65 boys) bone marrow samples of childhood B-ALL patients who were diagnosed in Bakirkoy Maternity and Children’s 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 were included in this study. Patients were diagnosed according to FAB classification. We evaluated the patients according to EGIL criteria in three subgroups: pro B ALL (EGIL I-B; CD79α+, CD19+, IgM−), common B ALL (EGIL II; CD79α+, CD19+, IgM−, CD10+ and pre B ALL (EGIL III; CD79α+, CD19+, IgM−, CD10+) [22]. Clinical parameters of the patients were shown in Table 1. In addition to 10 healthy bone marrow samples, to evaluate the patients with their stage specific controls (SSC) following subsets were sorted by FACS ARIA II. We compared the pro B-ALL patients with SSC-I (CD19+, CD22+, CD34+, IgM−), common B-ALL patients with SSC-II (CD34+−, CD19+, CD20+, IgM−) and pre B-ALL patients with SSC-III (CD34+, CD19+, CD20+, IgM−). The ethical committee of Istanbul Medical University (reference number and date: 2008/2696 and 22.10.2008) approved this study and written informed consents were obtained from all patients.

2.2. Flow cytometry

Healthy bone marrow subsets were sorted by flow cytometry (FACS ARIA II, Becton Dickinson). Samples labeled with CD19-FITC to separate CD19− and CD19+ B cell subsets. CD19+ cells were collected in 2 ml tubes and stained with CD34-PE (clone 563, all from BD Biosciences), and CD22-FITC (clone S-HCL-1) to sort CD34+, CD19− and CD22+ pro B cells. CD19+ cells stained with CD34-PE, CD20-PerCP-Cy5.5 (clone L27) and IgM-APC (clone G20-127) to isolate CD19 positive precursor B cell subsets (Supplementary Fig. 1). Antibody staining and washing of single-cell suspensions were done in sterile phosphate buffered saline solution (PBS) containing 5% heat-inactivated fetal calf serum (FCS). SSC-I, SSC-II and SSC-III cells were collected into 2 ml tubes with heat-inactivated FCS and RNA was isolated subsequently. Analyses were done by FACS Diva 6.1.2 (Becton Dickinson, USA) software.

2.3. cDNA synthesis and real time quantitative PCR (QRT-PCR)

Total RNA was isolated by QiaGen RNeasy Plus Mini Kit (Qiagen, GmbH, Germany) to eliminate the genomic DNA prior to RNA isolation. RNA quality and quantity was checked with Nanodrop 1000 (Thermo Fisher Scientific, Germany) and cDNA was synthesized by random hexamers and MMLV reverse transcriptase, from 1 μg of total RNA according to manufacturer protocol (MBI Fermentase Life Sciences, Lithuania). RQ-PCR was carried out on Light Cycler 480 Instrument (Roche Applied Sciences, Manheim, Germany). The specific primer-probe sets were designed using human universal probe library tool (probe no #36) and real time amplification was performed as described by the manufacturer (Roche Applied Sciences, Germany). The suitable reference gene was selected by GeNorm (V3.4, Belgium) software that is a system to determine the best candidate reference gene for each individual experiment. The underlying principles and formulas were described by Vandesompele et al. [23]. The most stable reference gene CyclinH1 was studied for normalization. Outliers discharged. Relative expressions were calculated according to the delta–delta Ct method, based on the mathematical model described by Livak et al. [24].
2.4. Mutation and deletion analysis

Primers, used for mutation and deletions, were designed by using CLC workbench 3.6.1 (Denmark) (Supplemental Fig. 2). B-ALL patients were analyzed for mutations of transactivator domain (exons 7, 8 and 9). Amplified samples of exons 7–9 were also analyzed for deletions on an agarose gel, which were also confirmed by sequencing (Supplemental Fig. 3).

2.5. Cell culture and Western blot analysis

Cell lines were cultured in RPMI 1640 (Invitrogen Life Technologies, USA), supplemented with 10% FCS, penicillin (50 U/ml), streptomycin (50 mg/ml; both Invitrogen Life Sciences, USA). Cells were lysed with Lysis buffer (20 mM Tris, 137 mM NaCl, 10 mM EDTA, and 100 mM NaF, 1% NP40, 10% glycerol) supplemented with protease inhibitor cocktail tablets (Roche, Germany). Lysates containing 20 μg of protein were separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred into PVDF membranes. Non-specific binding was blocked by incubation in blocking buffer (5% skimmed milk in PBS-Tween) followed by incubation with the primary antibodies and the appropriate secondary antibodies conjugated to horseradish peroxidase. PAX5 was detected by Anti-PAX5 (clone 24, mouse monoclonal IgG1, BD, USA). The Beta-Actin epitope specific rabbit antibody (Thermo Fisher Scientific, USA) is used for control. The signal was detected using the enhanced chemiluminescence (Lumi Light Western Blotting Substrate, Roche Applied Biosciences, Germany). NALM-6 (pre B-ALL cell line) was used for Pax5 positive control.

2.6. Statistical analysis

Differences between the relative expression levels of cases and controls were tested with Mann–Whitney test and differences between B-ALL patients’ subgroups were tested with Kruskal–Wallis test. p Value of 0.05 or less was considered statistically significant. To understand correlation between patients and clinical findings “Multiple regression” was used. The Kaplan–Meier method was used to estimate survival rates. All statistical analyses were done by Graphpad Prism V and Statgraphics Centurion XV.1 (USA).

3. Results

3.1. PAX5 expression in B-ALL patients

We studied the expression differences of the PAX5 in childhood B-ALL patients (n = 113) and healthy bone marrow samples (n = 10). Prominent PAX5 mRNA and protein expressions were detected in B-ALL patients compared to controls. Upregulated PAX5 mRNA levels were found in 90% of our childhood B-ALL patients when compared to controls (p = 0.0001) (Fig. 1A). In a group of patients (n = 15), PAX5 protein expression was also confirmed by western blot analyses and 13 out of 15, showed PAX5 protein expression. Previously characterized Pax5 variant (deletion of exon 8) was also detected on protein level (Fig. 1B). To gain further insights into the effects of PAX5 in B-ALL subgroups, patients were evaluated in three different immunological B-ALL subgroups (pro B, common B and pre B) according to EGIL criteria and expression differences of PAX5 mRNA

Fig. 1. PAX5 expression in B-ALL patients and controls. (A) Detection of relative PAX5 mRNA levels in leukemic and healthy B cells from 115 childhood B-ALL patients and 10 healthy bone marrow samples by RQ-PCR. Cyclophilin gene was used for normalization (p = 0.0001). (B) Protein expression of PAX5 in B-ALL patients by western blot (NALM6 was used as a positive control, P103, P21, P53, P58, P70, P96 have increased Pax5 protein levels, P21 and P53 have also A8 and P40 have no Pax5 protein levels). β-actin was used as a loading control of samples.

Fig. 2. Comparison of B-ALL subgroups [pro B-ALL (n = 11), common B-ALL (n = 87)] and pre B-ALL (n = 16) with their stage specific controls (SSC). Isolated subgroups by flow cytometry were used as a stage specific control (SSC) samples. SSC-I cells were CD19+, CD34+, CD22+, CD10−, IgM− cells. SSC-II cells were CD19+, CD34−, CD22+, IgM− cells. SSC-III cells were CD19−, CD34−, CD22−, IgM−. B cells. Statistical test was done by Mann–Whitney U test (p = 0.04, p = 0.04 and p = 0.55 respectively).

were characterized. Although the highest expression was detected at the pro B stage, PAX5 mRNA levels between patient’s subgroups were not significantly different (p = 0.33). Pro B, common B and Pre B patients were also compared to their stage specific healthy B-cell groups as controls, which were sorted by FACS. Pro B and common B patients have significantly high PAX5 mRNA levels compared to controls (p = 0.049 and p = 0.04 respectively). Relative PAX5 levels of pre B ALL patients were also high, but not significantly different from healthy controls (p = 0.55) (Fig. 2).

To demonstrate the specific expression of PAX5 in B cells, mRNA and protein expressions were studied in several leukemia cell lines (Fig. 3A and B). In concordance to patient’s results, pro B-ALL cell line NALM-6 had higher PAX5 expression levels from pre B-ALL cell line REH. High PAX5 levels did not show any association; sex (p = 0.50), age (p = 0.73), WBC count at diagnosis (p = 0.39), CD34 expression (p = 0.28) and CD10+CD19 expression (p = 0.77). Kaplan–Meier estimate of probability of survival according to PAX5 expression levels showed no significant difference (p = 0.45, data not shown).

3.2. Detection of PAX5 isoforms and mutations

Isoforms of PAX5 occurred by deletions of exons 7, 8 and/or 9 were detected in B-ALL samples and healthy controls. All of our healthy bone marrow samples (n = 10) had full length (FL) PAX5 and 5 of these had additional Δ8 isoform. In our childhood patients group, 41% of the patients showed multiple variants. 19% of these patients had the largest deletion defined by the absence of exon 7, 8 and 9, 13% of the cases had Δ8 and 9% had Δ8/9 variants (Fig. 4A). In four common B-ALL patients out of 115, the PAX5 deletion was homozygous. We checked PAX5 expression difference levels between these variants and found the homozygous deletion, Δ7, Δ7/8/9 had significantly higher expression of PAX5 mRNA than FL (p = 0.02, Fig. 4B). In addition, the correlation between CD34 positivity in patients and presence of deletion (Δ7/8/9) was significant (p = 0.05). Δ8 variant which was also detected in normal bone marrow samples, was more often in CD34 negative patients (18%).

All possible associations between the clinical features and deletions were checked, and no significant relations were found between patients subgroups among themselves (p = 0.08), sex (p = 0.37), WBC count at diagnosis (p = 0.10), and CD10+CD19 expressions (p = 0.95) (Table 2). Kaplan–Meier estimate of probability of survival according to PAX5 deletions showed no significant difference (p = 0.62, data not shown).

In addition to deletions, we screened the PAX5 gene mutations for transactivator domain (exons 7–9) and no mutation was found.

![Figure 3](image1.png)

**Fig. 3.** PAX5 expression in leukemic cell lines. (A) Relative PAX5 mRNA levels in B-ALL cell lines (FLEB14-4, NALM6, REH) and T-ALL cell lines (MOLT-4, JURKAT, RPMI8402, CEM); (B) Detection of protein levels in B-ALL and T-ALL cell lines by using western blot. β-actin was used as a loading control of samples.

![Figure 4](image2.png)

**Fig. 4.** (A) PAX5 deletions in selected childhood B-ALL patients. Deletions were analyzed on 2% agarose gel. M is a 100 bp DNA ladder, P48, P55 and P56 have FL and Δ7/8 and P3 have strong Δ7/8/9 expression besides full length (FL). P65 have only FL expression. P116 have FL, Δ8, Δ7/8/9. P17 have Δ7/8 but have no FL expression. (B) Differences of PAX5 mRNA levels between FL, Δ7, Δ7/8/9 and homozygous FL deletion patients (p = 0.02).

in our childhood B-ALL patients. Two previously identified SNPs (rs3780135 and rs35469494) were detected in the coding region of PAX5 gene (Supplement Fig. 4). Ninety percent of the patients had rs3780135 and 3.8% had rs35469494 variations. To detect the effects of SNPs on PAX5 gene expression, we compared the B-ALL patients with or without SNP and no significant correlation found for both two SNPs (p = 0.61).

### 4. Discussion

In this study, we aimed to determine the molecular abnormalities of PAX5 gene in childhood B-ALL patients and analyze the prognostic impact of these molecular findings. Although PAX5 is known to be expressed both in normal and malignant B cells [25], for the first time with this study the differential expression of PAX5 was shown in normal B cell maturation steps, revealing the differences between B-ALL subgroups and their stage specific control subsets.

PAX5 is a critical transcription factor in B cell development but the stage specific role in human has not been studied and the data in mice is not clear [26,27]. Here we showed that, the expression levels of PAX5 in normal human B cells were higher in cIgM positive B cells (SSC III) than cIgM negative cells (SSC I and SSC II). Moreover, up-regulated PAX5 expression was found in 90% of our childhood B-ALL patients, which is in concordance with the other studies with hematological malignancies [25,28,29]. When we analyzed the PAX5 expression among the B-ALL patients according to their maturation steps with their stage specific controls, the mRNA levels were significantly higher in pro B and common B ALL. Parallel with the increased mRNA levels, prominent PAX5 protein expressions were also detected. This finding suggests that high PAX5 expression in early stages may lead to abnormal malignant cell proliferation, either by inhibiting the proliferation of other blood cell types or triggering different pathways.

Here, we studied PAX5 isoforms occurring through the alternative splicing of exon 7, exon 8, and/or exon 9 that have been shown to result in different transactivation (TA) potentials [20,27]. The isoform PAX5-Δ8 was detected in healthy bone marrow samples as well as patients group which has been reported previously [29]. At least one deletion of exons 7–8 or 9 has been identified in the 41% of the patients. The largest deletion, including exons 7–9, was the most common deletion in our B-ALL patients. Large deletions in the transactivation domain of PAX5 might arrest B cell differentiation at the early stage and/or lead to abnormal expansion of pro B cells in children. Also, aberrant PAX5 isoforms with deletion of exon 7 and/or 8 might disrupt B cell hematopoiesis caused by the potential to increase expression of PAX5 target genes. There is also a need to study target genes to understand the downstream effects of these isoforms.

Major task of pro B cells, is to recombine VDJ rearrangements and/or express functional pre BCR receptor. Events, which are regulated by PAX5, have a critical importance for normal B cell maturation [30]. One of the interesting finding of our study is the association between the large deletions and maturation stages. When we evaluated patients according to the presence or absence of CD34 stem cell marker, CD34 positive cases significantly express Δ7/8/9 whereas CD34 negative cases express pax5–Δ8 which is an isoform that was also observed in healthy B cells. Early stage patients (pre B and common B) had larger deletions than more mature patients, supporting the idea that functional importance of PAX5 in B cell differentiation at early stages.

Recently, it was shown that mutations and structural rearrangements in PAX5 gene occur in 32% of childhood B-progenitor ALL [31]. Moreover, Familiades et al. determined that PAX5 mutations occur in 30% of adult pro B-ALL patients [32]. We also screened the point mutations in the transactivator domain of PAX5 (exons 7–9) and none of our patients showed PAX5 mutations, but two previously identified SNPs (rs35469494 and rs3780135) were detected. There is no previous data or clinical relevance between these polymorphisms and any malignancies. However, rs3780135 is located near the splice site of exon 9 and resides in the protein coding region. This polymorphism might change the alternative splicing motif and may explain the high frequency of deletions in our patient group.

The findings of this study showed that abnormal PAX5 expression and large deletions are characteristic for early stage patients. For the first time with this study, the differential expression of PAX5 in normal B cell maturation steps revealed stage specific effects in childhood B-ALL patients. This study suggests that PAX5 is a critical factor in B-ALL development and aberrant PAX5 expression especially at early stages may lead to leukemic transformation. These results need to be analyzed in larger cohorts with target gene analysis.

### Conflict of interest

All authors have no conflict of interest to declare.
Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.leukres.2011.07.017.

References