Incidence of *bcr-abl* fusion transcripts in healthy individuals

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Abstract. *Bcr-abl* fusion transcripts, resulting from translocation t(9;22), are hallmarks of Philadelphia chromosome positive (Ph+) leukemias. This translocation is detected in >90% of patients with chronic myelogenous leukemia and ~20% of acute lymphoblastic leukemia patients, which predominantly express the p210 and p190 proteins, respectively. Although the occurrence of t(9;22) in healthy individuals has been previously demonstrated, the number of studies is limited and the results are inconsistent. The present study screened for the presence of *bcr-abl* transcripts in the blood of a group of healthy individuals using a sensitive-nested reverse transcription polymerase chain reaction (RT-PCR) assay. Samples were collected from 189 healthy volunteers (145 adults and 44 children). RNA was reverse transcribed and amplified by two rounds of PCR, amplifying the two common variants of *bcr-abl* transcripts, p190 and p210. While the *bcr-abl* p190 transcript was not detected, the p210 transcript was detected in ~10% of samples. Notably, the incidence of p210 translocation was higher in males (12.2%) compared with females (7.7%) and males were 2.4 times more likely to have the translocation. A significant incidence was also observed in adults compared with children, where adults were 6 times more likely to have the translocation. The presence of *bcr-abl* transcripts in the blood of a significant proportion of healthy individuals should be considered in long-term investigations to establish its exact association with the risk of developing leukemia. Furthermore, the current assays should be revised to consider the proportion of normal samples carrying the p210 transcripts when making a differential diagnosis.

Introduction

At present, there are a number of different types/subtypes of human lymphomas and leukemias. These were originally classified according to histology and disease course, but are now being re-grouped based on genetic aberrations, including chromosomal translocations, oncogene mutations, and expression profiles (1).

The Philadelphia (Ph) chromosome, which results from translocation t(9:22), is one of the most characterized genetic abnormalities associated with leukemia. The association was initially described by Nowell and Hungerford (2) ~50 years ago, rendering it the first chromosomal rearrangement to be associated with a specific malignancy. It is now established that t(9;22) is observed in >90% of patients with chronic myelogenous leukemia (CML), which is a clonal bone marrow stem cell disorder characterized by the slow progression of proliferation of mature and immature granulocytes (neutrophils, eosinophils and basophils) that may eventually lead to acceleration towards a blast crisis (3). The Ph chromosome is also detected in ~20% of adult acute lymphoblastic leukemia (ALL), 5% of pediatric ALL, and rare cases of acute myelogenous leukemia (4).

The BCR-ABL fusion protein is the molecular consequence of the Ph chromosome translocation and is an active cytoplasmic tyrosine kinase (5). This fusion protein varies in size from 190 to 230 kDa, depending on the site of the breakpoint within the *bcr* gene. The majority of patients with CML express a 210-kDa BCR-ABL protein, while patients with Ph^+ ALL commonly express a 190-kDa BCR-ABL protein (6). A larger, 230-kDa BCR-ABL fusion protein is only rarely identified in a subgroup of patients with chronic neutrophilic leukemia.

It is widely believed that the breakpoint in the BCR sequence is correlated with the leukemic phenotype (7). In CML, the break on chromosome 22 is predominantly within a *BCR* gene region termed M-bcr. The majority of breaks occur immediately downstream of exon 2 or 3 of the M-bcr region and result in *b2a2* (*e13a2*) or *b3a2* (*e14a2*) fusion transcripts (8). In acute leukemia, however, the breakage also occurs outside M-bcr (in approximately half the cases), usually in intron 1 of the *BCR* gene (26,141), resulting in an *e1a2* fusion transcript (9). The difference in length of *BCR-ABL* transcripts is not only a reflection of the site of breakage but may also be a result of alternative splicing (10).

ABL is a non-receptor tyrosine kinase expressed in the majority of tissues. It transduces signals from cell-surface receptors for growth factors and adhesion receptors to regulate the cytoskeletal structure (11). BCR is also a signaling protein

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that contains multiple modular domains and its fusion to ABL is known to increase the tyrosine-kinase activity of ABL, and bring novel regulatory domains/motifs to ABL, such as the growth factor receptor-binding protein 2 and Src homolgy 2 domain-binding sites (12).

The development of leukemia is a complex process and the detailed involvement of BCR-ABL proteins in this process has been the subject of numerous studies. Apart from its diagnostic value, those studies focused on whether there is a prior genetic alteration to the t(9;22) translocation, its exact involvement in pathogenesis as well as progression of related leukemias and its involvement in cancer stem cells (13). However, there is controversy as numerous leukemia-specific genetic rearrangements, including *bcr-abl* are also observed in the peripheral blood and bone marrow of healthy individuals (14). The biological explanation of this observation remains largely unexplained (15).

The present study aimed to investigate the presence of *bcr-abl* fusion transcripts in the peripheral blood of healthy individuals. Nested reverse transcription polymerase chain reaction (RT-PCR) amplification was used to assure maximal sensitivity, and identify primers that distinguish the t(9:22) common variants including, *b2a2*, *b3a2* and *e1a2*. The results of the present study are discussed with regard to previous studies in a comprehensive overview focusing on the significance of these results.

Materials and methods

Sample collection. A single donation of 5 ml EDTA peripheral blood was drawn from 189 healthy subjects, of which 145 were adults and 44 were children. Informed consent was obtained from healthy volunteers. The study protocol was approved by the Institutional Review Board (IRB) of the University of Jordan. The adult group was composed of 73 males (50.3%) and 72 females (49.7%) aged between 20 to 86 years (mean ± SD=50.0±17.7), while the group of children was composed of 25 males (56.8%) and 19 females (43.2%) aged between 2 and 16 years (mean ± SD=8.4±3.9). The details of the age and gender distribution of the study population are listed in Table I. The subjects were recruited randomly from volunteers representing the different geographical and ethnic backgrounds of the Jordanian population. All the subjects were healthy and free of malignancy as demonstrated by a short medical history taken by qualified clinical professionals.

RNA extraction. Total RNA was extracted from peripheral blood samples collected in EDTA tubes using the cell lysis and RNA isolation reagent TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA was extracted within a few hours of blood collection. The quality of the RNA was analyzed by electrophoresis gels and the concentration was determined spectrophotometrically (Bio-Rad, Hercules, CA, USA). RNA was stored at -70˚C until use.

cDNA synthesis. Approximately 1 μg total RNA was converted into cDNA using an RT system kit according to the manufacturer's instructions (Promega, Madison, WI, USA). This kit utilized random hexamers and the MuLV RT enzyme.

Table I. Incidence of *bcr-abl* transcripts (p210 and p190) in leukocytes of healthy individuals, grouped according to age and gender.

Age group	No. of p210 positive samples (%)		No. of p190 positive samples (%)
	M	F	
Children	2/25 (8.0)	2/19 (10.5)	0 (0.0)
Adults	10/73 (13.7)	5/72 (6.9)	0 (0.0)
20-39 years	4/22	1/24	
40-59 years	2/24	2/23	
60-86 years	4/27	2/25	
Total	12/98 (12.2)	7/91 (7.7)	0 (0.0)

M, male; F, female.

Nested PCR amplification and transcript detection. Following cDNA synthesis, nested PCR was conducted to detect the presence of the three predominant *bcr-abl* fusion transcripts (*b2a2*, *b3a2* and *e1a2*). Primers and conditions used in this study have been adopted from previous studies with minor modifications (16,17). In this study, uniplex PCR was used instead of multiplex PCR. The RNA integrity, cDNA synthesis and PCR amplification were checked using internal control primers for the glyceraldehyde 3-phosphate dehydrogenase gene (18). A known positive CML sample for the *bcr-abl* transcript was used as a positive control. A non-template negative control was included in each PCR run to check for contamination. Nested PCR products (5 μl) were electrophoresed in 2% ethidium bromide stained agarose gels, visualized and images were captured (UVP, Cambridge, UK) under UV light.

Estimation of the minimal level of detection of the nested PCR. The minimal level of detection of the nested PCR was estimated using two methods. In the first method, cells from the K562 BCR-ABL-positive cell line were serially diluted in MCF-7 cells, which are BCR-ABL negative. Dilutions were prepared in 10-fold steps, from undiluted to a dilution of 10^{-7}. In the second method, BCR-ABL-positive RNA obtained from K562 cells was diluted in negative RNA from MCF-7 cells. The dilutions were also prepared in 10-fold steps, from undiluted to 10^{-7}.

The two methods showed a sensitivity level of 10^{-6} (1 in 1,000,000 dilution) using primers and conditions used in this study.

Statistical analysis. Stepwise logistic regression (SLR) was utilized to analyze gender and age as potential predictors of the presence of the t(9;22) translocation. Backward and forward SLR were investigated. The Hosmer-Lemeshow test was used to assess model fitness. $P<0.05$ was considered to indicate a statistically significant difference. Neither model analyzed contained a constant. Two types of statistical analysis, -2 log-likelihood and Wald statistics, were conducted with the first one being more reliable. If discrepancies were noted

Table II. Comparison between studies concerning the occurrence of *bcr-abl* fusion transcripts in samples obtained from healthy individuals.

Study (year)	Sample type (no.)	Positive results (%)	Method	Ref no.
Biernaux *et al* (1995)	Adult PB (73)	30.0 (p210)	RT-PCR (N)	(21)
	Children PB (22)	4.5 (p210)		
	Cord blood (22)	0.0 (p210)		
Bose *et al* (1998)	Adult PB (16)	25.0 (p210)	RT-PCR (N)	(22)
		69.0 (p190)		
	Non-CML cell lines[a] (7)	43.0 (p210)		
		100.0 (p190)		
	NIH3T3 murine fibroblasts	0.0		
Song *et al* (2011)	Adult PB (46, p210)	54.0 (p210)	RT-PCR (N)	(15)
	(53, p190)	77.0 (p190)		
	Child PB (28, p210)	32.0 (p210)		
	(27, p190)	67.0 (p190)		
	Cord blood (50)	16.0 (p210)		
		42.0 (p190)		
Current study	Adult PB (145)	10.3 (p210)	RT-PCR (N)	
		0.0 (p190)		
	Child PB (44)	9.1 (p210)		
		0.0 (p190)		

[a]Cell lines used were hematopoietic in origin (HL60, KG1, U937, Kasumi, Jurkat, JVM13 and JVM25). PB, peripheral blood; CML, chronic myelogenous leukemia; RT-PCR, reverse transcription-polymerase chain reaction; N, nested PCR.

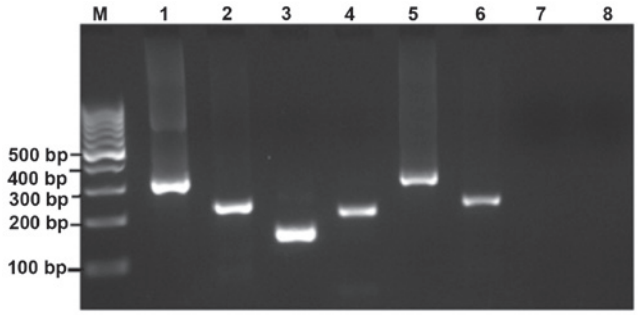

Figure 1. Representative nested reverse transcription polymerase chain reaction (RT-PCR) results for the amplified p190 and p210 *bcr-abl* fusion transcripts. Lane M, DNA molecular size marker (100 bp ladder); lane 1, positive control for p210 *b3a2* (*e14a2*) transcripts; lane 2, positive control for p210 *b2a2* (*e13a2*) transcripts; lane 3, positive control for p190 (*e1a2*) transcripts; lane 4, glyceraldehyde 3-phosphate dehydrogenase control gene; lane 5, a sample positive for p210 *b3a2* transcripts; lane 6, a sample positive for p210 *b2a2* transcripts; lane 7, negative PCR blank for p210 and lane 8, negative PCR blank for p190.

between the two analyses with regard to whether a predictor was useful to the model, the change was adopted in the -2 log-likelihood model. Cox and Snell's R^2 and Nagelkerke's R^2 were used to estimate the coefficient of determination and the strength of the prediction model.

Results

Amplification results for p190 and p210 fusion transcripts. The frequency of *bcr-abl* (p210 and p190) fusion transcripts in the peripheral blood samples obtained from a total of 189 healthy individuals, grouped according to age and gender, was analyzed using a highly sensitive nested RT-PCR assay (Fig. 1). The reactions were handled with care, and controls were included in every run to avoid false positive results due to contamination.

The amplification results are summarized in Table I. No expression of the *bcr-abl* p190 RNA transcript was identified in any of the samples tested. However, ~10% of healthy individuals were positive for the *bcr-abl* p210 transcripts. These p210 fusion transcripts were detected in 10.3% of adults and 9.1% of children (Table II). As regards, the incidence of translocation was higher in males (12.2%) compared with females (7.7%).

The *bcr-abl* p210 fusion transcripts detected in 19 individuals were approximately equally distributed between the *b3a2* (10) and b2a2 (9) subtypes. None of the tested samples contained the two transcripts together (Table III). This distribution is comparable to that observed in CML patients where the majority have either the *b3a2* (55.0%) or the *b2a2* (40.0%) transcripts, and in 5% of the cases *b3a2* and *b2a2* transcripts coexist as a result of alternative splicing (19,20). Furthermore, although it may not be statistically significant, 3 out of 4 children had the *b2a2* transcripts, and although the *b3a2* transcripts were detected equally in males (5) and females (5), the *b2a2* transcripts were detected in 7 males vs. 2 females (Table III).

Statistical analysis. Logistic regression analysis was conducted utilizing stepwise analysis in the forward and backward

Table III. Gender and age distribution of *b3a2* and *b2a2 bcr-abl* p210 fusion transcripts.

Sample no.	Age group (years)	Fusion transcript	Gender
1	Child (8)	*b3a2*	F
2	Child (9)	*b2a2*	F
3	Child (10)	*b2a2*	M
4	Child (12)	*b2a2*	M
5	Adult (20)	*b3a2*	M
6	Adult (22)	*b3a2*	F
7	Adult (46)	*b3a2*	F
8	Adult (49)	*b3a2*	M
9	Adult (55)	*b3a2*	F
10	Adult (61)	*b3a2*	M
11	Adult (63)	*b3a2*	M
12	Adult (71)	*b3a2*	F
13	Adult (73)	*b3a2*	M
14	Adult (23)	*b2a2*	F
15	Adult (25)	*b2a2*	M
16	Adult (39)	*b2a2*	M
17	Adult (49)	*b2a2*	M
18	Adult (65)	*b2a2*	M
19	Adults (70)	*b2a2*	M

M, male; F, female.

directions and without a constant in the model. Gender and age were consistently presented as a potential predictor of the t(9;22) translocation, irrespective of whether Wald or -2 Log likelihood statistics were used. As stated previously, the incidence of translocation was higher in males (12.2%) compared with females (7.7%). Males were 2.4 times [adjusted OR=2.4 95% confidence interval (CI), 1.2-4.8; P=0.008] more likely to have translocations. A significantly increased risk was also observed in adults compared with children, in which adults were 6 times (adjusted OR=6 95% CI, 3.3-10.7; P<0.001) more likely to have translocations. The model for gender and age was strong with Cox and Snell's R^2 and Nagelkerke's R^2 of 0.45 and 0.59, respectively.

Discussion

There are few studies concerning the the occurrence of bcr-abl fusion transcripts in healthy individuals (21,22,15). The results of these studies as well as the present study are summarized in Table II, which also shows the number and type of samples studied and the detection method used. Due to its superior sensitivity, all listed studies used a nested RT-PCR assay to detect the *bcr-abl* transcripts and primers specific for the p210 transcripts. In addition, certain studies also included primers for the p190 transcripts.

In regard to the detection of p210 fusion transcripts in the peripheral blood of healthy adults, Table II shows that the detection rate ranges from 10.3 (current study) to ≤54% (15) with two studies demonstrating results within this range, of 25 (22) and 30% (21). Notably, the current study recruited significantly more healthy volunteers (145 samples) than the three other studies (16, 46 and 73 samples). As for the occurrence of p190 transcripts in the peripheral blood of healthy adults, the variation was more significant and ranged from 0.0 (current study) to ≤69 (22) and 77% (15). The present study also used more samples for the p210 analysis (145 samples) compared with the two other studies (16 and 53 samples, respectively).

Regarding the *bcr-abl* p210 transcripts in the peripheral blood of healthy children, the results show a rate of 9.1% which is higher than that reported by Biernaux *et al* (21) in 1995 (4.5%) but much lower than that of Song *et al* (15) in 2011 (32%). With the p190 transcripts in children, while it was not possible to detect them in any of our 44 samples, the only other study which observed this rate detected transcripts in the blood of ~67% of the 27 children tested (15).

Contradictory results have been demonstrated in two studies that observed the *bcr-abl* transcripts in umbilical cord blood (CB) samples. Although Biernaux *et al* (21) failed to detect these transcripts in CB samples, Song *et al* (15) observed p210 and p190 transcripts at high frequencies (16 and 42%, respectively) in CB.

The abovementioned results of the three previous studies as well as the present study, are not consistent and show significant variation in the rate of *bcr-abl* transcript detection in healthy individuals.

The present study aimed to analyze the presence of the most common variants of the *bcr-abl* fusion transcripts in the peripheral blood of 189 healthy individuals. Highly sensitive nested PCR assays were used to separately detect each of the p190 and p210 fusion transcripts. The results demonstrated the presence of p210 fusion transcripts in ~10% of the tested samples, with gender and age associations, where older males

appeared more likely to have the transcripts in the peripheral blood. This correlation with advancing age was also demonstrated in two previous studies (15,21). For example in one of these studies (21), the *bcr-abl* transcripts were not detected in CB and were only detected in 1 of 22 children, but were detected in 18 of 52 adults. This increase may be due to the fact that, in adults an increased number of cell divisions had occurred and therefore, there was an increased chance of the accumulation of DNA rearrangements (21).

A number of other studies have demonstrated the occurrence of *bcr-abl* leukemic transcripts in the blood of seemingly healthy individuals (15,21,22). However, as demonstrated in Table II, there is considerable variation in the results. This variation was observed in the detection frequencies of p210 and p190 *bcr-abl* fusion transcription in peripheral and CB samples, as well as the frequency between children and adults. It is not easy to explain this discrepancy but it is suggested that the following factors may contribute. Only a small number of studies are available, and a number of these studies used a small number of samples. By comparison, the current study recruited the largest number of healthy volunteers. Ethnic, geographical or environmental elements may also contribute to the variation. In addition, another source of variation may be due to the methodology implemented in each study. As all studies shown in Table II used nested RT-PCR, this factor may hold less weight. However, there may still be variation due to laboratory to laboratory variation, assay sensitivity and experimental errors, of which false positives may be a predominant issue, particularly with a highly sensitive method, such as nested RT-PCR. One comparison that may highlight the importance of false-positive results concerns the occurrence of p190 transcripts in the peripheral blood of healthy adults. Although the present study failed to detect any such transcripts in the samples tested, other authors have demonstrated high frequencies of ~69 (22) and 77% (15). Notably, the present study used more samples (145 samples) compared with the two other studies (16 and 53 samples, respectively). Concordant with the results of the present study, no such high frequencies of *bcr-abl* p190 transcripts have been demonstrated even in ALL patients, where these transcripts are supposed to be a diagnostic hallmark for 20% of patients (4). In addition, our laboratory, which offers a diagnostic service to the Jordan University Hospital and numerous other nearby hospitals, has not detected p190 transcripts in such high frequencies in the hundreds of suspected ALL cases that have been screened, using the same highly sensitive nested RT-PCR assay (unpublished data). Therefore, there is a requirement for more studies recruiting larger numbers of healthy volunteers of all ages, from different geographical regions and ethnicities and preferably using different detection techniques.

Regardless of the variation between studies on the frequency of *bcr-abl* transcripts in normal individuals, the presence of the transcripts has been confirmed. This may suggest the requirement for diagnostic laboratories and clinicians worldwide to consider the presence of transcripts in healthy individuals when interpreting *bcr-abl* or t(9;22) positive results.

Although there have not been enough studies concerning the clinical value of the occurrence of *bcr-abl* transcripts in healthy individuals, numerous reports have focused on the involvement of t(9;22) and the resultant BCR-ABL fusion proteins on the oncogenesis of CML. One theory states that CML is, as the name indicates, a chronic disease of the hematopoietic stem cell where such cells, which harbor the p210 *bcr-abl* transcripts, remain quiescent for years prior to transforming into a malignant colony subsequent to acquiring extra mutations (15). Thus, in this theory, the presence of p210 *bcr-abl* transcripts in seemingly healthy individuals may be considered a risk factor for CML, which is consistent with the observation that the occurrence of p210 *bcr-abl* transcripts in healthy individuals and the incidence of CML is more commonly observed in older individuals. Furthermore, in line with this hypothesis, there are numerous reports that have demonstrated the development of CML-like conditions in mouse models following inoculation with *bcr-abl* transduced hematopoietic progenitor cells (23,24,25). These CML-like conditions developed with a certain latency which may also be due to the accumulation of additional mutations, required for clonal expansion (26).

However, the presence of *bcr-abl* transcripts in healthy individuals as a risk factor for CML is not a straightforward conclusion. The incidence of these transcripts is higher than that for CML, suggesting that a number of those healthy individuals are not likely to develop CML. One explanation for this is that the *bcr-abl* transcripts detected in healthy individuals may be generated in non-self-renewing terminally differentiated cells, and not in hematopoietic stem cells or early progenitors, which are known to be more capable of developing malignant clones (27). A recent study has suggested that patients who eventually develop CML may be suffering from a specific cytotoxic T-cell immune response deficit with respect to the BCR-ABL antigen (28). In addition, certain HLA types have been shown to be associated with reduced risk of CML (29). Such studies hypothesized that the immuno-competency of an individual is a determining protective factor against developing CML.

In conclusion, this study was consistent with previous studies concerning the occurrence of *bcr-abl* fusion transcripts in the peripheral blood of healthy individuals, and that this was more likely in adults compared with children. However, there are significant discrepancies in the reported frequencies of the various types of these transcripts between different studies. It is therefore recommended that further studies be conducted to reach a consensus on this issue, where larger numbers of healthy individuals are recruited, preferably from different geographical and ethnic backgrounds, and where different detection techniques are used to assess methodological variation. The collected samples may cover peripheral blood, bone marrow and CB. Moreover, a close and long-term follow-up of *bcr-abl*-positive cases is essential to assess the implication of these results on the development of related leukemias.

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