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Molecular Diagnosis & Therapy

ISSN 1177-1062
Volume 19
Number 5

Mol Diagn Ther (2015) 19:277-287
DOI 10.1007/s40291-015-0149-0

Springer

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ORIGINAL RESEARCH ARTICLE

High Frequency and Poor Prognosis of Late Childhood BCR-ABL-Positive and MLL-AF4-Positive ALL Define the Need for Advanced Molecular Diagnostics and Improved Therapeutic Strategies in Pediatric B-ALL in Pakistan

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Published online: 13 August 2015

Abstract

Background Fusion oncogenes (FOs) resulting from chromosomal abnormalities have an important role in leukemogenesis in pediatric B cell acute lymphoblastic leukemia (ALL). The most common FOs are BCR-ABL, MLL-AF4, ETV6-RUNX1, and TCF3-PBX1, all of which have important prognostic and drug selection implications. Moreover, frequencies of FOs have ethnic variations. We studied Pakistani frequencies of FOs, clinical pattern, and outcome in pediatric B-ALL.

Methods FOs were studied in 188 patients at diagnosis using reverse transcriptase-polymerase chain reaction (RT-PCR) and interphase fluorescent in situ hybridization (FISH). Data were analyzed using SPSS version 17 (SPSS Inc., Chicago, IL, USA).

Results FOs were detected in 87.2 % of patients. Mean overall survival was 70.9 weeks, 3-year survival was 31.9 %, and 3-year relapse-free survival was 18.1 %. Four patients died of drug toxicities. ETV6-RUNX1 (19.14 %) had better survival (110.9 weeks; $p = 0.03$); TCF3-PBX1

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(2.1 %) was associated with inferior outcome and higher central nervous system (CNS) relapse risk; MLL-AF4 (18.1 %) was more common in the 8- to 15-year age group (24/34; $p = 0.001$) and was associated with organomegaly, low platelet count, and poor survival; and BCR-ABL (47.9 %) was associated with older age (7–15 years, 52/90), lower remission rates, shorter survival (43.73 ± 4.24 weeks) and higher white blood cell count. Overall, MLL-AF4 and BCR-ABL were detected in 66 % of B-ALL, presented in later childhood, and were associated with poor prognosis and inferior survival.

Conclusions This study reports the highest ethnic frequency of BCR-ABL FO in pediatric ALL, and is consistent with previous reports from our region. Poor prognosis BCR-ABL and MLL-AF4 was detected in two-thirds of pediatric B-ALL and is likely to be the reason for the already reported poor survival of childhood ALL in South-East Asia. Furthermore, MLL-AF4, usually most common in infants, presented in later childhood in most of the ALL patients, which was one of the unique findings in our study. The results presented here highlight the need for mandatory inclusion of molecular testing for pediatric ALL patients in clinical decision making, together with the incorporation of tyrosine kinase inhibitors, as well as hematopoietic stem cell transplantation facilities, to improve treatment outcome for patients in developing countries.

Key Points

The poor prognostic BCR-ABL fusion oncogene is present in 47.9 % of pediatric B-acute lymphoblastic leukemia (ALL) patients in Pakistan, while MLL-AF4 occurs in 18.1 % of B-ALL patients.

Both of these poor prognostic genetic abnormalities (BCR-ABL and MLL-AF4) represent 66 % (approximately two-thirds) of Pakistani pediatric B-ALL patients, which may be the major reason for poor prognosis and overall inferior outcome of pediatric ALL in this region.

High frequencies of poor prognostic genetic subgroups in B-ALL define the need for advanced molecular diagnostics and improved therapeutic strategies in Pakistani pediatric B-ALL patients.

1 Introduction

Acute lymphoblastic leukemia (ALL) is a malignancy of immature lymphoid progenitors. It is the most common malignancy in children, with 5-year event-free survival (EFS) rates ranging between 76 and 90 % [1]. ALL can be classified as B-cell or T-cell disease, with B-ALL more common (80 %) than T-ALL [2]. B-ALL may be further subclassified on the basis of morphologic, immunologic, cytogenetic or molecular abnormalities [3]. Cytogenetic and molecular analyses of ALL have identified many genetic abnormalities, including chromosomal translocations giving rise to fusion oncogenes (FOs) which are directly involved in leukemogenesis [4]. The most common FOs in B-ALL are *BCR-ABL*, *MLL-AF4*, *ETV6-RUNX1* and *TCF3-PBX1* [5]; the presence of a particular FO influences differential diagnosis, prognostic stratification, and drug selection [6–8].

BCR-ABL, TCF-PBX1 and MLL-AF4 have poor prognosis, while *ETV6-RUNX1* has more favorable prognosis [9–11]. ALL patients with BCR-ABL have adverse outcomes despite intensive treatment strategies [12, 13], and the overall survival of patients with E2A-PBX1 can be improved with more intensive therapies [3, 13, 14]. MLL-AF4 is mostly found in infant ALL and is associated with high white blood cell (WBC) count, central nervous system (CNS) disease, poor prognosis, a lower response to therapy, and high risk of treatment failure [15, 16]. *ETV6-RUNX1* confers a favorable prognosis, long survival and lower rates of relapse following complete remission (CR) [17, 18]; therefore, patients with *ETV6-RUNX1* require less intensive therapy compared with other FOs [19]. The *ETV6-RUNX1* frequency varies considerably in different parts of

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the world [10, 19–22], possibly due to genetic and racial heterogeneity between different populations. Similarly, frequencies of other prognostically important FOs may also vary in different geographic regions [21–25]; therefore, it is important to determine frequencies of prognostically important FOs in a given population, and devise appropriate regional pediatric ALL treatment strategies accordingly.

Pakistani pediatric ALL patients have been reported to have an inferior outcome and poorer survival, even at specialized cancer centers with the best supportive care, compared with global treatment outcome [26, 27]; the underlying reasons for this difference are unknown. Studies carried out in Pakistan to investigate the genetics of pediatric ALL [23–25] have indicated high frequency of BCR-ABL [23, 25]; however, these reports either lacked clinical data [23], samples were analyzed using clinically nonvalidated assays such as solution hybridization [24], or the sample size was too small or not representative of the whole population [25, 28]. Therefore, the purpose of this study was to detect FOs of prognostic significance in a larger cohort of pediatric B-cell ALL patients from different cities of the country using clinically validated molecular cytogenetic protocols, and study their association with clinical presentation, remission induction and survival.

2 Materials and Methods

2.1 Patients

Peripheral blood samples were obtained from 188 clinically diagnosed pediatric B-cell ALL patients admitted to different oncology centers in Lahore, Faisalabad, Islamabad and Peshawar (Pakistan) from January 2004 to December 2013. Only patients between the ages of 2 and 15 years with a confirmed diagnosis of B-ALL were included. These patients did not have a prior severe physical or psychiatric illness, and their renal and hepatic functions were adequate. Clinical data were recorded at diagnosis and subsequently updated with patient progress. This study was approved by the Ethical Committees and Scientific Review Boards of the Department of Zoology, University of the Punjab, Lahore, Pakistan, and its collaborating hospitals. Written informed consent was obtained from all patients or their parents/guardians. All experiments were carried out at the Hematology Oncology and Pharmacogenetic Engineering Sciences (HOPES) Group, Health Sciences/Parasitology Laboratories (HSL), Department of Zoology, University of the Punjab (ZPU), Lahore, Pakistan. No animal research was involved.

2.2 RNA Extraction

Peripheral blood mononuclear cells (PBMNCs) were separated by Ficoll–Hypaque density gradient centrifugation. Total RNA was extracted from PBMNCs by TriZol reagent according to the manufacturer's instructions (Life Technologies, Grand Island, NY, USA).

2.3 Synthesis of Complementary DNA

RNA was reverse transcribed to complementary DNA (cDNA) for use as a template in the polymerase chain reaction (PCR) reaction. Reverse transcriptase (RT) reaction protocol and other reaction conditions were performed as previously reported [25]. Briefly, 10 µl of RNA was added to 10 µl of RT reaction mixture containing 5X RT buffer, 25 mM dNTPS, 10 mM random hexamer primers, RiboLock^{TM} RNase inhibitor, M-MuLV RT (Fermentas, Amherst NY, USA) and DEPC-treated water. Reaction was carried out by incubatinga mixture of template, random hexamer primers and DEPC-treated water at 70 °C for 10 min. The rest of the reagents were then added and incubated at 42 °C for 60 min, 70 °C for 10 min, and held at 4 °C in the last step. The integrity of cDNA was assessed by amplification of the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

2.4 Reverse Transcriptase-Polymerase Chain Reaction Amplifications

PCR primers and nested PCR protocols for the detection of four oncogene fusions were adopted from Awan et al. [25]. For the first round of nested PCR, a 50 µl PCR reaction was performed containing 5X PCR buffer with KCl, 25 mM $MgCl_2$, 25 mM dNTP mix, DEPC- treated water, Taq DNA polymerase primer (forward and reverse) and cDNA as a template. The same PCR conditions were used in PCR round 2 with internal primers, and using the PCR product round 1 as the template. Thermal cycling conditions for nested PCR were preliminary denaturation at 95 °C for 3 min followed by 35 cycles of denaturation of double-stranded DNA at 95 °C for 30 s, annealing of primers to the DNA template at 65 °C for 60 s, and extension to form multiple copies of DNA strands at 72 °C for 60 s, followed by a post-amplification extension at 72 °C for 7 min. Round 2 was carried out with the same conditions. The final products were visualized by gel electrophoresis, and all recommended precautions were taken to avoid contaminations. Appropriate negative and positive controls were included in each amplification experiment.

2.5 Interphase Fluorescent In Situ Hybridization

2.5.1 Selection of Material

Confirmation of RT-PCR results by interphase fluorescent in situ hybridization (FISH) was limited to two FOs (BCR-ABL and MLL-AF4) that yielded unexpectedly high frequencies. All probes and kits were purchased from Abbott Laboratories (Abbott Park, IL, USA), and FISH procedures were carried out according to the manufacturer's instructions.

2.5.2 Pre-Hybridization, Hybridization and Post-Hybridization

Whole WBCs cryopreserved at −80 °C (in 10 % dimethyl sulfoxide and 90 % fetal bovine serum) were thawed at 37 °C, pelleted by centrifugation and washed with 1X phosphate buffer saline. Cells were fixed in methanol/acetic acid, dropped on slides, and air dried. The slides were pretreated with '0.01 % pepsin + 0.02 M HCl' at 37 °C for 10 min. The cells and probes were denatured on a heating plate together at 78 °C for 10 min. Hybridization was performed overnight at 37 °C. Post-hybridization washing was done in 2x saline-sodium citrate (SSC) containing 50 % formamide for 7 min at 42 °C followed by two washes in 2x SSC (42 °C for 7 min). The slides were covered by Vectashield (Vector Laboratories, Burlingame, CA, USA) containing 0.5 g/ml DAPI (4', 6-diamidino-2-phenylindole).

2.5.3 Analysis

Stained slides were analyzed using a FISH analyzer system (Leica microscope; CytoVision 4.0, Applied Imaging, Biosciences Centre, Newcastle, UK).

2.6 Patient Treatment Protocol

All patients were treated using the UKALL2003 protocol.

2.7 Response Criteria

Patients were considered to be in CR when the results of the bone marrow examination were normal (including <5 % blasts and >25 % cellularity), the neutrophil counts were $>1.5 \times 10^9$/L, platelet count was $>100 \times 10^9$/L, and all extramedullary disease had resolved.

2.8 Statistical Analysis

Convenient sampling technique was used to collect the data, and nonparametric tests were used, as appropriate, to analyze the data. The association between different FOs and clinical and laboratory parameters of leukemia patients was studied using the Chi square test. The Kaplan–Meier method was used to calculate the median survival times, and Breslow's test was used to study the survival differences between various patient groups.

3 Results

3.1 Patient Characteristics

A total of 188 patients were included in the final analysis. Overall, 134 (71.3 %) patients were male and 54 (28.7 %) were female, with a median age of 7 (range 1–15) years. Thirty-two (17 %) patients were <2 years of age, 62 (33 %) were 2–7 years of age, and 94 (50 %) patients were 8–15 years of age. Twenty-four (12.8 %) patients had a mediastinal mass, 56 (29.8 %) had splenomegaly, and 80 (42.6 %) patients had hepatomegaly. Palpable lymphadenopathy was present in 72 (38.3 %) patients, and CNS disease, as confirmed by spinal cytology, was found in 12 (6.4 %) patients. Sixty-six (35.1 %) patients had a WBC count >30,000/μl, while 122 patients (64.9 %) had a WBC count <30,000/μl; the majority of patients had platelet counts >50,000/μl ($n = 120$, 63.8 %).

According to the French-American-British (FAB) criteria, 56 (29.8 %) subjects were L1, 100 (53.2 %) were L2, and 32 (17 %) were L3. All patients were classified as B-cell ALL on morphology (Table 1).

3.2 Molecular Cytogenetic Analysis

Of the 188 samples processed for molecular cytogenetics, FOs were detected in 87.2 % (164/188) of patients, *BCR-ABL* FO was detected in 90/188 (47.9 %) ALL patients, *ETV6-RUNX1* was detected in 36/188 (19.1 %) patients, *MLL-AF4* was detected in 34/188 (18.1 %) patients, *TCF3-PBX1*1 was detected in 4/188 (2.1 %) patients, and no FO was detected in 24/188 (12.8 %) patients (Table 1). There was 100 % concordance between RT-PCR and confirmatory interphase FISH results for BCR-ABL and MLL-AF4.

The comparative frequencies of FOs in pediatric ALL patients between the current study and the previous research work from Pakistan are shown in Table 2. A strikingly high frequency of *BCR-ABL* FO was observed in children with ALL in the present study. The frequencies of FOs in pediatric ALL from different continents, compared with the data from the present study, are depicted in Table 3. In our study, overall survival was approximately 140 weeks and mean survival was 70.9 weeks (95 % CI 60.3–81.6), 3-year overall survival was 31.9 % (60/188) and 3-year relapse-free survival (RFS) was 18.1 % (34/

Table 1 Comparison of clinical characteristics of pediatric B-ALL patients ($n = 188$)

Clinical and laboratory parameters	*BCR-ABL* [*N* (%)] *N* = 90	*ETV6-RUNX1* [*N* (%)] *N* = 36	*MLL-AF4* [*N* (%)] *N* = 34	*TCF3-PBX1*1 [*N* (%)] *N* = 4	No gene detected [*N* (%)] *N* = 24	*p* value
Male	70 (77.8)	22 (61.1)	24 (70.6)	0 (0)	18 (75.0)	0.142
Female	20 (22.2)	14 (38.9)	10 (29.4)	4 (100)	6 (25)	
Age, years						
<2	6 (6.7)	20 (55.6)	6 (17.6)	0 (0)	0 (0)	0.000
2–7	32 (35.6)	14 (38.9)	4 (11.7)	2 (50)	10 (41.7)	
8–15	52 (57.8)	2 (5.6)	24 (70.6)	2 (50)	14 (58.3)	
WBC						
$<30 \times 10^9$/L	48 (53.3)	34 (94.4)	20 (58.9)	2 (50)	18 (75.0)	0.032
$>30 \times 10^9$/L	42 (46.7)	2 (5.6)	14 (41.1)	2 (50)	6 (25)	
FAB type						
L1	28 (31.11)	6 (16.7)	14 (41.2)	0	8 (33.33)	0.711
L2	46 (51.11)	24 (66.7)	16 (47.1)	4 (100)	10 (41.7)	
L3	16 (17.8)	6 (16.7)	4 (11.8)	0	6 (25)	
Hepatomegaly						
No	46 (51.1)	30 (83.3)	16 (47.1)	0 (0)	16 (66.7)	0.049
Yes	44 (48.9)	6 (16.7)	18 (52.9)	4 (100)	8 (33.3)	
Splenomegaly						
No	70 (77.8)	30 (83.3)	18 (52.9)	0 (0)	14 (58.3)	0.031
Yes	20 (22.2)	6 (16.7)	16(47.1)	4 (100)	10 (41.7)	
Lymphadenopathy						
No	60 (66.7)	22 (61.1)	10 (29.4)	4 (100)	20 (83.3)	0.021
Yes	30 (33.3)	14 (38.9)	24 (70.6)	0 (0)	4 (2/16.7)	
Platelets						
$<50 \times 10^9$/L	28 (31.1)	12 (33.3)	18 (52.9)	4 (100)	6 (25)	0.146
$>50 \times 10^9$/L	62 (68.9)	24 (66.7)	16 (47.1)	0 (0)	18 (75.0)	
CR						
<4 weeks	26 (28.9)	30 (83.3)	8 (23.6)	2 (50)	16 (66.6)	0.001
>4 weeks	58 (64.4)	4 (11.1)	20 (58.9)	2 (50)	4 (16.7)	
No remission	6 (6.7)	2 (5.6)	6 (17.5)	0 (0)	4 (16.7)	
OS, weeks	58.15	110.93	52.49	87.00	82.66	0.002
RFS, weeks	26.17	51.74	18.68	22.00	45.33	0.000

ALL acute lymphoblastic leukemia, *CR* complete remission, *OS* overall survival, in weeks, according to *FOs* Fusion oncogenes, *RFS* relapse-free survival, *WBC* white blood cells, *FAB* French-American-British

188); 4 (2.12 %) patients died of treatment-related toxicities.

Patients with *ETV6-RUNX1* (36/188, 19.14 %) had significantly better survival than patients with other FOs (mean 110.9 weeks, 95 % CI 92.7–129.1; $p = 0.030$), followed by the *TCF3-PBX*1 [t(1;19)] FO (2.1 %, 4/188; mean overall survival 87 weeks), although small numbers of this FO preclude any definite conclusion (Fig. 1). Mean survival of patients in the <2 years age group (91.1 weeks, 95 % CI 66.7–115.5) was significantly better than patients in the 2–7 years age group (81 weeks, 95 % CI 62.2–99.8) and patients in the 8–15 years age group (57.5 weeks, 95 % CI 43.4–71.6; $p = 0.014$) (Fig. 2).

Overall, patients with the *ETV6-RUNX1* oncogene also had a favorable RFS (mean 53.20 weeks, 95 % CI 50.48–55.91), and median survival was 53.20 weeks (95 % CI 50.48–55.91) (Fig. 3).

3.3 Clinical Features of Patients with Different Fusion Oncogenes

3.3.1 BCR-ABL

This FO was detected in 47.9 % (90/188) of patients. There was a male preponderance (70/90, 77.8 %) in *BCR-ABL*+ patients, with a median age of 9 years. The frequency of

Table 2 Comparison of fusion oncogene frequencies in pediatric ALL patients between the current study and the previous reports from Pakistan

Serial no.	Total number	BCR-ABL+ [*n* (%)]	MLL-AF4+ [*n* (%)]	TCF3-PBX1+ [*n* (%)]	*ETV6-RUNX1* [*n* (%)]	Method used	References
1	188	90 (47.9)	34 (18.1)	4 (2.1)	36 (19.1)	RT-PCR and interphase FISH	Present study
2	103	51 (49.5)	16 (15.5)	2 (1.9)	17 (16.5)	Karyotyping and RT-PCR	Iqbal et al. [23]
3	85	3 (3.5)	4 (5)	0 (0)	3 (3.5)	Solution hybridization	Siddiqui et al. [24]
4	101	45 (44.5)	17 (16.8)	2 (2)	18 (17.8)	RT-PCR and interphase FISH	Awan et al. [25]
5	103	25 (24)	14 (14)	2 (2)	10 (9.7)	RT-PCR	Faiz et al. [28]

ALL acute lymphoblastic leukemia, *RT-PCR* reverse transcriptase-polymerase chain reaction, *FISH* fluorescent in situ hybridization

Table 3 Range of frequencies for prognostically important fusion oncogenes in different ethnic groups of the world

Country	Total no.	*BCR-ABL* [*N* (%)]	*MLL-AF4+* [*N* (%)]	*E2A-PBX1+* [*N* (%)]	*ETV6-RUNX1* [*N* (%)]	Hypodiploid [*N* (%)]	Hyperdiploid [*N* (%)]	Technique	References
Pakistan	188	90 (47.9)	34 (18.1)	4 (2.1)	36 (19.1)	NA	NA	RT-PCR, interphase FISH	
Malaysia	278	7.8 %	4.2 %	3.1 %	19.1 %	NA	NA	Real-time multiplex PCR assay	[21]
USA (California)	389	7 (1.8)	14 (3.6)	15 (3.9)	74 (19)	26 (6.8)	61 (15.7)	Karyotyping, FISH	[22]
India	23	7 (30)	NA	NA	NA	NA	NA	Karyotyping, RT-PCR	[27]
Pakistan	103	25 (24)	14 (14)	2 (2)	10 (9.7)	NA	NA	Karyotyping, RT-PCR	[28]
Serbia	70	7 (10)	NA	6 (8.6)	12 (17.1)	NA	NA	Karyotyping, RT-PCR	[29]
Mexico	261	19 (7.3)	11 (4.2)	15 (5.8)	39 (14.9)	NA	NA	Multiplex RT-PCR	[30]
China	1004	55 (5.5)	24 (2.4)	65 (6.5)	199 (19.8)	NA	NA	Karyotyping, RT-PCR	[31]
Italy	1744	38 (2.2)	18 (1.0)	NA	NA	NA	NA	PCR	[32]
Hungary	122	NA	NA	NA	25 (20.5)	NA	NA	Karyotyping, interphase FISH,	[33]
Czech Republic	5060	140 (2.8)	52 (1)	NA	NA	NA	NA	Karyotyping, FISH, RT-PCR	[34]
Korea	65	1 (1.8)	7 (11.3)	9 (14.1)	NA	NA	NA	Karyotyping, interphase FISH	[35]
Japan	741	32 (4.3)	NA	NA	NA	NA	NA	RT-PCR	[36]
Saudi Arabia	594	429 (4.2)	448 (2.5)	281 (3.6)	214 (21)	NA	403 (24.6)	Interphase FISH	[37]
Sudan	18	4 (22)	3 (17)	1 (5.5)	1 (5.5)	NA	NA	RT-PCR	[24]

Hypodiploid (<45 chromosomes), Hyperdiploid (47–50 or >51 chromosomes)

N not available for the Malaysian data set, *NA* not available, *RT-PCR* reverse transcriptase-polymerase chain reaction, *FISH* fluorescent in situ hybridization

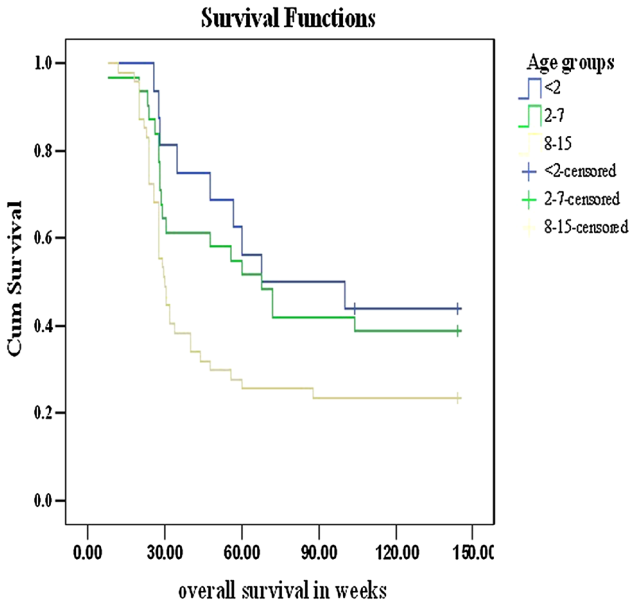

Fig. 1 Patient survival according to the fusion oncogenes. 'Censored' refers to those cases for which no event occurred, i.e. patient survived until the time the study/follow-up were conducted

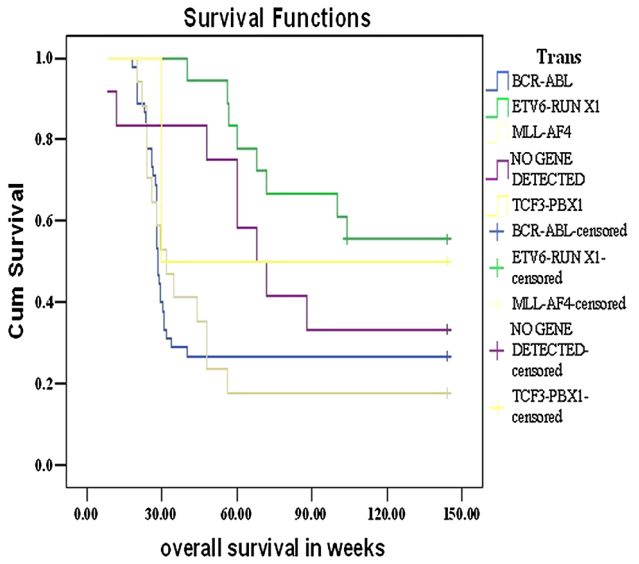

Fig. 2 Survival analysis according to age groups, in weeks. 'Censored' refers to all those cases for which no event occurred, i.e. patient survived until the time the study/follow-up were conducted

BCR-ABL positivity was directly proportional to age, with most patients being in the older age group. Six *BCR-ABL*+ patients were younger than 2 years, 32 patients were aged 2–7 years, and 52 patients were older than 7 years (Table 1). The leukocyte count in *BCR-ABL*+ patients was higher when compared with patients with other oncogenes ($p = 0.032$) (Table 1). Organomegaly was not more

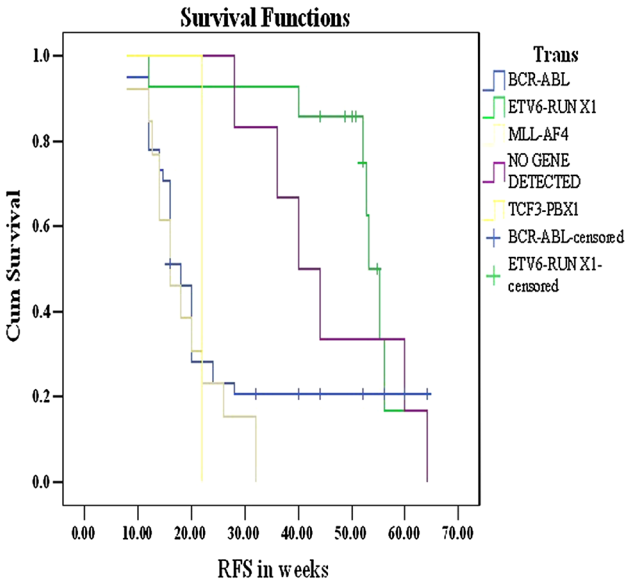

Fig. 3 RFS based on detection of oncogene fusion transcripts in B-cell ALL patients ($n = 188$). 'Censored' refers to those cases for which no event occurred, i.e. patient survived until the time the study/follow up were conducted. *RFS* relapse-free survival, *ALL* acute lymphoblastic leukemia

common in this patient group. *BCR-ABL* positivity was associated with a low remission rate and shortened survival (43.73 ± 4.24 weeks) (Fig. 3). A significant difference was seen between the survival of patients with *BCR-ABL* and other genotypes in all age groups ($p = 0.002$). Survival of patients according to different FOs and age groups are shown in Figs. 1 and 2, respectively.

3.3.2 *MLL-AF4*

A high frequency of the t(4;11) [*MLL-AF4*] FO was also observed in these children (34/188, 18.1 %). This group comprised 24 males and 10 females (Table 1). The *MLL-AF4* oncogene was predominantly found in the older (8–15 years) age group (24/34, 70.6 %; $p = 0.001$), with frequent organomegaly, low platelet count and poor survival. Mean survival of patients with the *MLL-AF4* fusion gene was 52.4 weeks (95 % CI 31.79–73.19) (Table 1; Figs. 1, 2, 3).

3.3.3 *ETV6-RUNX1*

In the present study, we detected *ETV6-RUNX1* in 36/188 (19.1 %) cases. Clinical analysis of 36 *ETV6-RUNX1*+ patients is shown in Table 1. This cohort consisted of 22 males and 14 females, with a median age of 1.85 years. Twenty patients were younger than 2 years, 14 were between 2 and 7 years of age, and 2 patients were in the 8–15 years age group. Most (94.4 %) *ETV6-RUNX1*+

patients had a WBC count $<30 \times 10^9$/L. The majority of patients with this FO were in the younger age group, in contrast to patients with *BCR-ABL* and *MLL-AF4* FOs. Most of the patients with *ETV6-RUNX1* achieved CR (30/36, 83.3 %) in ≤4 weeks of treatment, and had better prognosis overall than other FOs (Table 1; Figs. 1, 2, 3).

3.3.4 *TCF3-PBXI*

In this study, t(1;19) [TCF3-PBX1] was diagnosed in only four (2.1 %) female patients, with a mean overall survival of 87 weeks (Table 1; Fig. 1).

4 Discussion

Our results show BCR-ABL and MLL-AF4 FOs in two-thirds (66 %) of Pakistani pediatric B-ALL patients. This patient group was associated with late childhood, poor prognostic features, late remissions, and poor survival. Overall, Pakistani pediatric ALL patients had poor survival compared with global pediatric ALL patients [1].

Using RT-PCR, we screened for *BCR-ABL*, *MLL-AF4*, *ETV6-RUNX1* and *TCF3-PBX11* FOs in a group of 188 children with B-cell ALL, and confirmed important results by using interphase FISH analysis. A high (47.9 %) frequency of *BCR-ABL* FO was found in the present study, which is in accordance with previous studies from Pakistan [23, 25, 28]. Furthermore, BCR-ABL frequency in India has been reported to be 30.4 % among pediatric B-ALL patients [27], which indicates an overrepresentation of this poor prognostic genetic abnormality in the Indo-Pak region. Although global incidence of this genetic abnormality in childhood ALL has been reported to be 3–5 %, most of these studies were carried out in developed countries, such as Europe, the US and Japan [3]. In addition to Pakistan and India, the frequency of BCR-ABL in pediatric ALL from Sudan, Serbia, Malaysia, Mexico and China has been reported to be 22, 10, 7.8, 7.3 and 5.5 %, respectively [21, 24, 29–31], while in the US, Europe, Saudi Arabia, Korea and Japan, it has been reported to be approximately 2–5 % [32–37]. This shows variation in the frequencies of BCR-ABL FOs among different geographic regions; probably higher frequencies (7 % or more) in developing or third-world countries and lower frequencies (2–5 %) in developed countries.

In this study, BCR-ABL in ALL was associated with late childhood, with 52 (57.8 %) children in the 8–15 years age group. Moreover, BCR-ABL patients presented with poor prognostic clinical features, aggressive disease and poor clinical outcome, which is consistent with the global prognosis of BCR-ABL-positive pediatric ALL [1, 3, 7, 9, 21]. However, high BCR-ABL frequency may be one of the most important reasons for overall poor outcome of pediatric B-ALL patients from Pakistan, in both our study and previous reports [23, 25, 26, 28, 38]. This necessitates the mandatory inclusion of molecular diagnostics in routine clinical settings, provision of tyrosine kinase inhibitors (TKIs) and hematopoietic stem cell transplantation (HSCT) facilities at childhood cancer centers in Pakistan, the latter being proved to be standard of care in recent years for high-risk pediatric ALL patients [41]. Unfortunately, these advanced diagnostic and modern treatment facilities are currently not available at most oncology centers in Pakistan [7, 30].

The frequency of the *MLL-AF4* FO was also found to be higher (18.1 %) in our pediatric B-ALL patients, which is in accordance with previous studies from Pakistan [23, 25, 28]. Furthermore, it was more common in the 8–15 years age group (24/34, 70.6 %; $p = 0.001$). Like BCR-ABL, MLL-AF4 was associated with older childhood age in the majority of B-ALL patients. Globally, MLL-positive leukemias were detected in 60–80 % of infants [3, 7, 9, 21], which makes our observation of the association of MLL-AF4 with late childhood, as well as high overall frequency of this unfavorably prognostic genetic abnormality, unique to our population. However, it has been reported recently that MLL-AF4 frequency among ALL patients increases with age until the fourth decade of life [42], which supports our findings. Patients with MLL-AF4 were associated with frequent organomegaly, low platelet count and poor survival, which is in accordance with previous studies [3, 7, 16, 21, 42]. Along with BCR-ABL, a high frequency of MLL-AF4 may be the likely reason for the poor clinical outcome of pediatric B-cell ALL patients [23, 25, 26], which further necessitates the provision of molecular diagnostic services and advanced treatment and supportive care for pediatric ALL patients in our region.

TCF3-PBX1 is one of the most prevalent cytogenetic abnormalities in pediatric ALL—found in 5–6 % of patients [43]. The fusion gene TCF3-PBX1 was found in 2.1 % of our pediatric B-ALL population, which is in accordance with the frequency of this gene in our neighboring countries China [33] and Sudan [24]. Although this genetic abnormality was previously reported to be associated with poor prognosis and a higher risk of CNS relapse, recent reports indicate that this bad prognosis and poor outcome can be overcome by incorporating intensive treatment regimens [44, 45]. Therefore, molecular detection of TCF3-PBX1 at diagnosis can help with better treatment outcome and avoiding CNS relapse by adopting proper CNS-directed treatment strategies [43–45].

The frequency of *ETV6-RUNX1* FO (19.1 %) in Pakistani children with B-ALL is comparable with other parts of the world, including Asia (16.59 %), Europe (20–25 %),

and the US (17.85 %), but is higher than the Chinese and African populations (8.55 %) [21, 22, 24]. Interestingly, Western Spain has a very low frequency of *ETV6-RUNX1*, compared with up to 25 % in the rest of Europe. This also shows ethnic differences in pediatric ALL genetics as, ethnically, people in Western Spain are more likely to resemble the Indian subcontinent population [29]. Most of our *ETV6-RUNX1*-positive B-ALL patients had a favorable prognosis, achieved CR promptly, and showed better survival compared with other genetic groups studied, which is in accordance with earlier reports [3, 7, 21, 30, 31, 44–47].

ALL in children is a highly curable disease, with 76–90 % 5-year EFS rates in developed countries [1, 39–41]. However, outcomes remain poor in our patient population, which is in consistent with previous reports from different oncological centers of Pakistan which have reported a high frequency (51 %) of delayed or no remission [25], high degree of relapses [25, 26, 30], and high mortality [26, 30]. In addition to high BCR-ABL and MLL-AF4 frequencies, other factors contributing to this poor outcome include a delay in diagnosis and referral to a specialized center, poor socioeconomic conditions, suboptimal supportive care, as well as a shortage of trained pediatric hematologists, hematopathologists with advanced training, such as molecular hematology, and specialized treatment centers [26, 27, 48–51]. In addition, it is likely that the nonavailability of TKIs for BCR-ABL-positive ALL patients, and the lack of facilities for HSCT for high-risk patients also contributed to the poor survival of these patients [7]. In particular, the high incidence of BCR-ABL-positive pediatric ALL cases urgently needs further investigation and collaboration of local and international researchers to study the etiology and pathogenesis of this disease entity. Our studies also indicate ethnic and geographic variations in clinical parameters, genetic epidemiology and treatment outcome of pediatric B-ALL patients, which can be attributed to different ethnicity [1, 21, 52–54], lifestyle [55], socioeconomic status [48–51], and environmental factors, such as exposure to pesticides, fumes, dust, etc. [55–58], in different regions of the world [1, 21, 52–59].

This study has certain limitations. First, due to financial constraints we were not able to perform a comprehensive cytogenetic and FISH-based study to determine all genetic abnormalities. Second, the number of patients with certain FOs (e.g. TCF3-PBX1) was insufficient for their proper clinical characterization. We recommend carrying out further studies, possibly with international collaborations, with comprehensive genetic analysis in a larger number of patients to further investigate the genetic basis of poor prognosis and clinical outcome in Pakistani ALL patients.

5 Conclusions

Our research group reported the frequency of molecular markers and their correlation with disease biology and treatment outcome in the largest group of pediatric B-cell ALL patients from Pakistan. FOs with poor prognosis, namely *BCR-ABL* and *MLL-AF4*, were collectively detected in 66 % of patients, which is unique to our population and likely to be one of the major reasons for lower cure rates for children with ALL in our country. These findings support the notion that there are genetic, racial and geographic differences in the frequency of molecular markers of childhood ALL. This study has important clinical implications and indicates the need for the availability of molecular testing at all centers managing pediatric ALL patients, and to adopt strategies such as the incorporation of TKIs in pediatric ALL treatment protocols, as well as availability of more HSCT centers, to improve outcomes. Further studies are needed to find out the possible reason(s) for high *BCR-ABL* frequency among these patients.

Author contributions Zafar Iqbal, Ammara T. Gill and Mudassar Iqbal performed experiments, analyzed the data, and wrote the manuscript. Tanveer Akhtar, Mahmood Rasool, Aamir Mahmood and Muhammad Imran Irfan analyzed the data and critically reviewed the manuscript. Tashfin Awan, Noreen Sabir and Muhammad Farooq Sabar performed experiments and analyzed the data. Aamer Aleem and Muhammad Absar analyzed the data and wrote the manuscript. Afia M. Akram and Muhammad Hassan Siddiqi performed experiments. Masood A. Shammas, Abdullah Alanazi, Ahmad M. Khalid, Mehmood Hussain Qazi and Sajjad Karim critically reviewed the manuscript. Ijaz H. Shah, Muhammad Khalid, Abid S. Taj, Abid Jameel, Jamil Amjad Hashmi, Akhtar Hussain and Anjum Saeed analyzed the clinical data. Zafar Iqbal is guarantor of the overall content.

Compliance with Ethical Standards

Conflicts of interest Zafar Iqbal, Tanveer Akhtar, Tashfin Awan, Aamer Aleem, Noreen Sabir, Mahmood Rasool, Muhammad Absar, Afia M. Akram, Masood A. Shammas, Ijaz H. Shah, Muhammad Khalid, Abid S. Taj, Abid Jameel, Abdullah Alanazi, Ammara T. Gill, Jamil Amjad Hashmi, Akhtar Hussain, Muhammad Farooq Sabar, Ahmad M. Khalid, Mehmood Hussain Qazi, Sajjad Karim, Muhammad Hassan Siddiqi, Aamir Mahmood, Mudassar Iqbal, Anjum Saeed, and Muhammad Imran Irfan have no conflicts of interest to disclose.

Funding This study was partially supported by the College of Medicine Research Center, Deanship of Scientific Research, King Saud University, Riyadh, Saudi Arabia.

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