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Original Research

The correlation between Pax5 deletion and patients survival in Iranian children with precursor B-cell acute lymphocytic leukemia

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Abstract: Despite advances in treatment, children with acute lymphoblastic leukemia (ALL) still experience drug resistance and relapse. Several gene mutations are involved in the onset of this disease and resistance to therapy. The present study examines the incidence of IKZF1, CDKN2A/B, PAX5, EBF1, ETV6, BTG1, RB1, JAK2, and Xp22.33 gene deletions/duplications associated with pediatric ALL in Iran and investigates the possible effect of these mutations on drug resistance. Three-year disease-free survival (3DFS) was evaluated for children diagnosed with Philadelphia negative precursor-B-cell ALL hospitalized at Sayed-al-Shohada Hospital, Isfahan-Iran, from January 2009 until December 2012. DNA was extracted from bone marrow slides, and ALL correlated gene deletions and duplications were measured using Multiplex Ligation-dependent Probe Amplification (MLPA) method. The correlation between gene mutations and 3DFS was then assessed. Among the nine aforementioned investigated genes, 63% of samples showed at least one gene mutation. At least two concomitant genomic mutations were observed in 42% of samples. Pax5 deletion was the most prevalent gene mutation observed in 45% of cases, and showed significant negative impact on response to treatment. CDKN2A/B (9p21.3) gene deletion, and ETV6 (12p13.2) gene duplication also demonstrated negative effect on patient survival and contributed to a worse prognosis if concomitant with Pax5 gene deletion. ALL patients with one of the gene deletions including Pax5 and CDKN2A/B (9p21.3) or ETV6 (12p13.2) gene duplication are classified as high-risk patients and need more intensified protocols of treatment to improve their chance of survival.

Key words: Acute lymphocytic leukemia; Pax5 gene deletion; CDKN2A/B gene deletion; ETV6 gene duplication; Disease free survival.

Introduction

Acute lymphocytic leukemia (ALL) is a malignant disease in which immature lymphoid cells proliferate uncontrollably and replace normal bone marrow cells (1). Approximately 80% of these patients respond well to chemotherapy. However, 20% to 25% of the cases experience relapse or lack of response to treatment (2, 3). Drug-resistance is the main cause of relapse in these patients and depends on many constraints, including genetic mutations or environmental factors. The treatment severity of leukemia is determined based on the disease prognosis and risk of relapse. Therefore, intensified treatment protocols (including bone marrow transplant) contributing to higher complications, was restricted to the high-risk cases (4). Identifying the factors contributing to relapse risk, can help to select the more suitable protocol, and prevent the complications of unnecessary chemotherapy prescription(4) .

Certain gene mutations (including deletions or duplications) are reported in 80% of patients with ALL that may affect the progression and prognosis of the disease (5, 6). Pax5 and IKZF1 gene mutations are announced as the most common mutations in B-cell lymphocytic leukemia with a reported prevalence up to 31.5% (7-10). Although the leukemogenesis of these mutations is evident, they are not considered as the "sole cause" of leukemia; yet, several evidences suggest the negative role of some of these mutations in response to treatment and the subsequent chance of survival (5, 10, 11). Not only several known types of the gene deletions or duplications are involved in pediatric lymphoblastic leukemia, but also in many cases, two or multiple gene mutations may be concomitant (12).

The effect of the genetic mutations concomitance on the prognosis of ALL is yet to be determined. Identification of the factors that have a role in the patient's survival can help make better treatment decisions and therefore achieve better treatment outcomes. The present study therefore investigates the concomitance of a number of these mutations and its possible role in response to treatment.

Materials and Methods

Patients and sample collection

All the patients age one to fifteen years, with a final diagnosis of Philadelphia negative precursor B-cell ALL hospitalized from January 2009 to December 2012 at Sayed-al-Shohada Hospital, Isfahan-Iran, entered the study. All the patients who could not be followed up for at least 3 years, who had extramedullary involvement at the onset of the disease (including CNS or testis), infantile leukemia, or patients with Philadelphia positive

translocations observed in cytogenetic analyses were excluded from the study. Patients characteristics, including age, gender, WBC count and bone marrow flow cytometry results were extracted from their medical records and collected in different files.

DNA Isolation

The initial unstained bone marrow slides, prepared at diagnosis, were obtained from the patients' archives and used for DNA extraction. The genomic DNA was isolated from bone marrow slides using genomic DNA isolation kit (GeNet Bio, Korea). The concentration and purity of extracted DNA were estimated by spectrophotometry and electrophoresis in a 1% agarose gel stained with ethidium bromide and visualized directly under UV light. The DNA samples werekept at -20°C until PCR reactions were performed.

Genetic mutations analysis

IKZF1, CDKN2A/B, PAX5, EBF1, ETV6, BTG1, RB1, JAK2, and Xp22.33 gene deletions/duplications were investigated by multiplex ligation-dependent probe amplification (MLPA) using the Salsa MLPA P335-B1 ALL-IKZF1 kit (MRC-Holland, Amsterdam, the Netherlands) according to the manufacturer's instructions. This kit includes probes for each exon of IKZF1 (eight probes), CDKN2A/B (three probes), PAX5 (seven probes), EBF1 (four probes), ETV6 (six probes), BTG1 (four probes), RB1 (five probes), and SHOX-AREA, JAK2, P2RY8, ZFY, CSF2RA, IL3RA, and CRLF2 (one probe each) (figure 1).

Association of genetic mutations with three-year disease-free survival

Three-year disease-free survival (3-DFS) was evaluated for all the patients. Patients with evidence of treatment-resistance (failure of achieving remission in first induction), and patients with signs of relapse during any stage of treatment, were categorized as a negative 3DFS patients group. The frequency of 3-DFS was determined in each positive or negative previously mentioned gene mutations groups.

Statistical analysis

The Chi-square test was used to evaluate the relationship between mutation frequency and the 3DFS. To determine the effectiveness of the intervention, the paired t-test and the odds ratio (OR) were used at a confidence interval (CI) of 95%. The data obtained were analyzed in STATA-13 at a significance level of 5%.

Results

Patient samples

A total of 100 patients with pre-B linage ALL (55% male) with a mean age of 5.6 (±4.2) years entered the study. A treatment program based on ANZSTUDY intermediate risk protocol (http://www.anzctr.org.au/trial_view.aspx?ID=1568) was implemented for all children, and the 3-DFS was 74%.

The frequencies of 9 gene mutations (IKZF1, CDKN2A/B, PAX5, EBF1, ETV6, BTG1, RB1, JAK2, and Xp22.33) were determined, and the association between each gene mutation frequency with negative or positive 3DFS was investigated. Sixty three percent of patients displayed at least one of the aforesaid mutations, 22% of patients showed two mutations, and 19% of patients exhibited three mutations. Patients with at least one of the gene mutations, including Pax5 (9p13.2) deletion, CDKN2A/B (9p21.3) deletion, and ETV6 (12p13.2) duplication, showed negative effect on 3-DFS ($p< 0.05\%$) (Table 1). Three-year disease-free survival in patients without selected chromosomal abnormalities was similar to those with mutations other than those mentioned above. The incidence of Pax5 gene deletion, CDKN2A/B gene deletion and 12p13.2 gene duplication, was not significantly associated with gender or WBC count.

Since Pax5 deletion was the most frequent chromosomal abnormality and the prognostic factor affecting survival in our study, the concomitant effect of this mutation with other chromosomal aberrations was evaluated. Data showed that, dual deletions of Pax5 and CDKN2A/B genes contributed to a worse prognosis compared to Pax5 deletion alone (60.5% 3DFS vs 70.5%3DFS, p=0.0331) (Table2). Furthermore, The concomitance of Pax5 and 12p13.2 or 13q14.2 duplications demonstrated same results (44%3DFS vs 69%3DFS p=0.0166, and 47%3DFS vs 75% p=0.0215, respectively). There was not any significant correlation between Pax5 deletion and other additional mutations.

Discussion

In the current study, data showed that PAX5 mutation was the most prevalent gene deletion disorder in the pre-B ALL patients. Other studies have reported PAX5 deletion and sometimes CDKN2A/B deletion

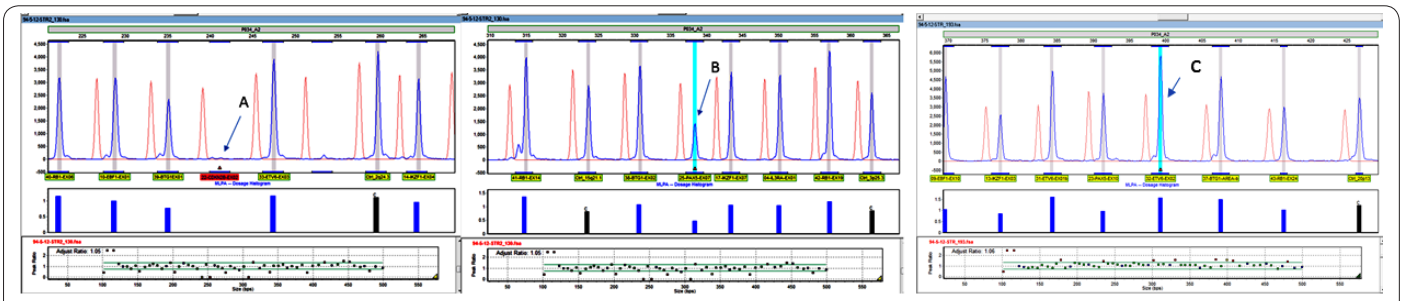

Figure 1. *Blue peak*: patient sample, *red peak*: control sample, *black column*: reference probe, *dark blue column*: gene probe (used according to SALSA MLPA 335 kit), *blue dot*: reference probe, *green dot*: normal gene probe, *red dot:* abnormal gene probe. Reference probes are selected according to SALSA MLPA 335 kit as mentioned in methods. MLPA gene analysis in a representative case of acute lymphoblastic leukemia; The indicated arrows depict deletions in (A) *CDKN2* (EX02) , and (B) *PAX5* (EX07) genes, and (C) *ETV6 (12p13.2)* gene duplication.

Table 1. The frequency of gene mutations (deletions and duplications) in pre B cell pediatric acute lymphoblastic leukemia, and their association with 3 year disease free survival (3DFS).

Mutation type		Percent of mutation in total patients	3-DFS positive (%) With mutation	3-DFS positive (%) without mutation	P value
Xp22.33	deletion	35%	71.5%	77.5%	N.S
	Duplication	24%	75%	75%	N.S
5q33.3 EBF1	deletion	28%	75%	75%	N.S
	Duplication	29%	86.2%	69%	N.S
7p12.2 IKZF1	deletion	34%	70.5%	78%	N.S
	Duplication	24%	79%	73%	N.S
9p24.1 JAK2	deletion	13%	77%	74.5%	N.S
	Duplication	32%	72.2%	77%	N.S
9p21.3 (CDKN2A/CDKN2)	deletion	35%	65.7%	81%	P=0.045
	Duplication	18%	77.5%	74%	N.S
9p13.2 (pax5)	deletion	45%	64.4%	87%	P=0.01
	Duplication	28%	85.5%	69.6%	N.S
12p13.2 ETV6	deletion	42%	75.5%	77%	N.S
	Duplication	23%	65%	78.5%	P=0.05
12q21.33 BTG1	deletion	14%	73%	78%	N.S
	Duplication	39%	76.5%	73.3%	N.S
13q14.2 RB1	deletion	30%	73%	78%	N.S
	Duplication	38%	71%	78%	N.S

Table 2. The Concomitance between PAX5 deletion and four other mutations; IKZF1 deletion, CDKN2A/CDKN2B deletion, ETV6 (12p13.2) duplication, and RB1(13q14.2) duplication, in pediatric B- linage ALL, and their impacts on 3-year disease free survival (3DFS). The concomitance between PAX5 deletion and any of the abovementioned mutations indicated worse outcome compared with samples with no indicated mutations. (P=0.025 [1a] , p=0.0331[1b] , p=0.0166 [1c] , p=0.0215 [1d]).

Gene mutation		Patient number	3DFS+
PAX5(deletion) and IKZF1(deletion) [1a]	PAX5 + IKZF1	27	63%
	No PAX5 No IKZF1	31	87%
	only PAX5	19	63%
	Only IKZF1	7	100%
PAX5(deletion) and CDKN2A/ CDKN2B (deletion)[1b]	PAX5 joint with CDKN2A/CDKN2B	28	60.5%
	No CDKN2A/CDKN2B No PAX5	48	87.5%
	PAX5 individually	17	70.5%
	CDKN2A/CDKN2B individually	7	85.5%
PAX5(deletion) and 12p13.2 (duplication) [1c]	PAX5 joint with 12p13.2	9	44%
	No PAX5 No12p13.2	41	92%
	PAX5 individually	36	69%
	12p13.2 individually	14	78%
PAX5(deletion) and 13q14.2 (duplication) [1d]	PAX5 joint with 13q14.2	17	47%
	No PAX5 No 13q14.2	34	83%
	PAX5 individually	28	75%
	13q14.2 individually	21	90%

as the most common gene mutations in pediatric ALL, with different frequencies from19% to 31.5% (12-14). It should be noted that in the present study, only non-burkitt Philadelphia negative ALL, age between one and 15 years old were included. Moreover, different frequencies of genetic mutations reported in various studies may be related to not only leukemia subtype and age of patients, but also to the race and geographical regions where patients live (1, 12, 15). These may justify the dissimilar prevalence of genetic abnormalities demonstrated in the current study compared to other reports. To our knowledge, the present study is the first effort in reporting the frequency of ALL related mutations in Iran and the first report to validate the poor prognostic value of PAX5 and CDKN2A/B gene deletions and 12p13.2 gene duplication in this country. This may pave the way for conducting more extensive studies on ALL cytogenetics in Iran, and possible development of improved ALL therapies, subsequently.

According to our data, the coincidence of some genetic deletions was very common. These results are supported by Schwab et al. who reported at least one deletion in 59%, two deletions in 19% and three in 9% of the pre-B ALL cases examined in their project (12). Many studies have examined the relationship between these deletions and survival, but the effect of the concomitance of these deletions on patients' survival has been less addressed. In the current study, From the nine selected mutations IKZF1, CDKN2A/B, PAX5, EBF1, ETV6, BTG1, RB1, JAK2, and Xp22.33 only PAX5

deletion, CDKN2A/B (9p21.3) deletion, and ETV6 (12p13.2) duplication are demonstrated to possess effective impact on 3DFS. In our study, patients with IKZF1 deletion had 70% 3DFS vs 78% in patients without deletion, but the difference was not significant. In review of literature, there are some studies that have introduced IKZF1 deletion as a negative prognostic factor for ALL (11, 12, 16). Our study is the first report of gene mutation frequency in pediatric ALL, from the center of Iran, and the difference in frequency and DFS may be related to the race and geographical regions. Our data also showed that although IKZF1 deletion was observed in 32 samples, but in 28% of cases had jointed with PAX5 deletion. In such patients, survival was significantly reduced, and 3DFS was 63% in cases with Pax5 deletion when was joined with IKZF1 deletion, vs 87% in cases without these mutations ($P<0.02$). In other words, the reduction in survival in cases with IKZF1 deletion, at least in some cases, may be due to the impact of concomitant PAX5 deletion, not the IKZF1 deletion itself.

Our result showed that, mutations including CDKN2A/B deletion and ETV6 (12p13.2) duplication had a negative influence on patient survival, but in several cases, they were jointed with PAX5 deletion. Also, the two-mentioned mutation had not any negative impact on survival when they were not jointed with PAX5 deletion, but the role of Pax5 deletion was far more intensified when had happened in concomitance with them. Further studies need to be applied in larger populations and the related mechanisms through which these mutations exert their prognostic impact awaits further investigation. Some mutations including EBF1 duplication and PAX5 duplication showed influences, although not significant, on patient survival (table 1). Evaluating larger populations in prospective cohort studies may clarify the exact prognostic value of these chromosomal abnormalities in pediatric ALL.

A growing body of evidence suggests a critical role for PAX5 in carcinogenesis. However, the effect of PAX5 on ALL prognosis and respond to treatment is not discussed properly, specifically in the presence of other chromosomal abnormalities. To the best of our knowledge, this is the first study to investigate the possible correlation between PAX5 deletion and concomitant genetic mutations considering their impact on ALL sensitivity to chemotherapy. The mechanism through which PAX5 may affect ALL prognosis awaits to be determined.

The present study showed that chromosomal modifications including PAX5 deletion, CDKN2A/B (9p21.3) deletion, and ETV6 (12p13.2) duplication have negative impact on ALL respond to therapy. A concomitance between PAX5 deletion and other aforementioned mutations contributed to a worse prognosis. Identification of such chromosomal abnormalities can help recognition of high-risk cases and improve their chances of cure through adopting more intensified treatment protocols.

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