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Molecular Characterization of 34 Children with Acute Leukemia and *MLL* gene Rearrangements in Argentina

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Abstract

Translocations involving the Mixed Lineage Leukemia (MLL) gene can be detected in de novo acute lymphoblastic leukemia (ALL), acute myeloblastic leukemia (AML), therapy-related acute leukemias, and are highly associated with infant acute leukemia. Analysis of these genetic rearrangements is required for stratifying patients into risk groups for treatment tailoring in current protocols. Their occurrence usually predicts a poor outcome. We report the biological and molecular characteristics of 34 pediatric acute leukemia cases with *MLL* rearrangements. Samples were further analyzed by LDI-PCR to characterize the respective fusion partner genes. *AFF1* was the main partner gene in ALL, while *MLLT10* was predominantly identified in AML in this series. Although RT-PCR allowed quick detection of most cases with MLL gene rearrangements, the routinely performed cytogenetic analysis combined with LDI-PCR was a powerful tool for accurate diagnosis and subsequent tailoring of treatment.

Keywords: *MLL*, LDI-PCR, infant acute leukemia, fusion genes

Introduction

Translocations involving the Mixed Lineage Leukemia gene (*MLL*, also known as Lysine-specific methyltransferase 2A, *KMT2A*), can be detected in *de novo* acute lymphoblastic leukemia (ALL), acute myeloblastic leukemia (AML) and therapy-related acute leukemia. The occurrence of abnormalities in the *MLL* gene, located on chromosome 11q23, is observed in more than 80% of infant ALL and more than 50% of infant AML cases [1-4] and most likely confers a poor prognosis for these patients [3, 5, 6]. At least 100 *MLL* rearrangements have been reported, of which 79 have been characterized at the molecular level [7-9]. *MLL/11q23* aberrations can be analyzed by G-banding, fluorescence *in situ* hybridization (FISH), reverse transcription followed by polymerase chain reaction (RT-PCR), or Long-Distance Inverse PCR (LDI-PCR), among others. Each technique has advantages and disadvantages but are complementary for accurately characterizing *MLL* alterations. LDI-PCR allows the detection of *MLL* rearrangements, specifically in cases where the fusion partner gene is yet unknown and improves the detection rate of *MLL* rearrangements [10]. Besides, the established DNA sequences of the fusion region of *MLL* rearrangements at the genomic level can be used as targets for Minimal Residual Disease (MRD) quantification [11]. Here, we report the characterization of *MLL* rearrangements by LDI-PCR in 34 pediatric patients from a single center in Argentina and describe cases in which discordances were found between results obtained by cytogenetics, RT-PCR and/or LDI-PCR.

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Patients and Methods

Sample collection

Bone marrow samples from 34 *de novo* acute leukemia cases with *MLL* rearrangements were characterized by LDI-PCR. Twenty-nine patients were younger than 1 year (infants). Five pediatric non-infant cases were included in the analysis by LDI-PCR due to the presence of *MLL/11q23* abnormalities detected by G-banding or FISH analyses. Immunophenotype of blasts was characterized in all cases according to standardized procedures described previously [12], and acute leukemia cases were classified according to the WHO 2008 definitions [13].

Conventional cytogenetics and Fluorescent in situ hybridization (FISH)

Chromosome G-banding analyses were performed by standard methods. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature [14]. FISH analysis for *MLL* rearrangements was performed using the LSI MLL Dual-Color, Break-Apart Probe, according to the manufacturer's instructions (Vysis, Abbott Molecular Inc., Des Plaines, IL, USA).

RT-PCR

RT-PCR was performed following the Biomed-1 protocol [15]. Samples from infant patients were routinely analyzed for the presence of *MLL-MLLT1, MLL-AFF1*, and *MLL-MLLT3* fusion transcripts using primers and procedures described previously [15, 16]. The presence of *MLL-MLLT10*, *MLL-MLLT11*, *MLL-MLLT6*, *MLL-MLLT4,* and *MLL-BTBD18* fusion transcripts was investigated using oligonucleotide MLL-A [15] as forward primer and the corresponding reverse oligonucleotides designed according to expected cleavage sites detected by LDI-PCR. Sequences of the oligonucleotides are available as **supplementary material S1**. The integrity and amplificability of samples were evaluated using the *ABL1* transcript as the control gene [16].

Long-distance inverse-polymerase chain reaction (LDI-PCR)

LDI-PCR was performed as previously described [10]. Briefly, 1 µg of genomic DNA was digested with restriction enzymes and re-ligated to form DNA circles before LDI-PCR using *MLL*-specific primers. Restriction polymorphic PCR amplimers were isolated from agarose gels and subjected to DNA sequence analysis to obtain the patient-specific fusion sequences. Unknown sequences were characterized using the human genome database (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Patient characterization

Clinical and hematological characteristics of the 34 cases are described in **Table 1**. Extramedullar compromise was observed in 14 cases (41%). The mean white blood cell (WBC) count was 190,100/mm^3 (range:2,100-760,000/mm^3), and hyperleukocytosis (>100,000/mm^3) was detected in 53% of cases. Diagnoses of these 34 cases were 20 ALL, 12 AML, and 2 ambiguous lineages acute leukemias (ALAL). ALL phenotype was Pro-B in 50% of cases; all AML cases were

Table 1: Clinical and hematological characteristics of the 34 patients with acute leukemia.

CASE #	AGE	SEX	WBC (x10^3/mm^3)	Hb (g/dl)	Plat (x10^9/L)	HEP	SP	EXTRAMEDULLAR
1	3 m	F	6.9	9.3	315	No	Yes	skin
2	2 m	M	550	7.7	30	No	No	Abdomen/ganglio
3	3 m	M	25	8	181	No	No	No
4	4 m	F	291	6.7	14	Yes	Yes	No
5	9 m	M	16	12.3	40	Yes	No	No
6	3 m	F	204	10	30	Yes	Yes	No
7	0.1 m	F	90	8	12	No	Yes	No
8	8 m	M	240	6	24	Yes	Yes	No
9	8 m	M	11	7.2	104	No	Yes	Skin
10	4 m	F	136	7.7	33	No	Yes	No
11	4.8 a	M	526	5	25	No	No	No
12	7 m	M	22.8	10.3	10	No	No	No
13	5 m	F	470	11	15	Yes	Yes	Kidney
14	4 m	F	700	9.9	88	Yes	Yes	CNS
15	2.3 a	F	4.1	4.4	213	Yes	Yes	No
16	1 m	F	249	9.1	30	No	Yes	Skin
17	2 m	F	300	6.8	10	Yes	No	No

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18	11 m	F	75.8	7.6	42	Yes	Yes	No
19	3 m	F	13	5.1	74	Yes	Yes	No
20	3 m	F	13.5	6.6	14	Yes	Yes	No
21	0.11 m	F	142	6.8	43	Yes	Yes	CNS, Kidney
22	8 m	F	217	6.4	67	Yes	Yes	No
23	11 m	F	217	7.4	135	Yes	Yes	Skin
24	3 m	M	3.6	10	118	Yes	Yes	Skin
25	2.8 y	F	3.8	7.9	27	Yes	Yes	Kidney
26	2.1 y	M	43	8.3	138	Yes	Yes	No
27	3 m	F	760	7.8	9	Yes	Yes	No
28	9 m	M	2.1	6.3	4	Yes	Yes	Testis
29	4 m	M	49.4	11.2	44	Yes	Yes	No
30	6 m	F	282	4	37	Yes	Yes	No
31	6 m	F	13.9	4.3	11	Yes	Yes	CNS
32	4 m	M	212.6	6.5	51	Yes	Yes	No
33	12 m	M	274	6.8	71	Yes	Yes	CNS, O, Kidney
34	5 m	M	300	7.1	47	Yes	Yes	CNS, Skin

M: male; F: female; y: years; m: months; WBC: White blood cells; Hb: hemoglobin; Plat: platelets; HEP: hepatomegaly; SP: Splenomegaly; CNS: Central Nervous System; O: orbit

monoblastic FAB-subtype, while both ALAL cases disclosed mixed Myeloid–B phenotype **(Table 2)**. Infants with ALL diagnoses were treated according to the Interfant-99 protocol [3]. ALL patients older than 1 year were treated according to the ALLIC-protocol [17]. Children diagnosed with AML received local BFM-based protocol (BFM) [18]. ALAL cases were treated according to the AML protocol based on the predominant myeloid blast population. Complete remission (CR) was achieved in 31 (91%) cases. In comparison, 2 patients died during the induction phase (6%), and 1 T-ALL patient did not respond to induction therapy at day 33, albeit showing a late response. Of the 32 patients (including the late responder) who achieved CR, 14 patients relapsed with a median of 11 (range: 3-64) months, 1 case developed a lineage switch, 6 patients died in CR, and 11 patients remained in continuous CR with a median follow up of 67 (38-121) months **(Table 2)**.

Cytogenetic and FISH analyses

Cytogenetic studies were successful in 33 (97%) of 34 patients included in this cohort **(Table 3)**. Abnormalities of the 11q23 region, suggesting an *MLL* involvement, were detected in 26 cases by G-banding. Following aberrations were found: t(4;11)(q21;q23) (n= 10), t(1;11)(q21;q23) (n= 3), t(11;19)(q23;p13) (n= 2), t(4;11;10)(q21;q23;q22) (n= 1), ins(11;4)(q23;q21q25) (n= 1), t(9;11)(p22;q23) (n= 1); inv(11)(q13q23) (n= 1), del(11)(q23) (n= 1), t(6;11)(q27;q23) (n= 1), t(11;17)(q23;q21) (n= 1), t(11;22)(q23;q13) (n= 1), t(10;11)(p12;q23) (n= 1). Two cases had a complex karyotype, where one showed alterations of 11q23 and 19p13.3 regions but did not have the typical t(11;19), while the other showed alterations of 9p22 and 11q23 regions. FISH analysis was performed for 21 cases, and the result disclosed a split signal in 20 of them, two with normal karyotypes and three with abnormal karyotypes not involving 11q23. Among the latter, one case disclosed a t(9;10)(q32;p11.2), and FISH revealed a cryptic insertion of the 5'MLL gene into the derivative chromosome 10. Another case showed a t(10;22;11)(p13;q11.2;q13) and FISH revealed MLL rearrangement with 5'MLL signal on 10p12. Finally, one case with del(1)(q21), add(10)(p11),-11 in which the split FISH study showed a split signal. In 1 case, the karyotype result was normal, and FISH did not show a split signal.

RT-PCR results

RT-PCR allowed the detection of *MLL* fusion transcripts in 33 (97%) of 34 cases **(Table 4)**. Recurrent rearrangements found were *AFF1* (n= 12), *MLLT1* (n= 4), and *MLLT3* (n= 3). Other partners were characterized by LDI-PCR and/or cytogenetic findings: *MLLT10* (n= 7), *MLLT11* (n= 3), *MLLT4* (n= 2), *MLLT6* (n= 1) and *BTBD18* (n= 1). In case #19, the expected *MLL-AFF1* fusion transcript was not detected.

LDI-PCR results

The identified *MLL* fusion partner genes **(Table 4)** detected by LDI-PCR on the derivative chromosome 11 were *AFF1* (n= 12), *MLLT10* (n= 6), *MLLT1* (n= 4), *MLLT3* (n= 3), *MLLT11* (n= 3), *MLLT4* (n= 2), *BTBD18* (n= 1), and *MLLT6* (n= 1). The fusion sequence on der11 (5′-3′) was detected in 32 of 34 cases. Only the reciprocal rearrangement could be detected in two cases, involving *AFF1* in one case and *SVIL* in the other. In 4 cases, LDI-PCR allowed the detection

Table 2: Immunophenotype, response to treatment and outcome of the 34 patients

CASE #	Immunophenotype/ FAB subtype	Induction response	Outcome (months)
1	FAB-M5	CR	Relapsed (3)
2	Pro-B	CR	CCR (+60)
3	Pro-B	CR	Death in CR (6)
4	Pre-B	CR	Relapsed (28)
5	Pro-B	CR	Relapsed (5)
6	FAB-M5	CR	CCR (+108)
7	Pro-B	CR	CCR (+121)
8	Pro-B	CR	Relapsed (12)
9	B Mature	CR	Relapsed (21)
10	FAB-M5	CR	Relapsed (15)
11	Pro-B	CR	CCR (+61)
12	Pre-B	CR	CCR (+71)
13	Pre-B	CR	Relapsed (3)
14	Pro-B	CR	Relapsed (1)
15	FAB-M5	CR	CCR (+87)
16	Pre-B	CR	Relapsed (98)
17	Pro-B	CR	Relapsed (9)
18	ALAL (myeloid + B)	CR	Relapsed (6)
19	Pre-B	CR	Death on CR (3)
20	Pro-B	CR	Death on CR (1)
21	ALAL (myeloid + B)	CR	Linage switch (1)
22	FAB-M5	CR	CCR (+67)
23	FAB-M5	CR	CCR (+66)
24	FAB-M5	CR	Death on RC (3)
25	FAB-M5	CR	CCR (+57)
26	T	Null response	Death on RC (6)
27	Pre-B	CR	Relapsed (4)
28	FAB-M5	CR	Relapsed (11)
29	Pro-B	Death	-
30	FAB-M5	CR	CCR (+52)
31	Pre-B	CR	Death on CR (1)
32	FAB-M5	CR	Relapsed (11)
33	FAB-M5	Death	-
34	Pre-B	CR	CCR (+38)

ALAL: acute leukemia of ambiguous lineage; CR: complete remission; CCR: Continuous complete remission.

Table 3: Results of cytogenetic and molecular cytogenetic studies of the 34 patients with Acute Leukemia

CASE #	KARYOTYPE	SPLIT-FISH
1	46,XX[20]	Positive
2	46,XY,t(4;11)(q21;q23)[9]/46,XY[11]	Not done
3	46,XY,t(4;11;10)(q21;q23;q22),9ph[20]	Positive
4	46,XX,t(4;11)(q21;q23)[20]	Not done
5	46 XY,inv(11)(q13q23)[20]	Positive
6	46,XY,t(1;11)(q21;q23)[20]	Positive
7	46,XX,t(4;11)(q21;q23)[3]/46,XX[17]	Not done
8	46,XY,t(4;11)(q21;q23)[14]/46,XY[6]	Positive
9	46~48,XY,+11,del(11)(q23),add(19)(p13),+mar[cp20]	Positive
10	46,XX,t(10;22;11)(p13;q11.2;q13)[20]	Positive
11	46,XY,t(4;11)(q21;q23)[15]/46,XY[5]	Not done
12	46XY,t(11;22)(q23;q13),add(12)(p12)[6]/47,idem,+X[11]/46,XY[3]	Positive
13	46,XX,t(11;19)(q23;p13)[20]	Positive
14	46,XX,t(4;11)(q21;q23)[14]/46,XX[6]	Positive
15	46,XX,del(1)(q21),add(10)(p11),-11,+mar[6]/46,XX[14]	Positive
16	46,XX[20]	Positive
17	46,XX,t(4;11)(q21;q23)[10]/46,XX[10]	Not done
18	46,XX,t(11;19)(q23;p13)[14]/46,XX[6]	Not done
19	45,XX,t(4;11)(q21;q23),der(13;21)(q10;q10)[7]/46,XX [13]	Positive
20	46,XX t(4;11)(q21;q23)[15]/46,XX[5]	Positive
21	46,XX,ins(11;4)(q23;q21q25)[14]/46,XX[6]	Not done
22	46,XX,t(1;11)(q21;q23)[20]	Not done
23	46,XX[20]	Positive
24	46,XY[20]	Negative
25	46,XX,t(1;11)(q21;q23)[2]/48,idem,+8,+21[18]	Not done
26	46,XY,del(11)(q23)[4]/46,XY[11]	Not done
27	No methapases were obtained	Not done
28	46,XY,t(9;11)(p22;q23)[17]/46,XY[3]	Not done
29	46,XY,t(4;11)(q21;q23)[16]/46,XY[4]	Not done
30	46,XX,t(6;11)(q27;q23)[20]	Positive
31	46,XX,t(11;17)(q23;q21)[4]/46,XX[16]	Positive
32	46,XY,t(10;11)(p12;q23)[17]/46,XX[3]	Positive
33	46,XY,t(9;10)(q32;p11.2)[18]/46,XY[2]	Positive
34	46,XY, der(3)(9pter-->9p22::11q23-->11q23::3p21-->3qter),der(9)(11qter-->11q23::9p22-->9qter),der(11)(11pter-->11q23::3p21-->3pter)[19]/46,XY[1]	Positive

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of *MLL* abnormalities that other techniques did not show at diagnosis, including 3 cases with normal karyotype and no FISH results (cases #1, #23, and #24). RT-PCR confirmed LDI-PCR results for the *MLL-MLLT10* fusion transcript. In 1 case with t(9;10) where G-banding did not detect 11q23 abnormality (case #31), LDI-PCR identified *MLL-MLLT6* that RT-PCR and split-FISH later confirmed. *MLL-MLLT1* rearrangement was detected in 4 patients, including the case with t(11;22)(q23;q13), add(12)(p12) detected by G-banding, which did not involve the 11q23 band. Three of them were spliced fusions, while in the other case, a 267 bp fragment of the *NDUFA9* gene was detected between *MLL* intron 9 and *MLLT1* intron 3 sequences (case #12). *MLL-AFF1* rearrangement was detected in 13 cases, 12 of which had been detected by RT-PCR, including a case with normal karyotype. In the remaining case (case #19), in which G-banding resulted in 45, XX,t(4;11)(q21;q23),der(13;21)(q10;q10), the *MLL-AFF1* transcript fusion was not detected by RT-PCR and neither by LDI-PCR. However, LDI-PCR detected the reciprocal fusion sequence between *AFF1* intron 10 and *MLL* intron 8. *MLL-MLLT3* was detected in 3 cases, 1 of whom showed a normal karyotype by G-banding. *MLL-MLLT4* rearrangement was observed in 2 cases, 1 of which presented as the only abnormal deletion of the band 11q23 by G-banding. In 3 cases where LDI-PCR detected *MLL-MLLT1* and *MLL-MLLT6,* the fusion transcripts were subsequently demonstrated by RT-PCR based on LDI-PCR results. According to G-banding results, *MLL-BTBD18* rearrangement was detected via LDI-PCR from the genomic DNA of a patient with inv(11q23). Based on LDI-PCR results, the MLL-MLLT10 fusion transcript was searched and detected in 6 patients (5 AML and 1 ALL). In 5 of these cases, LDI-PCR detected rearrangement at the molecular level, while in a further case of ALL and normal karyotype, *MLL-MLLT10* fusion transcript was detected by RT-PCR but not LDI-PCR. However, the reciprocal rearrangement involving the *SVIL* gene at chromosome 11p12 was detected by LDI-PCR.

Table 4: Results of molecular studies of 34 patients with Acute Leukemia

CASE #	RT-PCR	FUSED EXONS	LDI-PCR (der11)	MLL exon/ intron	PG exon/ intron	LDI-PCR (reciprocal)	PG exon/ intron	MLL exon/ intron
1	MLL-MLLT10*	ex9-ex8	NEGATIVE	-	-	SVIL-MLL	int3	int10
2	MLL-AFF1	ex9-ex4	MLL-AFF1	int9	int3	-	-	-
3	MLL-AFF1	ex11-ex4	MLL-AFF1	int11	int3	-	-	-
4	MLL-AFF1	ex10-ex4	MLL-AFF1	int10	int3	-	-	-
5	MLL-BTBD18*	ex10-ex3	MLL-BTBD18	ex10	ex3	BTBD18-MLL	ex3	int10
6	MLL-MLLT11*	ex10-ex2	MLL-MLLT11	ex11	int1	MLLT11-MLL	int1	int10
7	MLL-AFF1	ex11-ex4	MLL-AFF1	int11	int3	-	-	-
8	MLL-AFF1	ex11-ex4	MLL-AFF1	int11	int3	-	-	-
9	MLL-MLLT1	ex10-ex1	MLL-MLLT1	int10	*SJ*	-	-	-
10	MLL-MLLT10*	ex9-ex12	MLL-MLLT10	int9	int11	-	-	-
11	MLL-AFF1	ex9-ex4	MLL-AFF1	ex10	int3	-	-	-
12	MLL-MLLT1	ex9-ex4	MLL-NDUFA9-MLLT1	int9	int3	-	-	-
13	MLL-MLLT1	ex11-ex1	MLL-MLLT1	int11	*SJ*			
14	MLL-AFF1	ex9-ex4	MLL-AFF1	int9	int3	AFF1-MLL	int3	int9
15	MLL-MLLT10*	ex10-ex6	MLL-MLLT10	int10	int5	-	-	-
16	MLL-MLLT3	ex9-ex6	MLL-MLLT3	int9	int5	LMO2-MLL	int5	int9
17	MLL-AFF1	ex11-ex4	MLL-AFF1	ex11	int3	AFF1-MLL	int3	ex11

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18	MLL-MLLT1	ex9-ex5	MLL-MLLT1	int9	int4	-	-	-
19	NEGATIVE	NEGATIVE	NEGATIVE	-	-	AFF1-MLL	int10	int8
20	MLL-AFF1	ex12-ex4	MLL-AFF1	int11	int3	AFF1-MLL	int3	int11
21	MLL-AFF1	ex11-ex4	MLL-AFF1	int11	int3	4Q22-MLL	-	int11
22	MLL-MLLT11*	ex9-ex2	MLL-MLLT11	int9	int1	MLLT11-MLL	int1	int9
23	MLL-MLLT10*	ex10-ex11	MLL-MLLT10	int10	int8	-	-	-
24	MLL-MLLT10*	ex10-ex6	MLL-MLLT10	int10	int5	-	-	-
25	MLL-MLLT11*	ex10-ex2	MLL-MLLT11	int10	int1	-	-	-
26	MLL-MLLT4*	ex10-ex2	MLL-MLLT4	int10	int1	-	-	-
27	MLL-AFF1	ex11-ex6	MLL-AFF1	int11	ex5	-	-	-
28	MLL-MLLT3	ex9-ex6	MLL-MLLT3	int9	int5	-	-	-
29	MLL-AFF1	ex11-ex5	MLL-AFF1	int11	ex4	AFF1-MLL	int4	int11
30	MLL-MLLT4*	ex9-ex2	MLL-MLLT4	int9	int1	-	-	-
31	MLL-MLLT6*	ex10-ex7	MLL-MLLT6	int10	int6	-	-	-
32	MLL-MLLT10*	ex9-ex8	MLL-MLLT10	int9	int7	-	-	-
33	MLL-MLLT10*	ex9-ex6	MLL-MLLT10	int9	int5	-	-	-
34	MLL-MLLT3	ex10-ex6	MLL-MLLT3	int10	int5	-	-	-

**Detection based on LDI-PCR results; PG: partner gene; SJ: spliced sequence before MLLT1 exon 1*

Discussion

We report the genetic characterization of *MLL* rearrangements in 34 acute leukemia pediatric patients diagnosed and treated at a single institution in Argentina. *MLL* translocations are present in up to 80% of infant ALL cases and 50% of infant AML cases [19-21]. The presence of *MLL* rearrangements is an independent dismal prognostic factor requiring individualized therapy [3]. Therefore, identifying *MLL* fusions is mandatory for determining the most accurate treatment. Several techniques have been used in diagnosis to characterize this particular group of patients genetically. LDI-PCR was the most helpful technique for this analysis since it allowed the detection of almost any *MLL* rearrangements, particularly in cases where standard karyotyping or RT-PCR analyses failed. Although split-FISH appears to be the most reliable method for detecting MLL rearrangement, false-negative results can occur, and split-FISH alone does not provide information on the translocation partner. Moreover, LDI-PCR can be used when FISH probes are not available. Indeed, LDI-PCR is laborious, time-consuming, and may not detect all rearrangements. Nonetheless, it is the only tool that provides a patient-specific DNA target that can be directly used for designing patient-specific oligonucleotides for MRD analyses, especially in infant cases that usually disclose immature B phenotype and lack Immunoglobulins and T-cell receptors (Ig/TCR) rearrangements [22]. In the present report, *the MLL-AFF1* fusion gene was the predominant *MLL* aberration in infant ALL cases. In AML patients, *MLL-MLLT10* was the most frequently detected fusion transcript, probably due to the limited number of patients. Detection of *MLL-MLLT10* in our series allowed us to define the *MLL* status of one case with normal karyotype (case #24). Moreover, our findings led us to include *MLL-MLLT10* in the initial RT-PCR screening of AML cases without any other demonstrable recurrent abnormalities.

We described a case (#26) with deletion of 11q23 by conventional cytogenetics that showed an *MLL-MLLT4* fusion detected by LDI-PCR in a case not studied by FISH analysis. Two cases (#3 and #10) showed a three-way translocation, and in only one of them, it was possible to detect the 11q23 band alteration by conventional cytogenetics. However, in both cases, *MLL* abnormality was detected by split-FISH, RT-PCR, and LDI-PCR. Spliced fusions observed in two patients were generated by the genomic fusion of the 5'-*MLL* gene upstream of the *MLLT1* gene. In both cases, RT-PCR detected functional *MLL* fusion mRNA in which the 5'*MLL* exon 11 was spliced to exon 2 of the *MLLT1* gene. This is a well-described mechanism for *MLL-MLLT1* fusions [7, 22]. *MLL-MLLT3* is the most frequent rearrangement reported in AML. While limited in number, the high frequency of MLL-MLLT10 in our patients could be due to our focus on infants rather than pediatric patients. We neither found an

association between the recombination site within *MLL* and the immunophenotype or maturation stage of the blasts. Four cases in our series showed a normal karyotype, while FISH analysis in two cases revealed split signals in interphase nuclei. This could be due to a cryptic chromosomal abnormality, a minimal neoplastic clone not detected by cytogenetics, or a failure for cells to reach metaphases from the leukemic cells.

Patients with *MLL* abnormalities generally have poor prognoses and are treated according to high-risk protocols [5, 23]. This agrees with our data since patients in this study had poor survival, regardless of being ALL or AML. Six percent of deaths occurred during the induction phase, and 18% died while in complete remission. This therapy-related mortality is probably related to the high intensity of administered chemotherapies for these particular groups of children. Fourteen patients relapsed, and 11 (32%) remain in continuous complete remission. As expected, no association was found between survival and fusion partner gene involved in rearrangements, in agreement with all *MLL* abnormalities conferring poor prognosis to these particular leukemia cases as stated by Interfant-99 results [3]. MRD quantification by real-time PCR using rearrangements of Ig/TCR as a molecular target is a powerful predictor of outcome in ALL. This helpful tool for MRD determination in most pediatric leukemias may not apply to infant ALL patients because these rearrangements are highly polyclonal and thus not optically suited for qPCR. Even more, the lack of Ig/TCR rearrangements or incomplete Ig/TCR rearrangements can occur. This may be due to the immaturity of MLL-rearranged leukemic blasts [24] and raises the chances that the Ig/TCR rearrangements could be edited or completed during treatment, leading to false negative results in MRD quantification. Characterizing the breakpoint region at the genomic level enables the design of patient-specific probes or primers that recognize the fusion sequence of the leukemic clone. Based on the high frequency of *MLL* rearrangements in the infant population, the fusion gene constitutes an excellent target for MRD monitoring since it is specific for the leukemic clone, stable over time, and allows MRD analysis in cases with undetectable Ig/TCR rearrangements.

Conclusion

We report the molecular characterization of 34 pediatric patients with acute leukemia and *MLL* gene rearrangements treated at a single institution in Argentina. According to our data, RT-PCR allowed the identification of most cases with *MLL* rearrangements but could not completely characterize all cases. LDI-PCR helped detect *MLL* gene rearrangements even when the partner gene was unknown or could not be detected by other techniques. This technique had a good correlation with different methods for detecting *MLL* abnormalities. The high frequency of *MLL-MLLT10* rearrangements found in our AML population changed our diagnostic strategy since we incorporated *MLL-MLLT10* rearrangements in our RT-PCR screening panel. Although LDI-PCR is not known to be a screening technique, it can be used for cases where *MLL* abnormalities are highly suspected. Additionally, LDI-PCR constitutes a valuable diagnostic tool when Split-FISH is not easily available; however, it may not detect *MLL* rearrangements in some cases due to limitations of the technology or chromosomal breakpoints located outside of the *MLL* breakpoint cluster region [10]. Our results highlight the importance of using different and complementary techniques to precisely diagnose pediatric acute leukemia patients. An accurate characterization of *MLL* alterations allows optimal risk stratification and application of the most appropriate treatment for each patient. Additionally, LDI-PCR enables patient-specific MRD monitoring to adapt the corresponding therapy - if necessary - in cases with sub-optimal response to treatment or early relapse detection.

No Conflict Statement

All authors have participated in the planning, execution, and analysis of the study and have approved this version. The authors declare no potential conflicts of interest.

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SUPPLEMENTARY FILES

Supplementary Material S1

Sequences of the oligonucleotides for detection of fusion transcripts by RT-PCR: *MLL-MLLT10*, *MLL-MLLT11*, *MLL-MLLT6*, *MLL-MLLT4* and *MLL-BTBD18*

Primer		Complementary sequence	Sequence (5' → 3')
MLL-A	Fw	MLL exon 8	CCGCCTCAGCCACCTAC
AF10-A	Rv	MLLT10 exon 9	TGGACATTATCGGCACCATTAC
AF10-B	Rv	MLLT10 exon 12	TTGCCCTCTGACCCTCTAGTCT
BTBD18	Rv	BTBD18 exon 3	GCACTTGTTGGTTGTAAGCACT
MLLT4-ex2	Rv	MLLT4 exon 2	AGGACAGCATTCGCATATCAG
MLLT6-ex7	Rv	MLLT6 exon 7	AGATGAAACCACTGCCACCTC
MLLT11-ex2	Rv	MLLT11 exon 2	AGCTCCGACAGATCCAGTTCT

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