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Genetic and clinical characterization of 45 acute leukemia patients with *MLL* gene rearrangements from a single institution

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ABSTRACT

Chromosomal rearrangements affecting the *MLL* gene are associated with high-risk pediatric, adult and therapy-associated acute leukemia. In this study, conventional cytogenetic, fluorescence in situ hybridization, and molecular genetic studies were used to characterize the type and frequency of *MLL* rearrangements in a consecutive series of 45 Portuguese patients with *MLL*-related leukemia treated in a single institution between 1998 and 2011. In the group of patients with acute lymphoblastic leukemia and an identified *MLL* fusion partner, 47% showed the presence of an *MLL*–*AFF1* fusion, as a result of a t(4;11). In the remaining cases, a *MLL*–*MLLT3* (27%), a *MLL*–*MLLT1* (20%), or *MLL*–*MLLT4* (7%) rearrangement was found. The most frequent rearrangement found in patients with acute myeloid leukemia was the *MLL*–*MLLT3* fusion (42%), followed by *MLL*–*MLLT10* (23%), *MLL*–*MLLT1* (8%), *MLL*–*ELL* (8%), *MLL*–*MLLT4* (4%), and *MLL*–*MLLT11* (4%). In three patients, fusions involving *MLL* and a septin family gene (*SEPT2*, *SEPT6*, and *SEPT9*), were identified. The most frequently identified chromosomal rearrangements were reciprocal translocations, but insertions and deletions, some cryptic, were also observed. In our series, patients with *MLL* rearrangements were shown to have a poor prognosis, regardless of leukemia subtype. Interestingly, children with 1 year or less showed a statistically significant better overall survival when compared with both older children and adults. The use of a combined strategy in the initial genetic evaluation of acute leukemia patients allowed us to characterize the

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pattern of *MLL* rearrangements in our institution, including our previous discovery of two novel *MLL* fusion partners, the *SEPT2* and *CT45A2* genes, and a very rare *MLL*–*MLLT4* fusion variant.

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1. Introduction

MLL gene rearrangements are found in more than 70% of the cases of infant leukemia, both acute lymphoblastic leukemia (ALL) or acute myeloid leukemia (AML), but are less frequent in leukemia from older children (Daser and Rabbitts, 2005). *MLL* translocations are also found in approximately 10% of adult AML and in a small proportion of patients with therapy-related leukemia. Independently of their association with other high-risk features at presentation, *MLL* rearrangements are in most cases predictive of poor clinical outcome (Tamai and Inokuchi, 2010).

The *MLL* locus, which maps to 11q23, has been shown by conventional and molecular cytogenetic analysis to be involved in rearrangements up to 100 genetic loci, namely through chromosomal translocations, internal gene duplications, chromosome 11q deletions or inversions, and *MLL* gene insertions into other chromosomes or vice versa (Daser and Rabbitts, 2005; Meyer et al., 2009). Some rearrangements are produced only at the RNA level (spliced fusions) because the recombination occurred 5' of the involved partner gene. To date, 67 loci rearranged with *MLL* have been characterized at the molecular level and the respective fusion partner cloned (Daser and Rabbitts, 2005; Meyer et al., 2009; Cerveira et al., 2011; Coenen et al., 2011). Based on published data, the most frequent fusion partners in ALL, accounting for about 94% of all *MLL*-rearranged cases, are *AFF1*, *MLLT1*, *MLLT3*, and *MLLT10*, whereas in AML the *MLLT3*, *MLLT10*, *ELL*, *MLLT1*, *MLLT6*, and *SEPT6* genes account for nearly 77% of all reported cases (Meyer et al., 2009).

In this study, we report the frequency and type of *MLL* rearrangements present in a consecutive series of 45 patients that were diagnosed with acute leukemia in the Portuguese Oncology Institute, Porto, Portugal, over the last 13 years (1998–2011). These patients with *MLL*-related leukemia were identified as part of the routine diagnostic work-up by conventional and/or molecular cytogenetic analysis of all patients with acute leukemia diagnosed at this institution. In *MLL*-positive cases, this was followed by thorough molecular genetic analysis to characterize the respective fusion partner.

2. Material and methods

2.1. Patients

This study includes a consecutive series of 45 patients with acute leukemia diagnosed and treated at the Portuguese Oncology Institute, Porto, Portugal, which were subsequently shown to have a *MLL* gene rearrangement by fluorescence in situ hybridization (FISH) analysis. Data on patients 1–4 and

12 were previously published (Cerveira et al., 2006, 2008, 2010; Bizarro et al., 2007; Santos et al., 2010).

2.2. Chromosome banding and molecular cytogenetic analyses

The diagnostic bone marrow samples were cultured for 24 h in RPMI 1640 medium with GlutaMAX-I (Invitrogen, London, UK) supplemented with 20% fetal bovine serum (Invitrogen, London, UK). Chromosome preparations were made by standard methods and banded by trypsin-Leishman. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature (Shaffer et al., 2009). FISH analysis for possible *MLL* rearrangement was performed using the LSI *MLL* Dual-Color, Break-Apart Probe (Abbot Molecular/Vysis, Des Plaines, USA) according to the manufacturer's instructions.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from the diagnostic bone marrow sample of all patients using 1 ml of Tripure isolation reagent (Roche Diagnostics, Indianapolis, USA) and quantified in a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, USA). For cDNA synthesis, 1–2 µg of total RNA was subjected to reverse transcription with random hexamers using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, USA), according to the manufacturer's instructions. The final cDNA was diluted with 30 µl of H₂O. cDNA quantity and quality were assessed in a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies).

2.4. Reverse-transcription polymerase chain reaction (RT-PCR)

The RT-PCR assay for detection of *MLL* fusion transcripts was performed with forward primers in *MLL* breakpoint cluster region (BCR) and reverse primers located near the breakpoint junction of the putative fusion partner (Supplementary Table). When necessary, additional primers in (exons 9–13) or outside (exons 15–22) the *MLL* BCR and in the fusion partner open reading frame were used to exclude the presence of additional splice variants or to detect rare rearrangements occurring outside the *MLL* BCR (Supplementary Table). PCRs were performed in a 50 µl reaction volume containing 2 µl of synthesized cDNA, 5 µl of 10× GeneAmp PCR buffer II (100 mM Tris–HCl pH 8.3, 500 mM KCl) (Applied Biosystems, Foster City, USA), 5 µl of 25 mM MgCl₂, 0.4 µl dNTP mix (25 mM each dNTP) (Applied Biosystems), 0.4 mM of each primer (Metabion, Martinsried, Germany), and 1 unit of AmpliTaq Gold DNA Polymerase (Applied Biosystems). Reaction tubes were kept on ice

Table 1 – Clinical data on 45 acute leukemia patients with *MLL* fusions.

Case	Age	Sex	Diagnosis	Previous disease	BM blasts (%)	PB blasts (%)	Hb (g/dL)	Platelets ($\times 10^9/L$)	WBC ($\times 10^9/L$)	Hepato-megaly	Spleno-megaly	Adeno-megaly	CNS involvement	SI	GS	CR	Treatment protocol	BMT	EFS (months)	Survival (months)
1 ^a	6 yr	M	BAL	De novo	51.0	NA	5.4	278.0	1.4	–	–	+	–	–	–	Yes	ELAM 02	Allo	30	33
2 ^a	54 yr	F	t-AML-M4	Breast carcinoma	66.5	2.0	8.4	34.0	4.2	–	–	–	–	–	–	Yes	Mit + Cyt	Allo	0	18
3 ^a	1 yr	F	AML-M2	De novo	82.0	28.0	9.3	81.0	16.4	–	–	–	–	–	–	Yes	ELAM 02	Allo	58	59+
4 ^a	31 yr	M	AML-M0	De novo	79.8	80.0	12.0	15.0	99.0	–	+	–	–	–	–	Yes	Ida + Cyt	Allo	27	28+
5	47 yr	F	t-AML-M5	Papillary thyroid carcinoma	44.0	1.0	9.0	83.0	107.0	–	–	–	–	–	–	No	Palliative only	No	0	4
6	28 yr	M	AML-M5	De novo	88.0	74.0	10.0	30.0	16.0	–	–	–	–	–	–	Yes	Ida + Cyt	Allo	67	68+
7	32 yr	M	AML-M4	De novo	51.0	NA	10.0	251.0	4.0	–	–	–	–	–	+	Yes	Ida + Cyt	Allo	66	67+
8	5 mo	F	B-ALL	De novo	82.5	NA	6.1	13.0	382.7	+	+	–	–	–	–	Yes	INTERFANT 06	Allo	2	27+
9	5 mo	M	B-ALL	De novo	84.5	86.0	5.6	740.0	509.4	–	+	–	–	–	–	Yes	INTERFANT 06	Allo	5	6+
10	54 yr	M	B-ALL	De novo	69.0	13.0	8.4	148.0	1.2	–	–	–	–	–	–	Yes	HCVAD	Allo	30	40
11	10 yr	M	B-ALL	De novo	90.0	96.0	8.5	54.0	119.4	–	–	–	–	–	–	Yes	EORTC 58951	Auto	3	9
12 ^a	1 yr	F	B-ALL	De novo	83.5	44.0	6.6	81.0	79.2	–	–	–	+	–	–	Yes	EORTC 58951	Auto	29	63+
13	63 yr	F	B-ALL	De novo	90.0	41.5	13.4	18.0	21.3	–	+	–	–	–	–	Yes	Stanford	No	80	81+
14	59 yr	F	B-ALL	De novo	90.0	NA	11.2	70.0	100.7	–	–	–	–	–	–	Yes	Cyt + Ida + Eto	No	129	130+
15	14 yr	M	AML-M1	De novo	86.0	47.0	13.9	60.0	19.3	+	+	+	–	–	–	Yes	ELAM 02	Allo	6	11
16	1 yr	F	AML-M5	De novo	93.0	6.0	10.7	152.0	5.4	–	–	–	–	–	–	Yes	ELAM 02	Allo	4	5+
17	45 yr	F	AML-M5	De novo	97.5	66.0	8.0	88.0	16.0	–	–	+	–	–	–	Yes	Ida + Cyt	No	20	37
18	8 yr	F	AML-M5	De novo	93.0	80.0	9.5	30.0	248.6	+	+	–	–	–	–	No	ELAM 02	No	0	5 days
19	2 yr	M	AML-M5	De novo	66.0	78.0	11.9	138.0	34.2	–	–	–	–	–	–	Yes	ELAM 02	Allo	4	5+
20	2 yr	F	AML-M2	De novo	60.0	50.0	9.1	82.0	14.7	+	+	–	–	–	–	Yes	ELAM 02	No	59	60+
21	61 yr	M	t-AML-M5	Multiple myeloma	83.0	93.0	8.6	36.0	65.9	+	+	–	–	–	–	No	ELAM 02	No	0	1+
22	59 yr	M	AMDCL	De novo	81.0	NA	11.6	136.0	1.2	+	–	+	+	+	–	Yes	Ida + Cyt	Allo	11	13
23	68 yr	M	AML-M5	De novo	37.5	NA	8.1	9.0	0.1	–	–	–	–	–	–	No	Ida + Cyt	Allo	0	23
24	6 yr	F	B-ALL	De novo	86.0	NA	10.7	132.0	2.7	–	–	–	+	–	–	Yes	EORTC 58951	Auto	12	13+
25	12 yr	M	B-ALL	De novo	81.0	26.0	8.1	70.0	5.8	+	+	–	–	–	–	Yes	EORTC 58951	No	3	4
26	2 yr	F	B-ALL	De novo	89.3	0.00	7.7	38.0	4.71	+	+	–	–	–	–	Yes	EORTC 58951	Allo	4	40
27	5 mo	M	B-ALL	De novo	90.0	83.0	6.3	26.0	423.2	+	–	–	+	–	–	Yes	INTERFANT 06	Allo	44	48+
28	4 yr	F	AML-M5	De novo	98.0	NA	3.0	56.0	2.7	+	+	+	–	–	–	Yes	ELAM 02	Allo	23	24+
29	47 yr	M	AML-M4	De novo	88.0	70.0	8.0	16.0	19.0	–	–	–	–	–	–	Yes	Ida + Cyt	Allo	10	11
30	49 yr	F	t-AML-M4	Breast carcinoma	87.0	NA	9.0	40.0	1.0	–	–	–	–	–	–	Yes	Ida + Cyt	Allo	15	27
31	4 yr	M	AML-M5	De novo	22.0	20.0	7.6	91.0	11.7	–	–	+	–	–	–	Yes	ELAM 02	No	5	10
32	28 yr	F	AML-M4	De novo	82.7	32.0	7.0	37.0	2.0	–	–	–	–	–	–	Yes	Ida + Cyt	No	2	6
33	51 yr	M	t-AML-M5	Lymphoma	90.8	NA	12.0	28.0	154.0	–	–	–	–	–	–	Yes	Ida + Cyt	No	2	4
34	44 yr	M	AML-M4	De novo	45.5	NA	7.0	64.0	2.0	–	–	–	–	–	–	Yes	Ida + Cyt	No	13	20
35	3 mo	M	B-ALL	De novo	27.0	NA	9.8	400.0	11.2	+	+	–	+	+	–	Yes	INTERFANT 06	No	3	4
36	3 yr	M	T-ALL	De novo	96.8	82.0	8.9	34.0	238.0	+	+	+	–	–	–	Yes	EORTC 58951	No	13	24

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Table 1 – (continued)

Case	Age	Sex	Diagnosis	Previous disease	BM blasts (%)	PB blasts (%)	Hb (g/dL)	Platelets ($\times 10^9/L$)	WBC ($\times 10^9/L$)	Hepato-megaly	Spleno-megaly	Adeno-megaly	CNS involvement	SI	GS	CR	Treatment protocol	BMT	EFS (months)	Survival (months)
37	39 yr	F	AML-M5	De novo	NA	70.0	9.7	19.0	55.3	–	–	–	–	–	–	Yes	Ida + Cyt	No	6	8
38	40 yr	F	AML-M5	De novo	31.0	NA	10.0	124.0	6.0	–	–	+	–	–	–	Yes	Ida + Cyt	No	12	22
39	4 mo	M	B-ALL	De novo	61.0	40.0	8.3	59.0	11.9	+	+	–	+	–	–	Yes	INTERFANT 06	Allo	21	22+
40	61 yr	F	t-AML-M5	Breast carcinoma	90.4	11.0	7.0	27.0	52.0	–	–	–	–	–	–	Yes	Ida + Cyt	No	84	85+
41	11 yr	F	t-AML-M4	Ewing sarcoma	56.0	62.0	7.4	52.0	15.6	–	–	–	–	–	–	Yes	ELAM 02	Allo	42	44+
42	17 yr	F	T-ALL	De novo	94.3	96.0	7.6	26.0	48.4	–	–	–	+	–	–	Yes	HCVAD	No	8	15
43	27 yr	M	AML-M5	De novo	71.0	89.0	9.7	91.0	178.2	–	+	–	–	–	–	No	Ida + Cyt	Allo	0	1+
44	8 mo	F	B-ALL	De novo	21.0	NA	9.8	306.0	5.75	–	+	–	+	+	–	Yes	INTERFANT 06	No	30	31+
45	56 yr	M	AML-M4	Laryngeal carcinoma	27.0	NA	13.0	71.0	3.0	+	–	–	–	–	–	Yes	Mit + Cyt	No	1	2

BAL, biphenotypic acute leukemia; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; AMDCL, acute myeloid dendritic cell leukemia; t-AML, therapy-related acute myeloid leukemia; BM, bone marrow; PB, peripheral blood; Hb, Hemoglobin; WBC, white blood cells; DIVC, disseminated intravascular disease; CNS, central nervous system; SI, skin infiltration; GS, granulocytic sarcoma; CR, complete remission; EFS, event-free survival; NA, not available; Ida, idarubicin; Cyt, cytarabine; Eto, etoposide; Mit, mitoxantrone; Allo, allogeneic bone marrow transplantation; Auto, autologous bone marrow transplantation.

^a Previously published (see main text).

at all times to prevent non-specific amplification and incubated for 5 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 1 min at 56 °C, and 2 min at 72 °C, followed by a final elongation of 7 min at 72 °C on a GeneAmp PCR System 9700 (Applied Biosystems). Amplified products were analyzed on a 2% agarose gel (SeaKem LE Agarose) and the results were visualized in an image analyzer ImageMaster VDS (Amersham Biosciences, Little Chalfont, UK). When necessary, PCR amplimers were isolated from the gel and subjected to DNA sequence analyses to obtain the patient-specific fusion sequences.

2.5. Statistical analysis

Patient survival was evaluated using the Kaplan–Meier method. Differences between groups were assessed with the log-rank test. $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Patient characterization

A male–female ratio of nearly 1:1 (23 males vs. 22 females) was observed, with an age range of 3 months to 68 years (Table 1). Twenty-three patients were children (less than 18 years), including nine with 1 year or less. Twenty-seven patients (60%) had AML (nine children and 18 adults), with the following former FAB classification: 15 patients (five children and 10 adults) with M5 (56% of the cases), eight patients (one child and seven adults) with M4 (30%), two children with M2 (7%), one child with M1 (4%), and one adult with M0 (4%). Sixteen patients (36%) had ALL (13 children and three adults), including 14 patients (11 children and three adults) with B-cell ALL (88%) and two children with T-cell ALL (13%). One adult had acute myeloid dendritic cell leukemia (AMDCL), and one child had biphenotypic acute leukemia (BAL). Eight patients (one child and seven adults) developed secondary, MLL-related leukemia as a result of chemotherapeutic treatment from a previous neoplasia, the most frequent being breast carcinoma (three cases), followed by lymphoma, multiple myeloma, papillary thyroid carcinoma, Ewing sarcoma, and laryngeal carcinoma (one case each).

The majority of the patients had anemia (range 3.0–13.9 g hemoglobin/dL), a low platelet count (range 9.0–740.0 $\times 10^9/L$) and leukocytosis (range 0.1–509.4 $\times 10^9/L$) (Table 1). Splenomegaly was observed in 16 patients (36%), hepatomegaly in 14 cases (31%) and adenomegaly in eight patients (18%). Central nervous system involvement was observed in eight cases (18%), seven children and one adult. Skin involvement and granulocytic sarcoma were very rare events, occurring in three (two children and one adult) and one cases, respectively.

Induction therapy was performed with different treatment schemes (Table 1). One patient (case 5) refused treatment and received palliative care only. Twenty five patients (56%) received an allogeneic or autologous bone marrow transplantation (BMT). At the time of the last observation, patients that received a BMT had a better survival than patients that received chemotherapy alone, with 60% alive in the former group when compared with only 30% in the latter (Table 1).

Table 2 – Karyotype, FISH, and RT-PCR data on 45 hematological malignancy patients with *MLL* fusions.

Case	Karyotype	FISH	MLL fusion	Fused exons
1 ^b	45,XY,add(14)(q24),add(17)(p13),-18[8].ish ins(X;11)(q22-25;q23)(MLL5'+;MLL5'-,MLL3'+), add(14)(IGH-),der(17)(17qter→17p13::?:18q21::?) (BCL2+)/46,XY[12]	FS	MLL-CT45A2	Exon 9/exon 2
2 ^b	46,XX,t(2;11)(q37;q23)[30]	FS	MLL-SEPT2	Exon 10/exon 3
3 ^b	47,X,add(X)(p11),+6,add(11)(q23)[20].ish der(X)add(X)(p11)ins(X;11)(q?:q23q23)(5'MLL+), der(11)ins(11;?)(q22;?)ins(X;11)(5'MLL-,3'MLL+)	FS	MLL-SEPT6	Exon 10/exon 2 Exon 11/exon 2
4 ^b	46,XY,t(11;17)(q23;q25)[13]/46,XY[7]	FS	MLL-SEPT9	Exon 8/exon 2 Exon 8/exon 3
5	46,XX,t(11;19)(q23;p13.1)[22]	FS	MLL-ELL	Exon 10/exon 2
6	NM	FS	MLL-ELL	Exon 10/exon 3 Exon 11/exon 3
7	46,XY,t(1;11)(q21;q23)[18]/46,XY[4]	FS	MLL-MLLT11	Exon 10/exon 1
8	46,XX,t(4;11)(q21;q23)[5]/46,XY[15]	FS	MLL-AFF1	Exon 9/exon 4
9	47,XY,+X,-4,der(11)t(4;11)(q21;q23), +mar[9]/46,XY[11]	FS	MLL-AFF1	Exon 9/exon 4
10	46,XY,t(4;11)(q21;q23)[8]/46,XY[12]	FS	MLL-AFF1	Exon 9/exon 4
11	48,XY,+X,t(4;11)(q21;q23),+8,del(17) (p11)[12]/48,XY,+X,t(4;11)(q21;q23), +8,i(17)(q10)[3]/46,XY[8]	FS	MLL-AFF1	Exon 11/exon 4
12 ^b	46,XX,t(4;11)(q21;q23)[18]/46,XX[12]	FS	MLL-AFF1	Exon 9/exon 6
13	46,XX,t(4;11)(q21;q23)[13]/46,XX[11]	FG	MLL-AFF1	Exon 9/exon 4
14	46,XX,t(4;11)(q21;q23)[29]/46,XX[1]	FS	MLL-AFF1	Exon 9/exon 4
15	46,XY,t(1;6)(p32;p22),t(3;22)(q13.3; q12),t(10;11)(p12;q23)[18].ish der(10)inv(10)(p12p12)t(10;11)(p12; q23)(5'MLL+,3'MLL+),der(11)t(10;11) (p12;q23)(5'MLL-,3'MLL-)[8]	FS	MLL-MLLT10	Exon 9/exon 9
16	46,XX,t(10;11;18)(p12;q14; q21)ins(10;11)(p12;q23q23)[5].ish t(10;11;18)(5'MLL+,5'MLL-,3'MLL-; 3'MLL+)/47,idem,+8[13]/46,XX[2]	FS	MLL-MLLT10	Exon 9/exon 10
17	46,XX[20]	FS	MLL-MLLT10	Exon 10/exon 9
18	46,XX,add(10)(p12)x2,-11,+mar[20].ish der(10)add(10)(p12)ins(10;11)(p12; q23q23)(5'MLL+)x2,der(?) (?:11q23::?:11q23::?)(MLL+) (5'MLL sep 3'MLL)	FSGG	MLL-MLLT10	Exon 8/exon 15
19	46,XY,+1,der(1;15)(q10;q10),add(10) (p12),del(11)(q13q23)[3].ish add(10)(p12)(5'MLL+),del(11)(q13q23) (5'MLL-,3'MLL+)	FS	MLL-MLLT10	Exon 9/exon 7
20	46,XY,+8,add(10)(p11),-11[20].ish add(10)(p11)(5'MLL+)	FG	MLL-MLLT10	Exon 9/exon 15
21	46,XX,t(11;19)(q23;p13.3)[15]/46,XX[15]	FS	MLL-MLLT1	Exon 9/exon 6
22	46,XY,t(11;19)(q23;p13.3), add(12)(p12)[10]/46,XY[10]	FS	MLL-MLLT1	Exon 9/exon 2
23	47,XY,der(2)t(1;2)(q21;q37), +i(8)(q10),t(11;19)(q23;p13.3) [28]/47,idem,+8,-i(8)(q10)[3]	FSS	MLL-MLLT1	Exon 10/exon 2 Exon 11/exon 2
24	NM	FS	MLL-MLLT1	Exon 9/exon 2 Exon 10/exon 2
25	46,XY[20]	FS	MLL-MLLT1	Exon 10/exon 2 Exon 11/exon 2
26	NM	FS	MLL-MLLT1	Exon 10/exon 2 Exon 11/exon 2
27	46,XY[12]	FS	MLL-MLLT3	Exon 10/exon 6 Exon 8–10/exon 6 ^a Exon 11/exon 6
28	47,XX,t(9;11)(p22;q23),del(13)(q12q31),+r[20]	FS	MLL-MLLT3	Exon 10/exon 9
29	46,XY,t(9;11)(p22;q23)[3]	FS	MLL-MLLT3	Exon 10/exon 6 Exon 11/exon 6

(continued on next page)

Table 2 – (continued)

Case	Karyotype	FISH	MLL fusion	Fused exons
30	47,XX,t(9;11)(p22;q23),+der(9)t(9;11)[20]	FSO	MLL–MLLT3	Exon 11/exon 6
31	46,XY,der(9)t(9;11)(p22;q23)t(11;11)(p13;q23),der(11)t(9;11)(p22;q23),der(11)inv(11)(p13p15)t(11;11)(p13;q23)[20]	FS	MLL–MLLT3	Exon 10/exon 6 Exon 11/exon 6 Exon 10/exon 5 Exon 11/exon 5
32	46,XX,t(9;11)(p22;q23)[17]/46,XX[10]	FS	MLL–MLLT3	Exon 8/exon 6 Exon 9/exon 6
33	NM	FS	MLL–MLLT3	Exon 10/exon 6 Exon 8–10/exon 6 ^a Exon 11/exon 6
34	46,XY,t(9;11)(p22;q23)[5]/47,idem,+8[3]	FS	MLL–MLLT3	Exon 8/exon 9 Exon 9/exon 9
35	46,XY,del(7)(q22q31),t(9;11)(p22;q23)[8]/46,XY[12]	FS	MLL–MLLT3	Exon 8/exon 9 Exon 9/exon 9
36	46,XY,t(9;11)(p22;q23)[23]/46,idem,add(1)(p22)[4]/46,XY[3]	FS	MLL–MLLT3	Exon 10/exon 6 Exon 8–10/exon 6 ^a Exon 11/exon 6
37	46,XX,t(9;11)(p22;q23)[30]	FS	MLL–MLLT3	Exon 8/exon 6 Exon 9/exon 6
38	46,XX,t(9;11)(p22;q23)[14]/46,XX[16]	FS	MLL–MLLT3	Exon 10/exon 6 Exon 8–10/exon 6 ^a Exon 11/exon 6
39	46,XY[17]	FG	MLL–MLLT3	Exon 10/exon 6
40	46,XX[8]	FS	MLL–MLLT3	Exon 10/exon 6 Exon 8–10/exon 6 ^a Exon 11/exon 6
41	46,XX,t(9;11)(p22;q23)[2]/45,idem,–7[18]	FS	MLL–MLLT3	Exon 10/exon 6
42	48,XX,add(6)(q13),+10,del(11)(q23),+21,–22,+mar[20].ish der(6)(6pter→6q13::?:11q23→11qter)(3'MLL+),der(11)t(11;?)(q23;?)(5'MLL+,3'MLL–),+mar(5'MLL+)	FSG	MLL–MLLT4	Exon 23/exon 2
43	NM	FS	MLL–MLLT4	Exon 8/exon 2 Exon 9/exon 2
44	NM	FS	NA	NA
45	57,XY,add(11)(q23),+12,+18,inc[2]	FS	NA	NA

a Exon 8–10/exon 6, represents an mRNA fusion with skipping of MLL exon 9; NM, no metaphases; FS, fusion signal and split signal; FG, fusion signal and green signal; FSGG, fusion signal, split signal and two extra green signals; FSS, fusion signal and two split signals; FSO, fusion signal, split signal and an extra orange signal; FSG, fusion signal, split signal and an extra green signal; NA, not available.

b Previously published (see main text).

3.2. Genetic characterization

3.2.1. Conventional and molecular cytogenetics

Thirty-eight patients (84%) exhibited a simple split signal resulting from separation of the 3' and the 5' MLL probes, whereas the remaining cases showed more complex FISH patterns (Table 2). Cases 13, 20 and 39 revealed loss of the 3' MLL probe, whilst cases 18 and 42 had gain of the 5' MLL probe together with the split signal. Case 30, besides the split signal, also showed gain of 3' probe and case 23 had gain of both 3' and 5' probes.

In eight cases (17.8%), metaphase FISH was used to determine the chromosomal location of the MLL probes. Accordingly, in cases 1, 3, 15, 16, 18, 19, 20 and 42, FISH results were used for a more complete description of the karyotype (Table 2).

Bone marrow samples from all the 45 patients were subjected to conventional cytogenetic analyses (Table 2). In six

cases the karyotype could not be established and in five patients a normal karyotype was found. In the remaining 34 cases, the most frequently observed chromosomal rearrangements were reciprocal chromosomal translocations (27 patients), the majority being recurrent translocations allowing to presume the MLL gene partner. Among these, the most frequent chromosomal bands involved were 9p22 (11 cases) and 4q21 (seven cases). In case 31, in which a reciprocal translocation between chromosomes 9 and 11 was found, additional rearrangements of both chromosomes 9 and 11 were also observed. Chromosome 19 breakpoints were observed in four cases involving the sub-bands 19p13.1 (one case) and 19p13.3 (three cases). Three patients (cases 2, 4, and 7) presented with the rare translocations t(2;11)(q37;q23), t(11;17)(q23;q25) and t(1;11)(q21;q23), respectively. In one patient (case 15) a reciprocal translocation between chromosomes 10 and 11 was found, with FISH analysis revealing a concomitant inversion of chromosome 10. Another patient

(case 16) was found to have a complex three-way translocation involving chromosomes 10, 11, and 18, with FISH analysis showing the presence of 11q23 material inserted in the short arm of chromosome 10. In two patients (cases 19 and 42) a partial deletion of the long arm of chromosome 11 was observed (case 42 shown in Figure 1). Two patients (cases 3 and 45) presented with additional chromosomal material of unknown origin in the long arm of chromosome 11 and in the remaining three patients (cases 1, 18, and 20) no karyotypic evidence of an 11q23 rearrangement could be detected.

3.2.2. Molecular genetics

All *MLL* fusions identified in our series of pediatric and adult leukemia patients are summarized in Table 2. In two cases (patients 44 and 45), no *MLL* gene fusion could be identified by RT-PCR analysis for the most frequent *MLL* fusion partners. A subsequently performed Long-Distance-Inverse PCR (LDI-PCR) analysis also failed to identify the putative fusion

partner gene (results not shown). In the remaining 43 patients (96%) we could identify the fusion partner, the most common being the *MLLT3*, *AFF1*, *MLLT1*, *MLLT10*, *ELL*, and *MLLT4* genes, accounting for 88% of all cases. In two of these patients (cases 1 and 20) the fusion partner was first identified by LDI-PCR, with the fusion subsequently confirmed at the RNA level by RT-PCR.

In ALL, the most frequent rearrangement was the *MLL*–*AFF1* gene fusion (7/15 cases, 47%). All adult ALL patients presented the *MLL*–*AFF1* fusion (three cases), whereas in the 12 pediatric patients the distribution was as follows: *MLL*–*AFF1* (four cases, 33%), *MLL*–*MLLT3* (four cases, 33%), *MLL*–*MLLT1* (three cases, 25%), and *MLL*–*MLLT4* (one case, 8%).

In the 26 AML patients, 42% of all characterized *MLL* gene fusions involved the *MLLT3* gene (11 cases), followed by *MLLT10* (six cases, 23%), and *ELL* and *MLLT1* (two cases each, 8%). The *MLL*–*MLLT3* rearrangement was more frequent in adult (eight cases, 47%) than in pediatric (three cases, 33%)

Figure 1 – Cyto-genetic and molecular analysis of the bone marrow sample of a T-ALL patient with a rare *MLL*–*MLLT4* fusion variant (case 42). (A) Karyogram showing, besides a partial deletion of the long arm of chromosome 11, additional material of unknown origin on chromosome 6, trisomy of chromosomes 10 and 21, monosomy of chromosome 22, and a marker chromosome. (B) The same metaphase after G-banding and after destaining and processed for dual-color FISH analysis, showing the presence of the 3' *MLL* (red) in the add(6), the 5' *MLL* (green) in the abnormal chromosome 11, and an extra signal of the 5' *MLL* on the marker chromosome. (C) RT-PCR analysis with one *MLLT4* antisense primer located in *MLLT4* exon 2 and six *MLL* sense primers located in *MLL* exons 8, 15, 16, 17, 21, and 22 (lanes 1–6), showing the presence of a fusion between *MLL* exon 23 and *MLLT4* exon 2, that was confirmed by sequencing analysis (D). Lane 7 – 100 bp molecular marker. See [Supplementary Table](#) for details.

AML. In contrast, *MLL*–*MLLT10* was the most prevalent rearrangement in pediatric AML (four cases, 44%), but rare in adult patients (two cases, 12%). Two patients (one pediatric and one adult) presented with an *MLL*–*MLLT1* gene fusion. The *MLL*–*MLLT1* rearrangement could also be found in one adult patient with AMDCL. The *ELL* gene was found to be rearranged in two adult patients but not in pediatric cases. This was also the case of the *MLL*–*MLLT4* and *MLL*–*MLLT11* rearrangements, which were detected only in adult AML patients (one case each). The remaining three cases (two adults and one child) presented with rearrangements of the septin family of genes (*SEPT2*, *SEPT6*, and *SEPT9*), one of them (*SEPT2*) previously characterized by our group as a novel *MLL* fusion partner (Cerveira et al., 2006). Finally, the member of the Cancer/Testis (CT) gene family CT45A2 was also described by our group as a novel *MLL* fusion partner in a pediatric patient with BAL (Cerveira et al., 2010).

Breakpoint distribution analysis within *MLL* BCR revealed that in ALL patients breaks occurred predominantly in *MLL* intron 9 (seven cases, 47%), mostly in *MLL*–*AFF1* cases (six out of seven cases) (Table 2). In contrast with adult ALL cases, in which breaks occur solely in *MLL* intron 9, in pediatric patients *MLL* recombined equally in *MLL* introns 9 and 11 (four cases each), followed by *MLL* intron 10 (three cases). In *MLL*–*AFF1* pediatric patients the breaks predominate in *MLL* intron 9 (three out of four cases), in opposition to *MLL*–*MLLT1* and *MLL*–*MLLT3* fusions in which the breaks were more frequent in *MLL* intron 11. In one pediatric T-ALL patient the karyotype suggested a rearrangement involving chromosome 6q, where the *MLLT4* gene is located, but no *MLL*–*MLLT4* gene fusion could be detected when using *MLL* primers located in the *MLL* BCR. One of the hypotheses was that the break occurred outside the *MLL* BCR, so additional primers outside that region were designed. With this approach, a very rare break in *MLL* intron 23 was observed, generating a variant *MLL*–*MLLT4* gene fusion (Figure 1). The resulting fusion gene displays a fused open reading frame because intron 23 of the *MLL* gene is compatible with intron 2 of the *MLLT4* gene (see Suppl Figure 1 of Meyer et al., 2009).

AML patients showed a higher frequency of recombination events affecting *MLL* introns 9 and 11 (nine cases each), followed *MLL* introns 10 (six cases) and 8 (two cases) (Table 2). In pediatric patients breaks occurred more frequently in *MLL* intron 9 (four cases), followed by *MLL* introns 10 and 11 (two cases each) and 8 (one case), whereas in adult patients the breaks predominate in *MLL* introns 11 (seven cases) and 9 (five cases), followed by *MLL* introns 10 (four cases) and 8 (one case). Intron 11 breaks were mostly detected in AML *MLL*–*MLLT3* patients (six cases, one child and five adults), but could also be observed in two adult AML patients with *MLL*–*ELL* or *MLL*–*MLLT1* rearrangements, and in the only case of pediatric *MLL*–*SEPT6*. In addition, *MLL*–*MLLT3* AML cases also presented breaks in *MLL* introns 9 and 10 (three and two cases, respectively). None of the five *MLL*–*MLLT10* cases showed a break in *MLL* intron 11, with the breaks occurring more frequently in *MLL* intron 9 (four cases), followed by *MLL* introns 8 and 10 (one case each). Rearrangements affecting *MLL* intron 10 were also observed in the two patients with the *MLL*–*SEPT2* or *MLL*–*MLLT11* fusions. In the remaining four AML patients the breaks were mapped to *MLL* introns 8

(*MLL*–*SEPT9*), 9 (*MLL*–*MLLT1* and *MLL*–*MLLT4*), and 10 (*MLL*–*ELL*).

Finally, the adult patient with *MLL*–*MLLT1* AMDCL showed a rearrangement involving *MLL* intron 9. This breakpoint was the same as the one detected in the pediatric *MLL*–*CT45A2* BAL patient, but in this particular case the rearrangement was the result of a spliced gene fusion, with the breakpoint being located in the upstream region of the CT45A2 gene (Cerveira et al., 2010).

3.3. Prognostic significance of age and *MLL* rearrangements

At the time of writing, complete remission was achieved in 89% of the patients (40 out of 45 cases), but at the time of last observation 53% of the patients (24 out of 45) were deceased (Table 1). Survival data on selected patient subsets are represented in Table 3 and Figure 2. Although not statistically significant, a better overall survival was observed in patients with the *MLL*–*AFF1* rearrangement when compared with patients with other *MLL* rearrangements. This was also the case of patients with de novo disease or ALL when compared with patients with therapy-related disease or AML, respectively. On the other hand, patients with the *MLL*–*MLLT3* rearrangement showed a worse overall survival when compared with patients with other *MLL* rearrangements, although the difference has no statistical significance. Infant patients (≤ 1 year old) showed a statistically significant better overall

Table 3 – Survival data (5 years) on the 45 acute leukemia patients with *MLL* gene rearrangements.

	Median survival (months)	95% CI	P
<i>MLLT3</i> vs. Other			
<i>MLLT3</i>	22	6.5–37.5	0.286
Other	40	30.9–49.1	
<i>AFF1</i> vs. Other			
<i>AFF1</i>	Not reached		0.115
Other	23	14.3–31.7	
Previous disease			
De novo	33	18.7–47.3	0.375
Therapy-related	18	0.1–53.9	
Diagnosis			
AML	22	14.3–29.7	0.263
ALL	40	18.1–61.9	
<18 yr vs. ≥ 18 yr			
<18 yr	40	16.3–63.8	0.343
≥ 18 yr	22	15.4–28.6	
≤ 1 yr vs. >1 and <18 yr			
≤ 1 yr	Not reached		0.040
>1 and <18 yr	24	3.5–44.5	
≤ 1 yr vs. ≥ 18 yr			
≤ 1 yr	Not reached		0.036
≥ 18 yr	22	15.4–28.6	

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia.

Figure 2 – Kaplan–Meier plots illustrating overall 5 year survival according to diagnostic subset (A) and patient age subsets (B and C). (A) Acute lymphoblastic leukemia (ALL) vs. acute myeloid leukemia (AML) ($p = 0.263$); (B) Children (<18 years) vs. adult (≥ 18 years) ($p = 0.343$); (C) Infant (≤ 1 year) vs. children (>1 and <18 years) ($p = 0.040$), and infant (≤ 1 year) vs. adult (≥ 18 years) ($p = 0.036$).

survival when compared with both older children (>1 and <18 years old; $p = 0.040$) and adults ($p = 0.036$).

4. Discussion

In this study we report the clinical and genetic characterization of 45 Portuguese patients with acute leukemia and a MLL gene rearrangement treated at a single institution. According to our data, the most frequent MLL fusion partners in Portuguese patients with MLL-related acute leukemia are MLLT3, AFF1, MLLT1, MLLT10, ELL, and MLLT4, which are responsible for about 88% of all identified rearrangements. These results are in agreement with previously published larger series (Meyer et al., 2006, 2009) and indicates that laboratories that use RT-PCR as the only diagnostic method for the presence of MLL rearrangements will identify the vast majority of cases with MLL gene rearrangements by just testing for those few partner genes (Meyer et al., 2005). However, current recommendations indicate the systematic use of FISH as an initial approach in the evaluation of patients with acute leukemia, since it allows the detection also of rare MLL fusions or cases with an unknown MLL fusion partner (Meyer et al., 2009; De Braekeleer et al., 2011). In our series, this approach led to the identification of two novel MLL fusion partner genes: the SEPT2 gene, a member of the Septin gene family (Cerveira et al., 2006), and the CT45A2 gene, a member of the Cancer/Testis antigen family (Cerveira et al., 2010). Two other patients also presented MLL fusions with other septin family genes (SEPT6 and SEPT9), making the septins the gene family with more members involved in MLL rearrangements in our series. Two other members of the Septin gene family, SEPT5 and SEPT11, were also previously identified as MLL fusion partners, suggesting a relevant role of septins in leukemia induced by MLL–SEPT fusions (Cerveira et al., 2011). Moreover, a very rare MLL–MLLT4 fusion variant involving MLL exon 23 and MLLT4 exon 2 was also characterized in a pediatric T-ALL patient in the present study. To our knowledge, this is second case of a MLL exon 23 MLLT4 exon 2 fusion described in the literature and, interestingly, also in a T-ALL patient (Hayette et al., 2002). Breaks outside the MLL BCR seem to be a common feature of MLL rearranged T-ALL, and additional cases have been described with breaks in MLL exons 7 (MLL–MLLT10) and 21 (MLL–MLLT4) (Hayette et al., 2002), but the biological significance, if any, of this findings remains to be elucidated. As the break in MLL intron 23 is clearly outside the MLL BCR (introns 8–13), this fusion would be missed by a standard RT-PCR approach, highlighting the need for the combination of several techniques in the genetic characterization of these patients. However, even when both RT-PCR and LDI-PCR were used, no fusion partner could be identified in two patients. RT-PCR analysis has as the main drawback the need for the previous knowledge (or suspicion) of the putative fusion partner involved in the rearrangement. LDI-PCR, although an excellent method for the identification of unknown and known translocation partner genes and the establishment of patient-specific MLL fusion sequences, has several limitations, namely the size of amplicons or the fact that the chromosomal breakpoints of some der(11) alleles can be located outside of the MLL BCR (Meyer et al., 2005).

The *MLLT3* gene was the most frequent fusion partner detected both in adult and pediatric patients in our series, followed by the *AFF1* gene. This is in contrast with published data, in which the most frequently observed *MLL* fusion partner is *AFF1* followed by *MLLT3* (Meyer et al., 2006, 2009). This finding can be explained not only by the limited number of patients in our series but also by the higher frequency of AML in our series when compared with the literature. Indeed, whereas the frequency of fusions involving the *MLLT3* gene is higher in AML patients, rearrangements involving the *AFF1* gene predominate in ALL patients (Meyer et al., 2009). When the data is analyzed by leukemia lineage, our results are in accordance with published data, with *AFF1* and *MLLT3* being most frequently rearranged in ALL and AML, respectively. The higher frequency of AML in our series may reflect not only the size of our series but also a bias in the referral of patients to our institute.

Regarding breakpoint distribution, pediatric ALL patients have their chromosomal breakpoints in *MLL* introns 9 or 11, whereas adult ALL patients recombine more frequently in *MLL* intron 9. Pediatric AML patients show a preference for recombination events affecting *MLL* intron 9, whereas adult AML patients show a preference for rearrangements involving *MLL* intron 11. This is in line with published data, with the exception of adult AML patients that have previously been shown to recombine preferentially in *MLL* intron 9 (Meyer et al., 2009). However, the observed discrepancy can be explained by the over-representation of AML *MLL*–*MLLT3* patients in our series. In fact, these patients have previously been shown to be particularly prone to rearrangements involving *MLL* intron 11 (Meyer et al., 2009).

In general, patients with *MLL* rearrangements have a poor prognosis and are treated according to high-risk protocols (Biondi et al., 2000; Schoch et al., 2003; Harrison et al., 2010; Tamai and Inokuchi, 2010; Muntean and Hess, 2012). This is in agreement with our results, since the majority of the patients had poor survival, regardless of being ALL or AML, with 53% dead at the time of the last observation, with a mean follow-up of 57 months. No comparisons between treatment protocol and survival could be made with the literature because our series includes different leukemia subtypes treated with distinct treatment regimens. However, patients that received bone marrow transplantation showed better survival than those that did not. A significantly better overall survival was previously observed in *MLL* patients with de novo vs. therapy-related leukemia (Schoch et al., 2003). In our series we observed a trend for better overall survival in patients with de novo leukemia, although this study lacks statistical power to evaluate this matter due to the small number of patients (eight) with therapy-related leukemia. On the other hand, there are conflicting reports regarding the impact on prognosis of the *MLL*–*MLLT3* fusion, with several authors claiming a better prognosis for patients with this fusion (Mrózek et al., 1997; Rubnitz et al., 2002; von Neuhoﬀ et al., 2010) and others not finding any prognostic impact (Schoch et al., 2003; Balgobind et al., 2009). In our series, we did not observe a better overall survival for patients with *MLL*–*MLLT3* fusions, as a statistically insignificant trend toward worst overall survival was seen. Conversely, our

patients with *MLL*–*AFF1* seem to perform better in terms of overall survival when compared with patients with other fusions, although this was not statistically significant. Children seem to perform better than adults, with 57% alive at the time of the last observation when compared with only 36% of adult patients. Interestingly, this value rises to 89% in the group of children with 1 year or less (infants). As a consequence, in our series infants with *MLL*-related leukemia have statistically significant better overall survival when compared with both other children and adults. This seems in opposition to published data reporting that, despite recent improvements in treatment outcome, infants with *MLL*-related leukemia, in particular with the *MLL*–*AFF1* gene fusion, have a dismal prognosis (Pui and Campana, 2007; Muntean and Hess, 2012). However, due to the heterogeneity observed in our infant series regarding morphology (seven ALL and two AML) and *MLL* fusion partner (three *MLL*–*AFF1*, three *MLL*–*MLLT3*, one *MLL*–*SEPT6*, one *MLL*–*MLLT10*, and one unidentified *MLL* rearrangement), these results should be confirmed in a larger series of patients and over a longer follow-up.

With a frequency of 79%, reciprocal chromosomal translocations were the most frequent chromosomal rearrangements observed in the group of patients with an abnormal karyotype, whereas rearrangements of the long or short arms of chromosome 11 (deletions and inversions) and complex *MLL* rearrangements requiring three or more DNA double-strand breaks were very rare, detected in only two cases each. This is in agreement with previous published data in larger series, in which reciprocal chromosomal translocations are the predominant recombination event, followed by insertions, inversions and deletions (Meyer et al., 2009). In one patient (case 1) in which no karyotypic evidence of an 11q23 rearrangement could be detected, molecular analysis showed the presence of a spliced *MLL* fusion as a result of an insertion event (Cerveira et al., 2010). Spliced fusions are only generated at the RNA level, and can occur either by transcriptional read-through followed by a subsequent splice event or by trans-splicing (Meyer et al., 2005). In our series, five patients showed the presence of a normal karyotype, which can have two main interpretations. Since cells were cultured prior to analysis, this could be explained by the presence of non-neoplastic cells in the sample; alternatively if the studied cells are truly neoplastic, a chromosomal abnormality that is below the resolution level of cytogenetic analysis may be present (Gisselsson, 2009). In six cases the karyotype could not be established. Failure of cell culture can be related to several issues, including inadequate cell density ($<10^6$ cells/ml), bacterial overgrowth, or lack of viable cells in the sample.

In conclusion, our results support the use of FISH analysis as the initial screening method for the presence of *MLL* gene abnormalities in patients with acute leukemia. However, this initial approach should be systematically complemented with karyotype analysis that, although not informative in all cases, is nevertheless very useful in guiding molecular genetic analysis for the identification of the fusion partner. This, in turn, is crucial for an adequate patient follow-up through detection of minimal residual disease by RT-PCR after successful induction therapy.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.molonc.2012.06.004](https://doi.org/10.1016/j.molonc.2012.06.004).

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