Original article

Screening for *BCR-ABL* fusion gene among Iraqi children with ALL using real-time PCR

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Abstract

Background: Pediatric acute lymphoblastic leukemia (ALL) is a heterogeneous disease with diverse genetic subtypes and different clinical behavior. ALL with BCR-ABL is a subtype with distinctive response to treatment.
Objectives: This study aims to study the expression of BCR-ABL transcript in Iraqi pediatric ALL patients.
Patients, materials and methods: A case-control prospective study was conducted to study the expression of minor and major BCR-ABL fusion genes in bone marrow aspirates of 48 pre-treated ALL patients and 46 control subjects using qPCR technique. Those aspirates were collected from Children Welfare Teaching Hospital in Baghdad over a 1 year period from July 2013 to June 2014.
Results: From 48 patients with ALL 21 were males and 27 were females. Age ranged from 2 months to 13 years with a median age of 5 years. Twenty six of these patients aged between 1-5 years. Molecular screening detected minor BCR-ABL transcript in 2.1% of ALL patients whereas major BCR-ABL transcript was not expressed in any of ALL patients. Contrary to the control group, minor BCR-ABL transcript was detected in three samples.
Conclusion: The molecular prevalence of minor and major BCR-ABL fusion gene in Iraqi children with ALL is to somewhat similar to previous reports worldwide.
Key words: acute lymphoblastic leukemia, *BCR-ABL*, real-time PCR

Introduction:

ALL is a malignant 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 translocation is the hallmark of ALL.[3] The Philadelphia translocation is one of the identified chromosomal abnormalities among 25% of the adults with ALL, while only 3% of pediatric ALL is Philadelphia chromosome positive.[4,5] Philadelphia chromosome results from a reciprocal translocation between the long arms of chromosomes 9 and 22 [t(9;22)(q34;q11)], this translocation encodes a chimeric gene consisting of

portion of *BCR* fused to portion of *ABL1*.[6] Depending on the exact position of the translocated *ABL* gene (exon b2) within *BCR*, three different fusion products can be generated: p190 (molecular weight 190 kd, exon e1), p210 (exon b2 or b3), and p230 (exon e19). Patients with chronic myeloid leukemia express mostly the p210 protein and rarely p230, whereas ALL patients mainly express p190. Some of ALL patients present with p210 expression.[5] Such a transformation results in a constitutively active ABL tyrosine kinase that provokes abnormal signaling and activates several cellular pathways[3] such as the Crkl, AKT, Ras/Raf-1, Stat 1 and 5 pathways, platelet derived growth factor (PDGF), and the c-kit receptor tyrosine kinase.[5]

ALL Patients with *BCR-ABL* have poor prognosis with standard chemotherapy but show impressive response to imatinib mesylate.[6] It is possible that the prognosis in children is improved by stem cell transplantation in first remission.[7,8] This cytogenetic defect can be detected by a variety of techniques such as conventional cytogenetics, Fluorescence *in situ* hybridization (FISH) or reverse transcriptase polymerase chain reaction (RT-PCR).[9] RT-PCR and real-time polymerase chain reaction (qPCR) method in detection of fusion gene transcripts is highly sensitive and specific technique also needs very few tumor cells.[10]

Patients, Materials and Methods:

An ethical approval for conducting this study using two steps RT- qPCR technique was obtained. These aspirates were collected in the period from the 1st of July 2013 to the 31st of June 2014 from Children Welfare teaching hospital/ Medical city directory. The processing was done at Department of Pathology and the Communicable Diseases Research Center at 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 of ALL was made by senior hematopathologist and based on clinical features and findings of peripheral blood and bone marrow aspirates. Bone marrow aspirates obtained from 46 pediatric subjects who do not have malignant conditions but showed normality, anemia or isolated thrombocytopenia 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. All hematological findings regarding peripheral blood and bone marrow aspiration examinations were carefully documented from the patients' hospital and laboratory records.

Handling of these specimens was done with caution under aseptic measures plus using RNase decontamination solution in cleaning 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 tool and were purchased from Alpha DNA Ltd. and stored

lyophilized at (-20°C). These primers' sequences were GAACTCGCAACAGTCCTTCG and TGAGGCTGAAAGTCAGATGCT for minor *BCR-ABL* transcript, TCGTGTGTGAAACTCCAGACT and GGGTCCAGCGAGAAGGTTT for Major *BCR-ABL* transcript. For *GAPDH*, the primers sequences were as CTATAAATTGAGCCCGCAGCC and ACCAAATCCGTTGACTCCGA. After collecting the aspirates in EDTA tubes, a half ml of the EDTA blood was transferred into clean RNase free microfuge tube containing 1.3 ml of RNAlater®, Ambion (RNA Stabilization Solution) then stored at (-20 º C) until processing (usually within 1-2 days). RNA was extracted by phenol/ chloroform method using TRIzol® LS Reagent, Ambion as it was recommended by the TRIzol® LS Reagent protocol.[11] The extracted RNA was treated with DNA-free ™ kit, Ambion (DNA removal kit) to digest any contaminating residual DNA within the RNA pellet as it was advised by manufacturer.[12] After treating the extracted RNA with 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 having A_{260}/A_{280} ratio of 1.9 - 2.2 was considered to have good purity and was included in the study, otherwise it was excluded. RNA was then transferred to a new RNase free tube to be frozen deeply until further processing.

To be used in qPCR reaction, RNAs were reverse transcribed into cDNA using High capacity cDNA reverse transcription kit, Applied Biosystems after unification of their concentrations into (80 ng/ μL). Reverse transcription was conducted on ice according to the manufacturer's manual[13] then the tubes were loaded into the thermal cycler, Applied Biosystems and programmed as was proposed by the kit's protocol with a beginning step at 25°C for 10 minutes followed by a step at 37°C for 120 minutes then at 85°C for 5 minutes. The resultant cDNA (20 µL) was stored at (-20 º C) until using in qPCR. Real time PCR reaction was done on the cDNA template using KAPA SYBR® Fast qPCR kit Master Mix Universal, KAPA Biosystems in a Real time PCR machine, Stratagene, Agilnet according to manufacturers protocols[14,15]. Every reaction was done in a duplicate and included a non template control, non amplification control and non primer control as negative controls however no positive controls were used. *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 the above 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 to get a final reaction volume of 20 µL. The tubes were capped firmly to be loaded into the machine with the following thermal conditions: First, a hot start at 95º C for 10 minutes was done. Second, 40 cycles were performed consisted of denaturation at 95º C for 30 seconds,

annealing for 40 seconds [at appropriate temperature differs among primers based on the calculated melting temperature (Tm) of the primers used; for minor *BCR-ABL* and *GAPDH* it was 59º C while for Major *BCR -ABL* it was 61º C] followed by extension at 72º C for 30 seconds. At the end of each qPCR reaction, dissociation analysis was done to confirm the specificity of the PCR products by checking the melting temperatures. C_T values $\geq$ 38 were considered unreliable and neglected.[16]

All results were analysed 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 encountered at the age group of 1 - 5 years in 26 patients (55.1 %), 16 of them were males and 10 were females whereas the least frequency was found in the age group of less than 1 years old in only 2 patients (4.1 %) and both of them were females. In the ALL group, the most common presenting clinical feature was fever being observed in 43 patients (89.6%) followed by splenomegaly, pallor, hepatomegaly, mucocutaneous bleeding and lymphadenopathy in 79.2%, 77.1%, 70.9%, 62.5% and 58.3% of the patients, respectively. None of the male patients had testicular swelling suggestive of involvement. According to FAB classification criteria, 8 patients (16.7%) were classified as L1, 38 patients (79.2%) as L2 and only 2 patients (4.2%) as L3.

After treating the extracted RNA with DNA removal kit, assessing the purity of the extracted RNA and measuring the concentrations of that RNA and associated DNA (if any), for both of ALL and control groups, were done using micro-volume UV/Vis spectrophotometer. The extracted RNA concentrations ranged from 40 μg/ml to 295 μg/ml with a mean of 89 μg/ml and SE of $\pm$ 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 $\pm$ 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 lies within a range from 1.9 to 2.2 and thus considered to have good purity.

Real-time PCR

For all of the included cases in the ALL and control groups, RNA integrity was considered good by the positive qPCR amplification of the *GAPDH* housekeeping gene (internal control). In ALL group, *GAPDH* documented Ct values ranged from 21.14 to 31.00 with a mean of 25.23 and SE of $\pm$ 0.37 similarly in the control group, *GAPDH* Ct values ranged from 20.95 to 30.98 with a mean of 25.12 and SE of $\pm$ 0.38. Ct values of *GAPDH* amplification did not differ significantly between ALL and control groups (P = 0.360).

Only one female patient (2.1%) expressed minor *BCR-ABL* transcript (p 190) with a presenting age of 5 years. Mainly the

presentation was fever, pallor, and bone pain with splenomegaly, hepatomegaly and lymphadenopathy. *GAPDH* Ct value was 21.14 while *BCR-ABL* (p 190) Ct value was 25.20 with a melting temperature of 79.8°C (Figure 1).

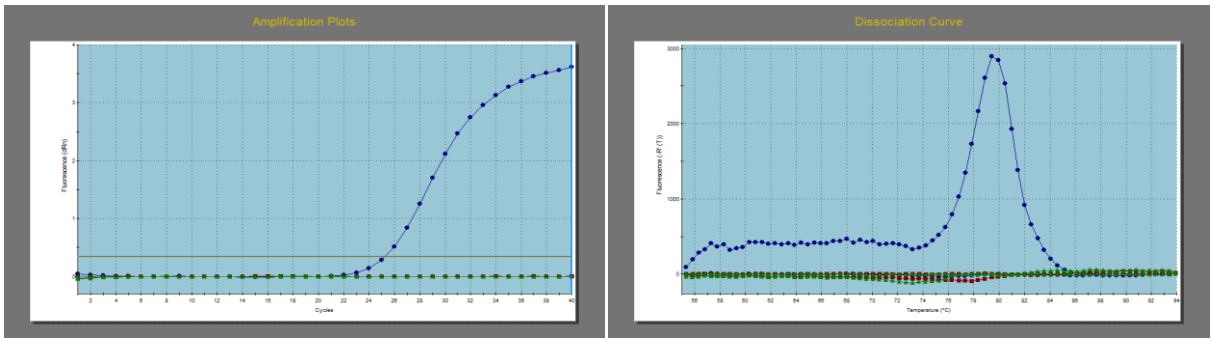

Figure 1. *BCR-ABL* p 190 amplification plot and dissociation curve by qPCR in ALL patient. Ct value was 25. Melting temperature was 80 °C. The photograph was taken directly from qPCR machine.

In contrast to the control group, most of the samples showed no positive amplification for the fusion gene transcripts except three samples. These samples had positive amplification for *BCR-ABL* p190 with a mean Ct value of 30.73 and SE of ± 0.96. When dissociation was done for each case of them, another sharp peak with a lower melting temperature than that of the product of the *BCR-ABL* p190 positive ALL case was observed, indicating a second product (Figure 2).

In current study, none of the cases in neither of the ALL group nor control group expressed *BCR-ABL* p210.

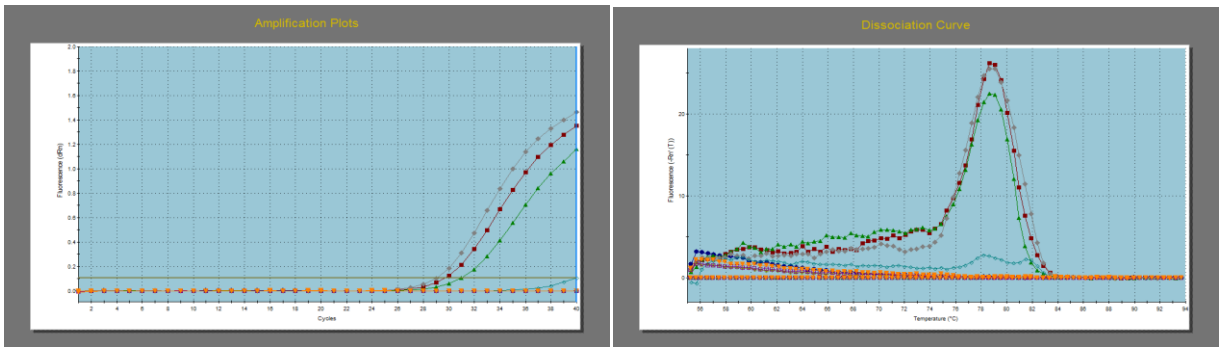

Figure 2. *BCR-ABL* p 190 amplification plots and dissociation curves by qPCR in controls. Ct values ranged from 29-31. Melting temperatures ranged from 78.5-79 °C. The photograph was taken directly from qPCR machine.

Discussion:

According to our knowledge, this is the first Iraqi study that used real time PCR technique to screen pediatric ALL patients for the expression of minor and major *BCR-ABL* 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 [3,17,18] where males dominated. That could be explained by differences in sample sizes between current study and the mentioned studies or due to the factor of randomized collection of cases.

The presenting median age was 5 years with 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.[19,20,21,22,23] However, an Indian study [24] demonstrated a higher presenting median age of 7.9 years probably due to ethnic variations between the two populations.

In relation to clinical presentation, fever was the most frequent presenting feature followed by splenomegaly, pallor, hepatomegaly and lymphadenopathy. These findings go in agreement with an Iraqi study [20]. Nevertheless, some studies like an Iranian study [25] documented lower presentation of splenomegaly and lymphadenopathy of 67.4% and 23.2%, respectively. Others, like in a Jordanian study[18], observed a lower presentation of hepatomegaly, mediastinal mass of 13% and 9%, respectively. It is well known that the presence of splenomegaly, hepatomegaly, lymphadenopathy and mediastinal masses indicates a high tumor burden in ALL, differences between Iraqi population and these populations could be attributed to late presentation of cases in Iraq due to current difficulties in the country.

Although FAB classification and due to its negligible clinical significance is substituted largely by the WHO classification worldwide in the last years, it remains the standard classification for ALL in Iraq due to shortage of immunophenotyping and lack of cytogenetic
and molecular studies.

In current study, the majority of the patients 79.2% were classified as L2 followed by L1 in 16.7% and L3 in 4.2%. To some extent, this goes in harmony with the observations of the Iraqi studies in 2005, 2012, 2013 and 2014 where L2 dominates other types [20,26,27,28] but distinct from findings of an Iranian study in 2013[19] where L1 dominates L2 and L3 in 74.6% of 63 patients. This distinction may be attributed to the different morphological features of ALL in Iraq or to the experience of the examining hematopathologist.

The Ph[1] ALL in children is considered as a high-risk leukemia with bad prognosis.[29] In existing study, we looked for both of the common and rare transcripts unlike majority of studies which searched only for the minor transcript. Minor *BCR-ABL* transcript was detected in only one (2.1%) patient and this was comparable to findings of many studies.[3,26,30,31,32] However, other studies pointed up to higher frequencies [20,33,34] and this may be caused by the ethnic and geographic variations in ALL genotypes. Few are the studies which searched for major *BCR-ABL* in pediatric ALL like in Netherland [35] and Malaysia [31] where they exhibit very low frequencies of 2.6% and 2.2% respectively. Close to these results, we did not detect this transcript in any of those patients.

Unexpectedly, three cases from the control group had positive amplification for *BCR-ABL* p190 and during melting showed a product with lower melting temperature than that in the positive *BCR-ABL* p190 ALL case, indicating probably a second product. A variant of this transcript is suspected and it would be useful if it is sequenced. Although detection of *BCR-ABL* transcript in healthy patients is unexpected, some studies illustrated similar finding.[36,37,38,39,40] Observing this transcript in asymptomatic individuals revealed an important biological suggestion: the detection of mRNA *BCR-ABL* does not indicate necessarily direct leukemic disease; instead, it could point to a possibility of malignant transformation.

Conclusion:

The molecular prevalence of minor and major *BCR-ABL* fusion gene in Iraqi children having ALL is to somewhat similar to previous reports worldwide.

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