Analysis of *GATA1* mutations and leukemogenesis in newborns with Down syndrome

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ABSTRACT. It has been reported that patients with Down syndrome (DS) frequently develop transient myeloproliferative disorder (TMD) and less commonly myeloid leukemia in DS (ML-DS). We examined the pathogenetic relationship of these conditions with somatic mutations of the *GATA1* gene in children with both TMD and ML-DS. To determine the incidence of *GATA1* mutations in a cohort of DS patients and the applicability of these mutations as a clonal marker to detect minimal residual disease, we screened 198 samples of 169 patients with DS for mutations in *GATA1* exon 2 by direct sequencing. Novel mutations were detected in four of the 169 DS patients (2 with TMD and 2 with ML-DS). We examined spontaneous remission and response to therapy in TMD and ML-DS patients and concluded that

these mutations can be used as stable markers in PCR analysis to monitor these events.

Key words: Down syndrome; Transient myeloproliferative disorder; *GATA1* mutation; Myeloid leukemia in DS; Minimal residual disease

INTRODUCTION

Individuals with Down syndrome (DS) have a higher probability of developing childhood acute leukemia. The condition of patients with DS has therefore stimulated a special interest in studies on leukemogenesis not only due to the unusually high prevalence of myeloid leukemia in DS (ML-DS) in children with DS (Lange, 2000; Zipursky, 2003) but also because of another clonal proliferation called transient myeloproliferative disorder (TMD), which affects 4 to 10% of newborns with DS (Zipursky et al., 1997; Pine et al., 2007). In about 60% of cases, TMD resolves spontaneously within the first 3 months of life (Massey et al., 2006; Kanezaki et al., 2010). However, approximately 20% of children diagnosed with TMD will develop ML-DS, after 2 to 3 years of TMD spontaneous remission, which does not regress without chemotherapy (Mundschau et al., 2003; Cabelof et al., 2009).

Acquired mutations in exon 2 of the hematopoietic transcription factor *GATA1* mapped at Xp11.23 are consistently present in the affected cells of children with TMD and ML-DS, leading to expression of N-terminally truncated GATA1s protein (Wechsler et al., 2002; Cabelof et al., 2009). These *GATA1* alterations are somatic and are therefore no longer detectable in remission samples (Ahmed et al., 2004). When TMD progresses to ML-DS, the same *GATA1* mutation is usually present in blasts of both, showing their clonal relationship (Hitzler and Zipursky, 2005). Alford et al. (2011) reported that the type of *GATA1* mutation is not prognostic for which patients with TMD will progress to ML-DS, since no difference is seen in types of mutation between patients with TMD and with ML-DS. Due to the fact that there is no way to predict which patient with TMD will develop ML-DS or which patient with ML-DS will relapse after chemotherapy, the somatic mutation of each patient may be used as a stable molecular target for minimal residual disease (MRD) to monitor the resolution of TMD and the therapy response in ML-DS patients (Hitzler and Zipursky, 2005; Pine et al., 2005; Alford et al., 2011).

The aims of the present study were to determine the presence of somatic mutations in exon 2 of the hematopoietic transcription factor *GATA1* gene in children with DS in two types of clonal proliferation, TMD and ML-DS, to consolidate it as a MRD molecular marker, and to determine the frequency of TMD in a cohort of DS children.

MATERIAL AND METHODS

Subjects

The reference population in this study was made up of all DS newborns and children of both sexes under four months old, with no clinical findings of TMD and ML-DS, who were seen at the Outpatient Department of Clinical Genetics, Hospital Universitário de Brasília (HUB) and the Center for Genetics (NuGEN), Hospital de Apoio de Brasília (HAB), from February 2008

to June 2011. These two public centers gather the entirety of the patients diagnosed with DS, coming from Brazil's Federal District and surrounding areas. DS children who presented with a picture of leukemia were followed by the staff of the Center for Pediatric Oncology and Hematology of the Department of State and Federal District Health (NOHP-SESDF), based in HAB, which is the regional benchmark in public health treatment of childhood leukemias.

One hundred and eighty-three peripheral blood samples (PB) corresponding to a total of 168 patients under four months old were analyzed. We included in this study a patient who presented with clinical signs of ML-DS at age one year and six months. A previous sample collected at age one year and one month for cytogenetic analysis was also tested. The other 29 samples corresponded to a follow-up of the patients with clinical suspicion of leukemia at different collection dates. Bone marrow (BM) samples were obtained for follow-up of six patients with clinical characterization of leukemia at different collection dates for a total of 15 samples. An age at diagnosis of less than four months was arbitrarily decided so as not to miss any possible TMD patient.

This study was approved by the ethics committees of Brasilia University Faculty of Medicine (UnB-FM 034/2008) and Secretary State for Health (FEPECS 034/2009). In all cases, informed consent was obtained from the parents in concordance with the Declaration of Helsinki.

Cytogenetic analysis was performed for every case by GTG banding according to standard procedures.

Analysis of *GATA1* mutations

Genomic DNA was extracted from PB and BM using the Genomic Blood DNA Purification kit (Amersham Biosciences) or PureLink Genomic DNA kit (Invitrogen).

Polymerase chain reaction (PCR) of the *GATA1* exon 2 was carried out using primers PR307 (5'-TGAGGTGATGGAGTGGGAGGAGG-3') and PR310 (5'-GGTCGGCACATCC ATTTGAGAAGC-3') (479-bp amplicon). PCR was performed on an iCycler™ thermocycler (Bio-Rad) in a reaction mixture containing 50 ng chromosomal DNA and 10 pmol each primer. PCR conditions were: 35 cycles of 30 s at 94°C, 60 s at 64°C and 60 s at 72°C, initiated by 5 min of denaturation at 95°C, and completed by 7 min of final extension at 72°C. Negative controls were included in all experiments. After agarose gel electrophoresis analysis, PCR products were purified and sequenced.

Sequencing was performed directly on purified PCR products using internal primers: PR351 (5'-GAAGGATTTCTGTGTCTGAGG-3') and PR366 (5'-CAATGCCAAGACAGCC ACTC-3') on an ABI 3730XL automatic DNA sequencer (Applied Biosystems). Sequencher 4.9 software was used for the sequence analysis, and the electropherograms obtained were compared with the reference sequence for *GATA1* (GenBank NM_002049).

To accurately determine the size and location of the mutations from patients 1 and 2, purified PCR products were cloned into a pGEM-T vector system (Promega). Fifteen and thirty-one recombinant clones from patients 1 and 2, respectively, were selected by digestion with *Sac*II and *Nde*I restriction enzymes and submitted to sequencing.

Detection of minimal residual disease (MRD)

Two patients with *GATA1* mutations were monitored for MRD by PCR using muta-

tion-specific oligonucleotides for each *GATA1* mutation. a) Samples from patient 1 with TMD were submitted to multiplex PCR with primers PR349 (5'-CACTGGCCTACACTCCCCAGGTA-3') and PR310 (156-bp amplicon) and control *GAPDH* gene with primers PR194 (5'-CCCATC ACCATCTTCCAGG-3') and PR195 (5'-AGTGAGCTTCCCGTTCAGC-3') (472-bp ampli-con). b) Samples from patient 2 with ML-DS were submitted to nested PCR with primers PR307 and PR382 (5'-GCAGCTGCAGCAGGCCAGTGC-3') (318-bp amplicon). The PCR analysis was performed as described above. In the TMD patient, the relative pixel intensity in grayscale at 156-bp PCR/472-bp GAPDH ratio was measured by ImageJ software to confirm the decrease in the mutated population.

RESULTS

Among the 168 DS patients under four months old evaluated, with no clinical findings of TMD and ML-DS, 92 (54.8%) were male and 76 (45.2%) were female, whose ages at the time of diagnosis ranged between 1 day and 3 months and 26 days, with a mean age of 26.5 days and median of 14 days. A subset of only neonates was represented by 125 (74%) patients, with a mean age of 12.7 days and median of 10 days. Cytogenetic analysis showed that 159 (94.6%) had simple trisomy 21 (47,XY,+21 or 47,XX,+21) and nine (5.4%) were identified with Robertsonian translocations [46,XY,+21,rob(21:21)(q10;q10) or 46,XY,+21,rob(21:21)(q10;q10)]. No case of mosaicism was identified.

To detect *GATA1* mutations in exon 2, where more than 95% of all mutations occur, samples from all DS patients were analyzed by direct sequencing of the purified PCR products. The detection rate of *GATA1* mutations in exon 2 was 2.36%, corresponding to four out of 169 DS patients as shown in Table 1. All mutations identified were novel and inserted a frameshift with premature translation termination, possibly resulting in the expression of GATA1s as a consequence of an alternative translation initiation site.

Table 1. *GATA1* mutations in DS patients with transient myeloproliferative disorder (TMD) and myeloid leukemia in Down syndrome (ML-DS).

Patient	Gender	Age	Diagnose	Protein alteration	Mutation	Karyotype
Patient 1	M	18 days	TMD	p.Tyr63fs66	c.187_211delTACAGGGACGCTGAGGCCTACAGAC	47,XY,+21
Patient 2	M	2 years 7 months	ML-DS	p.Ala62fs13	c.185_206dupGCTGCAGCTGCGGCACTGGCCT	47,XY,del(5)(p13~14), del(6)(q?),+21[12]/ 47,XY,+21[46]
Patient 3	F	7 days	TMD	p.Thr53fs82	c.160_166delACCGCTGinsGTA	47,XX,+21
Patient 4	M	1 year 1 month 7 days	ML-DS	p.Asp20fs117	c.58delG	47,XY,+21

Considering only DS newborns with 30 days of life or less, the frequency of *GATA1* mutations was 1.6%, two out of 125 DS newborns. In two patients, both four days old, who presented with hematological abnormalities of TMD, we did not identify any *GATA1* muta-tions. Considering these two TMD patients and those two with TMD and *GATA1* mutations (patients 1 and 3), the frequency of TMD would be 3.2% (four out of 125 DS newborns).

Patients 1 and 3 (Table 1) were characterized as TMD, and patient 1 was further monitored for MRD by multiplex PCR in four sequential samples (PB and BM): 18 days, 26 days, 1 month 3 days and 1 month 17 days (Figure 1A). The quantification of relative pixel intensity of the bands from the agarose gel showed a decrease in *GATA1* mutation in relation

to the GAPDH control as shown in Figure 1B. This probably corresponded to a decrease in the *GATA1* mutated population, and progression to spontaneous remission. The complete remission could not be documented as the patient died as a consequence of congenital heart disease at the age of three months.

Patient 2 arrived at the genetics clinic at the age of three months and was underdiagnosed for TMD. However, at the age of one year and eight months, he returned with suspicion of leukemia. Approximately five months later, cytogenetic analysis showed clonal alteration at BM aspirate that confirmed the diagnosis of ML-DS (Figure 2). PCR analysis of 16 samples (PB and BM) obtained at different collection dates, revealed multiple bands on the agarose gel (Figure 3). After direct PCR sequencing of the eight initial samples, from 3 months 2 days until 2 years 1 month 4 days, no mutation was detected. The PCR product from the sample of two years and six months was cloned, and after sequencing, a 22-bp duplication was detected in the recombinant clones. Using a specific primer for this duplication in a nested PCR, we monitored all 16 samples for MRD and observed the persistence of the *GATA1* mutation in all samples, including the first ones collected prior to ML-DS manifestation (Figure 3).
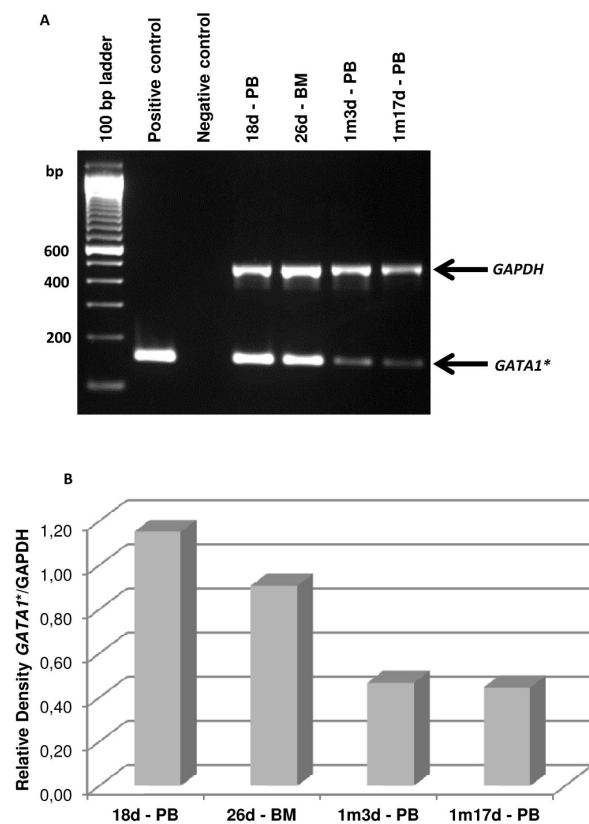
Figure 1. Monitoring spontaneous remission of transient myeloproliferative disorder in Down syndrome patient 1. **A.** Multiplex PCR analysis of MRD from samples (PB and BM) at different ages. GATA1* = *GATA1* mutation specific amplicon (156 bp); GAPDH = internal control (472 bp). **B.** Relative quantification of GATA1 mutation in relation to GAPDH, showing the progression to spontaneous remission.
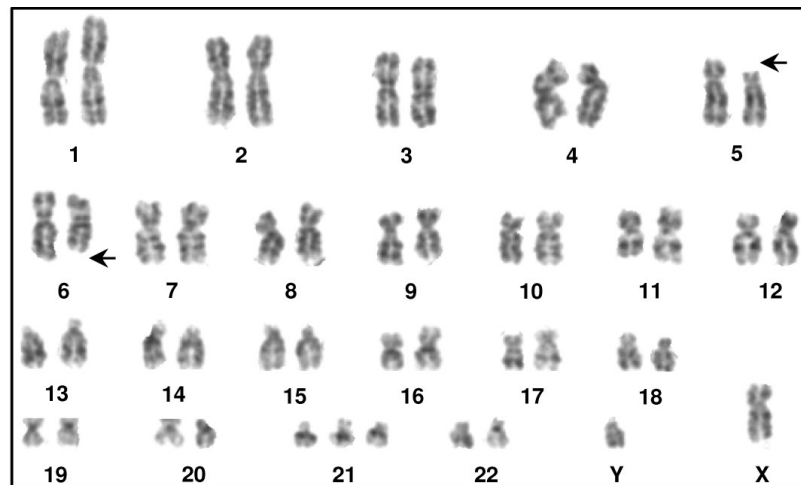
Figure 2. Cytogenetic analysis from Down syndrome patient 2 with clonal alteration at BM aspirate. Karyotype: 47,XY,del(5)(p13~14), del(6)(q?),+21[12]/47,XY,+21[46].
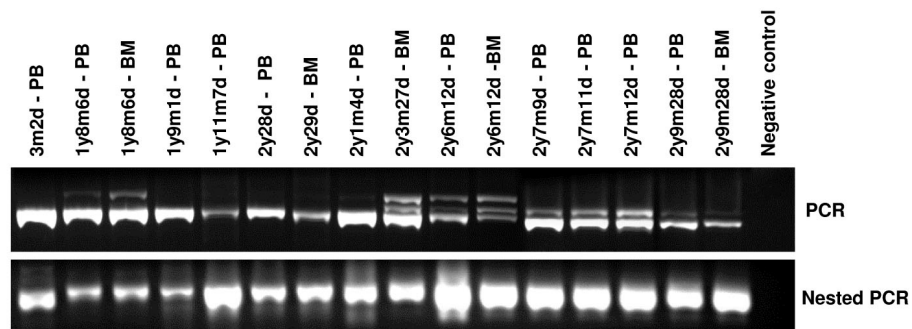
Figure 3. *GATA1* mutation detected in Down syndrome (DS) patient 2 with myeloid leukemia in DS. Sixteen samples (PB and BM) in chronological order since 3 months and 2 days until 2 years and 9 months and 28 days. PCR analysis of *GATA1* exon 2 (479-bp fragment) showed multiple clones with higher molecular weight. After identification of 22-bp duplication in *GATA1* exon 2, the therapy response was monitored by nested PCR, showing the 318-bp fragment in all samples, which represented poor prognostic of MRD.

Patient 4 was referred to us at age one year one month and six days, to confirm the DS diagnosis. Five months later, he presented with hematological abnormalities and ML-DS was suspected. The identification of the mutation corroborated the diagnosis.

DISCUSSION

Screening for *GATA1* mutations in DS newborns without clinical findings of TMD have been reported by Ahmed et al. (2004) and Pine et al. (2007) using PCR direct sequencing. Here, we screened 168 DS patients without clinical manifestations of TMD and one DS

patient with suspicion of ML-DS for mutations in exon 2 of *GATA1*. We detected four novel mutations indicative of TMD and ML-DS, corresponding to a frequency of 2.36% (four out of 169). Considering only the newborns, under 30 days of life, the frequency of mutations was 1.6% (two out of 125). Previous studies by Zipursky et al. (1997), Ahmed et al. (2004) and Pine et al. (2007) showed higher TMD frequencies of 10.4% (eight out of 77), 9.5% (two out of 21) and 3.8% (22 out of 585), respectively. However, if we include in the frequency the two TMD patients in whom we failed to identify a *GATA1* mutation, the frequency would be 3.2% (four out of 125 DS newborns), close to the frequency published by Pine et al. (2007).

In two DS patients (patients 1 and 3), *GATA1* mutations were detected in samples collected in the first weeks of life (Table 1) before any hematological evidence of TMD. This finding supports the literature data that the peak of TMD occurs in the second and third weeks of life (Lange, 2000; Malinge et al., 2009). Patient 1 showed clinical signs of leukemia at 26 days of life, eight days after the first visit at genetic center for DS diagnose. Patient 3 did not return to the genetics clinic for follow-up.

Most children with DS are referred for cytogenetic studies soon after birth and screening for *GATA1* mutations should be done at that time. In our study, we included 44 patients with ages between 31 days and 3 months 26 days and did not identify mutations in any of them. However, patient 2 presented with ML-DS at age 2 years 29 days, but we could detect the mutation using specific primers in a sample collected at 3 months. This case is particularly interesting because he never entered complete remission after treatment for ML-DS, and the detection of mutated clones at age three months indicated that he did not achieve spontaneous remission of TMD either, as shown in Figure 3.

Our failure to detect a *GATA1* mutation in the two DS newborns who were later TMD diagnosed by clinical signs of hematologic abnormalities and in the eight initial samples of patient 2 may reflect the rarity of the mutant clone, which results in the low sensitivity to detect a low blast count by direct sequencing. Alford et al. (2011) also reported failure to detect *GATA1* mutations in 16 patients with TMD (12%) due to low blast count. They documented 0.5% as the lower limit of blasts for successful mutation detection. However, they recorded one patient (blast count 42%) in whom detection of mutation failed, suggesting an uncommon mutation involving sequence outside the genomic area, spanning the PCR, or a deletion inside this area, affecting the primer annealing site.

According to Alford et al. (2011), direct sequencing of DNA from blasts submitted to FACS was successful in identifying sequence mutation in patients, whereas direct sequencing of DNA from unfractionated samples failed. In some cases, it was necessary to subclone the *GATA1* PCR product to pinpoint mutation sequence. They suggested that high-resolution melt analysis would be more sensitive in cases that failed to detect mutation.

Despite the low frequency of *GATA1* mutations detected, all mutations identified here were insertion/deletion/duplications, all resulting in a premature stop codon. These data are consistent with the frequencies of 78% by Alford et al. (2011), 74% by Cabelof et al. (2009), 83% by Hitzler et al. (2003) and 100% by Wechsler et al. (2002).

We had the opportunity to evaluate a simple tool in monitoring the development of MRD in TMD and ML-DS patients (Figures 1 and 3) and to confirm that blasts are stable clonal markers suitable for the measurement of MRD. Several authors have suggested the use of quantitative PCR for monitoring MRD, but here, we demonstrate that even semi-quantitative PCR can be used due to the high frequency of mutant clones.

Only one case of clonal alteration in ML-DS patient 2 was identified by cytogenetic analysis. According to cytogenetic data in neonates with TMD reported by Alford et al. (2011), no significant correlation was observed between not progressing to ML-DS and presenting with trisomy 21 as the only cytogenetic abnormality. Therefore, karyotype does not predict patients who will progress to ML-DS. In the present analysis, simple molecular techniques were shown to be an ideal tool for detecting and monitoring *GATA1* mutation, while cytogenetic studies contributed to identifying numerical and structural abnormalities.

The frequency of *GATA1* mutations in newborns in this and previous studies is probably highly underestimated due to the inability of the methods to detect low frequency clones. New methods such as next generation sequencing with high coverage may shed light on the true frequency of *GATA1* mutations and give new insights into DS leukemogenesis.

On the basis of our observations, we recommend that all DS newborns be screened routinely in clinical evaluations to detect *GATA1* mutations early, thereby determining the real frequency of TMD and allowing follow-up of these patients for possible progression to ML-DS by MRD monitoring in TMD and subsequently in therapy response of ML-DS patients.

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