Original Article

Molecular Detection of Fusion Oncogenes in Zambian Patients with Acute Lymphoblastic Leukemia

Pauline Okuku[1,2], Geoffrey Kwenda[3], Mulemba Samutela[3], Panji Nkhoma[3], Hamakwa Mantina[2]

[1]Department of Pathology and Microbiology, School of Medicine, University of Zambia,
[3]Department of Biomedical Sciences, School of Health Sciences, University of Zambia,
[2]Department of Pathology and Microbiology, University Teaching Hospital, Lusaka, Zambia

Abstract

Introduction: Chromosomal aberrations play a significant role in the pathogenesis of acute lymphoblastic leukemia (ALL) with prognostic and therapeutic implications. Despite the availability of molecular tools, low-resource settings struggle to diagnose the disease due to limited diagnostic capacity. The objective of this study was to detect common chromosomal aberrations in patients with ALL attending the University Teaching Hospital (UTH) in Lusaka, Zambia. **Materials and Methods:** In this prospective study, 19 blood samples from patients with ALL were screened for the presence of BCR-ABL, E2A-PBX1, MLL-AF4, and ETV6-RUNX1 fusion oncogenes using reverse transcriptase-polymerase chain reaction assay. Blood counts and clinical characteristics of patients were also assessed. **Results:** The age of patients ranged from 1½ to 72 years and comprised 57.9% of males and 42.1% of females. The majority of these patients were children (68%), and adults only comprised 32%. Only BCR-ABL and E2A-PBX1 oncogenes were detected in 3/19 of cases. The BCR-ABL gene was detected in a 4-year-old female child and a 15-year-old child. Both cases were associated with hepatomegaly and anemia coupled with low hemoglobin, white blood cell, and platelet counts. E2A-PBX1 was detected in a 12-year-old child with lymphadenopathy and splenomegaly, coupled with low hemoglobin, white blood cell, and platelet counts. All the three patients who harbored these fusion oncogenes died. **Conclusion:** This is the first study from Zambia to investigate the presence of fusion oncogenes in leukemia patients, which were found only among the older children population. Based on these findings, we recommend that molecular diagnosis be made a priority for the younger leukemia patient population at UTH.

Keywords: *Acute lymphoblastic leukemia, fusion oncogenes, reverse transcriptase-polymerase chain reaction, Zambia*

Introduction

Acute lymphoblastic leukemia (ALL) is a malignant disease most common in children and accounts for about 80% of all pediatric leukemia cases.[1] It is less common in adults and represents about 12% of all leukemia cases in this group.[2,3] It is characterized by an accumulation of malignant immature lymphoid cells in the bone marrow.[4] Genetic studies have identified a number of aneuploidies and chromosomal aberrations associated with ALL.[5,6] These genetic abnormalities play a critical role in the prognosis and treatment of the disease.[6,7]

Previous studies have shown that ALL is a heterogeneous disease with different clinical and genetic abnormalities in different ethnic groups.[8,9] This suggests that the distribution of genetic lesions of ALL may not be the same among different ethnic groups. It is, therefore, important to understand the biology of the disease, including the molecular mechanisms involved, in order to provide appropriate treatment to affected populations. In limited-resource countries such as Zambia, the management of ALL proves to be a challenge because of limited diagnostic and treatment capacity. For diagnosis of ALL, the World Health Organization recommends the use of molecular methods for the detection of genetic lesions.[10] At the University Teaching Hospital (UTH) in Lusaka, Zambia, the current diagnostic approach for leukemia involves only clinical and cell morphological assessments. The use of this approach implies that most of the cases are not correctly diagnosed, thereby rendering physicians to manage patients inappropriately. This study was, therefore, aimed at detecting common chromosomal aberrations in ALL patients attending the UTH.

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Address for correspondence:
Mrs. Pauline Okuku
Department of Pathology and Microbiology, School of Medicine, University of Zambia, P. O. Box: 50110, Lusaka 15101, Zambia.
E-mail: aniekanokuku@gmail.com

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Materials and Methods

Patients and samples

This was a prospective study in which 19 blood samples were obtained from both male and female patients with ALL being managed at the Onco-Haematology Clinic of the UTH in Lusaka, Zambia, for diagnostic purposes. These samples were received in the hospital Haematology Laboratory between July 2015 and April 2016 as part of the routine laboratory analyses. Four milliliters of blood was collected from each patient in an EDTA vacutainer through venepuncture. Patients were excluded if they had acute myeloid leukemia or severe systemic illness. Data on demographics, medical history, clinical manifestations, and symptoms for each patient were abstracted from medical records at the hospital. Physicians performed all the physical examinations on the patients.

Full blood count analysis and peripheral blood examination

Full blood counts were performed on the Sysmex XT 4000i Hematology Analyzer (Sysmex Corporation, Japan). For preparation of blood smears, a drop of blood was placed on one end of a clean glass slide then a smear of a single layer of cells was made using a glass spreader held at a 45 degrees angle. The blood film was then air-dried and labeled on the frosted end of the slide. The film was fixed in absolute methanol for 5 min and then transferred into a jar of May-Grunwald stain (Merck, California, USA) freshly diluted with an equal volume of 0.1M Söresen's phosphate buffer (pH 7.2–7.4). The film was then allowed to stain for 15 min and then transferred into a jar of Giemsa's stain (Merck, California, USA) freshly diluted with 4 volumes of the Söresen's phosphate buffer for 15 min. The slide was then transferred into a jar of fresh buffer for 5 min and allowed to air-dry. The film was finally examined under a microscope for malignant cells by a qualified hematologist as a means of determining the ALL phenotype.

Detection of chromosomal aberrations

RNA extraction

Total RNA was extracted from whole blood using a QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Synthesis of complementary DNA and polymerase chain reaction amplification

Reverse transcription and polymerase chain reaction (PCR) amplification were performed in the same tube using the AgPath-ID One-Step RT-PCR Kit (Applied Biosystems, Foster City, CA, USA) which simultaneously converts RNA into complementary DNA and performs DNA amplification. Briefly, 2 µl of RNA was added to 23 µl of PCR master mix containing 12.5 µl 2X RT-PCR buffer (Applied Biosystems, Foster City, CA, USA), 1 µl 25X PCR Enzyme Mix (Applied Biosystems, Foster City, CA, USA), 4.5 µl water, and 2.5 µl of 5 µM of each pair of the primers targeting one of the four fusion ALL genes: E2A-PBX1, MLL-AF4, BCR-ABL (p190), or TEL-AML1 [Table 1]. The reverse transcriptase reaction was carried out at 45°C for 10 min, followed by inactivation and initiation denaturation at 95°C for 10 min. For DNA amplification, the reaction tube was first heated to 95°C for 10 min and then subjected to 40 cycles of amplification at 94°C for 30 s, 68°C for 30 s, and 72°C for 1 min. This was finally followed by a 10-min extension step at 72°C and holding at 4°C on a GeneAmp 2700 PCR thermocycler (Applied Biosystems, Foster City, CA, USA). The presence of amplified products was detected by electrophoresis of 5 ml of the amplicon on a 1.5% SeaKem LE agarose gel (Lonza, Rockland, ME, USA). After electrophoresis, the gels were imaged on a Biotop SC-645 Gel Documentation System (Biotech Co. Ltd., Shanghai, China). The expected gene fusion product sizes are shown in Table 1.

Ethics approval

All specimens were de-identified and given study-specific identification codes. Informed consent was obtained in a local language from a parent or legal guardian of the children and adult patients. Permission to conduct the study was sought from the UTH Management in Lusaka, Zambia, while ethics clearance was obtained from the University of Zambia Biomedical Research Ethics Committee (Clearance Certificate Number 005-06-15).

Results

Patient characteristics

Out of the 19 cases enrolled in the study, 57.9% (11/19) were male, while 42.1% (8/19) were female [Table 2]. The male-to-female ratio was 1.4:1. The age for the patients was from 1½ to 72 years, with a median age of 14. The majority of patients were children (68%, 13/19) and adults comprised only 32% (6/19). Children were defined as patients aged 15 years and below. Males among the children comprised 46% (6/13), while females were 54% (7/13). In the adult category, 83% (5/6) were male, while only one was a female.

The main clinical features identified among the patients were fever, hepatomegaly, lymphadenopathy, splenomegaly, and anemia. The proportion of patients with fever was 36.8% (7/19), the majority of which were children (85.7%, 6/7) and only one adult (14.3%, 1/7). Hepatomegaly was seen in 26.3% (5/19) of the patients, all of which were children. Lymphadenopathy occurred in 15.8% (3/19) of the patients, and this was only seen in children. Splenomegaly was seen in 10.5% (2/19) of the patients: one female child and one male adult. Anemia occurred in 52.6% (10/19) of the patients, and 60% (6/10) of which were children. The remaining 40% (4/10) were adults [Table 2]. They

Table 1: Fusion oncogene targets and primers used in the multiplex polymerase chain reaction assays

Fusion transcript	Fusion Gene	Primer name	Primer sequence	Size (bp)
t(1;9}(q23;p13)	t(1;9}(q23;p13)	E21A-A	5'-CACCAGCCTCATGCACAAC-3'	373,400,504
		PBX-B	5'-TCGCAGGAGATTCATCACG-3'	
t(4,11)(q21;q23)	MLL-AF4	MLL-A	5'-CCGCCTCAGCCACCTAC-3'	184,353,427
		AF4-B	5'-TGTCACTGAGCTGAAGGTCG-3'	
t(9;22)(q34;q11)	BCR-ABL(p190)	BCR-e1-A	5'-GACTGCAGCTCCAATGAAC-3	347,413,521
		ABL-a3-B	5'-GTTTGGGCTTCACACCATTCC-3'	
t(12;21)(p13;q22)	TEL-AML1	TEL-A	5'-TGCACCCTCTGATCCTGAAC-3'	259,298
		AML1-B	5'-AACGCCTCGCTCATCTTGC-3'	

Table 2: Correlation of clinical characteristics of patients to genetic alterations

Clinical parameter	Frequency, *n* (%)	Fusion oncogene	
		TCF3/PBX1, *n* (%)	BCR/ABL, *n* (%)
Sex			
Male	57.9 (11/19)	9.1 (1/11)	9.1 (1/11)
Female	42.1 (8/19)	-	12.5 (1/8)
Age			
≤5	31.6 (6/19)	-	-
6-15	36.8 (7/19)	-	14.3 (1/7)
>15	31.6 (6/19)	16.7 (1/6)	16.7 (1/6)
Fever	36.8 (7/19)	14.3 (1/7)	14.3 (1/7)
Hepatomegaly	26.3 (5/19)	20 (1/5)	
Lymphadenopathy	15.8 (3/19)	-	-
Splenomegaly	10.5 (2/19)	-	-
Anemia	52.6 (10/19)	-	10 (1/10)

all presented with normocytic normochromic anemia. The majority of the participants presented with high white blood cell counts, low levels of hemoglobin, and low platelet counts. White blood cell counts ranged from 0.24 to 657 × 10^9/l (mean value, 131 × 10^9/l) and platelet counts ranged from 7 to 289 × 10^9/l (mean value, 99.6 × 10^9/l), while that of hemoglobin levels ranged from 2.2 to 13 g/dl (mean value, 6.4 g/dl). Leukocytosis was detected in 63.2% (12/19) of the patients, while the proportion with leukopenia and pancytopenia and thrombocytopenia was 26.3% (5/19) and 68.4% (13/19), respectively [Figure 1].

Detection of fusion oncogenes

Only two fusion oncogenes, BCR-ABL (p190) and E2A-PBX1, were detected in three different patients (15.8%, 3/19) by the reverse transcriptase-PCR [Figure 2]. BCR-ABL was detected in two of the 19 (10.5%) patients: one in a 4-year-old female child, while the other one in another child male child aged 15 years who had fever, hepatomegaly, and anemia with low hemoglobin level (4.5 g/dl), white blood cell count (1.73 × 10^9/l), and platelet (13 × 10^9/l) count. E2A-PBX1 was detected only in one 12-year male child (5.3%) with fever and hepatomegaly and also low hemoglobin (6.9 g/dl), white blood cell (0.24 × 10^9/l), and platelet (53 × 10^9/l) counts. Lymphadenopathy and splenomegaly occurred only in patients who did not have detectable ALL fusion oncogenes [Table 2]. All the patients who harbored these fusion oncogenes died.

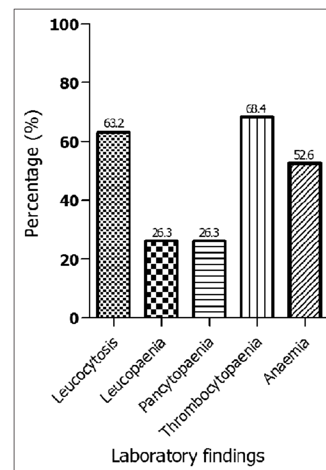

Figure 1: Laboratory findings of suspected acute lymphoblastic leukemia cases at diagnosis

Discussion

Molecular genetic abnormalities and the cytogenetic features of leukemic cells are highly prognostic of treatment outcome, permitting more precise risk assessment of this disease in order to avoid overtreatment or undertreatment of individual patients.[11]

This study showed that there were more male than female patients, and this was in line with studies from other countries. A study conducted in southern India showed

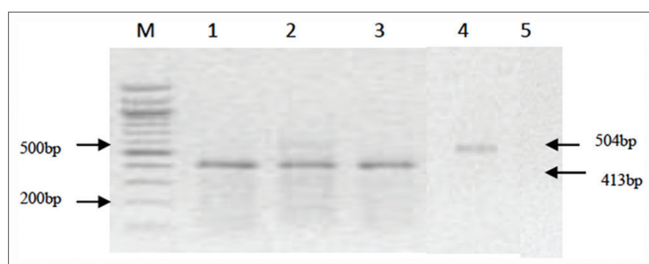

Figure 2: Detection of fusion genes; A representative gel electrophoresis showing samples positive for gene fusion. M, 100 bp marker; 1, positive control; 2 and 3, samples positive for BCR/ABL (p190) gene fusion; 4, sample positive for E2A/PBX1 fusion gene and 5, negative control

a male-to-female ratio of 1.6:1, while a similar study conducted in China reported a 1.84:1 male-to-female ratio.[12,13] The reason for the observed male preponderance of the ALL cases is unclear. Most of the cases were associated with children, and this finding is consistent with findings in other countries. Similar studies conducted in the United States and India demonstrated that the incidence of ALL was higher in children and adolescents under 15 years of age than in individuals above this age.[14,15]

Fever, anemia, and hepatomegaly were the predominant clinical manifestations at presentation. Other manifestations presented included lymphadenopathy and splenomegaly, but these were not associated with cases. Laboratory findings also showed high levels of leukemic cells which resulted in most of the patients having high white blood cell count, low hemoglobin level, and low platelet count. A study conducted in Brazil showed that hepatomegaly, splenomegaly, fever, and lymphadenopathy were the most frequent clinical features among ALL patients.[16] A similar study carried out in India showed that hepatosplenomegaly and lymphadenopathy were ranked higher than other clinical features.[17]

Molecular genetic abnormalities and the cytogenetic features of leukemic cells are highly prognostic of treatment outcome, permitting more precise risk assessment of individual patients.[18] Our results show that two of the children harbored the BCR-ABL1 fusion oncogene (t9;22). Previous studies have shown that the most frequent chromosomal translocation in older children and adults is t (9;22).[19,20] A study conducted in Guatemala showed that the incidence of BCR-ABL transcript was high, accounting for 7% of the cases among older children aged between 5 and 14 years.[21] The explanation for the association between this gene fusion and this age group could be as a result of the fact that the incidence of the BCR-ABL gene transcript increases with age from approximately 3% of children with ALL up to approximately 25% in adult ALL cases.[22]

The only other fusion oncogene detected was E2A-PBX1, and this was detected a 12-year-old child. Similar studies conducted in Guatemala and Mexico showed a low prevalence of this oncogene.[21,23,24] However, in our study, we cannot arrive at this conclusion as our sample size was relatively small compared with other studies. The transcript resulting from this gene fusion leads to the expression of chimeric transcription factors that block differentiation and apoptosis of cells by interfering with the function of their wild-type counterparts.[25]

Both BCR-ABL1 and E2A-PBX1 fusion transcripts have been found to be associated with poor prognoses at all ages.[26-28] ALL patients with these fusion gene transcripts often present with aggressive leukemia that is resistant to standard therapies resulting in high relapse rates.[29] It is worth noting that in our study, the patients who were positive for these fusion genes died in the course of the study. However, many factors could have contributed to their death, one of which could be attributed to the poor outcomes associated with these gene fusions.

Other fusion oncogenes, MLL-AF4 and ETV6-RUNX1, were not detectable in our patients. This could be attributed to the fact that MLL-AF4 is more prevalent among infants <6 months of age,[30] but our study had patients older than this age. It has been documented that ETV6/RUNX1 is the most common genetic lesion in childhood ALL,[31] but we did not detect this oncogene in our study. The probable explanation for this is that our sample size was small. However, patients harboring this oncogene have been shown to respond favorably to treatment.[32]

Conclusion

This is the first study from Zambia to investigate the presence of leukemia fusion oncogenes among ALL patients and provides some baseline data for the country. Studies from other countries have indicated that molecular evaluation of ALL at diagnosis and its integration with clinical characteristics can improve patient outcome through the intensification of treatment protocols.[33,34] More comprehensive and large-scale studies using molecular tools such as real-time PCR and whole-genome sequencing are needed to detect more cases of ALL or other leukemia cases in order to enhance the identification of genetic lesions and contribute to the improved management of affected patients.

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Conflicts of interest

There are no conflicts of interest.

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