Upregulation of T-Cell-Specific Transcription Factor Expression in Pediatric T-Cell Acute Lymphoblastic Leukemia (T-ALL)

Abstract

Objective: T-cell acute lymphoblastic leukemia (T-ALL) is associated with recurrent chromosomal aberrations and abnormal ectopic gene expression during T-cell development. In order to gain insight into the pathogenesis of T-ALL this study aimed to measure the level of expression of 7 T-cell oncogenes (LMO2, LYL1, TAL1, TLX1, TLX3, BMI1, and CALM-AF10) in pediatric T-ALL patients.

Material and Methods: LMO2, LYL1, TLX1, TLX3, BMI1, TAL1, and CALM-AF10 expression was measured using quantitative real-time PCR in 43 pediatric T-ALL patients.

Results: A high level of expression of LMO2, LYL1, TAL1, and BMI1 genes was observed in a large group of T-ALL. Several gene expression signatures indicative of leukemic arrest at specific stages of normal thymocyte development (LYL1 and LMO2) were highly expressed during the cortical and mature stages of T-cell development. Furthermore, upregulated TAL1 and BMI1 expression was observed in all phenotypic subgroups. In all, 6 of the patients had TLX1 and TLX3 proto-oncogene expression, which does not occur in normal cells, and none of the patients had CALM-AF10 fusion gene transcription. Expression of LYL1 alone and LMO2-LYL1 co-expression were associated with mediastinal involvement; however, high-level oncogene expression was not predictive of outcome in the present pediatric T-ALL patient group, but there was a trend towards a poor prognostic impact of TAL1 and/or LMO2 and/or LYL1 proto-oncogene expression.
Conclusion: Poor prognostic impact of TAL1 and/or LMO2 and/or LYL1 proto-oncogene expression indicate the need for extensive study on oncogenic rearrangement and immunophenotypic markers in T-ALL, and their relationship to treatment outcome.

Key Words: T-ALL, Pediatric, Transcription factor, Expression, Prognosis

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is a rare, aggressive malignancy of thymocytes and corresponds to a heterogeneous group of leukemia arrested at various stages of lymphoid development. T-ALL constitutes 15% of all childhood ALL and 25% of adult ALL; approximately 30% of patients relapse within the first year of treatment and the outcome is usually death [1]. T-ALL patients characteristically have recurrent and rare cytogenetic alterations that affect the gene expression profile of involved genes. These proto-oncogenes are associated with the pathways involved in T-cell development, such as differentiation, proliferation, survival, the cell cycle, and self-renewal. Most chromosomal translocations associated with T-ALL result in juxtaposition of T-cell antigen receptor (TCR) loci (α/δ or β) regulatory elements to proto-oncogenes, thus deregulating expression of the latter [2]. TCR promoters and enhancers are juxtaposed to a number of developmentally important transcription factor genes, including HOX11/TLX1, TLX3/HOX11L2, TAL1/SCL, TAL2, LYL1, bHLHBI1, LMO1, and LMO2. Deregression of these genes primarily arrest differentiation at specific stages of T-cell development [3-7].

TAL1, TAL2, LYL1, and bHLHBI1 genes are part of the basic helix-loop-helix (bHLH) protein family and function as transcriptional co-factors that form complexes with E2A/HEB. bHLH protein family members also bind to members of the LMO gene family. During normal human T-cell development, TAL2 and LMO1 are not expressed, whereas Lyl1, Tal1; and Lmo2 expression in mice are restricted to the earliest double-negative stages of T-cell maturation. TLX1 is a class II homeobox gene normally involved in spleen development that is not activated during normal T-cell development. TLX1-positive T-ALL cases share a similar gene expression profile characterized by arrest at the early cortical, CD1-positive thymocyte stage. Translocation/ectopic expression of these transcription factors have been reported with different percentage in pediatric T-ALL [8,9]. More recently, evidence of NOTCH1 mutations in 25%-50% of pediatric T-ALL patients has further enhanced the biologic heterogeneity of T-ALL [10,11]. An alternative mechanism of increased NOTCH1 activation.
via loss-of-function mutations of FBXW7 leads to inhibition of ubiquitin-mediated degradation of the activated form of NOTCH1 [12].

These transcription factors and their oncogenicity have been well documented in mouse models; however, few of these known oncogenetic markers have been shown to have prognostic significance in humans. Conflicting outcomes have been associated with TLX3 and TAL1 deregulation, and NOTCH1 mutations. TLX1-positive T-ALL patients frequently have activating NOTCH1 mutations, and TLX1 over expression and/or translocation confers a better prognosis, but this association varies by study [13-16]. In an earlier study we determined the NOTCH1 and FBXW7 mutation status in a pediatric T-ALL cohort [10]. In the present study we examined the transcription factor genes LMO2, LYL1, TAL1, TLX1, TLX3, and BMI1, and gene fusion of CALM-AF10 in an effort to identify T-cell-specific oncogenic transcription factors and their association with prognosis in pediatric T-ALL patients.

Materials and Methods

Participants

At the time of diagnosis of T-ALL, bone marrow (BM) (n = 33) and peripheral blood (PB) (n = 10) samples were obtained from 43 pediatric patients. The study included pediatric T-ALL patients diagnosed at Istanbul University, Cerrahpaşa School of Medicine (n = 15) and Istanbul School of Medicine (n = 12), Bakirköy Maternity and Children’s Hospital (n = 5), Ministry of Health Şişli Etfal Teaching Hospital (n = 5), Kocaeli University, Kocaeli School of Medicine (n = 2), Ministry of Health Göztepe Teaching Hospital (n = 2), and Ministry of Health Haseki Teaching Hospital (n = 2). Patients were diagnosed based on French-American-British (FAB) Group criteria and their clinical characteristics are shown in Supplemental [17]. Mean age of the 17 female and 26 male patients was 8.8 ± 4.1 years, and the median white blood cell (WBC) count was 68,400 x 10^8.8 ± 4.1 years, and the median white blood cell (WBC)

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In all, 20 of the patients were in complete remission (CR), 14 died, and 9 were lost to follow-up. T-ALL subgroups were classified according to in vivo response to induction therapy on d 33 (<5% BM blasts). Based on the 33-d response, patients were classified as good (<5% BM blasts) and poor (>5% BM blast) responders. CR was defined as the absence of leukemic blasts in the peripheral blood and cerebrospinal fluid, <5% lymphoblasts in BM aspiration smears, and no evidence of localized disease. Primary treatment failure was defined as persistence of PB blasts or ≥25% blasts in BM after induction therapy. Relapse was defined as the

Anonymous control thymocyte subsets were obtained from pediatric thymus tissues, using the same guidelines described for T-ALL subgroup classification [19]. Sorted CD34+ CD38– CD1a–, CD34+ CD38+ 1a–, CD34+ CD38+ 1a+, ISP (immature single positive), DP (double positive) CD3–, DP CD3+, SP (single positive), CD4+, and SP CD8+ cells were used as T-cell stage specific controls. Thymus cells were kindly provided by Dr. Frank J.T. Staal, Erasmus Medical Center, Department of Immunology, Rotterdam, The Netherlands. The Istanbul School of Medicine Ethics Committee (reference number and date: 2008/305 and 20.02.2008) approved the study protocol and informed consent was provided by all the patients.

**cdDNA synthesis and quantitative real-time PCR (QRT-PCR)**

Total RNA was isolated using a Qiagen RNeasy Plus Mini Kit (Qiagen, GmbH, Germany), so as to eliminate the genomic DNA prior to RNA isolation. RNA quality and quantity were measured using a Nanodrop 1000 (Thermo Fisher Scientific, Germany), and cDNA was synthesized from 1 μg of total RNA using a random hexamer and MMLV reverse transcriptase, according to the enzyme manufacturer’s instructions (MBI Fermentase Life Sciences, Lithuania). Quantitative real-time PCR (QRT-PCR) was performed using an ABI 7700 (Applied Biosystems, Foster City, CA, USA) with specific primer-probes, as described by van Grotel et al. [20]. The level of expression of LMO2, LYL1, TLX1, TLX3, BMI1, TAL1, and CALM-AF10 was normalized to ABL gene expression. The threshold value, which is the maximum level of expression of each of the normal thymic subsets, was evaluated for each gene.

**Statistical analysis**

Relative gene expression was calculated according to the delta-delta Ct method-based mathematical model [21]. Analyzed gene expression was categorized as high and low, as compared to controls. Categorical variables were compared using Fisher’s exact test and comparison of medians was performed using the Mann-Whitney U test. Remission status was assessed after completion of induction chemotherapy. Treatment efficacy was analyzed according to in vivo response to induction therapy on d 33 (<5% BM blasts). Based on the 33-d response, patients were classified as good (<5% BM blasts) and poor (>5% BM blast) responders. CR was defined as the absence of leukemic blasts in the peripheral blood and cerebrospinal fluid, <5% lymphoblasts in BM aspiration smears, and no evidence of localized disease. Primary treatment failure was defined as persistence of PB blasts or ≥25% blasts in BM after induction therapy. Relapse was defined as the
reappearance of PB blasts, >5% blasts in BM, or the appearance of extramedullary manifestations after CR was achieved.

The Kaplan-Meier method was used to estimate survival rates. Median follow-up was 13.28 months (range: 1-130 months). Overall survival (OS) was defined as the interval from the date of diagnosis to the date of last follow-up or death. Relapse-free survival (RFS) was the time from the start of CR 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). Differences were compared using the 2-sided log-rank test. Multivariate survival analysis was estimated according to the Cox regression model, and included the variables of gene expression, gender, age, WBC count, and immunophenotype. The level of statistical significance was set at P = 0.05. Statistical analysis was performed using SPSS v.12.0 for Windows (SPSS, Inc, Chicago, IL, USA).

**Results**

**Up-regulated oncogene expression found in pediatric T-ALL patients**

The patients were grouped according to EGIL criteria (immature, cortical, and mature stages) and the levels of

![Figure 1: QRT-PCR analysis of oncogenes in the pediatric T-ALL patient subgroups (according to EGIL criteria) and normal thymic control subsets. A. LYL1 expression was significantly higher in the cortical and mature stage subgroups, as compared to their specific controls (cortical versus control: P = 0.02; mature versus control: P = 0.007). B. TAL1 expression was significantly higher in all the phenotypic subgroups than in the controls (immature versus control: P = 0.01; cortical versus control: P = 0.01; mature versus control: P = 0.02 [Mann-Whitney U test]). C. LMO2 expression was significantly higher in the cortical and mature stage subgroups than in the controls (cortical versus control: P = 0.05; mature versus control: P = 0.007). D. BMI1 expression was higher in all the phenotypic subgroups than in their specific controls (immature versus control: P = 0.01; cortical versus control: P = 0.01; mature versus control: P = 0.01).
gene expression were compared to normal thymic subsets. A group of patients had an up-regulated gene expression profile for \textit{LYL1}, \textit{TAL1}, and \textit{LMO2}. In all, 25 of 41 patients (60.9\%) had high-level \textit{TAL1} gene expression, 30\% of the 43 T-ALL patients had \textit{LMO2} overexpression, and 10 patients (25.6\%) had a high level of \textit{LYL1} gene expression, as compared to the controls. Individual gene expression levels did not significantly differ between the T-ALL phenotypic subgroups (immature, cortical, and mature). Comparison of the phenotypic subgroups and their stage-specific counterparts showed that \textit{LYL1} expression was significantly higher both in the cortical and mature stage subgroups than in their specific controls (P = 0.02 and P = 0.007, respectively). \textit{TAL1} expression was significantly higher in all the phenotypic subgroups (immature versus control: P = 0.01; cortical versus control: P = 0.01; mature versus control: P = 0.02 [Mann-Whitney U test]), and \textit{LMO2} expression was significantly higher in the cortical and mature stage subgroups (P = 0.05 and P = 0.007, respectively) (Figure 1A-C). \textit{LYL1} and \textit{TAL1} co-expression was significantly higher in the cortical (P = 0.008 [Mann-Whitney U test] and immature (P = 0.01) stage subgroups.

Three of the patients had \textit{TLX1} expression and 3 others had \textit{TLX3} expression. \textit{BMI1} expression was observed in all of the patients and 53.4\% of the 43 patients exhibited over-expression, as compared to the controls. \textit{BMI1} expression was higher in all the phenotypic subgroups, as compared to their specific counterparts (immature versus control: P = 0.01; cortical versus control: P = 0.01; mature versus control: P = 0.01 [Mann-Whitney U test]) (Figure 1D). One of the recurrent translocations in T-ALL patients is t(10;11)(p13;q14-21), which results in the CALM-AF10 fusion transcript; none of the pediatric patients were carrying CALM-AF10 fusion.

\textbf{Oncogene expression and outcome in the pediatric T-ALL patients}

To assess the prognostic significance of the above findings we compared the levels of oncogene expression and patient clinical characteristics, and then analyzed survival in the T-ALL patients. High-level \textit{LYL1} expression was correlated with mediastinal masses (P = 0.01) and other organ involvement (P = 0.04). \textit{LMO2-LYL1} co-expression was strongly associated with mediastinal involvement (P = 0.02 [Fisher’s exact test]). The patients with \textit{TLX1} expression had mediastinal masses (P = 0.003 [Fisher’s exact test]) and \textit{TLX1} expression was correlated with organ involvement, including the kidneys and heart P = 0.05 [Fisher’s exact test]). \textit{TLX3} expression was associated with central nervous system (CNS) involvement (P = 0.04 [Fisher’s exact test]).

Preliminary comparison of the Kaplan-Meier plots showed that there wasn’t a significant difference between the individual oncogene expression groups, except for the \textit{TLX3} gene (patients with high-level \textit{TLX3} expression versus patients with no or low-level expression [Cox regression <0.0001; 95\% CI: 1.6-18.6]). Additionally, we combined the oncogenes, such as \textit{TAL1} and/or \textit{LMO2} and/or \textit{LYL1}, \textit{TLX1} and \textit{TLX3}, \textit{TAL1} and/or \textit{LMO2} and/or \textit{LYL1} and/or \textit{TLX1}, and \textit{TLX3} and/or \textit{BMI1}. The outcome of these synergistic subgroups versus patients with normal expression of the oncogenes did not reach the level of statistical significance (Figure 2); however, the obtained data showed a trend toward longer RFS in the patients with normal \textit{TAL1} and/or \textit{LMO2} and/or \textit{LYL1} expression (P = 0.08 [Mantel-Cox test]). NOTCH1/FBXW7 mutations were observed in all the genetic subgroups.

Although significant differences were not observed in RFS or OS between the NOTCH1/FBXW7 wild-type and mutated cases, as previously described, we noted a trend toward longer RFS in the mutant patients (Supplemental Figure 1) [10]. Hence, we considered NOTCH1/FBXW7 mutation to be a marker of good prognosis and re-analyzed the survival data for the oncogene expressions. As a result, we observed an independent correlation between a high oncogene expression profile and short OS and RFS in the patients with normal \textit{TAL1} and/or \textit{LMO2} and/or \textit{LYL1} expression (P = 0.08 [Mantel-Cox test]). NOTCH1/FBXW7 mutations were observed in all the genetic subgroups.

\textbf{Discussion}

Deregulation of signaling pathways that control normal T-cell development in the thymus plays a crucial role in T-ALL leukemogenesis. These pathways—under normal circumstances—are strictly regulated by transcription factors, which are also proto-oncogenic proteins. A few molecular mechanisms suggested for T-ALL pathogenesis including the mutations in \textit{NOTCH1} and \textit{FBXW7} genes leading to NOTCH pathway activation and ectopic expressions of the specific transcription factors such as \textit{LYL1}, \textit{TAL1}, \textit{LMO2}, \textit{TLX} genes. Currently, there are no genetic markers that can be used to reliably predict treatment response and/or outcome in pediatric T-ALL patients [22].

\textit{LMO2}, \textit{LYL1}, and \textit{TAL1} genes are members of the bHLH protein family. Among the phenotypic subgroups in the present study (immature, cortical, and mature), \textit{LYL1} and \textit{LMO2} exhibited the highest level of expression in immature cases, as reported by Meijerink et al. [23].
Recognition of specific T-cell developmental subgroups may have prognostic relevance in T-ALL. Among pediatric T-ALL patients, a pro-T immunophenotype was strongly correlated with poorer outcome than other T-cell phenotypes [24]. In contrast, comparison of LYL1 and LMO2 gene expression with their specific counterparts in the present study showed that the most significant differences occurred in mature stage cases. Moreover, LYL1 alone and LMO2-LYL1 co-expression were strongly associated with mediastinal involvement. We classified gene expression in the cases as TAL1/LYL1/LMO2 up-regulated or normal (Figure 2A and B), and analyzed the Kaplan-Meier estimates of OS and RFS. Although it was not significant but TAL1/LYL1/LMO2 up-regulated patients showed poor RFS rates. To date, it is unclear if TAL1/LYL1/LMO2 expression can be used to predict treatment outcome, although some studies suggest that it can [25].

The CALM-AF10 (MLLT10) fusion gene t(10;11) (p13;q21) is a common transcript in acute leukemia. It has been reported that 9% of adult T-ALL patients have
CALM-AF10 fusion and it is restricted to immature lineage [26]. CALM-AF10-positive ALL is associated with TLX family members and their transcriptional regulator, BMI1 [26]. The BMI1 gene determines the proliferation capacity of normal and leukemic stem cells [27]. In the present study none of the T-ALL patients carried the CALM-AF10 fusion gene, whereas 53.4% of the cases had elevated BMI1 mRNA levels. Increased BMI1 expression in pediatric T-ALL cases is generally ectopic and independent of CALM-AF10 fusion. It was also suggested that up-regulated expression of BMI1 is responsible for the aggressive nature of T-ALL. Although a specific relationship between the present patient’s clinical features and high-level BMI1 expression was not observed, T-ALL is the most aggressive form of ALL.

TLX genes are normally not expressed in adult tissues [28]. Approximately 7%-20% of childhood T-ALL patients have ectopic TLX1 and TLX3 expression [15,16]. In the present study 12% of the patients had ectopic expression of TLX1 and TLX3. Some studies indicate that ectopic TLX1 or TLX3 expression confers a poor response to treatment, whereas others report that they do not [5,13]. All the present study’s patients with TLX1 expression had mediastinal masses and those with TLX3 expression had significant CNS involvement, which may consider a possibility of poor prognosis. TLX gene expression may figure out TLX1 or TLX3 translocations but unfortunately these patients lack of cytogenetic data to validate.

NOTCH1 activating mutations occur in 30%-60% of T-ALL patients [10,11]. Additionally, inactivating mutations in the E3-ubiquitin ligase gene FBXW7 contribute to aberrant expression of NOTCH1. TLX1 and NOTCH was reported to be synergistically activated to regulate transcription in T-ALL [29]. These mutations were observed in all the present study’s genetic subgroups, but weren’t correlated with oncogene expression or OS ans DFS, but the number of patients with TLX1 expression was insufficient for reaching any conclusion.

The present findings offer some clues about the effects of activated transcription factors in pediatric T-cell leukemogenesis, prognostic parameters, and therapeutic applications in different thymic subsets. The most significant finding of the present study is that deregulation of multiple transcription factors (LMO2, LYL1, TAL1, TLX1, TLX3, and BMI1) was involved in the differentiation of T-cells, which is in agreement with other reports [1-5]. The present findings are relevant to 3 main topics. The first is the etiological point of view that the development of pediatric T-ALL is associated with up-regulation of several oncogenic transcription factors in a stage-specific manner. T-cell-specific oncogene expression has a greater impact in mature stage patients than in immature stage patients. The second is the prognostic implication of which identification and validation of oncogenic transcription factors in T-cell leukemia may lead to the development of new prognostic markers; these may then be useful for patient follow-up in the future. Lastly, identification of specific expression profiles in pediatric T-ALL subgroups may aid the development of new therapeutic applications and protocols.

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Conflict of interest statement
The authors of this paper have no conflicts of interest, including specific financial interests, relationships, and/or affiliations, relevant to the subject matter or materials included.

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