Molecular diagnosis of Iraqi chronic myeloid leukemia patients using quantitative real-time PCR

التشخيص الجزيئي لمرضى سرطان الدم النخاعي المزمن في العراق								
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Abstract

Thronic myeloid leukemia (CML), also known as chronic granulocytic leukemia, is a form of leukemia characterized by the increased and unregulated growth of myeloid cells in the bone marrow and the accumulation of these cells in peripheral blood. Total RNA extraction, cDNA and quantitative real-time PCR (Q-RT-PCR) were done for thirty CML Iraqi patients. Hematology tests (hemoglobin, platelets and WBCs counts) were done for the same samples in the same time. The results of this study show that some samples with normal hematology values have BCR-ABL (p210) fusion transcript with molecular analysis by Q-RT-PCR. This indicates the importance of this technique in the diagnosis and monitoring the therapy of CML patients.

المستخلص

سرطان الدم النخاعي المزمن احد أنواع السرطانات المنتشرة في العراق تتميز بالنمو غير المسيطر عليه لخلايا نخاع العظم وتراكم هذه الخلايا في الدم المحيطي . شملت هذه الدراسة ثلاثين عينة دم للمرضى تم استخلاص الرنا وتحويله الى cDNA ثم تم تضخيمه بجهاز Q-real time PCR . تم إجراء فحوصات الدم للمرضى وشملت اعداد كريات الدم البيضاء والهيموكلوبين وأعداد الصفائح الدموية . أظهرت نتائج الدراسة تعبير الجين قيد الدراسة BCR-ABL بنسب مختلفة لكنها لم تتناسب مع قيم فحوصات الدم .

Introduction

Chronic myelogenous (or myeloid) leukemia (CML), also known as chronic granulocytic leukemia, is a form of leukemia characterized by the increased and unregulated growth of myeloid cells in the bone marrow and the accumulation of these cells in peripheral blood [1]. CML is associated with a cytogenetic abnormality known as Philadelphia (ph) chromosome. This chromosome, a shortened chromosome 22, resulted from a t(9;22)(q34;11) reciprocal translocation, allowing the fusion of the 3' region of the proto-oncogene c-ABL (9q34) with the 5' region of the BCR (breakpoint cluster region) gene on chromosome 22q11 [2]. The different fusion proteins encoded by BCR-ABL vary in size depending on the breakpoint in the BCR gene but share a high tyrosinase activity, in part responsible for the leukemogenesis [3]. Three breakpoint cluster regions in BCR gene have been described to date; Major (M-BCR), minor (m-BCR), and micro (μ -BCR) (4). More than 95% of ph positive CML patients present a breakpoint in the M-BCR region. Two major breakpoints are found after the 13th exon resulting in b2a2 (e13a2) fusion, or after the 14th exon

resulting in b3a2 (e14a2) fusion. Both fusions mRNA are translated into 210^{BCR-ABL} protein [5].

Sequential monitoring of the quantitative levels of BCR/ABL mRNA in CML correlates with the activity of malignant clones and predicts impending clinical relapse; and therefore, is useful in guiding clinical therapeutic decisions [6].

Quantitative Real time-PCR permits the accurate quantification of PCR products during the exponential phase of the PCR amplification process. Quantitative PCR data can be rapidly obtained without post-PCR processing by real-time detection of fluorescent signals during and/or subsequent to PCR cycling, thereby drastically reducing the risk of PCR product contamination [7].

The BCR-ABL tyrosine kinase is a well-validated therapeutic target in Chronic Myeloid Leukemia (CML). Imatinib mesylate (formerly STI-571), a tyrosine kinase inhibitor is highly effective at the hematological, cytogenetic and molecular level in CML.

The aim of this study is to evaluate molecular and hematological response of CML Iraqi patients to the drug used in the treatment of CML, Imatinib Mesylate Gleevec ST1571 [8]. Molecular response to imatinib was evaluated by the measurement of BCR-ABL transcripts level in blood using real time reverse transcriptase quantitative polymerase chain reaction (RT Q-PCR).

Materials and methods

In this study thirty Iraqi patents were diagnosed as CML by morphological examination of peripheral blood and bone marrow. All CML patients were treated with imatinib standard protocol. In order to determine the effectiveness of the imatinib, quantitative RT-PCR was done along with hematological examination of peripheral blood for each patient.

RT Q-PCR

A. RNA extraction

Total RNA was extracted from peripheral blood samples using (*ONE STEP RNA REAGENT-* Bio Basic Inc.) reagent. Twenty five micro liters of CML patient peripheral blood was mixed properly with 750 μ l of Trizol incubated for 5min at room temperature, then 200 μ l of chloroform were added, mixed gently then left for 10min at room temperature. After that centrifugation was done 12000rpm for 15min to separate the sample to three layers, the upper layer was transferred to fresh eppendorf tube then 500 μ l of 75% ethanol was added, the tubes then incubated at -20 for 30min. then 500 μ l of 75% ethanol was added, centrifugation at 12000rpm for 10min, the supernatant removed and the precipitate dried on filter paper then suspended with 50 μ l of autoclaved distilled water and stored at -20C°. The integrity of RNA was determined by gel electrophoresis prior to reverse transcription.

B. cDNA synthesis

For cDNA synthesis the concentration of RNA was first measured by a spectrophotometric method and then the cDNA was synthesized using first strand cDNA synthesis kit (invitrogen). One microgram/10 μ l distilled water RNA was mixed with 1 μ l random hexamer primers (promega) and 1 μ l 100mM dNTPs (promega), the reaction incubated at 65C for 5min then mixed with second reaction

contain; 4 μ l 5x first strand buffer (invitrogen), 1 μ l 1 0.1M DTT (invitrogen), 1 μ l RNasin (promega) and 1 μ l superscript III reverse transcriptase (invitrogen) then incubated at 50C° for 50min and 70C° for 15min. After that 30 μ l of distilled water was added and the integrity of cDNA was determined by gel electrophoresis. Then cDNA was stores at -20C° until used.

C. Real time PCR

Quantification of BCR-ABL p210 transcripts in the peripheral blood samples of Chronic Myeloid Leukemia (CML) patients was done according to the instruction of BCR-ABL Mbcr Fusion*Quant*® Kit (*IPSOGEN Cancer Profiler*) and performed on Bio-Rad machine. Real time PCR reaction contain 5 μ l cDNA, 12.5 μ l Taqman 2xPCR master mix, 1 μ l primer–probe mix, and 6.5 μ l distilled water. PCR condition were, step1 (50 C° for 2min, 1 cycle); step2 (95 C° for 10min, 1 cycle); step3 (95 C° for 15sec, and 60 C° for 1min, 50 cycles).

D. Data analysis

In the TaqMan® technology, the number of PCR cycles necessary to detect a signal above the threshold is called the Cycle threshold (Ct) and is directly proportional to the amount of target present at the beginning of the reaction.

Using standards with a known number of molecules, one can establish a standard curve and determine the precise amount of target present in the test sample. IPSOGEN's standard curves are plasmid-based; we use three plasmid standard dilutions for the control gene (CG), and five standard dilutions for the fusion gene (FG), in order to ensure accurate standard curves.

For each gene (ABL and BCR-ABL), raw Ct values obtained on plasmid standard dilutions are plotted according to the Log Copy Number (3, 4 and 5 for C1, C2 and C3; 1, 2, 3, 5 and 6 for F1, F2, F3, F4 and F5).

The ABL standard curve equation should be used to transform raw Ct values (obtained with ABL-PPC) for the unknown samples, into ABL copy numbers (ABLCN).

The BCR-ABL standard curve equation should be used to transform raw Ct values (obtained with BCR-ABLPPF) for the unknown samples, into BCR-ABL copy numbers (BCR-ABLCN).

The ratio of these CN values gives the normalized copy number (NCN):

NCN = BCR-ABLCN / ABLCN

Hematological examination

Hemoglobulin, Platelets and white blood cells count were done according to standard methods [9].

Results and discussion

Thirty adult hematology malignancy cases were screened for the presence and quantification of BCR-ABL (p210) fusion transcript.

Total RNA were extracted from patients' blood using Trizol method, three bands were revealed as shown in Figure (1). Total RNA extracted from mammalian cells should give tow sharp, clear bands (28S, 18S) rRNA in denatured agarose gel electrophoresis. In this study, nondenatured agarose gel electrophoresis was used to determine the integrity of total RNA extracted. Nondenatured conditions results in

bands that are not as sharp and even multiple bands representing different structures of a single RNA species.

Fig (1): Nondenatured agarose gel electrophoresis (1%) of total RNA isolated from CML patients' blood cells. Electrophoresis was conducted at (60 V) for 2 h and the gel was stained by 0.5 µg/ml ethidium bromide. Samples numbers (3, 4, 7, 8, 9, 12, 13 and 14) give multiple bands, while other samples did not.

Agarose gel electrophoresis of cDNA product, which generated from total RNA gave single band and faint smear as shown in Figure (2). Random primers usually used in order to improve the yield of cDNA. We did not find gel photo for single strand cDNA in the literature and kits manuals in order to compare with our results, some peoples suggest the result may be rRNA and a smear (personal contact).



Fig (2): Agarose gel electrophoresis of cDNA. Electrophoresis was conducted at (60 V) for 2 h and the gel was stained by 0.5 μg/ml ethidium bromide. Samples numbers (1, 2,3,6,7,8 and 9) give multiple bands, while other samples did not.

Real-time PCR amplification graph shown in Figure (3) reveal the threshold cycle and exponential amplification of standards and samples. PCR efficiencies were more than 90%, amplification of standards gave good standard curve which determined as log starting quantity in Figure (4). These results are taken in account for validation the test.



Fig (3): PCR Amp/Cycle Graph for FAM-490



Fig (4): Standard Curve Graph for FAM-490

The cut-off value will represent the limit of detection and in this study used the lowest *BCR-ABL* plasmid dilution that can be amplified reliably (ie, 5-10 molecules) [10] and also the samples showing no amplification curve for BCR-ABL but shows amplification curve for control gene is considered as negative for the test and reported as 0.0% expression. Hematology tests (hemoglobin, platelets and WBCs counts) (table1) which are important parameters for evaluation of CML patients for therapy and diagnosis were done in parallel with real time PCR. CML patient's shows increased in WBC count and decrease in HB and either low or high platelets count [11]. No relation between Q-RT-PCR ratios and these parameters can exist as shown in Figure (5). These results indicate that hematological remission is different than molecular remission, in patients with normal hematology still detect copies transcript fusion [12].



Fig (5). The Q-R-I CK distribution of CML pau

Hematological parameter	(Q-RT-PCR ratio (%)	
	<0.50	0.50	=>1.00	P value
Hb (g/dL)	11.45±1.41	11.10±0.98	11.44±2.03	0.900
	(8.60-14.30)	(9.80-12.3)	(8.20-13.80)	
WBC (109/L)	64.89±74.07	48.78±45.91	61.77±67.06	0.903
	(3.80-185.0)	(5.50-120.0)	(4.80-182.0)	
Platelets count (109/L)	296.56±108.68	443.20±242.94	381.44±237.32	0.236
	(137.0-469.0)	(212.0-779.0)	(116.0-720.0)	

*Significant using ANOVA test at 0.05 level of significance

-Results expressed as mean±standard deviation (minmum-maximum)

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