Frequency of BCR-ABL fusion transcripts in Sudanese patients with chronic myeloid leukemia using real-time reverse transcription-polymerase chain reaction

Abdel Rahim M. Muddathir, MSc, PhD, Anwaar A. Kordofani, MD, MRCPath, Imad M. Fadl-Elmula, MD, PhD.

ABSTRACT

الأهداف: ننشر معدل تكرارالجينات الملتحمة بى سي ار–اي بى ال عند مرضى سرطان الدم الميلودى المزمن لدى السودانين.

الطريقة: أجريت دراسة وصفية مقطعية اشتملت على 112 مريض مصاب بسرطان الدم الميلودى المزمن ترددوا على عدة معامل اكلينيكية بولاية الخرطوم، السودان خلال الفترة من 2007م إلى 2010م حيث نجح التحليل للجينات الملتحمة فى 109 عينة من الدم ونخاع العظم باستخدام تقنية تفاعل البلمرة التسلسلسلي الكمي المعكوس.

النتائج: أظهر تحليل الجينات الملتحمة فى 109 مريض بأن (35/109) 32.1% لديهم أحد او الاثنان من بروتين 210 بى سي ار–اي بى ال (ب2 ا2 و ب3 ا2)، من (32.1% 35 مريض 21 مريض لديهم ب2 ا2 فقط ,6 لديهم ب3 ا2 فقط و 8 لديهم الاثنان (ب2 ا2 و ب3 ا2)، 74 من المرضى المتبقين لديهم بروتين 210 و بروتين 190 بى سي ار–اي بى ال (ب2 ا2 / ب3 ا2 / ج2ا1)، حيث ان 19 من المرضى لديهم جميع الجينات الملتحمة (ب2 ا2 / ب3 ا2 / ج2ا1)، كما أن 33 منهم لديهم الجينان (ج2ا1 / ب2 ا2) والمتبقى من 22 المرضى لديهم (ج2ا1 / ب3 ا2).

خاتمة: أظهرت نتائج الدراسة اختلافات فى معدل تكرارالجينات الملتحمة بى سي ار–اي بى ال عند مرضى سرطان الدم الميلودى المزمن لدى المرضى السودانين بالمقارنة بالدراسات المنشورة سابقاً فى مناطق جغرافية أخرى، وهذا ربما يبدو لوجود المجموعة العرقية المشاركة فى هذه الدراسة كما يعضض هذا الاختلاف وجود التميز الجينى لدى مرضى سرطان المثانة والثدى السودانين فى بعض الدراسات المنشورة.

Objectives: To report the frequencies of BCR-ABL transcript variants studied in Sudanese patients with chronic myeloid leukemia (CML).

Methods: This is a descriptive cross-sectional study carried out in 112 CML patients who attended at different clinics of Khartoum state, Sudan from February 2007 to December 2010. Transcript analysis was successful in 109 venous blood and bone marrow samples using quantitative real-time-polymerase chain reaction (RT-PCR).

Results: The transcripts analysis of 109 patients showed that 32.1% (35/109) expressed one or both of the P210 BCR/ABL rearrangements (b2a2 and b3a2). Of those 35 (32.1%), 21 patients expressed b2a2, 6 expressed b3a2, and 8 expressed both transcripts b2a2/b3a2. The remaining 74 patients revealed transcripts combination of P190 BCR/ABL and P210 BCR/ABL (e1a2/b2a2/b3a2), of which 19 patients had all the transcripts (e1a2/b2a2/b3a2), 33 revealed 2 transcripts (e1a2/b2a2), and the remaining 22 patients had (e1a2/b3a2).

Conclusion: The frequencies of BCR-ABL rearrangements in the Sudanese population demonstrated distinct profiles in contrast with the frequencies reported in similar studies conducted in other geographical regions.This might be due to the distinct ethnic group involved in this study which has been supported further by reported distinct genetic profiles in Sudanese patients with bladder and breast cancer.

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From the Faculty of Medical Laboratory Sciences, Department of Hematology and Blood Transfusion (Muddathir), Alzaiem Alazhari University, Department of Pathology (Kordofani), Medical Sciences Campus, and the Faculty of Medicine (Fadl-Elmula), Al-Neelain University, Khartoum, Sudan.

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Address correspondence and reprint request to: Dr. Abdel Rahim M. Muddathir, Department of Hematology and Blood Transfusion, Faculty of Medical Laboratory Sciences, Alzaiem Alazhari University, PO Box 845, Khartoum 11111, Sudan. Tel. +249 912351688. Fax. +249 (183) 243747. E-mail: abdelrahimm@gmail.com

In the last 20 years, chronic myeloid leukemia (CML) became the predominant hematological malignancy among males and the second most common frequently diagnosed among Sudanese females.[1] Most of CML patients have BCR/ABL transcripts that are dominated by b3a2 (55%) and b2a2 (40%), which is corresponding to the major BCR gene breakpoint cluster region (M-BCR). However in 5% of cases, both b3a2 and b2a2 transcripts may be present as a result of alternative splicing.[2] A few CML patients rarely express a shortened BCR/ABL transcript with an e1a2 transcript (m-BCR) (minor breakpoint cluster region) and e19a2 transcript (micro-BCR).[3] It has been observed that most of scarce CML cases are associated with a distinct clinical phenotype (an obvious monocytosis for e1a2 junction and a clinical picture of neutrophilic-CML for the e19a2 junction).[4] In addition, some of the previous reports performed showed that the frequencies of BCR-ABL mRNA transcripts in CML patients differ in different ethnic groups.[5,6] To our knowledge, one single study addresses among Sudanese population with small sample size as a part of diagnostic service for CML patients.[7] There is no published data concerning to what extent the frequency of co-expression in BCR-ABL fusion transcripts among Sudanese CML patients using sensitive molecular technique such as real-time reverse transcription PCR (qRT-PCR). Thus, the present study was designed to determine the frequency of BCR-ABL transcript variants and their co-expression among Philadelphia-positive (Ph-positive) Sudanese CML.

Methods. A total of 3-5 ml of venous blood and bone marrow samples were obtained from 112 Ph-positive CML patients presented in different clinics in Khartoum state, Sudan from February 2007 to December 2010. All patients have been diagnosed as Ph-positive CML, either by conventional nested reverse transcriptase polymerase chain reaction (RT-PCR) or conventional cytogenetic analysis. Their age ranged between 2 and 70 years (median 38 years) were processed for RNA extraction and BCR/ABL transcripts detection using nested RT-PCR.

The study was conducted according to the principles of the Helsinki Declaration, and was approved by the Al Neelain University Ethical Review Board. Each patient has an assigned written informed consent.

Ribonucleic acid (RNA) extraction. Ribonucleic acid was extracted from the bone marrow and/or peripheral blood using Trizol (Invitrogen, Grand Island, USA) according to the manufacturer instructions. The RNA quantity and quality was assessed using a Nanodrop spectro-photometer (NanoDrop Technologies, Wilmington, Delaware, USA) and if 260/280 ratio in the NanoDrop monitor less than 1.8, repeated RNA extraction can be carried out.[8]

Complementary DNA (cDNA) synthesis for nested RT-PCR. A total of 1 µg of RNA was used for reverse transcription using an avian myeloblastosis virus reverse transcriptase (AMV-RT) Kit (Roche, Basel, Switzerland), and the reaction medium was made up to 20 µl with UltraPure™ DEPC-treated Water invitrogen-(Grand Island, USA). The cDNA synthesis was carried out at 25°C for 10 min and then at 42°C for 45 min. Avian myeloblastosis virus-RT was denatured by incubating the reaction at 99°C for 5 min, and then cooling at 4°C for 5 min at the end of the RT step. The formed cDNA was then stored at -20^{0}C until analysis was started. The methodology used have been recommended by standardized RT-PCR analysis of fusion gene transcripts, a report of the BIOMED-1 concerted action.[9]

Nested RT-PCR. Nested RT-PCR was carried out with 2 pairs of (nested) primers corresponding to BCR and ABL exons in 2 rounds to amplify residual CML cells that remain after treatment to reach the sensitivity of 1 in 106 cells. The final PCR product was run in 1.5% agarose and the PCR product was visualized directly on ethidum bromide-stained-gel and photographed. The band of PCR product in the nested RT-PCR was considered between 111-360 bp.[9]

Complementary DNA synthesis for qRT-PCR. Approximately 1.5 µg of total RNA in 10 µl of H_2O was heated at 65^{0}C for 5 min in oven. Complementary DNA master mixture was prepared in cold condition (cooled on crushed ice), and the reaction medium was made up of 20 µL with moloney murine leukemia virus reverse transcriptase (MMLV RT) enzyme (200 units superscripts) (Invitrogen®). RNAse free distilled autoclaved H_2O, first stand (FS) buffer 5x (master mix), dithiothreitol (DTT) 0.1 mM, and deoxy ribonucleotide triphosphate (dNTP) 10 mM, random hexamer (N) 6 0.5 mM were mixed together. All reagent were supplied with invitrogen (Grand Island, USA). After that 10ul from the cDNA mixture was added to the eppendorf tubes and incubated for 37^{0}C for one hour; 30 µl of H_2O were added to the cDNA micro tubes then stored at -20^{0}C until required. The methodology used was recommended by the standardization and quality control studies of qRT-PCR carried out by the Europe Against Cancer Program.[10]

Real-time reverse transcription PCR. Approximately 5 µl from patient's cDNA has been mixed with 20µl of TaqMan universal master mix, H_2O, mixed primers and probes of the major and minor BCR-ABL transcripts

in addition to GUS and ABL as housekeeping genes probes provided by the Applied Biosystems, Carlsbad, California, USA. The steps were program on the LightCycler® 480 (Roche Applied Science, USA) instrument (50°C for 2 min, 95°C for 10 min) followed by 50 cycles at 95 for 15 seconds and 60°C for 1 min.[10]

All qRT-PCR reactions were performed on LightCycler® 480 instrument using primers and TaqMan probes from Applied Biosystems in conjunction with the TaqMan Universal Master Mix (purchased from the same manufacturer). The qRT-PCR was performed in duplicate using LightCycler® 480 Software, the threshold cycle (Ct) values representing the PCR cycle number at which fluorescence signal increase above an arbitrary threshold were exported into Microsoft Excel software for further analysis.[10]

Determination of the transcript types in agarose gel. To determine the type of BCR/ABL within the major break cluster region (BCR) and to confirm the presence of the minor BCR, we run the final PCR product in 1.5% Agarose gel and read by the bands were visualized under UV light. The fragment size for the PCR products were 148 bp for the b3a2 rearrangement, 73 bp for the b2a2 rearrangement, and 92 bp for the e1a2 rearrangement (**Figure 1**).

Statistical analysis. Chi-square test was carried out to test the significance difference between the obtained Sudanese frequency distribution and the other reported population frequencies using SPSS version 13. The confidence interval (CI) was set at 95% and the results were considered statistically significant at $p<0.05$.

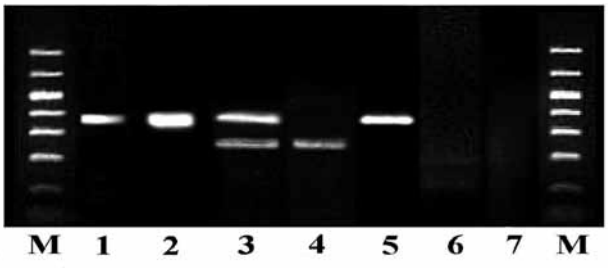

Figure 1 - Determination of BCR-ABL fusion transcript product obtained by real-time reverse transcription-polymerase chain reaction (qRT-PCR) in agarose gel. M: 50 bp DNA marker, Lane 1 +ve: Positive control for b3a2 BCR-ABL transcript, Lane 2: 148 bp PCR product of b3a2 BCR-ABL transcript, Lane 3: PCR products of both b3a2 and b2a2 BCR-ABL transcripts, Lane 4: 73 bp PCR product of b2a2 BCR-ABL transcript, Lane 5: PCR product of b3a2 BCR-ABL transcript, Lane 6 -ve: Negative control from healthy individual, and Lane 7: water as negative control.

Results. Although nested RT-PCR was successful in 112 patients; qRT-PCR was possible in 109 cases due to bad quality of RNA in 3 cases. The transcripts analysis of 109 patients showed 32.1% (35/109) expressed one or both of the P210 BCR/ABL rearrangements (b2a2 and b3a2). Of those 35 (32.1%), 21 patients expressed b2a2, 6 expressed b3a2, and 8 expressed both transcripts b2a2/b3a2. The remaining 74 patients revealed transcripts combination of P190 BCR/ABL and P210BCR/ABL (e1a2/b2a2/b3a2), of which 19 patients had all the transcripts (e1a2/b2a2/b3a2), 33 revealed 2 (e1a2/b2a2), and the remaining 22 had e1a2/b3a2. Of the cases positive for P210, 74.3% (81/109) expressed b2a2, whereas 50.4% (55/109) expressed b3a2 and 67.8% (74/109) expressed e1a2. The transcripts co-expression showed also combination of b2a2/b3a2 in 24.7% of cases (27/109), b2a2/e1a2 in 47.7% of patients (52/109), b3a2/e1a2 in 20.1% of cases (22/109), and finally triple co-expression b3a2/b2a2/e1a2 in 17.4% of patients (19/109) (**Table 1**).

Discussion. In the present study, the frequencies of BCR-ABL rearrangements in Sudanese demonstrated distinct profile in contrast with the frequencies reported in similar studies conducted in Sudan and other geographical regions[7] (**Table 2**). Our study showed that there is an increased in the frequencies of co-expression of both P210 (b3a2, b2a2) transcripts frequencies and P190 (e1a2) frequencies. The co-expressed b2a2/b3a2 transcripts frequencies in our Sudanese population (7.3%) showed significant statistical different from Korean (2%; $p=0.002$), while there is no statistical difference observed in Eastern Indian (5.4%; $p=0.622$), and Mexican (7%; $p=0.963$).[11-13] Furthermore, the b2a2/b3a2/e1a2 transcript frequency in our studied population (17.4%) showed significant statistical differences compared with Mexican (6%; $p=0.001$) (**Table 2**). For b2a2 transcript frequencies, the Sudanese patients revealed 19.3% in contrast to Caucasian (40%; $p=0.009$), Ecuadorian (94.6%; $p=0.001$), Korean (32.3%; $p=0.007$), Mexican (48%; $p=0.001$), while

Table 1 - Frequencies of BCR/ABL transcripts and their co-expression in 112 Philadelphia-positive chronic myeloid leukemia patients presented in different clinics in Khartoum, Sudan.

Type of BCR-ABL fusion transcript	Frequencies (%)
b2a2	74.3
b3a2	50.5
b2a2/b3a2	24.7
b2a2/b3a2/e1a2	17.4
e1a2	67.8

Table 2 - Frequencies of BCR/ABL transcripts in Sudanese population compared to other published studies.[11-14]

Type of BCR-ABL fusion transcript	Sudanese %	Caucasian[11] Sample size (n=37)	Ecuadorian[14] Sample size (n=40)	Korean[11] Sample size (n=548)	Mexican[13] Sample size (n=250)	Eastern Indian[12] Sample size (n=122)
b2a2	19.3	40	94.6	32.3	48	29.5
P-value		0.009	0.001	0.007	0.001	0.071
b3a2	5.5	55	5.4	67.7	35	61.6
P-value		0.001	0.904	0.001	0.001	0.001
b2a2/b3a2	7.3			2	7	5.4
P-value		-	-	0.002	0.963	0.622
b2a2/b3a2/e1a2	17.4				6	
P-value		-	-	-	0.001	-
b2a2/e1a2	30.3					
P-value		-	-	-	-	-
b3a2/e1a2	20.2					
P-value			-	-	-	-
e1a2	-	5				1.8
P-value		-	-	-	-	-

there is no statistical difference observed with Eastern Indian (29.4%, p=0.071).[12,14] For b3a2 transcript frequencies, our patients revealed 5.5%, which was statistically different from Caucasian (55%), Korean (67.7%), Mexican (35%), and Eastern Indian (61.6%) (p=0.001 for each), while no significant difference with the Ecuadorian (5.4%; p=0.904) (**Table 2**). Although the difference might be due to the high sensitivity of our used qRT-PCR technique in comparison to the standard used nested RT-PCR which have been used in the published reported studies,[7] ethnic back ground differences might have a role in BCR-ABL fusion transcripts frequencies variation. In European studies, the populations were mostly Caucasian, whereas the population of our study was mostly Sudanese; namely, Afro-Asiatic or Nilo-Saharan.[15] Although the present study was the second in African population, its results may explain the distinct CML clinical course observed in Sudanese patients compared with the natural history observed in Caucasian population. In addition, our results suggest that the different BCR/ABL transcript variants in Sudanese may reflect different clinical characteristics of the disease. Thus, our findings are in complete accordance with the hypothetical claimed that the geographical differences may be translated into distinct genetic profile and natural disease. Support for such hypothesis has arrived from reports on bladder cancer among Sudanese population in which distinct genetic profile has led to distinct natural history of the disease in contrast with what was seen in European bladder cancer patients.[16] Previous study from a cohort of 20 Sudanese breast cancer patients was tested for germ line and somatic mutation in their BRCA2 exon 11 as well as the main conserved area of the p53 tumor suppressor gene. The results indicate that both regions may play a limited role in the pathogenesis of breast cancer in those patients. The fact that there are no somatic mutations detected in p53 was particularly surprising as the expected rate for mutations in breast cancer is 30-50% in Caucasian population.[17] Same support for the hypothesis has come also from distinct genetic profile of cystic fibrosis in Ecuadorian compared with the genetic profile of the same disease in Europeans. The distinctive genetic profile can be seen even at molecular level since co-expression may be caused by alternative splicing or phenotypic variation, leading to unusual clinical course of the diseases.[14] The geographical variation in CML transcripts frequencies is extremely important for designing diagnostic strategies in each geographic/ethnic population.

Study limitations. One of the limitation of this study were the small sample size. Although Sudanese patients with CML are not seldom, most of them were diagnosed in non-specialized hematology clinics, which made CML patients samples collection difficult. We did not include other rare fusion transcripts such as micro BCR-ABL transcript; therefore, other studies should include a larger number of patients including detection for the other seldom BCR-ABL transcripts.

In conclusion, although the frequencies of BCR-ABL rearrangements in Sudanese population demonstrated distinct profile in contrast with the frequencies reported in similar studies conducted in other geographical regions, our results may explains the distinct CML clinical course observed in Sudanese patients compared with the natural history observed in Caucasian population.

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