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Clinical and molecular epidemiology of neonatal leukemia in Brazil

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Abstract

The clinical and molecular findings of 77 cases of neonatal leukemia (NL) and 380 of infant leukemia (IL) were selected to distinguish features between NL and IL. Somatic gene mutations associated with acute leukemia including *FLT3*, *RAS* and *PTPN11* were revisited. There were 42 cases of congenital leukemia associated with Down syndrome (DS) and 39 of these cases presented features of acute myeloid leukemia (AML)-M7. Twenty-seven of the DS cases underwent spontaneous remission and were reclassified as a transient myeloproliferative disorder. *GATA1* mutations were found in 70% of these cases. In non-DS, frequent abnormalities were *MLL* rearrangements, mainly *MLL–AFF1* in acute lymphoblastic leukemia and *MLL–MLLT3* in AML. The *FLT3* mutation was not found, while *RAS* ($n = 4$) and *PTPN11* ($n = 2$) mutations were identified and reported for the first time in NL. There was substantial evidence to support that somatic abnormalities occur *in utero*. Thus, congenital leukemia is a good model for understanding leukemogenesis.

Keywords: Congenital acute lymphoblastic leukemia, acute myeloid leukemia, *FLT3*, *KNRAS*, *PTPN11*, *MLL*, *GATA1*

Introduction

Congenital or neonatal leukemia (NL) is diagnosed up to 31 days after birth and is a very rare malignancy that occurs at a rate of 1 per 5 million live births in developed countries. It represents less than 1% of all childhood leukemia [1]. Acute myeloid leukemia (AML) is more common than acute lymphoblastic leukemia (ALL) in NL [2,3]. Complete characterization of the worldwide NL cohort is lacking, likely because many patient descriptions have been published as isolated case reports. In low- and middle-income countries, there is no clear picture of NL occurrence due to unstable case documentation or a lack of proper investigations for leukemia. Nevertheless, since 1999 in Brazil, three basic treatment tenets have been established for pediatric oncology improvements: (i) the recognition of neoplasia at an early age with immunomolecular diagnostic procedures; (ii) public access to treatment protocols and health care to assist children with long-term treatments; and (iii) extensive pediatric oncology training [4]. As a result, the recognition of NL has improved over time.

In neonates, ALL is characterized by a trend toward a higher incidence of *MLL* gene rearrangements (*MLL*-r), whereas in AML the subtypes are diverse, and more studies are needed to determine consistent and specific associations in neonates with and without Down syndrome (DS) [5]. The etiology of congenital leukemia is still unknown. However, some factors may be associated with the development of a leukemia clone during the fetal period and chromosomal instability. The remarkably brief preclinical features associated with the somatic mutation profile of hematopoietic cells may be a unique *in vivo* model for correlating cooperative molecular events in the malignant pathways. This study aimed to add to the clinical and molecular findings in NL, which may lead to a better understanding of the diverse pathogenesis mechanisms started during fetal life.

Materials and methods

Subjects

The Brazilian Collaborative Study Group of Infant Leukemia (BCSGIAL) included patients from a network of hospitals located in different Brazilian states. Its purpose was to confirm the immunophenotypic and molecular findings in children younger than 24 months old diagnosed with acute leukemia [4,6]. Thirty onco-centers from different Brazilian cities enrolled patients in the BCSGIAL. Of the 457 cases enrolled, 77 (17%) neonates (younger than 1 month of

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life) and 380 (83%) infants (age ≥ 32 days up to 12 months after birth) were selected for the present study (shown in Table I). Myeloid leukemias (AML) with DS were also included. Data collection and laboratory procedures at diagnosis were evaluated and approved by the Ethics Committee of all participating institutions. Data analysis was approved by the Research Ethics Committee, Instituto Nacional de Câncer (CEP #024/10; CONEP # 707/2010).

Leukemia diagnosis

ALL and AML diagnoses were first established through morphological examinations according to standard criteria, followed by immunophenotyping and molecular tests. ALL was categorized as B-cell precursor ALL (Bcp-ALL) based on the immunophenotyping profile, and the CD10 status was determined. AML was defined by the World Health Organization (WHO) classification [7]. The immunophenotype was assessed by flow cytometry with a panel of monoclonal antibodies, using a FACSCalibur or FACSCanto (Becton, Dickinson and Company, San Jose, CA) machine as described previously [8].

Banding cytogenetics and FISH

The karyotypes of leukemic cells were obtained at the time of diagnosis prior to any treatment using a standard G-band cytogenetic technique. Chromosomes were identified and analyzed in accordance with the International System of Human Cytogenetic Nomenclature 2005 [9]. To detect possible cryptic and/or fusion gene translocations, fluorescence *in situ* hybridization (FISH) was performed to complement conventional cytogenetic analysis [10].

Molecular tests

Genomic DNA and total RNA were isolated from mononuclear cells isolated from bone marrow samples with a QIAamp® DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) or TRIzol reagent kit (Invitrogen, Carlsbad, CA), respectively.

Detection of recurrent fusion genes

Detection of somatic fusion genes commonly associated with the major childhood ALL subtypes (*ETV6-RUNX1*, *TCF3-PBX1*, *BCR-ABL1*, *MLL*-r) and with AML (*RUNX1-RUNX1T1*, *CBFB-MYH11*, *MLL*-r) was systematically performed by reverse transcriptase polymerase chain reaction (RT-PCR) according to standard techniques. Detection of *MLL*-r was performed by conventional cytogenetics, RT-PCR (*MLL-AFF1*, *MLL-MLLT3*, *MLL-MLLT1ENL*) and/or by fluorescence *in situ* hybridization (FISH) as previously described [6,11].

Detection of *GATA1* mutations

In samples from neonates with DS, *GATA1* mutations were analyzed by direct sequencing in diagnostic samples prior to any treatment and when there were more than 10% blast cells. Genomic DNA was extracted from bone marrow (BM) aspirate or peripheral blood (PB) cells following standard protocols. Exon 2 of *GATA1* was amplified by PCR using the

Table I. Demographic and clinical features of neonatal compared with infant leukemia, Brazil 1990–2013*.

Feature	All leukemias, *n* (%) (*n* = 457)	Neonatal leukemias, *n* (%) (*n* = 77)	Infant leukemias, *n* (%) (*n* = 380)	*p*-Value†
Gender				
Female	219 (48)	38 (49)	181 (48)	0.783
Male	238 (52)	39 (51)	199 (52)	
Type of leukemia				
ALL	270 (59)	26 (34)	244 (64)	< 0.001
AML	187 (41)	51 (66)	136 (36)	
Down syndrome				
Yes	53 (12)	42 (55)	11 (3)	< 0.001
No	404 (88)	35 (45)	369 (97)	
Initial findings				
Hepatomegaly	315 (69)	44 (57)	271 (71)	0.014
Splenomegaly	331 (72)	47 (61)	284 (75)	0.014
Enlarged nodes	126 (28)	10 (13)	116 (31)	0.002
CNS involvement	85 (19)	11 (14)	74 (20)	0.286
Leukemia cutis/chloroma	27 (6)	09 (12)	18 (5)	0.030
Therapy‡				
No	35 (12)	28 (49)	7 (3)	< 0.001
Yes	247 (88)	29 (51)	218 (97)	
Status‡				
Alive	188 (48)	33 (47)	155 (48)	0.933
Dead	207 (52)	37 (53)	170 (52)	
		Median (min-max)	Median (min-max)	
WBC count ($\times 10^9$/L)	53 (1-913)	63 (2-730)	56 (1-913)	0.601
Hemoglobin (g/dL)	7 (2-50)	12 (4-24)	7 (2-50)	< 0.001
Platelets ($\times 10^9$/L)	54 (5-568)	57 (159-490)	49 (5-568)	0.437
Blasts (%)	63 (0-100)	60 (0-100)	80 (0-100)	0.374

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CNS, central nervous system; WBC, white blood cell.
*Results not shown in table: one biphenotypic case (both lymphoid and myeloid markers) (age 0-1 month), one biphenotypic case (both lymphoid and myeloid markers) (age 1-12 months), two cases of juvenile myelomonocytic leukemia (JMML) (age 1-12 months).
†*p*-Value corresponds to neonatal leukemia vs. infant leukemia.
‡Differences due to missing values.

primers 2F = GTCCTCGCAGGTTAATCCCC, 2R = GCCAAG GATCTCCATGGCAACCC [12]. PCR products were purified using a GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, UK) and sequenced in both directions with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA) using a 3500 Genetic Analyzer (Applied Biosystems). Sequence analysis was performed with BioEdit 7.0.9 software, comparing the obtained electropherograms with the reference sequence for *GATA1* (GenBank NM_002049) [12].

Detection of *FLT3* mutations

The presence of *FLT3* mutations was screened according to methods previously described [13]. Briefly, *FLT3*-D835 mutations were detected by restriction fragment polymorphism-mediated PCR assay and *FLT3*-internal tandem duplication (ITD) was examined by amplification of the JM domain in exons 11 and 12. Samples with altered pattern were sequenced. PCR products were purified with a GFX PCR DNA and Gel Band Purification kit (GE Healthcare) and sequenced in both directions with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) using a 3500 Genetic Analyzer (Applied Biosystems). Sequence analysis was performed with BioEdit 7.0.9 software, comparing electropherograms with the reference sequence for *FLT3*, which was accessed from the National Center for Biotechnology Information (NCBI) (NG_007066.1; NM_004119.2; NP_004110.2).

Detection of *KRAS* and *NRAS* mutations

Genomic DNA was screened for mutations in exon 1 (codons 12 and 13) of the *KRAS* and *NRAS* genes (denoted *K/NRAS*) with PCR amplification followed by direct sequencing [14,15], comparing electropherograms with the reference sequences of *KRAS* (NG_007524.1; NM_004985.3; NP_004976.2) and *NRAS* (NG_007572.1; NM_002524.4; NP_002515.1).

Detection of *PTPN11* mutations

Exon 3 of *PTPN11* was screened for mutations by PCR amplification [16], followed by direct sequencing, comparing electropherograms with the reference sequence of *PTPN11* which was accessed from the NCBI (NG_007459.1; NM_002834.3; NP_002825.3).

Treatments

The children recruited in the study received different chemotherapy treatments in accordance with the Brazilian AML and ALL Berlin–Frankfurt–Münster (BFM) treatment protocol group (GBTLI-ALL93, GBTLI-ALL99) or Interfant-based protocols [17–19].

Statistical analysis

The χ^2-test (two-sided) was used (or Fisher's exact test when expected values were fewer than five) to compare the frequency of neonates and infants (children less than 12 months of age), as well as distinct variables in the neonate group (gender, ALL versus AML, clinical findings). p-Values < 0.05 were considered statistically significant. For a subset of patients ($n = 318$) with a minimum of 2 years' follow-up, the Kaplan–Meier method was used to estimate survival rates [20], and differences were compared with a two-sided log-rank test [21]. All statistical analyses were performed using the Statistical Product and Services Solutions statistical package, version 18.0 (SPSS Inc., Chicago, IL).

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Results

Differences and similarities in the demographics and clinical features of 77 neonates and 380 infant leukemia (IL) cohorts, from 1990 to 2013, are provided in Table I. There was a similar distribution regarding gender (ratio males:females, 0.93) in both the NL and IL groups. Differences were observed in the leukemia subtypes, as AML was more frequent than ALL in neonates, and ALL was more frequent than AML in infants ($p < 0.001$). Forty-two (55%) of the neonates had DS ($p < 0.001$). There were statistically significant differences in clinical signs, such as hepatosplenomegaly, enlarged nodes and leukemia cutis/chloroma ($p = 0.014$, 0.002, 0.030, respectively). Thirty-five children were not treated with conventional chemotherapy, the majority from the NL group ($p < 0.001$). For all NL and IL, white blood cell counts (WBCs) ranged from 1 to 913×10^9/L (median 53), hemoglobin levels from 2 to 50 g/dL (median 7) and platelet counts from 5 to 568×10^9/L (median 54). Absolute blast counts varied, with a median value of 63. Differences were observed in hemoglobin levels between the two groups ($p < 0.001$).

Because clonal transient myeloproliferative disorder (TMD) in newborns with DS is a separate entity, the clinical characteristics and biological findings of 77 patients with NL with or without DS are shown in Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.938327. There was a predominance of NL with DS and AML characteristics ($p < 0.001$). Newborns with DS and non-DS showed the same frequencies of leukemia cutis and/or chloroma. Statistically significant differences were observed for hepatomegaly, splenomegaly, enlarged nodes and central nervous system involvement ($p = 0.021$, < 0.001, < 0.001, 0.001, respectively). The WBCs and hemoglobin levels were statistically different in DS and non-DS. WBCs ranged from 3 to 270×10^9/L (median 30) and from 7 to 730×10^9/L (median 120), and hemoglobin levels from 8 to 24 g/dL (median 13) and from 4 to 14 g/dL (median 10) ($p < 0.001$), respectively.

The most frequent ALL subtype was CD10 − pro-B ALL. Amongst the AML characteristics, the megakaryoblastic (AML-M7) subtype was the most frequent (52%), followed by AML-M4 and -M5 (12%). Myelomonocytic leukemia was positive for CD13/CD33, CD14 and CD117, and AML-M7 was positive for CD41/61/42. Other than constitutive trisomy 21, the most common chromosomal

somatic aberrations were *MLL*-r in 71% of ALL and 57% of AML (excluding neonates with DS) and *MLL–AFF1* in ALL. The most frequent abnormal fusion gene in AML was *MLL–MLLT3*. Less frequent chromosomal alterations were hyperdiploidy and t(2;19)(q31;q13.2) and t(8;21)(q22;q22), which generate the *NUP98–BCL3* and *RUNX1–RUNX1T1* fusion genes, respectively. Additionally, submicroscopic mutations such as *PTPN11* ($n = 1$; 8%) and *RAS* ($n = 4$; 21%) were found. All of these mutations were found in *MLL*-r cases. No mutation was found in the *FLT3* gene.

As DS was associated with TMD, the clinical and laboratory findings were analyzed separately. The time of diagnosis ranged from 3 to 31 days, with a mean age of 8 days and median age of 14 days. Males, white children and AML-M7 blast cell morphology and immunophenotype ($n = 39$) predominated. *GATA1* mutations were found in 20 of the 29 (70%) cases tested. All mutations in exon 2 were recurrent, inserted as a frame shift that results in a stop codon. The spontaneous remission observed in more than 50% of the cases of NL was documented as a TMD. The initial diagnosis of AML remained in five cases, because the NL was treated due to persistence of blast cells and hepatic failure. Death rates were calculated separately in congenital leukemia with and without DS. The frequency of mortality in acute leukemia in neonates without DS was 65.7%, whereas it was 35.9% in neonates with DS ($p = 0.001$). The relative risk of 0.39, 95% confidence interval (CI), 0.20–0.75, conferred a protection factor on death risk in neonates with DS.

The 5-year overall survival (OS) after diagnosis according to NL subtypes in DS and non-DS is shown in Figure 1. The results were significantly better for patients with DS than non-DS. The median OS for non-DS patients with ALL and AML was 6.2 months (95% CI, 3.3–9.2) and 0.3 months (95% CI, 0–1.0), whereas the median in patients with DS with TMD or ML-DS (myeloid leukemia) was not reached. The log-rank test showed a significant difference ($p < 0.001$).

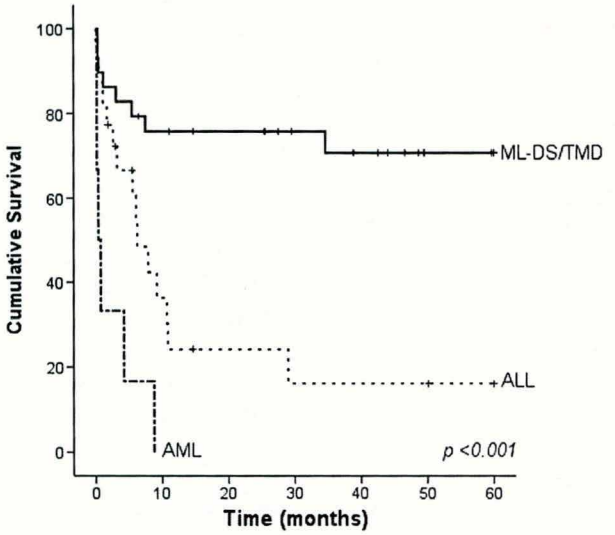

Figure 1. Overall survival of neonatal (age ≤ 31 days) leukemia subtypes, Brazil 1990–2011. Median OS for non-DS patients with ALL and AML was worse than for patients with DS. Log-rank test showed significant difference ($p < 0.001$). ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia (in patient without Down syndrome [DS]); TMD, transient myeloproliferative disorder; ML-DS, myeloid leukemia in patient with DS.

Discussion

Both medicine-based evidence and a descriptive analysis of rare diseases are important for understanding malignancies. Ten years ago, Hart Isaacs published a very extensive literature review (dating back to the 1970s) which included clinical and laboratory (cytology, molecular and cytogenetic) findings of congenital leukemia. Almost at the same time, Bresters *et al.* reported the Dutch experience along with a review of the literature [2,3]. These descriptive analyses included a huge number of cases of NL and included enough data to determine the differences in these subsets. Neonatal CD10 − Bcp-ALL is the most common subtype (in non-DS), and AML is more frequent in newborns with DS, whereas T-cell ALL is rare. Furthermore, the *MLL–AFF1* fusion gene is the most frequent genetic abnormality found in non-DS cases of NL.

In both the Bresters 2002 and Isaacs 2003 reviews, spontaneous remission of TMD was observed for patients with DS [2,3]. Since then, new cases of congenital leukemia have been reported, revisited and updated with new insights (Table II [2,3,22–34]). Cases of TMD have been better characterized, and are positively associated with trisomy 21 (DS) and somatic *GATA1* mutations [35]. Nowadays, it is known that *GATA1* mutations are required for the development of TMD. Recently, van der Linden *et al.* recommended that the diagnosis and management of NL should be distinguished from TMD-DS and other clinical conditions that mimic leukemoid reactions [5]. The cases of NL in our series were divided into two subgroups: (i) neonates without DS or any other genetic syndrome ($n = 35$) and (ii) neonates with DS ($n = 42$). In this study, we included both groups, which comprised an unselected series of congenital leukemia with Bcp-ALL, AML without DS and TMD, which were analyzed separately. The groups are representative of a heterogeneous population. Our analyses took into account the clinical and demographic characteristics of the patients, which showed similar distributions to those commonly observed in previous reported series [2,3,5,31]. One of the major concerns with the recognition of NL is the differential diagnosis to exclude congenital infections, vaccine reaction, hemolysis and primary immune deficiency. For example, the immunization schedule recommended by the Brazilian Ministry of Health includes vaccination of newborns with bacillus Calmette–Guérin (BCG, single dose) and the first dose of hepatitis B while still in the maternity ward, and occasionally the child will present adverse reactions [36]. All of these conditions present similar clinical signs, including leukocytosis and circulating immature cells, and involve the natural immunity system; thus, there are overlapping clinical signs with NL. It is recommended that immunophenotyping, cytogenetics and molecular analyses should always be performed in neonates with leukemoid reactions, to distinguish these entities from other serious diseases that affect this population [37]. *MLL* gene rearrangements, most commonly with *AFF1* and *MLLT3*, were observed in

Table II. Results of systematic review of recent data in the literature, including Brazilian cases, 1970–2013.

Leukemia subtype	Type of study	Period	No. of cases	Karyotype and/or molecular result	*n* (%)	Reference
ALL + AML	Review	1970–2002	55/145	Cytogenetic abnormalities	115 (79)	Issacs, 2003 [3]
			81/145	With *MLL*-r	42 (37)	
				t(4;11) (q21;q23)	11 (10)	
				t(9;11) (p22;q23)	7 (6)	
				t(11;19)(q23;p13)	17 (15)	
				Other	7 (6)	
				t(11;22) (p13;q13)	8 (7)	
ALL	Single center experience		1	t(4;11) (q21;q23)	1	Prigogina *et al.*, 1979 [29]
ALL + AML	Single center experience	1975–2000	04/15	*MLL*-r	14 (20)	Bresters *et al.*, 2002 [2]
			11/15	t(4;11) (q21;q23)	5 (7)	
				t(9;11) (p22;q23)	2 (3)	
				t(11;19)(q23;p13)	7 (10)	
ALL	Clinical trial	1999–2005	30	*MLL* germ line	2 (7)	van der Linden *et al.*, 2009 [31]
				MLL-r	25 (93)	
				t(4;11) (q21;q23)	12 (48)	
				t(9;11) (p22;q23)	1 (4)	
				t(11;19)(q23;p13)	8 (32)	
				Other *MLL* translocations	2 (8)	
				Undefined *MLL* translocations	2 (8)	
AML	Clinical trial	1988–1990	2	t(l;22)(pl3;q13)	2	Carroll *et al.*, 1991 [23]
ALL	Case report	Un	2/4	*FLT3* mutations	2	Chang *et al.*, 2013 [24]
				MLL-r	3	
				t(4;11) (q21;q23)	2	
				t(11;19)(q23;p13)	3	
Mixed phenotype	Case report	Un	1	*MLL* germ line	0	Ergin *et al.*, 2013 [26]
AML	Case report	Un	1	t(11;19)(q23;p13)	1	Borkhardt *et al.*, 2003 [22]
ALL	Case report	Un	1	t(4;11) (q13;q22)	1	Van den Berghe *et al.*, 1979 [30]
ALL	Case report	Un	1	t(4;11) (q21;q23)	1	Frontanes *et al.*, 2012 [28]
AML	Case report	Un	1	t(11;19)(q23;p13.1)	1	Chantrain *et al.*, 2009 [25]
AML	Case report	Un	1	t(8;16)(p11;p13) *MYST3–CREBBP*	1	Wong *et al.*, 2008 [32]
AML	Case report	Un	1	46,XY,t(6;17)(q23;q11.2)	1	Ferguson *et al.*, 2005 [27]
ALL + AML	Retrospective study	Un	3/443	*KRAS/NRAS*	3	Liang *et al.*, 2006 [33]
ALL	Clinical trial	Un	1/109	*KRAS/NRAS*	1	Driessen *et al.*, 2013 [34]
AML + ALL	Retrospective study	1990–2013	77/457	*MLL*-r	29 (68)	Present report
				RUNX1/RUNX1T1	2 (7)	
				KRAS/NRAS	4 (21)	
				GATA1	20 (70)	

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; *MLL*-r, *MLL* gene rearrangement; Un, unknown.

ALL (71%) and AML (57%) without DS. One of the 21 cases of ALL presented with hyperdiploidy. This numerical chromosomal abnormality has not been described in other NL studies, although hyperdiploidy has been identified in neonatal blood spots, providing definitive evidence that this chromosomal event occurred before birth [38]. One case of AML presented with the novel cytogenetic abnormality t(12;19)(q31;q13.2), which has not been reported in NL. The fusion transcript *RUNX1–RUNX1T1* was identified in another case of AML. Despite the rarity of these findings, it is likely that they arose *in utero*.

The *MLL* fusion gene is sufficient in itself to cause leukemia, which may explain the difference between the frequencies of *MLL*-r and those of other abnormalities; in addition, essential secondary mutations may be rapidly acquired, which is especially important if the protein product has an effect on chromatin structure or the stability of gene expression [39]. The disruptive effect of *MLL* gene fusion on DNA repair or cell-cycle regulation could facilitate the rapid acquisition of additional, genetic changes, particularly when continuously exposed to genotoxic substances during fetal hematopoiesis [40]. Gilliland suggested that additional genetic changes in the cell signaling pathway of the clone with abnormal fusion genes are required, mainly throughout kinase activation, to provide proliferative advantage to abnormal cells [41]. Since then, candidate genes in the Ras/mitogen activated protein (MAP) kinase signaling pathway have been constantly examined. In the present study, *KRAS* mutations ($n = 4$) were found in NL, as well as *PTPN11* mutations (8%), suggesting that such mutations act as cooperative leukemogenic events along with recurrent fusion genes. *KRAS* mutations have been previously found in NL, especially in those cases with *MLL*-r [33,34]. Although somatic mutations in *FLT3* were recently identified using whole exome sequencing of samples from patients with congenital ALL, we failed to demonstrate *FLT3* mutations in our NL series [24].

Neonates with DS frequently have a TMD, with clonal proliferation of immature myeloid-megakaryocytic cells due to *GATA1* mutations (reviewed in [42]). However, TMD is self-limiting, and proliferation decreases spontaneously without leukemia-specific treatment. *GATA1* encodes a transcription factor essential for erythroid and megakaryocyte differentiation. The majority of *GATA1* mutations prevent synthesis of full-length GATA1, leading to a truncated protein, GATA1s [43]. A total of 20 (70%) mutations in the *GATA1* gene were identified in this cohort of DS neonates. This lower frequency compared to previous studies [44] may be explained by technical issues, which include limitations of the screening method and blast cell proportion at the beginning of the analyzed samples, discussed in detail previously

[12]. *GATA1* mutations were exclusively found in TMD and/or AML-M7. The pathogenesis of TMD-DS and development to true AML-M7 are still unclear, and the estimation of the *GATA1* expression level at diagnosis in the neonate period may be a predictive factor.

As in all retrospective analyses, there are some negative and positive points that should be discussed. The weakness of this study is that we might have missed important variables in the broad spectrum of some NL subtypes at the time of the diagnosis, or missed unrecognized cases during the period of study. However, the strong point of the study is the increased recognition of NL over time, and the clear, defined diagnosis and treatment of TMD-DS. In addition, molecular abnormalities other than *MLL*-r and *GATA1* mutations, such as *RAS* and *PTPN11* mutations, were identified and reported.

In conclusion, molecular aberrations are highly frequent among cases of NL. These critical somatic abnormalities that occur in early hematopoietic precursors *in utero* are good models to demonstrate the distinct set of gene mutations that explain leukemogenesis.

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Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

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Supplementary material available online

Supplementary Table I showing characteristics of neonates.