RESEARCH ARTICLE

Molecular Genetic Studies on 167 Pediatric ALL Patients from Different Areas of Pakistan Confirm a Low Frequency of the Favorable Prognosis Fusion Oncogene TEL-AML1 (t 12; 21) in Underdeveloped Countries of the Region

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Abstract

TEL-AML1 fusion oncogene (t 12; 21) is the most common chromosomal abnormality in childhood acute lymphoblastic leukemia (ALL). This translocation is associated with a good prognosis and rarely shows chemotherapeutic resistance to 3-drug based remission induction phase of treatment as well as overall treatment. Thus, the higher the frequency of this fusion oncogene, the easier to manage childhood ALL in a given region with less intensive chemotherapy. Although global frequency of TEL-AML1 has been reported to be 20-30%, a very low frequency has been found in some geographical regions, including one study from Lahore, Punjab, Pakistan and others from India. The objective of present study was to investigate if this low frequency of TEL-AML1 in pediatric ALL is only in Lahore region or similar situation exists at other representative oncology centers of Pakistan. A total of 167 pediatric ALL patients were recruited from major pediatric oncology centers situated in Lahore, Faisalabad, Peshawar and Islamabad. Patients were tested for TEL-AML1 using nested reverse transcription polymerase chain reaction (RT-PCR). Only 17 out of 167 (10.2%) patients were found to be TEL-AML1 positive. TEL-AML1+ALL patients had favorable prognosis, most of them (82.4%, 14/17) showing early remission and good overall survival. Thus, our findings indicate an overall low frequency of TEL-AML1 in Pakistan pediatric ALL patients, in accordance with lower representation of this prognostically important genetic abnormality in other less developed countries, specifically in south Asia, thus associating it with poor living standards in these ethnic groups. It also indicates ethnic and geographical differences in the distribution of this prognostically important genetic abnormality among childhood ALL patients, which may have a significant bearing on ALL management strategies in different parts of the world.

Keywords: Childhood acute lymphoblastic leukemia - genetic epidemiology - TEL-AML1 - geographic differences

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Introduction

The cellular components of blood are formed from a pool of stem cells in the bone marrow, through an organized pathway called the hematopoietic system (Grech et al., 2014). The pluripotent stem cells are capable of differentiation to form any of the elements of blood, but overproliferation by these cells can lead to a condition called blood cancer or leukemia (Prasanthi et al., 2010; Li et al., 2012). This case, unregulated and abnormal white blood cells division leads to production of large number of immature blood cells (blasts), leading to impairment of various body functions, resulting in different clinical manifestations often seen in leukemia patients (Jemal et al., 2008; Senyuk et al., 2012). The exact cause of most leukemia types is unknown, as is the hematopoietic abnormality underlying its development (Cheok and Evans, 2006) Various studies from different clinical settings have described various risk factors and causes of leukemia, including congenital genetic disorders, exposure to radiations, chemicals or drugs and virus-associated leukemias (Lewis, 2007). The pathogenesis is often multistep including inherent chromosome instabilities, abnormal DNA repair and altered immune functions (Jefford and Irminger-Finger, 2006).

Acute lymphoblastic leukemia is the most common type of childhood cancer, involving 25% of all leukemic patients and approximately 80% of acute leukemia cases in childhood (Sokol and Loughran, 2006; Urayama et al., 2013). Peak prevalence of ALL is between the ages

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of 2 and 5 years. Patients have white blood cell (WBC) counts less than 50×10^9/L at the time of diagnosis (Voute et al., 2005; Lazarus and Laughlin, 2010). Based on the immunophenotyping, ALL can be classified as T-lineage (10-20% of ALL), B-cell precursor (80-90% of ALL) or mature B-cell disease expressing clonal surface immunoglobulins (2% of ALL). In addition, B-lineage ALL can be subdivided into pro-B, early pre-B and pre-B, but this subdivision is not generally used in the risk group and treatment stratification (Belurkar et al., 2013). ALL subtypes show considerable differences in terms of clinical features, laboratory value, treatment response, relapse sites, and relapse kinetics (Pui et al., 2008; Belurkar et al., 2013).

Pediatric ALL is associated with many genetic abnormalities which have huge clinical implications in prognostic stratification, anti-leukemic drug selection at different treatment phases and follow-up of the treatment (Mangolini et al., 2013; Urayama et al., 2013) A common acquired genetic lesion in ALL patients is TEL-AML1 fusion oncogene, resulted due to t (12; 21) (p13; q22), that have previously been described among chromosomal translocations specific for lymphoid malignancies (Tsuzuki and Seto, 2013). In several studies, the application of molecular and cytogenetic tools such as fluorescent in situ hybridization (FISH), southern blot analysis, karyotyping and RT-PCR have shown TEL-AML1 fusion transcripts in up to 20-30% of childhood ALL, making it the most common molecular cytogenetic abnormality in pediatric ALL (Lewis, 2007; Hong et al., 2008). TEL and AML-I genes involved in this translocation play important role in the pathogenesis of human leukemia (Woerden et al., 2000; Tsuzuki and Seto, 2013). TEL-AML1 translocations may occur by non-homologous recombination involving imprecise, constitutive repair processes following DNA double-strand breaks (Wiemels, 2012).

Advances in understanding of the pathobiology of acute lymphoblastic leukemia have shown that drugs specifically targeting the genetic defects of leukemic cells could revolutionize management of this disease (Wiemels and Greaves, 1999). Significant use of the drug L-asparaginase can benefit the patients with TEL-AML1 acute lymphoid leukemia Iqbal, (2006) and nearly all TEL-AML positive pediatric patients can be cured using intensive treatment protocols containing intensive asparaginase and high-dose methotrexate (Pui et al., 2008; Bhojwani et al., 2013). Furthermore, it is clinically established that, as compared to other genetic abnormalities, TEL-AML1 positive pediatric ALL patients shows excellent prognosis and outcome to standard treatment protocols being used in different regions of the world for treatment of this disease (Bhojwani et al., 2013; Mangolini et al., 2013). Thus, determination of TEL-AML1 fusion oncogene status at diagnosis and its percentage among other genetic abnormalities in pediatric ALL patients in a given population can significantly affect the outcome of treatment and can have a significant bearing on disease management policies related to pediatric ALL in a geographic region.

Although, global frequency of TEL-AML1 positivity has been reported to be 20-30% in pediatric ALL (Hong et al., 2008), some authors have reported very lower frequencies of TEL-AML1in some geographical regions (Kwong and Wong, 1997; Eguchi-Ishimae et al., 1998; Garcia-Sanz et al., 1999; Tsang et al., 2001; Rahman et al., 2006; Chung et al., 2010; Mazloumi et al., 2012) including one study from a single centre at Lahore, Punjab, Pakistan indicating 6% (3/50) frequency of TEL-AML1 (Faiz and Qazi, 2010), while others have reported frequency of this gene to be at other single Centres to be about 16% (Iqbal et al., 2007; Sabir et al., 2012). However, all of these studies were carried out at single centre or locations in Pakistan and therefore does not provide a comprehensive view of TEL-AML1 frequency in Pakistani population.

The primary objective of our study was to find out frequency of TEL-AML1 fusion oncogene in pediatric acute lymphoblastic leukemia patients from different cities of Pakistan. As TEL-AML1 fusion oncogene is difficult to be detected by routine cytogenetic analysis while reverse transcriptase PCR (RT-PCR) can even detect TEL-AML1 in apparently normal karyotype ALL (Fears et al., 1996), a sensitive and clinically validated RT-PCR protocol at many laboratories (van-Dongen et al., 1999; Armstrong and Look, 2005; Iqbal and Tanveer, 2006; Awan et al., 2012; Sabir et al., 2012) was used to study the frequency of this genetic abnormality in our childhood ALL patients.

Materials and Methods

Sample collection

Peripheral blood samples (3-5ml) were collected from 167 clinically diagnosed pediatric ALL patients (age ≤15 years) at diagnosis in EDTA coated tubes (BD Diagnostics, NJ 07417 USA) from Children Hospital Lahore Pakistan, Allied Hospital Faisalabad Pakistan, Pakistan Institute of Medical Sciences (PIMS) Islamabad Pakistan and Institute of Radiotherapy and Nuclear Medicine (IRNUM), Peshawar, Pakistan. Study was approved from ethical and institutional review boards of all institutes. Accordingly, all patients or their parents/guardian signed written informed consent.

Isolation of RNA

Total RNA was isolated using TriZol LS reagent using manufacturer's instructions (Ambion®, Life Technologies) with a little optimization and modifications (Awan et al. 2012). Quality and quantity of RNA was determined spectrophotometrically as well as by running on 1% Agarose gel.

Complementary DNA (cDNA) synthesis

All reagents for CDNA preparation and PCR were purchased from Fermentas (USA). RNA was reverse transcribed to cDNA using reverse transcriptase (RTase) enzyme. RT-reaction protocol and other reaction conditions were opted from Van-Dongen et al. (1999). Briefly, 10μl of RNA was added to 10μl of RT-reaction mixture containing 5x RT buffer, 25mM dNTPs, 10mM Random hexamer primers, RibolockTM RNase inhibitor, M-MuLV Reverse transcriptase and DEPC-treated water. Reaction was carried out by incubating mixture of template, random hexamers and DEPC treated water

at 70˚C for 10min and hold at 4˚C in the last step. The integrity of cDNA was assessed by amplification of housekeeping gene ABL.

RT-PCR amplifications

PCR primers and nested PCR protocols for the detection of TEL-AML1 fusion gene in ALL patients were adopted from (Armstrong and Look, 2005). A 50μl reaction mixture was prepared containing 3μl of cDNA, 1μl each of forward and reverse primers, 5μl 5xPCR buffer with KCL, 5μl $MgCl_2$ (25mM), 0.5μl dNTP Mix (10mM), 34.2μl DEPC treated water, 0.3μl Taq DNA Polymerase. In second round of nested PCR, the product of First round was used as template.

Thermal conditions for PCR

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 at 65°C for 60 sec and extension 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. PCR products were electrophorized on 1.5% agarose gel. To avoid contamination, standard precautions were taken including use of aerosol-resistant tips, dedicated pipettes for pre-PCR, PCR and post-PCR steps and physical separation of pre-PCR, PCR and post-PCR. Appropriate negative and positive controls were included in each amplification experiment.

Statistical analysis

Data was analyzed using Statistical Package for the Social Sciences (SPSS version 19).

Results

Characteristics of childhood ALL patients

Out of 167 pediatric ALL patients, 116 (69.5%) were males and 51 (30.5%) were females, with 2.27:1 male to female ratio. One hundred and forty patients out of 167 (83.8%) were B-ALL and 37 (16.2%) were T-ALL. Mean survival was 32 months (95% CI: 14-42.5) and while 3-years overall survival was 31.7% (53/167) and 3-year relapse free survival was 17.9% (30/167) while 4 patients (2.4%) died of treatment-related toxicities. About 65% patients had WBC count <30000/μl (64.9%) while 35 % had more >30000/μl; about 64% had platelet counts

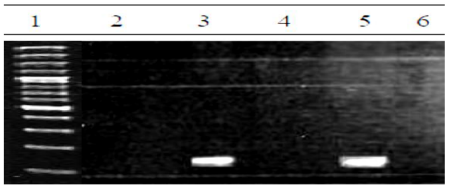

Figure 1. Nested RT-PCR Followed by Agarose Gel Electrophoresis Showing 180bp PCR Fragment Corresponding to TEL-AML Fusion gene in Pediatric acute Lymphoblastic Leukemia Patients (Lane 1; 100bp Plus Ladder, Fermentas, USA, Lane 2; Negative Control, Lane 3; Positive Control Showing 180bp PCR Product Corresponding to TEL-AML1, Lanes 4-6: Pediatric ALL Patients)

Table 1. Demographic Data, Clinical Characteristics and Treatment Outcome of TEL-AML1 Positive Pediatric ALL Patients from Four Major Oncology Centers in Pakistan

Clinical characteristics		TEL-AML No (%)
Total number of patients		17 (10.2)
Gender	Male	11 (64.7)
	Female	6 (35.3)
Age Median (Range)		3.85 (2.3-14.5)
Immunophenotyping	B-ALL	17 (100)
	T-ALL	0 (0)
WBC	<30×10^9/l	14 (82.4)
	>30×10^9/l	3 (17.6)
Hepatomegaly	No	13 (76.5)
	Yes	4 (23.5)
Spleenomegaly	No	13 (76.5)
	Yes	4 (23.5)
Platelets	>50×10^9/l	12 (70.6)
	<50×10^9/l	5 (29.4)
CR	<4weeks (early)	14 (82.4)
	>4weeks (late)	3 (17.6)
OS (months)		32.2
RFS (months)		17.5

*WBC: White Blood Cells; CR: Complete Remission; OS; Overall Mean Survival; RFS; Relapse Free Survival

>50000/μl. Twenty two (13.2%) patients had a mediastinal mass, 50 (about 30%) had splenomegaly, and 71 (42.5%) patients had hepatomegaly. CNS disease, as confirmed by spinal cytology, was found in 11 (6.6%) patients.

Detection of TEL-AML1 fusion oncogene

Patient samples were analyzed by nested RT-PCR. A specific PCR-amplified band of 180 bp corresponding to TEL-AML was seen after gel electrophoresis (Figure 1). TEL-AML1 detected in only 17 out of 167, thus indicating an overall 10.2% frequency of this genetic abnormality in Pakistani pediatric ALL patient population.

Characteristics of TEL-AML1+ALL patients

Out of 17 patients, 11 were male and 6 were females. Median age was 3.85 years. All patient presented with B-cell ALL. Most of the patients had WBC count less than 30×10^9/l (82.4%), platelet count more than 50×10^9/l (70.6%) and no hepatomegaly/spleenomegaly (76.5%), all indicating favorable prognosis. Accordingly, 82.4% of the TEL-AML1 positive patients achieved remission within 4 weeks of treatment. Overall survival of TEL-AML1+ patients was 32.2 months and relapse free survival was 17.5 months (Table 1). Only one (5.9%) patient died due to treatment-related toxicities. Thus, TEL-AML1 positive patients showed favorable prognosis and good treatment response, although their overall frequency among pediatric ALL patients was very low.

Discussion

TEL-AML1 t (12; 21) (p13; q22) fusion gene, resulting from 12; 21 chromosomal translocation, is believed to be the most common molecular genetic abnormality in childhood acute lymphoblastic leukemia (ALL).

This translocation is difficult to detect by conventional cytogenetic analysis. Multicolor FISH can be used to reveal complex rearrangement that can lead to fusion gene. The case detection rate is higher with RT-PCR as compared to any other molecular assay, (Bain, 2010). Molecular diagnosis of t (12; 21)-positive ALL may identify a subgroup of patients which do not require intensive treatment for cure. TEL-AML1 fusion is reported to be 20-25% in childhood ALL (Shaker et al., 2001; Zelent et al., 2004; Coppola, 2010). Majority of patients display a precursor B-cell immunophenotype, low WBC count, and good response to treatment (Zaza et al., 2004; Riccio et al., 2010). However, most of the comprehensive studies related to TEL-AML1 frequency are from Europe and US (Shaker et al., 2001; Zelent et al., 2004; Coppola, 2010). Due to its prognostic significance and implication in drug selection, a detailed account of TEL-AML1 frequency among childhood ALL is required for proper treatment strategies in a given population.

In the present study, frequency of oncogene under consideration was studied using nested RT-PCR using the clinical specimens from four major oncology treatment centers in the country. Seventeen out of 167 (10.2%) samples were found to be positive for TEL-AML1, which is lower than the frequencies reported from developed countries (Zelent et al., 2004). Previously, the prevalence of TEL-AML1 fusion oncogene in Pakistani population has been reported by to be 11% (Iqbal and Tanveer, 2006) which is in accordance with our findings, although that study lacked clinical outcome of TEL-AML1 positive patients. In another study from Lahore, Punjab, Pakistan, 6% (3/50) frequency of TEL-AML1 has been reported (Faiz and Qazi., 2010), while others have reported frequency of this gene to be to be about 16% (Iqbal et al., 2007; Awan et al., 2012). However, all of these studies were carried out at single centre or locations in Pakistan and does not represent a major subset of childhood ALL population of the country. Our finding support the findings of Mesquita et al. (2009) that TEL-AML1 has low incidence in developing countries which may be associated with poor living standards in these countries. This observation is further strengthened by the observation that neighboring countries of Pakistan with more or less similar socio-economic and environmental factors, like India, Iran and China show somewhat parallel incidence of TEL-AML1 oncogene among acute lymphoblastic leukemia patients. In India, TEL-AML1 was detected in 5-7% of pediatric ALL patients (Rahman et al., 2006; Mazloumi et al., 2012). Likewise, Rahman et al. (2006) reported 3% adults and 7% pediatric ALL presented with TEL-AML1 gene at diagnosis. However, in two different studies in China, frequency of this fusion oncogene was detected to be 3.3% (Tsang et al., 2006), and 17.9% (Chung et al., 2010), an observation which further supports geographic differences in pediatric ALL genetics, as China is a large country with a lot of geographical variations.

Different frequencies of TEL-AML1 have been reported in pediatric ALL patients in other countries of South East Asia and Southern Asia. A 17.1% TEL-AML1 frequency among ALL Korean children has been reported (Chung et al., 2010). Similarly a studies on ALL population in Taiwan, screened for the TEL-AML1 rearrangement by reverse transcription-polymerase chain reaction (RT-PCR), revealed 17% positive cases (Liang et al., 1996), as compared to 19% in 88 Malaysian pediatric ALL patients (Gill et al., 2005). In Egyptian population, 11.6% frequency of TEL-AML1 transcript was reported in newly diagnosed precursor B-ALL cases of acute lymphoblastic leukemia (Shaker et al., 2001). Frequency of TEL-AML in pediatric ALL patients from Hiroshima Japan was reported to be 10% (Eguchi-Ishimae et al., 1998; Takahashi et al., 1998), while it was reported to be 19% in Nagoya, Japan (Takahashi et al., 1998). Nevertheless, a higher frequency of TEL-AML1 has been reported in pediatric ALL patients in many of the European countries. In United States this is estimated to be 17-25% among pediatric patients (Loh et al., 1998; Jamil et al., 2000). The incidence of TEL-AML1 fusion in 334 Italian and German children with ALL was 18.9% (Borkhardt et al., 1997) and 22-27% in German children alone (Papadhimitriou et al., 2008), as compared to 22.5% in 617 children from UK (Harrison, 2000). Similarly the prevalence of TEL-AML1 transcript in acute lymphoblastic leukemia patients in Serbia is 17.1% (Lazic et al., 2010) and 20% in Brazil (Magalhaes et al., 2000). Frequency of TEL-AML1 in Greek pediatric patients seems comparable to that in other European countries, found as 24.3% in 120 ALL children (Papadhimitriouet al., 2008). Unlike most of European countries, the frequency of TEL-AML1 among pediatric ALL in Spain was reported to be 2% (Garcia-Sanz et al., 1999). All of these observations indicate geographic differences in frequency of this fusion oncogene with prognostic value. It will be very interesting to explore the racial, ethnic and geographical reasons behind variations in genetic epidemiology of TEL-AML1 fusion oncogene in different parts of the word. Interplay of genetic elements, environmental factors and life-style of the people living in these geographic locations may be involved in this interesting phenomenon which is needed to be explored using advanced cellular and molecular biological techniques in larger patient populations.

Conclusively, results of the present study are in agreement with the previously reports of TEL-AML1 frequencies from less developed countries. Although our childhood ALL patients with TEL-AML1 fusion oncogene showed good prognosis and treatment outcome, their overall low frequency may be one of the important factor in overall poor response of pediatric ALL in Pakistan. Further large scale studies are required to figure out the reasons for low frequency of TEL-AML1 in our pediatric ALL patients.

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