RESEARCH ARTICLE

Five Most Common Prognostically Important Fusion Oncogenes are Detected in the Majority of Pakistani Pediatric Acute Lymphoblastic Leukemia Patients and are Strongly Associated with Disease Biology and Treatment Outcome

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Abstract

Background and Objectives: Acute lymphoblastic leukemia (ALL) is a complex genetic disease involving many fusion oncogenes (FO) having prognostic significance. The frequency of various FO can vary in different ethnic groups, with important implications for prognosis, drug selection and treatment outcome. Method: We studied fusion oncogenes in 101 pediatric ALL patients using interphase FISH and RT-PCR, and their associations with clinical features and treatment outcome. Results: Five most common fusion genes i.e. BCR-ABL t (22; 9), TCF3-PBX1 (t 1; 19), ETV6-RUNX1 (t 12; 21), MLL-AF4 (t 4; 11) and SIL-TAL1 (del 1p32) were found in 89/101 (88.1%) patients. Frequency of BCR-ABL was 44.5% (45/101). BCR-ABL positive patients had a significantly lower survival (43.7±4.24 weeks) and higher white cell count as compared to others, except patients with MLL-AF4. The highest relapse-free survival was documented with ETV6-RUNX1 (14.2 months) followed closely by those cases in which no gene was detected (13.100). RFS with BCR-ABL, MLL-AF4, TCF3-PBX1 and SIL-TAL1 was less than 10 months (8.0, 3.6, 5.5 and 8.1 months, respectively). Conclusions: This is the first study from Pakistan correlating molecular markers with disease biology and treatment outcome in pediatric ALL. It revealed the highest reported frequency of BCR-ABL FO in pediatric ALL, associated with poor overall survival. Our data indicate an immediate need for incorporation of tyrosine kinase inhibitors in the treatment of BCR-ABL+ pediatric ALL in this population and the development of facilities for stem cell transplantation.

Keywords: Acute lymphoblastic leukemia - pediatric ALL - fusion oncogenes - Pakistan

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Introduction

The incidence of acute lymphocytic leukemia (ALL) is not uniform around the world. It varies from 0.9-4.7 per 100,000 children per year (Zhang et al., 2011).The infant mortality rate in Pakistan is 71% which is one of the highest in the world (Ashraf, 2012). ALL comprises of eighty percent of childhood acute leukemias (Cheok and Evans, 2006) and there is a striking incidence peak during the ages two to seven years where the incidence

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is as high as 10 per 100,000 children (Pui et al., 2008).

The genetic lesions in ALL play a key role in the abnormal development of lymphoid cells (Kuiper et al., 2007; Treviño et al., 2009; Iacobucci et al., 2012; Xu et al., 2012). The diagnosis of ALL is based on morphology, immunophenotype and cytogenetic analysis of the leukemic blast cells in the peripheral blood and bone marrow (Bhojwani et al., 2012; McGregor et al., 2012). Molecular analysis of the common genetic alterations in leukemic cells has contributed greatly to our understanding of the pathogenesis and prognosis of ALL (Iqbal et al., 2007; Pui et al., 2008). The most common fusion oncogenes in pediatric ALL are BCR-ABL, ETV6-RUNX1, MLL-AF4, SIL-TAL1 and TCF3-PBX1 (Pui et al., 2008; Schrrape et al., 2012). The frequency of particular genetic subtypes differs in children but the general mechanisms underlying the induction of ALL are likely to be similar to adult ALL (Pui et al., 2004).

Chromosomal translocations are powerful prognostic indicators in pediatric ALL (Kuiper et al., 2007; Iacobucci et al., 2012). The presence of recurrent genetic markers represents subtypes of the disease which may have different etiologies (Iqbal et al., 2007; Kuiper et al., 2007; Treviño et al., 2009; Pui et al., 2011). There is little information available about the molecular epidemiology of pediatric ALL in Pakistan. According to the best of our knowledge this is the first community-based study from Pakistan focused on exploring the prevalence of five common ALL fusion oncogenes in children with ALL and their correlation with the disease biology and treatment outcome.

Materials and Methods

Peripheral blood samples were obtained from 148 pediatric ALL patients admitted to different hospitals of Lahore, Pakistan from January 2009 to December 2011. Patients between the ages of<one to fifteen years, with a confirmed diagnosis of ALL were included. These patients did not have a prior severe physical or psychiatric illness and their renal and hepatic function were adequate. Twenty samples were unfit for further processing while 27 were negative for GAPDH ("housekeeping" genes). The remaining 101 samples were processed for molecular cytogenetics. We studied 5 fusion genes in 101 pediatric ALL patients using Interphase FISH and RT-PCR at the time of diagnosis. The clinical data were recorded at diagnosis and subsequently.

RNA extraction

Total RNA was extracted from leukemic cells by TriZol reagent according to the manufacturer's instruction.

Synthesis of complementary DNA (cDNA)

RNA was reverse-transcribed to cDNA for using as template in PCR reaction. RT reaction protocol and other reaction conditions were adopted from Van Dongen (Van Dongen et al., 1999). Briefly, 10μl of RNA was added to 10 μl of RT-reaction mixture containing 5X RT buffer, 25 mM dNTPS, 10 mM random hexane primers, RiboLockTMRNase inhibitor, M-MuLV reverse transcriptase (Fermentas, USA) and DPCE- treated water. Reaction was carried out by incubating mixture of template, random hexamers and DEPC treated water at 70ºC for 10 min. Then rest of the reagents were 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 housekeeping genes GAP-DH.

RT-PCR amplifications

PCR primers and nested PCR protocols for the detection of five fusion genes were adopted from Van Dongen et al. (1999). 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 water, Taq DNA polymerase primer (forward and reverse) and cDNA as a template. The same test was run in round 2 with the template being the product of round 1. 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 sec, annealing of primers to DNA template at 65°C for 60 sec and extension to form multiple copies of DNA strands at 72°C for 60 sec, followed by a post amplification extension at 72°C for 7 minutes. Round 2 was carried out with the same conditions. The final products were visualized by gel electrophoresis. All recommended precautions were taken to avoid contaminations. Appropriate negative and positive controls were included in each amplification experiment.

Statistical analysis

For this study we used convenient sampling technique to collect the data, and used non-parametric tests, as appropriate, to analyze the data. Chi Square test was used to study the association between different oncogenotypes and clinical and laboratory parameters of leukemia patients. Kaplan and Meier method was used to calculate the median survival times, while Breslow's test was used to study the survival differences between various patient groups.

Results

Patients' characteristics

A total of 101 patients were included in the final analysis. A total of 101 patients were included in the final analysis. There were 70 (69.3%) male and 31 (3o.6%) females with a median age of 7 (range1-15) years. Eighteen patients (20.2%) were<2 years old. There were 33 (32.6%) patients between the ages of 2-7 years and fifty (49.5%) patients were in the age range of 7-15 years. Median WBC count was 16.3×10^9/l (range 0.4-465), median platelet count was 54.5×10^9/l (range 7-525), and median Hb level was 6.5 g/dl (range 3.5-13.7). Sixty five (64.3%) patients had WBC counts<30×10^9/l while 36 (35.6%) had WBC counts >30×10^9/l. Sixty five (64.3%) patients had a fever before starting chemotherapy. Twenty one (21.7%) patients had a mediastinal mass, 30 (29.7%) had a splenomegaly, and 43 (42.5%) patients had hepatomegaly. Palpable lymphadenopathy was present in 39 (38.6%) patients. CNS disease, as confirmed by

spinal cytology, was found in 7 (9.3%) patients. Seventy nine patients could be classified by the pathology review committee using the FAB criteria; 36 (35.6%) cases were L1, 48 (47.5%) were L2. Immunopphenotyping was reported in 77/101 cases. Majority of the patients expressed B-lineage antigens (70 cases) and 7 patients expressed T-lineage antigens (Tables 4, 5).

Molecular cytogenetic analysis

Of the 101 samples processed for molecular cytogenetics, BCR-ABL FO was detected in 45/101 (44.5%) patients, ETV6-RUNX1 in 18/101 (17.8%) patients, MLL-AF4 in 17/101 (16.8%) patients and SIL-TAL1was found in 7/101 (6.9%) patients. Only 2/101 (2.0%) ALL patients were positive for TCF3-PBX1 (Table 1, Figure 1). This study found a low prevalence of ETV6-RUNX1, MLL-AF4, SIL-Tal1 and TCF3-PBX1 fusion

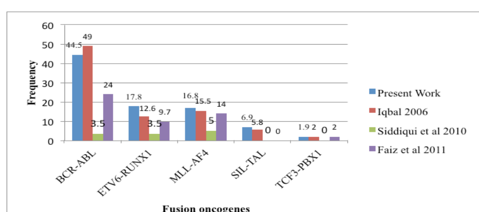

Figure 1. Comparison of Frequency of Fusion Oncogenes in Pediatric ALL Patients between Present Study and the Previous Research Work from Pakistan

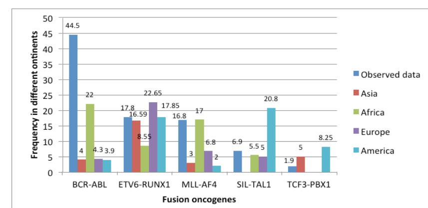

Figure 2. Bar Chart Showing Comparison of Frequencies of Fusion Oncogenes in Different Continents of the World

Table 1. Comparison of Observed Data with Previous Studies of Pediatric ALL Conducted in Pakistan (Percentages)

Fusion Oncogene	Present study	Iqbal et al. (2006)	Siddiqui et al. (2010)	Faiz et al. (2011)
BCR-ABL	44.5	49.0	3.5	24.0
ETV6-RUNX1	17.8	12.6	3.5	9.7
MLL-AF4	16.8	15.5	5.0	14.0
SIL-TAL	6.9	5.8	*	*
TCF3-PBX1	1.9	2.0	0	2.0

*Not detected. Present data is not significantly different as compared to Iqbal (p-value 0.754), Siddique et. al, (p-value 0.085) and Faiz et al (p-value 0.806)

Table 2. Average Percentage of the Fusion Oncogenes in Pediatric ALL from Different Continents Compared with Present Data from Pakistan

Fusion Gene	Present study	Asia	Africa	Europe	America
BCR-ABL	44.5	4.00	22.00	4.30	3.90
ETV6-RUNX1	17.8	16.59	8.55	22.65	17.85
MLL-AF4	16.8	3.00	17.00	6.80	2.00
SIL-TAL1	6.9	*	5.50	5.00	20.80
TCF3-PBX1	1.9	5.00	*	*	8.25

*Not detected

oncogenes in Pakistani children with ALL but there was a relatively high occurrence of BCR-ABL rearrangement. Table 2 shows the comparison of BCR-ABL frequency in pediatric ALL from different parts of the world.

Frequency of most of the fusion genes was approximately the same as compared to average frequency in Asia (p=0.221), Europe (p=0.462) and America (p=0.917), whereas it was significantly different from

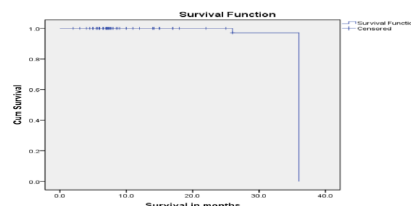

Figure 3. Overall Survival. Overall 33 Out of 101 Completed Their Study Period, the Average Survival of Peads was 35.71 Months

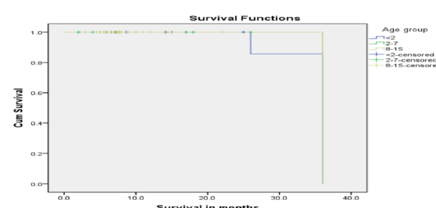

Figure 4. Survival of the Patient with Respect to Age. Overall survival of patients in age group ≤2 is less than other age groups but it is not significantly different p-value 0.145

Table 3. Overall Frequency, Median, 95%CI and Statistical Significant Differences of Numeric Variables (Age Groups, TLC, Platelets and Complete Remission) in Patients with Leukemia

Variable		Frequency	Median	95%CI	P-Value
Age	<2	18	1.5	1.11-2.3	<0.001
	2-7	33	5	4.7-5.9	
	8-15	50	12	10.7-12.7	
TLC	<30,000	65	5210	6213.35-10175.42	
	>30,000	36	70300	52100-131000	<0.001
Platelets	<50,000	40	16000	15100-24900	<0.001
	>50,000	61	109000	75700-14200	
CR	<4	69	4	1.22-1.84	<0.001
(Weeks)	>4	20	17	16.33-33.99	

Table 4. Overall Frequency, Percentage and Statistical Significance of Categorical Variables of Leukemia Patients

Variable		No. of patients	%	P-Value
FAB	L1	36	35.60	<.001
	L2	48	47.50	
	unknown	17	16.80	
Mediastinal Mass	Present	21	20.70	<.001
	Absent	80	79.20	
Hepatomegaly	Present	43	42.50	<.001
	Absent	58	57.40	
Splenomegaly	Present	30	29.70	<.001
	Absent	71	70.00	
Lymphadenopathy	Present	39	38.60	<.001
	Absent	62	61.30	
CNS involvement	Present	7	6.90	<.001
	Absent	94	93.00	
Testicular involvement	Present	3	1.98	<.001
	Absent	98	97.00	

African countries (p<0.001). However, the frequency of BCR-ABL was much higher as compared to reported from rest of the world.

Clinical features of patients with different fusion oncogenes

BCR-ABL: There was a male preponderance (34 male, 75.5%; 11 females, 24.5%) in BCR-ABL+ patients with a median age of 9 years (Table 5). There were 3 BCR-ABL+ patients in the less than 2 year age group, 16 patients in the 2-7 year age group and 26 patients in the older than 7 group. These figures show that the frequency of occurrence of BCR-ABL positivity is directly proportional to age. The leukocyte count in BCR-ABL+ patients was higher when compared to patients with other oncogenes (Table 5). Organomegaly was not more common in this patients group. There was a significant difference between the survival of patients with BCR-ABL and other genotypes in all age groups (p=0.004) (Figures 6, 7A-C). BCR-ABL positivity was associated with low remission rates and shortened survival.

ETV6-RUNX1: Clinical analysis of 18 ETV6-RUNX1 positive patients is shown in Table 5. This cohort consisted of 11 male and 7 females with a median age of 1.85 years. The gene frequency was highest in patients younger than 2 years. The WBC count in ETV6-RUNX1+ patients was not very high and they had a good prognosis.

MLL-AF4: Seventeen patients had MLL-AF4 gene rearrangement with a median age of 9 years. There were 12 male and 5 female patients (Table 5). Five patients were younger than 2 years, two between 2 and 7 years, and ten patients were in the 7-15 age group. Majority of

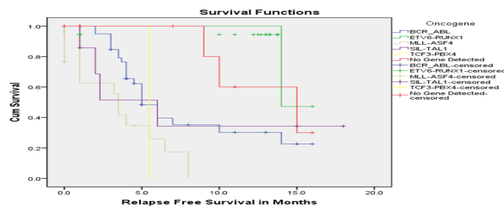

Figure 5. Overall Survival with Respect to Fusion Oncogenes. The highest relapse-free survival was documented in ETV6-RUNX1 (14.167 months) followed closely by those cases in which no gene was detected (13.100). Survival in BCR-ABL, MLL-ASF4, TCF-PBX4 and SIL-TAL1 was less than 10 months (7.994, 3.559, 5.500 and 8.080 months respectively)

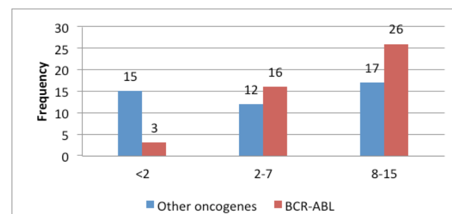

Figure 6. Comparison of Number of Patients with BCR-ABL and Other Oncogenes According to Age Groups. BCR-ABL is detected in comparatively older pediatric ALL patients

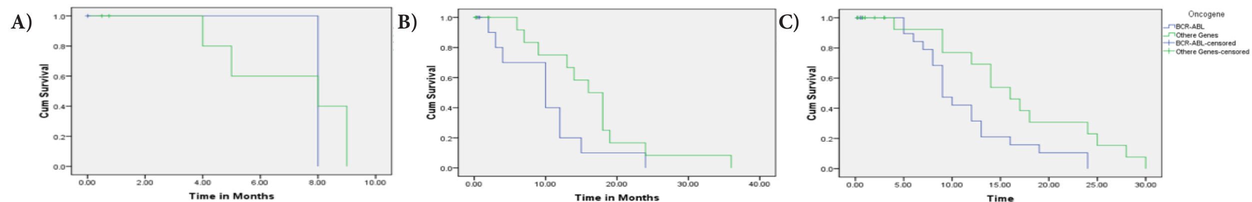

Figure 7. Comparison of Patient Survival with BCR-ABL and Other Oncogenes. A) Age less than 2 years, B) Age 2-7 years), C) Age 8-15 years. There is a significant difference between the survival of patients with BCR-ABL and other four oncogenes in all age groups (p=0.004)

Table 5. Comparison of Clinical Characteristic of Pediatric ALL Patients with Different Fusion Oncogenes

Clinical and laboratory parameters		BCR-ABL No (%) N=45	ETV6-RUNX1 No (%) N=18	MLL-AF4 No (%) N=17	SIL-TAL1 No (%) N=7	TCF3-PBX1 No (%) N=2	FO not detected No (%) N=12
Sex	Male	34 (75.0)	11 (61.0)	12 (70.5)	3 (42.8)	0	9 (75.0)
	Female	11 (24.4)	7 (38.8)	5 (29.4)	4 (57.1)	2 (100)	3 (25.0)
Age	<2	3 (6.7)	10 (55.5)	5 (29.4)	0	0	0
	2-7	16 (35.6)	7 (38.8)	2 (11.8)	2 (28.6)	1 (50)	5 (41.6)
	8-15	26 (57.8)	1 (5.5)	10 (58.8)	5 (71.4)	1 (50)	7 (58.4)
WBC	<30,000	24 (53.3)	17 (94.4)	10 (58.8)	4 (57.1)	1 (50)	9 (75.0)
	>30,000	21 (46.6)	1 (5.5)	7 (41.1)	3 (42.8)	1 (50)	3 (25.0)
Hepatomegaly	No	23 (51.1)	15 (83.3)	8 (47.1)	3 (42.9)	0	9 (12.0)
	Yes	22 (48.9)	3 (16.7)	9 (53)	4 (57.1)	2 (100)	3 (25.0)
Splenomegaly	No	35 (77.8)	15 (83.3)	9 (53)	5 (71.4)	0	7 (58.3)
	Yes	10 (23.3)	3 (17.7)	8 (47.1)	2 (28.6)	2 (100)	5 (41.7)
Lymphadenopathy	No	30 (66.7)	11 (61.1)	5 (29.4)	5 (21.0)	2 (100)	0
	Yes	15 (33.3)	7 (38.9)	12 (70.5)	2 (28.0)	0	2 (16.7)
Platelets	<50,000	14 (31.1)	6 (33.3)	9 (52.9)	6 (85.7)	2 (100)	3 (25.0)
	>50,000	31 (68.9)	12 (66.7)	8 (47.1)	1 (14.7)	0	9 (75.0)
CR	<4weeks	13 (28.9)	16 (94.4)	4 (23.5)	5 (71.4)	1 (50)	8 (66.7)
	>4weeks	29 (64.4)	1 (5.6)	10 (82.4)	1 (14.2)	1 (50)	2 (16.6)
	No remission	3 (6.7)	1 (5.6)	3 (13.6)	1 (14.2)	0	2 (16.6)

our patients were older unlike the usual occurrence where most of the patients are infants.

TCF3-PBX1: Translocation t (1; 19) occurs in around 2% of patients and involves the fusion of E2A genes on chromosome 19 to the PBX1 gene on chromosome 1. Only two female patients were diagnosed with this translocation. Both the patients were over 2 years of age. This translocation is associated with the inferior outcome in the context of response to chemotherapy with poor prognosis. It was associated with higher risk of CNS relapse although small numbers preclude any firm conclusion (Table 5).

SIL-TAL1: This gene was found in 7 patients, 3 male and 4 females. All the patients were older than 2 years, with the majority falling in the age range 7-15 years. The immunophenotype data were available in all SIL-TAL1 patients showing this fusion gene was associated with T-ALL. Organomegaly was frequently observed in these patients.

Discussion

Acute lymphoblastic leukemia (ALL) is a heterogeneous disease and comprises of many different genetic subgroups as identified by various chromosomal and molecular abnormalities (Treviño et al., 2009; Xu et al., 2012). This heterogeneity is likely to be due to genetic, racial and geographic variations that exist among different populations (Pui et al., 2003; 2009; Treviño et al., 2009; Iacobucci et al., 2012; Schmiegelow et al., 2012; Xu et al., 2012). Therefore, the distribution of genetic and molecular subtypes may not be uniform in different parts of the world (Romana., 1995; Ariffin et al., 2007). Moreover, gene-environment interactions, which are critical in leukemogenesis, may differently contribute in defining the relative proportions of molecular subgroups in different geographic regions (Iqbal et al., 2006; Siddique et al., 2010; Faiz et al., 2011; Schmiegelow et al., 2012; Schrappe et al., 2012). The most common oncogenes found in leukemia patients are the fusion genes, which are formed as a result of different genetic abnormalities at the chromosomal level (Zelent et al., 2004). Chromosomal anomalies resulting from fusion oncogenes create hybrid transcripts that usually encode transcription factors (Mesquita et al., 2009). Different oncogenes are believed to be a different molecular entity and they act by different molecular pathways (Iqbal et al., 2012).

Cytogenetic studies of the leukemia patients to identify the presence of fusion oncogenes are extremely important in the prognostication and management planning of this disease (Ariffin et al., 2007). The five major risk-stratifying translocations in patients with ALL are BCR-ABL, ETV6-RUNX1, MLL-AF4, SIL-TAL1 and TCF3-PBX1 (Lazic et al., 2010; Iacobucci et al., 2012). The frequency of some of the FO in this study is comparable to the previous studies from Pakistan and other parts of the world (Gaynon et al., 1997; Iacobucci et al., 2012). A strikingly high frequency of BCR-ABL FO was found in this study which is in keeping with the previous reports from Pakistan (Iqbal and Tanveer, 2006, Iqbal et al., 2007; Faiz et al., 2011).

The overall prevalence of fusion oncogene shows consistency between population under study and the global reports (Gaynon et al., 1997; Van Dongen et al., 1999; Pui et al., 2008; Iacobucci et al., 2012; Schrrape et al., 2012). Most notable was the high frequency of the BCR-ABL that was 44.5% as compared to population study in Europe where it is not so high (Van Dongen et al., 1999; Lazic et al., 2010; Iacobucci et al., 2012) except in Sudan, Africa (Siddique et al., 2010) while BCR-ABL has been reported to be totally absent in Saudi Arabian pediatric ALL patients (El-sissy et al., 2006; Siraj et al., 2006). The Philadelphia chromosome is present in children with ALL and leads to the production of BCR-ABL fusion protein with tyrosine kinase activity. This subtype of ALL is common in older children than in infants. Pediatric patients are mostly precursor B-Cell ALL and have a high leukocyte count. It is associated with poor prognosis due to poor response to initial therapy especially in case of high leukocyte count. Complete remission in 4 weeks can lead to increase in EFS rate. The Mexican study found highest prevalence of TCF3-PBX1 11.5% (Jimenez et al., 2008.) while this gene is only 1.9% in population under study. The frequency of ETV6-RUNX1 is 17.8% and it is in agreement with the prevalence of this chimerical gene in the affluent societies like France where it is reported 19.7% in pediatric ALL population (DeBraekeleer., 2010; Reichard, 2011) Patients with t (4; 11) MLL-AF4 are usually infants with high WBC count. They are more likely than other children with ALL to have CNS disease and to have a poor response to therapy and a poor prognosis. They are at high risk of treatment failure. Children with t (4; 11) have better outcome than infants (Raimondi et al., 1996) This reflects ethnic and geographic differences in biology and genetics of pediatric ALL. Similar reports about ethnic differences in the disease biology, genetics and treatment outcome have been reported in adult ALL (Sabir et al., 2012).

A remarkable progress has been made in the treatment of ALL in children with cure rates of around 80% (Bowman et al., 2011; Hunger et al., 2012). Unfortunately, the survival of pediatric ALL patients in Pakistan remain poor which needs urgent attention. There are possibly several factors which contribute to this poor outcome and these include a delay in diagnosis and referral to a specialized centre, lack of understanding and awareness on the part of the parents, poor socio-economic conditions where many patients need to buy the drugs and other themselves which may lead to compromises and suboptimal treatment, and possibly suboptimal supportive care. In addition, the non-availability of tyrosine kinase inhibitors for BCR-ABL+ cases and lack of facilities for hemopoietic stem cell transplantation for high risk patients very likely contributed to the overall and this group's poor survival (Schultz et al., 2009; Leung et al., 2011; Pulsipher et al., 2011; Rives et al., 2011). High incidence of BCR-ABL positive pediatric ALL cases needs urgent attention for further investigation and collaboration of the local and international researchers to study the etio-pathogenesis of this disease entity.

In conclusion, this is the first study from Pakistan correlating molecular markers with disease biology and

treatment outcome in pediatric ALL. Our study revealed the highest reported frequency of BCR-ABL FO in pediatric ALL which, consequently, was associated with poor overall survival. Our data indicate an immediate need for incorporation of tyrosine kinase inhibitors in the treatment of BCR-ABL+ pediatric ALL in this population and the development of facilities for stem cell transplantation.

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