Open Journal of Hematology

ROSS Science Publishers

Research Article

OPEN ACCESS

Molecular genetics and Cytogenetics data of 317 patients with de novo acute leukemia

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Published: 4th May, 2015 Accepted: 4th May, 2015
Received: 19th February, 2015

Open Journal of Hematology, 2015, **6**-3

ABSTRACT

Background/Aims: *Cytogenetic and molecular genetics play a pivotal role in treatment of acute leukemias. We prospectively evaluated genetic alterations in Brazilian patients with acute myeloid leukemia (AML) or acute lymphoblastic leukemia (ALL) and their association with clinical and laboratorial data.*

Methods: *Flow cytometry, conventional cytogenetics (CC), FISH, PCR, RT-PCR and sequencing were performed on samples from 161 de novo ALL and 155 AML.*

Results: *Main CC findings in AML were t(15;17) (19.4%), +8 (17.4%), complex karyotype (14.6%), t(8;21) (7.6%); in ALL main CC findings were high hyperdiploidy (18.7%), low hyperdiploidy (9.7%), t(1;19) (9.7%), t(9;22) (8.2%). Frequencies of gene fusions and mutations in AML were PML-RARa 21.9%, RUNX1-RUNX1T1 7.1%, CBFB-MYH11 and MLL-AF9 2.6%, FLT3-ITD 14.2%, NPM1mut 13.6%. In ALL, ETV6-RUNX1 and BCR-ABL were present in 11.5% of the cases, TCF3-PBX1 in 10.8% and MLL-AF1 in 1.5%. Results were discordant between CC and RT-PCR in 3.6% of the cases. PML-RARa was associated with younger age, lower WBC and platelet; FLT3-ITD with higher hemoglobin and WBC; NPM1mut with higher platelet and WBC, older age and normal karyotype. BCR-ABL was associated with higher age; MLL-AF1 with higher WBC and EGIL BI-subtype.*

Conclusions: *The incidence of some aberrations in AML differed from international literature. Discrepancies found between methodologies reinforce the importance of both CC and PCR in the diagnosis of leukemias.*

INTRODUCTION

The precise diagnosis of acute leukemia depends on clinical, morphological, immunophenotypic, cytogenetic and molecular information. In the past few decades, it has become increasingly clear that genetic tests provide essential data, not only for diagnosis confirmation, but also for treatment planning. The detection of specific aberrations, such as t(9;22)(q34;q11.2) or t(15;17)(q24;q21), is used to assign patients to specific drug therapy [1, 2]. In addition, some translocations and mutations are clearly associated with high risk of relapse and are therefore important to define patients in need of transplant or more therapeutically aggressive regimens. Indeed, the present World Health Organization (WHO) leukemia classification system is largely based upon genetically defined subgroups [3, 4, 5].

Acute leukemic cells may carry a variety of genetic alterations, which can be detected using Conventional Cytogenetics (CC) and molecular genetic techniques. CC provides a complete spectrum of chromosomal abnormalities and is mandatory for prognosis evaluation. It presents, however, pitfalls: it misses submicroscopic mutations and cell culture is unsuccessful in about 10 to 30% of Lymphoblastic Acute leukemias (ALL) [6] and in 5% of Myeloid Acute leukemias (AML). Molecular methods, on the other hand, are more sensitive and apply to the majority of patients while allowing the detection of point and length mutations. However, they target defined alterations only. As a result, cytogenetics and polymerase chain reaction (PCR) complement each other when diagnosing and managing acute leukemias.

The paucity of published data on large groups of patients from Latin America prompted us to investigate, in this study, cytogenetic and molecular aberrations present in Brazilian patients with the diagnosis of de novo acute leukemia. Here we report our findings on the detection of the main chromosomal abnormalities and genetic mutations with prognostic impact, and their association with clinical and laboratorial data. We also discuss the usefulness of each detection method in the light of the newest hematological disorders classification system.

MATERIALS METHODOLOGY

Patients

Patients suspected of having de novo acute leukemia were referred to seven treatment centers from February 2011 to September 2013: Serviço de Oncologia from Hospital Santa Casa de Misericórdia de Belo Horizonte (Belo Horizonte - MG, Brazil); Serviço de Hematologia e Transplante de Medula Óssea from Hospital Universitário da Universidade Federal de Juiz de Fora (Juiz de Fora - MG, Brazil); Departamento de Hematologia Pediátrica from Hospital Pequeno Príncipe (Curitiba – PR, Brazil); Departamento de Pediatria from Hospital Erasto Gaertner (Curitiba – PR, Brazil); Departamento de Hematologia from Hospital São João de Deus (Divinópolis – MG, Brazil); Serviço de Hematologia Adulta from Hospital de Clínicas da Universidade Federal do Triângulo Mineiro (Uberaba – MG, Brazil); and Centro de Oncologia Dr. Oswaldo Leite from Hospital de Urgência de Sergipe Governador João Alves Filho (Aracaju – SE, Brazil)]. One hospital canceled its participation after six months because of logistic difficulties. The Ethic Committees of each institution approved the study. Patients or guardians gave their written informed consent to participate in the research. Exclusion criteria were previous occurrence of other malignancy or insufficient bone marrow material collected (less than 3ml). Diagnosis of acute leukemia was based on the WHO classification system [2, 3, 4]. EGIL classification was also defined for ALL patients [7]. French-American-British (FAB) classification [8] was hampered by lack of central morphology revision, but was defined whenever possible in AML. Cytogenetics and molecular tests were conducted in a central laboratory (BIOCOD-Hermes Pardini).

Immunophenotype analysis

Flow cytometry was performed on bone marrow (BM) and peripheral blood (PB) samples using commercially available reagents (Becton-Dickinson Biosciences) following recommendations by Davis et al. [9] and Craig & Foon [10]. The panel of monoclonal antibodies used to determine leukemia lineage consisted of: CD45, CD34, HLA-DR, CD38, TdT, CD10, CD19, CD22, IgM, CD79a, CD2, CD3, CD4, CD7, CD8, CD13, CD14, CD15, CD33, CD64, CD117, MPO, CD16 and CD56.

Conventional cytogenetics

G banding karyotype was performed on short-term BM non-stimulated cultures as described in Czepulkowski [11]. Two protocols were carried out simultaneously, one with RPMI 1640 (Gibco) and another with Marrowmax (Gibco). In most cases, 20 metaphases were analyzed. Results were described using the International System for Human Cytogenetics Nomenclature [12]. Cytogenetic risk group classification of AML was based on the refined Medical Research Council proposal [13, 14]. For ALL, cytogenetic high risk group was defined by the presence of t(4;11)(q21;q23) or t(9;22))(q34;q11), hypodiploid <45 chromosomes or near tetraploid; favorable risk was defined by high hyperdiploid karyotype (>50).

FISH

Fluorescent in situ hybridization (FISH) was performed on interphase nuclei of BM aspirates in cases in which RT-PCR and cytogenetic results were discordant, and in cases carrying an 11q23 translocation. Dual-color dual-fusion commercially available probes were used for the t(15;17)(q24;q21) and t(9;22)(q34;q11.2) (Cytocell) and t(1;19)(q23;p13.3) (Abbott Molecular/Vysis). For rearrangements involving CBFB or MLL gene, break apart probe kits were used (Abbott Molecular/Vysis). Protocol was carried out according to the manufacturers' instructions; 200 interphases were analyzed in each case.

Molecular Biology

For molecular biology analysis, BM and PB were collected in PAXgene™ tubes (QIAGEN/BD, Valencia, CA, USA). RNA was extracted using PAXgene Bone Marrow RNA Kit (QIAGEN/BD)™ and PAXgene Blood RNA Kit (QIAGEN/BD)™, DNA was extracted with phenol-chloroform from PAXgene pellet. The concentration and the quality of nucleic acids were assessed using Nanovue™ (GE) spectrophotometer. 0.5 µg of BM RNA was reverse transcribed into cDNA with Improm II® Reverse Transcription System (Promega). cDNA quality was confirmed with a multiplex RT-PCR amplifying four genes (ABL, BCR, PBGD and B2M) according to Watzinger & Lion [15]. Most frequent translocations/inversions in each leukemia type were tested using single RT-PCR, no nested reactions were performed.

For myeloid leukemias, transcripts were detected as described by van Dongen et al. [16] for PML-RARa t(15;17), RUNX1-RUNX1T1 t(8;21) and CBFB-MYH11 type A inv(16) and as described by Mitterbauer et al. [17] for MLL-AF9 t(9;11). FLT3 internal tandem duplication (ITD) mutations were evaluated according to Kiyoi et al. [18] and Meshinchi et al. [19]. Mutations in NPM1 exon 12 were assessed through direct sequencing [20]. For lymphoblastic leukemias, BCR-ABL t(9;22), TCF3-PBX1 t(1;19), ETV6-RUNX1 t(12;21), MLL-AF1 t(4;11) were studied [16].

Statistical analyses

Differences in the distribution of categorical variables between subsets of patients according to each genetic alteration detected using molecular biology were assessed using Qui-square and Fisher exact tests. Mann-Whitney test was used for the continuous variables. The following characteristics observed at diagnosis were tested: sex, age (as both continuous variable and group category), white blood cell count (WBC), hemoglobin (Hb) concentration, platelet count, FAB cell morphology in AML and EGIL subtype in ALL. NPM1mut and FLT3-IDT mutations were also tested for cytogenetic status (normal versus aberrant). The significance level adopted was $p<0.05$. Analyses were performed with R software (version 3.0.1).

RESULTS AND OBSERVATIONS

Patients

From a total of 374 patients prospectively enrolled in the study, 155 (41.4%) were classified as AML, 161 (43.1%) as ALL, and one (0.3%) had a mixed phenotype acute leukemia; the remaining presented other conditions. From the 317 acute leukemia patients, 153 were adults and 164 were children or adolescents. Only four children were infants (two AML and two ALL). The distribution of the acute leukemia patients per age group and cell lineage is described in table 1. Demographic and blood count data at diagnosis are shown in table 2.

Cytogenetics

Cell culture for cytogenetics was performed for 158 (98.1%) ALL patients and 153 (98.7%) AML patients, with a karyotype success rate of 84.8%

(134/158) and 94.1% (144/153), respectively. The frequencies of the cytogenetic findings that have prognostic significance in AML are shown in table 3.

Table 1: Distribution of 317 acute leukemia patients according to cell lineage and age

Lineage	*Adults (>18 years)*	*Children / adolescents (<18 years)*	*Total*
AML	117 (75.5%)	38 (24.5%)	155
ALL	36 (22.4%)	125 (77.6%)	161
B-cell precursor	27	100	127 (78.9%)
Burkitt-cell leukemia	2	2	4 (2.5%)
T-cell precursor	7	22	29 (18.0%)
B/T-cell precursor[a]	0	1	1 (0.6%)
Mixed phenotype	0	1	1
Total	153 (48.3%)	164 (51.7%)	317

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia

[a] Lineage could not be defined

Based on the usual cytogenetic risk group classification [13, 14], 44 (30.5%) of AML patients were considered to have favorable karyotype, 75 (52.1%) intermediate and 25 (17.4%) unfavorable. Median age of AML patients with unfavorable CC prognosis was 52.4 years, while those with favorable karyotypes had a median age of 24.6 (p 0.018).

The main cytogenetic findings in ALL patients are described in table 4. Favorable cytogenetic risk group consisted of 23 (17.2%) patients and unfavorable consisted of 16 (11.9%). Median age of ALL patients with unfavorable CC prognosis was 20.1 years, while favorable karyotypes had a median age of 4.1 (p 0.001).

Molecular Biology

PCR and RT-PCR were performed on 153 bone marrow samples of AML patients and 2 peripheral blood samples because bone marrow was not available. For ALL, BM from 157 patients were tested. In one case, there was only PB available and in the remaining, there was not sufficient RNA for the analyses.

Table 2: Patient demographics and blood count data for AML and ALL

Variable	*ALL*			*AML*		
	Total	< 18y	≥ 18y	Total	< 18y	≥ 18y
Sex						
Female	85 (52.8%)	65 (52.0%)	20 (55.5%)	65 (41.9%)	11 (28.9%)	54 (46.2%)
Male	76 (47.2%)	60 (48.0%)	16 (44.5%)	90 (58.1%)	27 (71.1%)	63 (53.8%)
Age (years)[a]	10.4 (0.6-64.2)	6.5 (0.6-17.7)	32.4 (18-64.2)	40.9 (0.4-91.2)	10.2 (0.4-17)	50.6 (18.5-91.2)
WBC x 10^9/L[a]	8.6 (0.36-690)	9.9 (0.4-690)	6.3 (0.9-237)	15 (0.5-268)	10 (0.9-268)	17 (0.5-229)
Platelets x 10^9/L[a]	35 (2.0 – 374)	39 (2-351)	30 (14-374)	43.5 (1-520)	46 (4-213)	43.5 (1-520)
Hb g/dL[a]	8.0 (3.0-14.0)	8.2 (3.2-13.9)	7.9 (3-14)	8.0 (2.6-13.4)	7.7 (2.6-12.3)	8.0 (3-13.4)

[a] median values (range)

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; WBC, white blood cells count; Hb, hemoglobin concentration; < 18y, children or adolescents; ≥ 18y, adults

In terms of gene fusions found in AML patients, the most frequent was PML-RARa, the long PML-RARa isoform bcr1/bcr2 was detected in 21 (62%) and the small bcr3 in 13 (38%). Most patients with M3 FAB morphology were positive for the PML-RARa transcript (94.4%). RUNX1-RUNX1T1 was present in 11 (7.1%) cases; most patients carrying this transcript had an undefined FAB subtype. MLL-AF9 and CBFB-MYH11-A were detected in 4 (2.6%) cases each. Three out of four MLL-AF9 carriers had a M5 FAB morphology. From CBFB-MYH11-A patients, 2 were classified as FAB M4 and 2 had no FAB subtypes available.

Mutations NPM1 and FLT3-ITD were present in 13.6% and 14.2% of the AML cases, respectively, being more frequent in normal karyotype patients (42% and 17.8%, respectively). All 19 NPM1mut carriers with a successful CC had

a normal karyotype and none of the carriers had gene fusions detected. 90% of the NPM1 exon 12 mutations consisted of a TCTG insertion, known as type A mutation. Frequencies of genetic alterations tested by molecular biology in AML are shown in table 5.

Concerning acute lymphoblastic leukemia, the most frequent gene fusions were ETV6-RUNX1 and BCR-ABL; ETV6-RUNX1 was detected only in children, while BCR-ABL was more prevalent in adults (table 6). Among BCR-ABL carriers, the major breakpoint cluster region (M-bcr or p210 isoform) was present in 3 adults, while the minor breakpoint region (m-bcr or p190 isoform) was found in 12 patients (80.0%), adults and children. MLL-AF1 was present in only 2 cases (1.5%), one adult and one child, both with BI EGIL subtype.

Table 3: Frequencies of the main prognostically significant cytogenetic abnormalities in AML

Cytogenetic abnormality	***AML <18y (n=37)*** ***N (%)***	***AML ≥18y (n=107)*** ***N (%)***	***Total AML (n=144)*** ***N (%)***
None	7 (18.9)	37 (34.6)	44 (30.6)
Complex karyotype[a]	3 (8.1)	18 (16.8)	21 (14.6)
Balanced abnormalities			
t(15;17)(q24;q21)	10 (27.0)	18 (16.8)	28 (19.4)
t(8;21)(q22;q22)	3 (8.1)	8 (7.5)	11 (7.6)
11q23[b]	3 (8.1)	4 (3.7)	7 (4.9)
t(9;11)(p22;q23)	3 (8.1)	2 (1.9)	5 (3.5)
t(6;11)(q27;q23)	0	1 (0.9)	1 (0.7)
t(1;11)(q21;q23)	0	1 (0.9)	1 (0.7)
inv(3)(q21;q26)/ t(3;3)(q21;q26)	0	3 (2.8)	3 (2.1)
inv(16)(p13q22)/t(16;16)(p13;q22)	1 (2.7)	2 (1.8)	3 (2.1)
t(9;22)(q34;q11.2)	0	0	0
t(6;9)(p23;q34)	0	0	0
Unbalanced abnormalities			
+8/+i(8)(q10)	7 (18.9)	18 (16.8)	25 (17.4)
-Y	3 (8.1)	10 (9.4)	13 (9.0)
+21	6 (16.2)	4 (3.7)	11 (7.6)
-7	2 (5.4)	6 (5.6)	8 (5.6)
-5/del(5q)	0	8 (7.5)	8 (5.6)
-17/del(17p)	1 (2.7)	4 (3.7)	5 (3.5)

[a] ≥ 3 abnormalities.

[b] rearrangement involving MLL gene was confirmed by FISH

AML, acute myeloid leukemia; <18y, children or adolescents; ≥ 18y, adults

Table 4: Frequencies of the main prognostically significant cytogenetic abnormalities in ALL

Cytogenetic abnormality	***ALL < 18y (n=102)*** ***N (%)***	***ALL ≥18y (n=32)*** ***N (%)***	***Total ALL (n=134)*** ***N (%)***
None	32 (31.4)	8 (25.0)	40 (29.9)
High hyperdiploidy >50	22 (21.6)	3 (9.4)	25 (18.7)
Low hyperdiploidy 47-50	12 (11.8)	1 (3.1)	13 (9.7)
Hypodiploid <46	6 (5.9)	3 (9.4)	9 (6.7)
Balanced abnormalities			
t(1;19)(q23;p13.3)	12 (11.8)	1 (3.1)	13 (9.7)
t(9;22)(q34;q11.2)	3 (2.9)	8 (25.0)	11 (8.2)
11q23[a]	2 (2.0)	2 (6.3)	4 (3.0)
t(4;11)(q21;q23)	2 (2.0)	1 (3.1)	3 (2.2)
t(7;11)(q22;q23)	0	1 (3.1)	1 (0.8)
14q32	1 (1.0)	3 (9.4)	4 (3.0)
t(8;14)(q24;q32)	1 (1.0)	1 (3.1)	2 (1.5)
t(12;14)(q13;q32)	0	1 (3.1)	1 (0.8)
t(14;19)(q32;q13)	0	1 (3.1)	1 (0.8)
Unbalanced abnormalities			
+21	28 (27.5)	3 (9.4)	31 (23.1)
+X	25 (24.5)	4 (12.5)	29 (21.6)
+8	17 (16.7)	2 (6.3)	19 (14.2)
+4, +10, +17	10 (9.8)	0	10 (7.5)
del(5q)	4 (3.9)	1 (3.1)	5 (3.7)

[a] MLL gene rearrangement confirmed by FISH in 3 patients, the fourth had no material available for analysis.

ALL, acute lymphoblastic leukemia; <18y, children or adolescents; ≥ 18y, adults

Comparison between cytogenetics and RT-PCR

Considering 278 acute leukemia patients for whom karyotype was available, there were 10 (3.6%) discordant results between cytogenetics and RT-PCR (Table 7). In 4 of these cases, it was possible to carry out FISH analysis.

Three AML patients presented conflicting results. One 46,XX patient with cell morphology characteristic of FAB M3 subtype had an RT-PCR positive for PML-RARa, but FISH turned out negative. Flow cytometry showed that the pattern of surface antigen expression was compatible with acute promyelocytic leukemia (APL) and the patient was clinically classified accordingly. The other two AML discrepancies involved inv16(p13;q22) (table 7).

Table 5: Frequencies of the main prognostically significant genetic lesions in AML detected by PCR/RT-PCR

Genetic alteration	***Genes involved***	***AML < 18y (n=38)*** ***N (%)***	***AML ≥18y (n=117)*** ***N (%)***	***Total AML (n=155)*** ***N (%)***
None				
t(15;17)(q22;q21)	*PML-RARa*	11 (28.9)	23 (19.7)	34 (21.9)
t(8;21)(q22;q22)	*RUNX1-RUNX1T1*	3 (10.5)	8 (6.8)	11 (7.1)
inv(16)(p13;q22)-A	*CBFB-MYH11*	0	4 (3.4)	4 (2.6)
t(9;11)(p22;q23)	*MLL-AF9*	2 (5.3)	2 (1.7)	4 (2.6)
FLT3-ITD	*FLT3*	5 (13.2)	17 (14.5)	22 (14.2)
NPM1exon12mut	*NPM1*	1 (2.6)	20 (17.1)	21 (13.6)

AML, acute myeloid leukemia; <18y, children or adolescents; ≥ 18y, adults

Table 6: Frequencies of the main prognostically significant genetic lesions in ALL B lineage detected by RT-PCR

Genetic alteration	***Genes involved***	***ALL < 18y (n=102)*** ***N (%)***	***ALL ≥18y (n=28)*** ***N (%)***	***Total B-ALL (n=130)*** ***N (%)***
t(12;21)(p13;q22)	*ETV6-RUNX1*	15 (14.7)	0	15 (11.5)
t(9;22)(q34;q11.2)	*BCR-ABL*	5 (4.9)	10 (35.7)	15 (11.5)
t(1;19)(q23;p13.3)	*TCF3-PBX1*	12 (11.8)	2 (7.1)	14 (10.8)
t(4;11)(q21;q23)	*MLL-AF1*	1 (1.0)	1 (3.6)	2 (1.5)

ALL, acute lymphoblastic leukemia; <18y, children or adolescents; ≥ 18y, adults

Seven ALL patients presented discrepant results. Three were negative for t(9;22)(q34;q11.2) in CC and positive for BCR-ABL in the RT-PCR analysis. FISH was carried out in one case and the translocation was confirmed. The remaining 2 were tested for BCR-ABL with a different primer pair and PCR protocol [21], and were positive. The other discordances involved three cases of t(1;19)(q23;p13.3)/TCF3-PBX1 and one t(4;11)(q21;q23)/MLL-AF1.

Clinical and laboratorial associations

In AML, PML-RARa was associated with M3 morphology (p <0.001), younger median age (p=0.002), lower WBC (p=0.009) and platelet count (p=0.018). This translocation was more frequent among adolescents and younger than 60 (p=0.003). CBFB-MYH11-A and MLL-AF9 were associated with FAB morphology M4 (p=0.002) and M5 (p=0.002), respectively (Table 8).

FLT3-ITD mutation in AML was associated with higher Hb concentration (p=0.012), WBC count (p=0.003) and M1 morphology (p=0.043). NPM1mut patients had a higher platelet count (p=0.015), older median age (p=0.019) and higher WBC (p=0,039). Moreover, this mutation was significantly more frequent in patients presenting normal karyotype (p<0.001) (Table 8).

In ALL, BCR-ABL was associated with higher median age at diagnosis for the whole group (p<0.001) and for older children (p=0.012). MLL-AF1 was associated with higher mean leukocyte count (p=0.017) and EGIL BI subtype (p=0.001). Results of univariate analysis of continuous variables in ALL patients are shown in table 9.

Our data confirmed the different distribution of gene fusion and mutations through age groups (table 10), such as absence of CBFB-MYH11-A, FLT3-ITD and NPM1mut in young AML children (< 9y), low frequency of PML-RARa in adults older than 60, occurrence of ETV6-RUNX1 only in children and adolescents, and higher frequency of BCR-ABL in adults between 18 and 60 years.

DISCUSSION

In view of the most recent leukemia classification systems, the diagnosis of genetic abnormalities is essential for leukemia subtype

definition. Most reports, however, refer to populations outside Latin America. Considering the geographical differences in the distribution of subtypes of acute leukemias, we drew a genetic profile of Brazilian patients using conventional cytogenetics and molecular techniques.

The frequency of most cytogenetic aberrations detected in AML was in line with that of literature, although there were exceptions. There was a higher frequency of t(15;17)(q24;q21) in both young adults (< 60 y) and pediatric groups when compared to data from non-Latin countries (7.6% and 9.9%, respectively) [22]. This observation is in accordance with previous descriptions of a higher frequency of APL, of which this translocation is a hallmark, among Latin American patients [23]. An additional chromosome 21 appeared more frequently in children when compared to other reports [22]. On the other hand, inv(16) (p13q22) and t(16;16)(p13;q22) were rarely detected in adults, despite being more frequent in other studies, even when only Brazilian patients were evaluated [24].

Table 7: Cases presenting disagreement between CC and RT-PCR analysis

AML	***FAB subtype***	***Cytogenetics***	***RT-PCR***	***FISH***	***Immunophenotype***
008	AML-M3	46,XX[30]	t(15;17) positive	t(15;17) negative	CD45+, MPO+, CD13+, CD33+, CD117+
128	AML-M2	46,XY,inv(16)(p13q22)[18]/46,XY[2]	inv16A negative	*CBFB* break apart positive	(I) [a] CD45+, HLA-DR+, CD34+, CD7+, CD13+, CD117+, MPO+/ (II) CD45+, HLA-DR+, CD4+, CD13+, CD14+, CD15+, CD33+, CD64+
311	AML-M4	46,XX,?del(16)(q12q22)[4]/46,XX[12]	inv16A positive	ND	(I) [a] CD45+, HLA-DR+, CD38+, CD34+, CD13+, CD33+, MPO+, CD117+; (II) CD45+, HLA-DR+, CD38+, CD4+, CD13+, CD33+, MPO+, CD14+, CD15+, CD64+
ALL	*EGIL subtype*	*Cytogenetics*	*RT-PCR*	*FISH*	*Immunophenotype*
030	B-ALL II	46,XX[20]	t(1;19) positive	ND	CD79a+, TdT+, CD10+, CD19+, CD20+, CD22+, CD38+, CD45+, HLA-DR+
040	B-ALL III	46,XX,der(19)t(1;19)(q23,p13)[2]/46,idem,t(2;9)(p13,?q22)[15]	t(1;19) negative	t(1;19) negative [b] t(12;21) positive	CD45+, HLA-DR+, TdT+, CD38+, CD10+, CD19+, CD22+, CD79a+, IgMc+
267	B-ALL III	53, XX, +X, +4, +6, +10, +14, +add(17)(p13), +21[5]/54, idem, +5[7]/46, XX[8]	t(1;19) positive	ND	HLA-DR+, CD34+, CD38+, TdT+, CD10+, CD33+, CD79a+, IgMc+
066	B-ALL II	46,XY,9qh+c[30]	t(9;22) positive	t(9;22) positive	HLA-DR+, CD34+, TdT+, CD10+, CD10+, CD22+, CD79a+
104	B-ALL III	46,XY,1qh+c[29]	t(9;22) positive [c]	ND	HLA-DR+, CD34+, TdT+, CD10+, CD19+, CD22+, CD79a+, IgMc+
205	B-ALL II	46, XX[16]	t(9;22) positive [c]	ND	CD45+, HLA-DR+, CD34+, CD38+, TdT+. CD10+, CD19+, CD22+, CD79a+
140	T-ALL	47, XX, t(4;11)(q21;q23), +8,21pstk+c[5]/47, idem, t(3;10)(q27;q22), del(6)(q?13q21), t(8;14)(q24.1;q32)[3]/95, XXXX, +4, t(4;11)(q21;q23)x2, +8, +8, 21pstk+cx2[4]/46, XX, 21pstk+c[8]	t(4;11) negative	ND	CD45+, CD38+, CD7+, CD13+ [d]

[a] Patients had two different blast cell populations

[b] FISH was negative for t(1;19) but showed gain of 1q and loss of 19p in 65% of the cells analyzed.

[c] RT-PCR was positive using two protocols with different primer pairs.

[d] Patient had immunophenotype of biopsy compatible with T-ALL/T lymphoma (CD10-, CD20-, CD3+, TdT-) and relapsed with a t(4;11) on the karyotype.

The translocations commonly found in AML were more frequent in children than in adults. On the other hand, complex karyotypes were more frequent among adults. It is well known that balanced translocations tend to be found in younger AML patients, while older ones usually have unbalanced aberrations [25]. The significant older mean age observed in the high-risk group is probably a reflection of the higher frequency of complex karyotypes in patients older than 50.

In acute lymphoblastic leukemias, the frequency of abnormal karyotypes in adults was slightly higher than in children, as described by others [20], while hyperdiploid and unbalanced abnormalities were more common in children, as in previous reports [6, 22]. The older mean age of patients with poor prognosis compared to that in patients with good prognosis can be partially explained by the higher frequency of t(9;22) in the adult group.

RT-PCR confirmed that the translocation involving PML and RARa genes (chromosomes 15 and 17, respectively) was the most common genetic lesion in AML. The frequency of PML-RARa among M3 FAB morphology patients (94.4%) is in accordance with previous publications [26]. One M3 patient positive in the RT-PCR for this gene fusion was negative in both CC and FISH. In some reports, PML-RARa was only detectable by RT-PCR or sequencing analysis [27]. There was a significant association of PML-RARa with lower age and lower median WBC count at diagnosis for the overall group of patients and for adults. These findings are in line with reports showing that, unlike other AMLs subtypes, APL is generally characterized by low leukocyte counts at diagnosis and occurs most frequently between 15 and 60 years of age [28].

The second most common gene fusion in AML was RUNX1-RUNX1T1 resulting from t(8;21), which showed no associations with the clinical and laboratorial variables tested. MLL-AF9, resulting from t(9;11), had a significant association with M5 morphology. CBFB-MYH11-A, resulting from inv16, showed association with FAB M4, as described by others [29]. RT-PCR confirmed a low prevalence of inv16, as observed in CC, although other CBFB-MYH11 variants were not tested.

The incidence of FLT3-ITD in children (13.2%) was in the range already described (5-22%), although the incidence in adults (14.5%) was not as high as in previous works (17-25%) [30, 31]. The mutation was not detected in young children, but occurred evenly among the other age groups, in agreement with Bacher et al. [25]. Although ITDs were more frequent in normal karyotype patients, the association was not significant, unlike previous studies [32], probably because our cases included a high frequency of APL in whom FLT3-ITD is common. Higher WBC was found in FLT3-ITD carriers for both children and adults groups analyzed separately, which does not corroborate the observation that FLT3-ITD is associated with high WBC in adults only [33, 34]. As for high hemoglobin levels, the association was significant in adults only.

As expected, an NPM1 mutation was much more frequent in adults than in children, since the prevalence of the mutation increases with age [34, 35]. The frequency found in adults (17%) is lower than that reported by others (25-41%); in children the frequency (2.6%) is within the range reported (0-12%) [35]. The most common NPM1mut (type A) [35], corresponded to 90% of AML NPM1mut cases.

The association of NPM1 mutations and normal karyotype is well known [35], and was also observed in the present study. NPM1 carriers had higher platelet counts at diagnosis; however, when analyzing age groups separately, this association was significant in adults only, as already observed by Braoudaki et al. [36] and Chauhan et al. [34], although the group of positive children is too small for a conclusion to be drawn. There was an inverse relation between NPM1 and M3 FAB subtype; in fact, the NPM1 was observed neither in PML-RARa carriers nor in any of the patients carrying the gene fusions tested.

In B-ALL, there was a significant difference in the BCR-ABL distribution according to age group, with a higher frequency in adults between 18 and 60 years (38.5%). The major breakpoint region (p210) was seen in 20% of all BCR-ABL carriers and was only present in adults; indeed, this isoform is rare among children [37]. The MLL-AF1 fusion was associated with immature B cell leukemia and higher WBC at diagnosis; although the number of patients was too small, the findings agree with previous reports [38].

Table 8: Univariate analysis of clinical/laboratorial data and genetic alterations detected by PCR/RT-PCR in AML patients

Genetic alteration	*Age (years)*			*Hb (g/dL)*			*Platelets (x10^9/L)*			*WBC (x10^9/L)*		
	Median	*N*	*p-value*	*Median*	*N*	*p-value*	*Median*	*N*	*p-value*	*Median*	*N*	*p-value*
PML-RARα												
Negative total	46.0	121	0.002	7.8	117	0.780	49.5	118	0.018	17.7	114	0.009
Positive total	22.5	34		8.3	33		23.5	34		5.0	33	
Negative <18y	9.1	29	0.188	7.9	29	0.249	42.0	29	0.832	22.7	29	0.104
Positive <18y	11.7	11		7.5	11		53.0	11		4.8	10	
Negative ≥18y	55.9	92	0.000	7.8	88	0.196	50.0	89	0.002	17.4	85	0.037
Positive ≥18y	35.6	23		8.4	22		23.0	23		6.4	23	
RUNX1-RUNX1T1												
Negative total	43.1	144	0.071	8.0	139	0.138	45.0	141	0.165	14.5	136	0.357
Positive total	24.7	11		7.0	11		34.0	11		24.0	11	
Negative <18y	10.1	36	0.417	7.9	36	0.558	47.5	36	0.735	10.0	35	0.694
Positive <18y	12.1	4		7.0	4		43.5	4		26.5	4	
Negative ≥18y	52.1	108	0.028	8.0	103	0.150	45.0	105	0.113	17.0	101	0.473
Positive ≥18y	30.0	7		7.0	7		25.0	7		24.0	7	
CBFB-MYH11[a]												
Negative total	40.6	151	0.340	8.0	146	0.187	43.0	148	0.849	15.8	143	0.431
Positive total	47.3	4		6.8	4		46.0	4		20.8	4	
Negative ≥18y	51.0	111	0.945	8.0	106	0.167	43.0	108	0.796	17.0	104	0.454
Positive ≥18y	47.3	4		6.8	4		46.0	4		20.8	4	
MLL-AF9												
Negative total	40.9	151	0.097	8.0	146	0.958	43.0	148	0.592	15.8	143	0.524
Positive total	18.6	4		7.4	4		72.5	4		17.2	4	
Negative <18y	10.4	38	0.385	7.8	38	0.514	40.5	38	0.082	10.0	37	0.324
Positive <18y	6.7	2		9.4	2		113.0	2		53.1	2	
Negative ≥18y	51.0	113	0.219	8.0	108	0.538	43.5	110	0.398	17.0	106	0.037
Positive ≥18y	34.4	2		7.4	2		32.0	2		1.3	2	
FLT3-ITD												
Negative total	41.2	134	0.945	7.8	130	0.012	43.0	132	0.670	11.9	126	0.003
Positive total	40.2	22		9.5	21		48.0	21		34.9	22	
Negative <18y	10.2	35	0.223	7.7	35	0.279	40.5	35	0.332	9.1	34	0.031
Positive <18y	12.2	5		8.5	5		75.0	5		69.9	5	
Negative ≥18y	51.4	99	0.416	7.9	95	0.021	43.5	97	0.889	13.7	92	0.036
Positive ≥18y	48.4	17		9.7	16		28.0	16		30.1	17	
NPM1mut												
Negative total	36.2	134	0.019	8.0	129	0.342	39.0	131	0.015	12.4	126	0.039
Positive total	55.9	21		7.4	21		61.0	21		20.2	21	
Negative <18y	10.1	38	0.163	7.7	38	0.733	40.5	38	0.203	10.0	37	0.426
Positive <18y	14.0	2		8.2	2		108.5	2		44.3	2	
Negative ≥18y	49.2	96	0.221	8.0	91	0.220	37.2	93	0.038	12.9	91	0.045
Positive ≥18y	56.0	19		7.2	19		58.0	19		20.2	17	

WBC, white blood cells count; Hb, hemoglobin

[a] There were no CBFB-MYH11 in children

Table 9: Univariate analysis of clinical/laboratorial data and genetic alterations detected by RT-PCR in B-ALL patients.

Gene Fusion	*Age (years)*			*Hb (g/dL)*			*Platelets (x10^9/L)*			*WBC (x10^9/L)*		
	Median	*N*	*p-value*	*Median*	*N*	*p-value*	*Median*	*N*	*p-value*	*Median*	*N*	*p-value*
ETV6-RUNX1[a]												
Negative total	10.6	116	0.147	7.9	116	0.914	32.0	115	0.785	8.2	114	0.743
Positive total	5.4	15		8.0	15		41.5	14		12.4	14	
Negative <18y	6.0	88	0.937	7.9	88	0.859	35.0	87	0.930	8.6	86	0.960
Positive <18y	5.4	15		8.0	15		41.5	14		12.4	14	
BCR-ABL												
Negative total	7.6	116	<0.001	8.0	116	0.544	84.6	114	0.705	8.4	114	0.936
Positive total	27.1	15		7.5	15		64.5	15		5.2	14	
Negative <18y	5.5	98	0.012	8.0	98	0.741	34.5	96	0.731	8.6	96	0.147
Positive <18y	14.0	5		7.5	5		60.0	5		45.9	4	
Negative ≥18y	31.1	18	0.303	8.1	18	0.666	23.5	18	0.502	13.8	18	1.000
Positive ≥18y	39.0	10		7.7	10		30.0	10		20.0	10	
TCF3-PBX1												
Negative total	9.3	117	0.295	7.9	117	0.512	32.0	115	0.633	7.7	114	0.071
Positive total	8.4	14		7.8	14		35.5	14		16.1	14	
Negative <18y	5.9	91	0.438	7.9	91	0.140	35.0	89	0.769	8.5	88	0.042
Positive <18y	5.9	12		6.1	12		31.4	12		33.4	12	
Negative ≥18y	33.4	26	1.000	7.7	26	0.082	25.0	26	0.099	4.4	26	0.964
Positive ≥18y	34.1	2		10.7	2		173.5	2		7.9	2	
MLL-AF1[b]												
Negative total	8.9	129	0.836	7.9	129	0.567	33.0	127	0.587	8.0	126	0.017
Positive total	33.3	2		9.9	2		26.0	2		368.5	2	

WBC, white blood cells count; Hb, hemoglobin

[a] ETV6-RUNX1 did not occur in adults

[b] MLL-AF1 was not analyzed separately in adults and children because of the reduced n.

Table 10: Analysis of age categories and main genetic abnormalities detected by PCR/RT-PCR in 155 AML and 131 B-ALL patients.

	Age subgroup					
AML subgroup	*< 1 year (n=2) N (%)*	*≥1-< 9 years (n=13) N (%)*	*≥9-18 years (n=25) N (%)*	*≥18-<60 years (n=79) N (%)*	*≥ 60 years (n=36) N (%)*	*p-value*
t(15;17) *PML-RARα*	0	2 (15.4)	9 (36.0)	22 (27.8)	1 (2.8)	0.003
t(8;21) *RUNX1-RUNX1T1*	0	1 (7.7)	3 (12.0)	6 (7.6)	1 (2.8)	0.564
Inv(16)A *CBFB-MYH11*	0	0	0	3 (3.8)	1 (2.8)	1.000
t(9;11) *MLL-AF9*	0	1 (7.7)	1 (4.0)	2 (2.5)	0	0.362
FLT3-ITD	0	0	5 (20.0)	13 (16.5)	4 (10.8)	0.636
NPM1mut	0	0	2 (8.0)	15 (19.0)	4 (11.1)	0.349
B-ALL subgroup	*< 1 year (n=2) N (%)*	*≥1-< 9 years (n=63) N (%)*	*≥9-18 years (n=38) N (%)*	*≥18-<60 years (n=26) N (%)*	*≥ 60 years (n=2) N (%)*	
t(12;21) *ETV6-RUNX1*	0	11 (17.5)	4 (10.5)	0	0	0.147
t(9;22) *BCR-ABL*	0	1 (1.6)	4 (10.5)	10 (38.5)	0	<0.000
t(1;19) *TCF3-PBX1*	0	7 (11.1)	5 (13.2)	2 (7.7)	0	0.918
t(4;11) *MLL-AF1*	0	1 (1.6)	0	0	1 (50.0)	0.060

When we compared conventional cytogenetics and PCR in 278 patients who had a karyotype, there was a good correlation between both techniques. Still, we found six cases (2.2%) in which CC missed the translocation detected by RT-PCR. If we consider specifically the 11 patients who were positive for BCR-ABL in RT-PCR and had a karyotype, three were negative in CC. FISH or a

different PCR system confirmed the presence of the fusion gene in these cases, yielding a false-negative rate for this aberration of 21.4%. No nested reactions were done, which greatly minimized the chances of false-positives RT-PCR. In a previous comparative study, t(9;22) was also the most frequently abnormality missed by CC [39].

There are two main reasons for the detection of fusion genes in normal karyotype patients. Either a clone selection caused by higher proliferative activity of normal cells in culture, or a low percentage of blasts carrying the translocation. Alternatively, patients may have a submicroscopic gene rearrangement that CC does not detect [40].

In one case described in CC as del(16), CBFB-MYH11-A gene fusion was detected by RT-PCR, highlighting the importance of performing RT-PCR and/or FISH assays in all patients with del(16)t(16;16), as suggested by Mrózek et al. [22]. On the other hand, there was one false-negative RT-PCR patient who was positive both in CC and FISH. Probably this patient had a non-A CBFB-MYH11 fusion not detected by the primers used. This may also be the case of a patient who was shown to carry a t(4;11) by CC but had a negative PCR for MLL-AF1 at diagnosis, but later on relapsed bearing a t(4;11) on the karyotype.

Another case of notice was a t(1;19) detected by CC likely to be a false-positive since the patient was negative for TCF3-PBX1 and positive for ETV6-RUNX1 in the RT-PCR test. One explanation could be the occurrence of mimetic translocations, involving or not genes already detected in the "classical" translocations [41]. FISH analysis should resolve diagnostic uncertainty when equivocal markers are detected.

There were 37 patients harboring abnormalities that were not targeted by PCR or RT-PCR, but were essential for the classification system. Most of these (34) were ploidy alterations in ALL (hypodiploidy or high hyperdiploidy); a few (3) were AML which bore rare structural abnormalities of the long arm of chromosome 3 that have been described as having extremely poor prognosis [42]. Thus, CC has the advantage of identifying ploidy and numerical chromosome alterations, as well as a broad spectrum of rearrangements not usually tested by RT-PCR.

These results clearly illustrate the need of using CC and molecular approaches in the diagnosis of acute leukemias. The failure to detect some genetic abnormalities may lead to erroneous stratification of the patient, leading to inappropriate therapy allocation. Patients who could benefit from the use of specific drugs, such as all-trans retinoic acid for t(15;17) and tyrosine kinase inhibitors for t(9;22) carriers may have a reduced survival chance if the translocations are missed. In this study, we detected four patients that were candidates for specific therapy who had not been detected in CC.

There are some limitations to our study. The main limitation is the non-consecutive inclusion of all patients treated in each institution. Out of the seven treatment centers that participate in this research, only three contributed both pediatric and adult patients. As for the remaining hospitals, only adult hematology (2) or pediatric divisions (2) were enrolled. In addition, some patients whose bone marrow aspirates were insufficient for genetic analyses were excluded from the cohort. We could not retrieve information on how many patients were missed during the course of the research. Therefore, the prevalence of leukemia subtypes in the present report is just an estimate of the real one.

CONCLUSIONS

To our knowledge, this study comprises the largest series of Latin-America acute leukemia patients in whom an extensive genetic characterization has been performed. There were differences in the incidence of some gene fusions and mutations found in AML when compared to that from developed countries. As reported by others, we observed clear-cut patterns of distribution of abnormalities when comparing children and adults, which indicates differences in the pathogenesis of the disease in these groups. Most of the significant associations found between clinical and laboratorial data and genetic alterations were similar to previous reports. Finally, the results obtained when comparing techniques reinforce the importance of relying on both conventional cytogenetic and molecular genetic tests as complementary tools to diagnose and classify acute leukemias, together with clinical, morphological and immunophenotypic data.

CONFLICT OF INTEREST

The authors have no conflict of interests to declare.

ACKNOWLEDGMENTS

The authors thank patients, their families, nurse and medical teams for agreeing to participate in this research. We thank Cristiane Lommez de Oliveira, Márcia Caldeira Brant Neves Campos, Valéria Matarelli Calijorne for the management of research projects, and Ludmila Muniz and Paloma Gonçalves da Silva for technical support. This study was supported by FINEP-Inovação e Pesquisa, Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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