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RESEARCH ARTICLE

Expression of TEL-AML1 and E2A-PBX1 by real-time PCR in pediatric ALL in Baghdad

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Abstract

Background: Acute lymphoblastic leukemia (ALL) is a heterogeneous disease that has multiple genetic subtypes with different prognostic significance.
Aim of study: To study the expression of TEL-AML1 and E2A-PBX1 fusion genes in Iraqi pediatric ALL patients.
Patients, materials and methods: A prospective case-control study was conducted using RTqPCR to study the expression of TEL-AML1 and E2A-PBX1 fusion genes in bone marrow aspirates of 48 pre-treated ALL patients and 46 control subjects. These aspirates were collected from The Children's Welfare Teaching Hospital in Baghdad from July 2013 to June 2014.
Results: 21/48 ALL patients were males and 27/48 were females. Age ranged from 2 months to 13 years with a median age of 5 years. Twenty six of these patients were aged between 1-5 years. Molecular screening detected TEL-AML1 transcript in 20.8% of ALL patients whereas E2A-PBX1 transcript was detected in 16.7% of those patients.
Conclusion: The molecular prevalence of TEL-AML1 and E2A-PBX1 fusion genes in Iraqi children with ALL parallels previous reports worldwide.

Introduction:

Acute lymphoblastic leukemia (ALL) is a neoplastic disease characterized by distinct morphologic, immunophenotypic, cytogenetic, and molecular features, some of which have important clinical implications for diagnosis and/or prediction of response to a particular treatment.[1,2] Chromosomal translocations are the hallmark of ALL.[3] The most frequent chromosomal aberrations found in ALL are t(12;21), t(1;19), t(9;22) and t(4;11) producing fusion transcripts for *TEL-AML1*, *E2A-PBX1*, *BCR-ABL* and *MLL-AF4* respectively.[4] These cytogenetic defects can be detected by variety of techniques like conventional cytogenetics, fluorescence *in situ* hybridization (FISH) or reverse transcriptase polymerase chain reaction (RT-PCR).[5] RT-PCR and real-time polymerase chain reaction (qPCR) detection of fusion gene transcripts in leukemia is highly sensitive and specific.[6] This study aims to determine if ALL in Iraqi's pediatric patients has molecular features that are similar to other countries.

Patients, materials and Methods:

Ethical approval from the Iraqi Ministry of Health was obtained for a prospective case control study using two-step RTqPCR to study the expression of TEL-AML1 and E2A-PBX1 transcripts in bone marrow aspirates of 48

pediatric ALL patients. These aspirates were collected over a 12 months period from July 2013 to the June 2014 from The Children's Welfare Teaching Hospital/ Medical City Center. Processing was performed at the Department of Pathology and the Communicable Diseases Research Center at the College of Medicine, Baghdad University.

Forty eight pediatric newly diagnosed pre-treated ALL patients aged less than 14 years were included in this study. Diagnosis as ALL was made by a senior hematopathologist and based on clinical features and findings of peripheral blood and bone marrow aspirate examinations. Bone marrow aspirates obtained from 46 pediatric subjects who did not have cancer but showed either normality, isolated thrombocytopenia or anemia during evaluation of their bone marrow aspirates, were used as controls. The patients' personal and clinical information were carefully documented from the patients' parents, guardians or hospital records. Findings of hematological investigations regarding peripheral blood and bone marrow aspiration examinations were carefully documented from the patients' laboratory and hospital records. All of the 48 ALL patients were assessed periodically for between 4 to 15 months to assess the clinical outcome.

Handling of the specimens proceeded with caution under aseptic measures plus use of RNase decontamination (RNaseZap, Ambion) of the working surfaces and apparatus. Forward and reverse oligonucleotide primers for the mRNA (spanning introns) of each genetic target were designed using NCBI/ primer-BLAST online software and were purchased from Alpha DNA Ltd. (Quebec, Canada) and stored lyophilized at (-20°C). The sequences of the used primers for *TEL-AML1* transcript were 5'TGTCTCCCCGCCTGAAGA'3 and 5'TCGTGGACGTCTCTAGAAGGATT'3. For *E2A-PBX1* transcript, the sequences were 5'CACCAGCCTCATGCACAAC'3 and 5'TCGCAGGAGATTCATCACG'3 while for *GAPDH*, the primers sequences were 5'CTATAAATTGAGCCCGCAGCC'3 and 5'ACCAAATCCGTTGACTCCGA'3. After collecting aspirates in EDTA tubes, 500 µl of the EDTA blood was transferred into a clean RNase free microfuge tube containing 1.3 ml of RNA Stabilization Solution (RNAlater®, Ambion) then stored at (-20ºC) until processing (usually within 1-2 days). Total RNA was extracted by phenol/ chloroform using TRIzol® LS Reagent (Ambion) as recommended by the reagent protocol. The extracted RNA was treated with a DNA removal kit (DNA-free ^{TM} kit, Ambion) to digest any contaminating residual DNA within the RNA pellet as advised by the manufacturer. After treating the extracted RNA with the DNA removal kit, RNA concentrations and purity were measured automatically by BioDrop µLite, Micro-Volume UV/Vis Spectrophotometer. The purity was checked for each specimen by means of reading the absorbance at 260nm and 280nm and calculating the A_{260}/A_{280} ratio. Every RNA had A_{260}/A_{280} ratio of 1.9 - 2.2 was considered to have acceptable purity and was included in the study, otherwise it was excluded. RNA was then transferred to a new RNase free tube to be frozen at -20^{o}C until further processing.

Prior to use in qPCR, RNA samples were reverse transcribed into cDNA using a reverse transcription kit (High capacity cDNA reverse transcription kit, Applied Biosystems) after unification of RNA concentrations into 80 ng/ µL. Reverse transcription was conducted on ice according to the manufacturer's manual then the tubes were loaded into an Applied Biosystems thermal cycler and programmed using 25^{o}C for 10 minutes followed by a step at 37^{o}C for 120 minutes then at 85^{o}C for 5 minutes. The resultant 20 µL cDNA was stored at (-20 º C) until use in qPCR. Real time PCR reaction was performed on the cDNA template using KAPA SYBR® Fast qPCR kit Master Mix Universal (KAPA Biosystems) in a Stratagene real time PCR machine according to manufacturers' protocols. Every reaction was produced in a duplicate and included a non-template control, non-amplification control and no primer control served as negative controls. *GAPDH* housekeeping gene was used as an internal control to check the integrity of the reverse transcribed RNA. On ice and in each tube, 19 μL of a mixture (consisting of 10 µL of 2X KAPA SYBR® Fast qPCR kit Master Mix, 1.2 µL of 5 µM forward primer and 1.2 µL of 5 µM reverse primer, 6.2 µL of nuclease free water and 0.4 µL of ROX/ low) was added to a 1 µL of each cDNA in separated tubes to get a final reaction volume of 20 µL. The tubes were capped and loaded into the thermal cycling machine with the following thermal conditions: a hot-start at 95º C for 10 minutes was followed by 40 thermal cycles consisting of denaturation at 95º C for 30 seconds, annealing for 40 seconds at 64º C, 63º C and 59º C, respectively for *TEL-AML1*, *E2A-PBX1* and *GAPDH*, followed by extension at 72º C for 30 seconds. At the end of each qPCR cycle, dissociation melting curve analysis was programmed to confirm the identity of the PCR products by checking the melting temperatures. Dissociation thermal conditions were: a step at 95º C for 1 minute followed by a step at 55º C for 30 seconds then at 95º C for 30 seconds. C_q values $\geq$ 38 were considered unreliable and neglected.[7]

All results were analyzed and calculated using the Statistical Package for the Social Sciences (SPSS) 20.0 software and the Microsoft Office Excel software 2007. Only *P* values less than 0.05 were considered statistically significant.

Results:

From a total of 48 patients with ALL, 21 (47.9%) were males and 27 (56.3%) were females with a male to female ratio (M: F) of 0.78:1. Age at diagnosis ranged between 2 months to 13 years; median age was 5 years and mean age was 5.9 years with standard error (SE) of $\pm$ 0.51 years. ALL was more prevalent in the age group of 1 - 5 years in 26

patients (55.1 %), 16 of them were males and 10 were females whereas the least frequent occurrence of ALL was found in the age group of less than 1 years old in only 2 patients (4.2 %).
The extracted RNA concentrations ranged from 40 µg/ml to 295 µg/ml with a mean of 89 µg/ml and SE of ± 4 µg/ml. DNA concentrations ranged from 0 µg/ml to 1 µg/ml with a mean of 0.01 µg/ml and SE of ± 0.001 µg/ml. The purity was checked for each specimen by means of reading the A_{260} and A_{280} and calculating the A_{260}/A_{280} ratio. Every RNA sample included in this study had a ratio within a range from 1.9 to 2.2, and thus was considered to have a useable purity.
Commonly used internal controls are ß-actin and *GAPDH* mRNAs and 18S rRNA.[8] For all of the included cases in the ALL and control groups, RNA integrity was assessed through the qPCR amplification of the *GAPDH* housekeeping gene as an internal control. In the ALL group, *GAPDH* documented Cq values ranged from 21.14 to 31.00 with a mean of 25.23 and SE of ± 0.37. Similarly in the control group, *GAPDH* Cq values ranged from 20.95 to 30.98 with a mean of 25.12 and SE of ± 0.38. Cq values of *GAPDH* amplification did not differ significantly between ALL and control groups ($P = 0.360$) (Figure1).

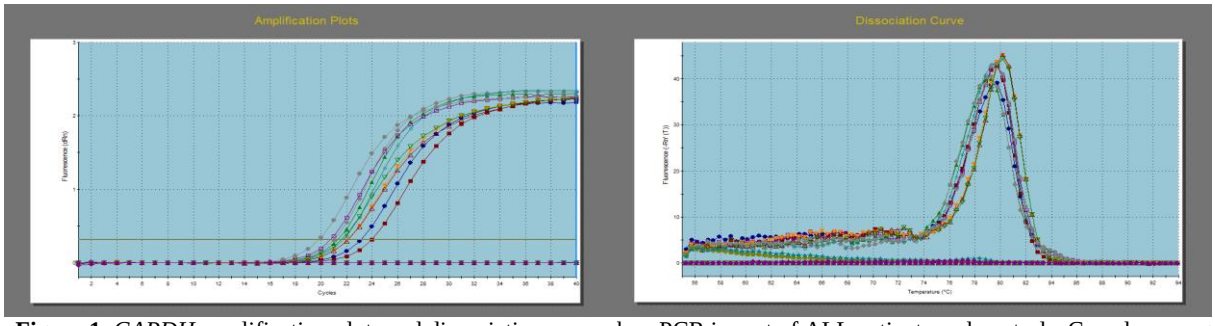

Figure 1. *GAPDH* amplification plots and dissociation curves by qPCR in part of ALL patients and controls. Cq values ranged from 20-24. Melting temperatures ranged from 79-80 ^{o}C. The photograph was taken directly from qPCR machine.

TEL-AML1 was the most frequent detected transcript found in 10 ALL patients (20.8%), 2 were males and 8 were females. Age at presentation ranged between 2 months and 11 years with a mean of 5.1 ± 1.1 years.
In this group, *GAPDH* Cq values ranged between 21.14 and 31.00 with a mean of 25.57 and SE of ± 0.36. *TEL-AML1* transcript Cq values ranged between 21.76 and 27.84 with a mean of 23.95 and SE of ± 0.36. Melting temperature of the products ranged between 79^{o}C to 80^{o}C (Figure 2).

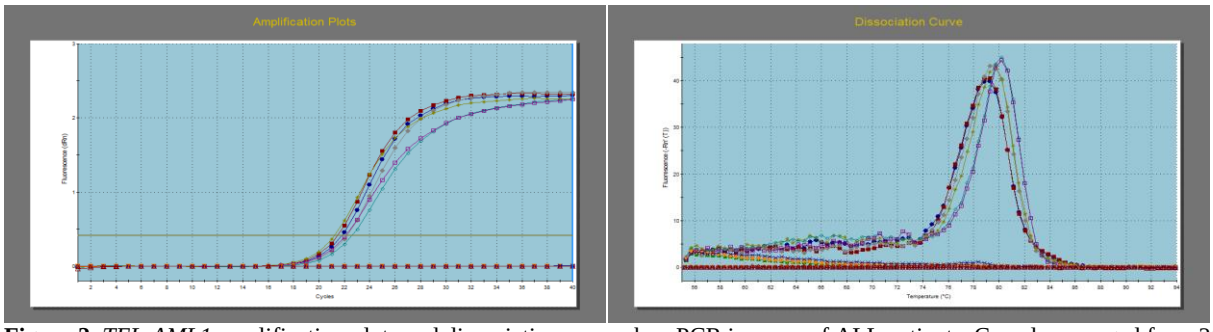

Figure 2. *TEL-AML1* amplification plots and dissociation curves by qPCR in some of ALL patients. Cq values ranged from 21-23. Melting temperatures ranged from 79-80 ^{o}C. The photograph was taken directly from qPCR machine.

ΔCq = (mean Cq of *TEL-AML1* ± SE) – (mean Cq of *GAPDH* ± SE).
ΔCq = (23.95 ± 0.36) – (25.57 ± 0.36).
ΔCq = – 1.68 ± 1.19.

After following these *TEL-AML1* 10 positive patients many months post treatment, a good response with continuation complete remission (CCR) was observed in all of them (100%).
Among 6 followed up *TEL-AML1* patients, only one patient showed positive expression after induction. This patient was a slow early responder, however he achieved CCR.
The Cq results for this patient were as the following:
Post-treatment *GAPDH* Cq was 27.07 and *TEL-AML1* Cq was 31.00

Pre-treatment *GAPDH* Cq was 28.12 and *TEL-AML1* Cq was 26.12
ΔΔCq = Post-treatment ΔCq – Pre-treatment ΔCq
ΔΔCq = (31 – 27.07)– (26.12 – 28.12)
ΔΔCq = 3.93 – (-2) = 5.93
$2^{-\Delta\Delta Cq} = -3.93$
This $2^{-\Delta\Delta Cq}$ value represents a reduction of *TEL-AML1* expression by 3.93 folds after treatment. The outcome of this patient was CCR.

E2A-PBX1 transcript expression was detected in 8 ALL patients (16.7%), 3 were males and 5 were females. Age at presentation ranged between 2.5 months and 7 years with a mean of 3.7 ± 0.8 years.
In this group, *GAPDH* Cq values ranged between 21.19 and 28.07 with a mean of 24.44 and SE of ± 0.78. *E2A-PBX1* transcript Cq values ranged between 26.73 and 30.69 with a mean of 28.45 and SE of ± 0.51. Products melting temperature ranged from 84°C to 85° C (Figure 3).

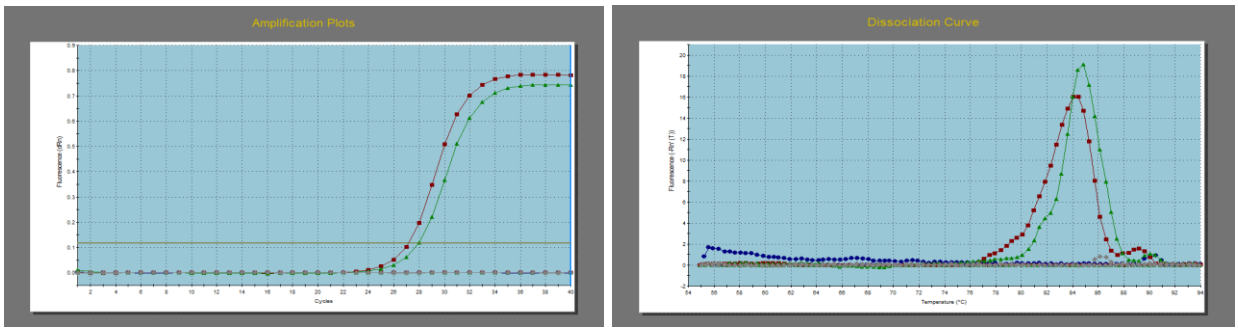

Figure 3. *E2A-PBX1* amplification plots and dissociation curves by qPCR in some of ALL patients. Cq values ranged from 27-28. Melting temperatures ranged from 84-85 ° C. The photograph was taken directly from qPCR machine.

ΔCq = Cq gene of interest – Cq internal control.
ΔCq = (mean Cq of *E2A-PBX1* ± SE) – (mean Cq of *GAPDH* ± SE)
ΔCq = (28.45± 0.51) – (24.44 ± 0.78)
ΔCq = 4.01 ± 0.69

After following the 8 *E2A-PBX1* positive patients many months post treatment, a CCR was observed in 5 of them, 2 patients died after relapse and 1 patient was lost to follow-up.
Among 6 followed-up patients with *E2A-PBX1,* only one patient showed positive expression after induction, he was not in remission status during BM examination. The Cq values for this patient were as the following:
Post-treatment *GAPDH* Cq was 28 and *TEL-AML1* Cq was 32.51
Pre-treatment *GAPDH* Cq was 28.07 and *TEL-AML1* Cq was 30.69
ΔΔCq = Post-treatment ΔCq – Pre-treatment ΔCq
ΔΔCq = (32.51 – 28) – (30.69 – 28.07)
ΔΔCq = 4.51 – 2.62 = 1.89
$2^{-\Delta\Delta Cq} = 0.11$
This $2^{-\Delta\Delta Cq}$ value illustrates an increase of expression by 0.11 fold after treatment, this patient died.
In contrast the control group, none of the samples showed positive amplification for *TEL-AML1* or *E2A-PBX1* fusion genes transcripts.

Discussion:

To our knowledge this is the first Iraqi study that used qPCR to screen Iraqi pediatric ALL patients for the expression of *TEL-AML1* and *E2A-PBX1* transcripts.
Out of 48 ALL patients included in this study, 43.8% were males and 56.3% were females. This was to some extent different from what was reported in other studies [3,9,10] where males dominated. That could be explained by differences in sample sizes between current study and the other mentioned studies or due to the factor of randomized collection of cases. The presenting age ranged from 2 months to 13 years; with a median age of 5 years and mean of 4.7 ± 0.57 years. More than half of the patients aged between 1 – 5 years old. These findings were compatible with

other studies.[11,12,13,14,15] However, an Indian study [16] demonstrated a higher presenting median age of 7.9 years probably due to ethnic variations between the two populations that might caused such drift.

RNA analysis yielded good RNA concentrations with high purity and negligible detected amount of genomic DNA. Using DNA removal kit plus designing primer sequences targeting mRNA, eliminated amplification of genomic DNA sequences. With using *GAPDH* housekeeping gene as an internal control, it was possible to assess the integrity of the extracted RNA by positive *GAPDH* amplification. Cq values of *GAPDH* amplification did not differ significantly between ALL and control groups which suggests *GAPDH* to be suitable stably expressed internal control for comparative quantification in similar studies.

This study demonstrated detection of *TEL-AML1* in 20.8% of ALL patients which was absent in the control group. This finding was to some extent comparable to findings in other studies. [12,17,18,19,20,21,22] As it was mentioned in many studies, the application of molecular and cytogenetic tools has demonstrated *TEL-AML1* fusion transcripts in about to 20-30% of pediatric ALL, making it the most frequent molecular cytogenetic abnormality in childhood ALL.[23] However, other studies demonstrated slightly higher or lower frequencies than our study.[3,23,24] These variable frequencies could be attributed to the diverse methodologies used with dissimilar levels of sensitivity and specificity, also differences in sample size, geographic heterogeneity and population divergence in these studies in addition to the environmental contamination that characterized Iraq due to conflicts over 30 years as was suggested by an Iraqi study in 2010 [25], all may play a role in such frequency variations.

Mean of presenting age associated with *TEL-AML1* was 5.1 $\pm$ 1.1 years and this closely resembled observations of other Iraqi, Indian and Pakistani studies where *TEL-AML1* is known to be associated with younger favorable age group.[3,12,23,24]

The *E2A-PBX1* transcript was observed in 16.7 % of ALL patients whilst it was not detected in the control group. This makes it the second most frequently detected fusion gene among ALL patients similar to findings of many other studies regardless of the varying frequencies.[12,19,26] However, the frequency of *E2A-PBX1* detection in the current study was higher ($P > 0.05$) than what was reported in some studies. [10,20,21,24,27] Besides differences in used methodologies, ethnic and numerical diversity among these studies, possible environmental conditions that characterized Iraq from other countries due to repeated wars and conflicts with probable high pollution level, may be the reasons culpable for such discrepancies.

Concerning presenting age, the mean age for patients with *E2A-PBX1* was 3.7 $\pm$ 0.8 years similarly to finding of another Iraqi study [24] but lower than what was reported in other studies.[12,27] Such differences could be explicated by the dissimilarities in sample size among mentioned studies.

Conclusion:

Molecular screening detected TEL-AML1 transcript in 20.8% of ALL patients whereas E2A-PBX1 transcript was detected in 16.7% of those patients. The molecular prevalence of TEL-AML1 and E2A-PBX1 fusion genes in Iraqi children having ALL is to somewhat similar to previous reports worldwide.

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