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GATA1 mutations in a cohort of Malaysian children with Down syndrome-associated myeloid disorder

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INTRODUCTION Children with Down syndrome (DS) are at increased risk of developing distinctive clonal myeloid disorders, including transient abnormal myelopoiesis (TAM) and myeloid leukaemia of DS (ML-DS). TAM connotes a spontaneously resolving congenital myeloproliferative state observed in 10%–20% of DS newborns. Following varying intervals of apparent remission, a proportion of children with TAM progress to develop ML-DS in early childhood. Therefore, TAM and ML-DS represent a biological continuum. Both disorders are characterised by recurring truncating somatic mutations of the *GATA1* gene, which are considered key pathogenetic events.

METHODS We herein report, to our knowledge, the first observation on the frequency and nature of *GATA1* gene mutations in a cohort of Malaysian children with DS-associated TAM (n = 9) and ML-DS (n = 24) encountered successively over a period of five years at a national referral centre.

RESULTS Of the 29 patients who underwent *GATA1* analysis, *GATA1* mutations were observed in 15 (51.7%) patients, including 6 (75.0%) out of 8 patients with TAM, and 9 (42.9%) of 21 patients with ML-DS. All identified mutations were located in exon 2 and the majority were sequence-terminating insertions or deletions (66.7%), including several hitherto unreported mutations (12 out of 15).

CONCLUSION The low frequency of *GATA1* mutations in ML-DS patients is unusual and potentially indicates distinctive genomic events in our patient cohort.

Keywords: Down syndrome, GATA1, ML-DS, TAM

INTRODUCTION

Down syndrome (DS) is the most common congenital cytogenetic abnormality, with an incidence of one in 600–800 live births.[1] The syndrome is characterised by recognisable physical features, cognitive disability, and a varying frequency of cardiac, gastrointestinal, skeletal and endocrine defects. DS is also characterised by an increased susceptibility to childhood leukaemia,[2-4] including an increased frequency of distinctive clonal myeloid disorders in early childhood. A spontaneously resolving congenital myeloproliferative state designated as transient abnormal myelopoiesis (TAM) is encountered in 10%–20% of DS newborns.[5] After a period of apparent remission (1–4 years), 20%–30% of DS children with TAM progress to develop a characteristic, treatment-responsive megakaryoblastic form of acute myeloid leukaemia termed myeloid leukaemia of DS (ML-DS).[6,7]

TAM and ML-DS thus represent a continuum of clonal myeloproliferation in DS. Fundamental to both disorders is the gene-dosage imbalance of the human chromosome 21.[8] An additional shared, critical pathogenetic event involves the acquisition of characteristic somatic mutations in the *GATA1* gene.[9] The gene, located on chromosome X (*Xp11.23*), encodes a key haematopoietic transcription factor involved in erythroid and megakaryocyte differentiation. These mutations, involving exons 2 or 3 of the *GATA1* gene, result in synthesis of an aberrant truncated isoform (termed short *GATA1* or *GATA1s*) that is putatively oncogenic.[10] The mechanistic basis for somatic *GATA1* mutations and the additional molecular events that determine progression from TAM to ML-DS are the focus of intense research.[11,12]

This observational study attempts to characterise, for the first time, to our knowledge, the frequency and nature of somatic *GATA1* mutations in DS children with TAM and ML-DS, encountered consecutively over a period of five years at a national paediatric oncology referral centre in Malaysia.

METHODS

From January 2007 to December 2011, children with DS who were consecutively diagnosed to have TAM and/or ML-DS at University Malaya Medical Centre, Kuala Lumpur, Malaysia, were included in the study. The diagnosis of TAM was based on observation of unequivocal blasts on peripheral blood microscopy. ML-DS was confirmed by marrow studies, including microscopy, flow cytometry and, where indicated, bone marrow histology. Parental consent was obtained in all cases. Institutional approval was granted to collect, store and study peripheral blood and/or bone marrow samples from children in the study (University of Malaya Ethical Approval Reference MEC 2008/678.13). Clinical and laboratory data were retrieved from patient medical records.

Mononuclear cells were isolated from peripheral blood (TAM) and bone marrow (ML-DS) by erythrocyte lysis, followed by serial centrifugation. Genomic DNA was extracted from mononuclear cells using standard chloroform-phenol isolation. Screening for *GATA1* mutations was carried out by selective stepwise analysis of exons 2 and 3, with exon 3 sequences analysed in samples

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Table I. Clinical characteristics and laboratory data of the transient abnormal myelopoiesis (TAM) patients (n = 9).

Parameter	No. (%)/ Median (range)
Gender	
Male	6 (66.7)
Female	3 (33.3)
Gestation age (wk)	38 (32–40)
Birth weight (kg)	2.73 (1.80–3.10)
Age at diagnosis (day)	1 (0–15)
Clinical signs	
Liver size (cm)	4 (0–5)
Spleen size (cm)	2 (0–4)
Diagnostic laboratory examination	
White blood cell (× 10^9/L)	41.8 (13.0–308.8)
Blast (%)	25 (1–79)
Haemoglobin (g/dL)	13.4 (7.9–22.7)
Platelet (× 10^9/L)	127 (71–546)
Aspartate aminotransferase (IU/L)	43 (18–197)
Alanine aminotransferase (IU/L)	75 (17–357)
Follow-up (mth)	14 (6–47)
Time to TAM resolution (day)	46 (21–188)
Treatment	
Yes	3 (33.3)
No	6 (66.7)
Outcome	
Remission	7 (77.8)
Progressed to ML-DS	1 (11.1)
Death	1 (11.1)

ML-DS: myeloid leukaemia of Down syndrome

Table II. Clinical characteristics and laboratory data of the myeloid leukaemia of Down syndrome (ML-DS) patients (n = 24).

Parameter	No. (%)/ Median (range)
Gender	
Male	10 (41.7)
Female	14 (58.3)
History of TAM	2 (8.3)
History of myelodysplastic phase	6 (25.0)
Age at diagnosis (mth)	24 (10–60)
Presenting symptom*	
Fever	17 (70.8)
Pallor	18 (75.0)
Bleeding	11 (45.8)
Clinical signs	
Liver size (cm)	3 (0–9)
Spleen size (cm)	1 (0–10)
Lymphadenopathy	5 (20.8)
Diagnostic laboratory examination	
White blood cell (× 10^9/L)	9.5 (4.4–200.0)
Blast (%)	16 (0–93)
Haemoglobin (g/dL)	7.1 (3.8–12.4)
Platelet (× 10^9/L)	25 (5–936)
Aspartate aminotransferase (IU/L)	36 (14–98)
Alanine aminotransferase (IU/L)	50 (22–159)
Bone marrow blast (%)	42 (10–75)
CNS disease	0
Treatment	
Yes	22 (91.7)
No	2 (8.3)
Follow-up (mth)	11 (0.1–55.0)
Outcome	
Remission	14 (58.3)
Died	9 (37.5)
Resistant disease	2 (8.3)
Relapse	4 (16.7)
Sepsis	3 (12.5)
Defaulted	1 (4.2)

*Patients may have presented with ≥ 1 symptom. No ML-DS patients had central nervous system (CNS) leukaemia. TAM: transient abnormal myelopoiesis

bearing wild-type exon 2. The exon 2 sequence was amplified using primers (forward 5′-GGAAGGATTTCTGTGTCTGAG-3′ and reverse 5′-GCACTCAGCCAATGCCAAGA-3′) and polymerase chain reaction (PCR) cycling conditions reported by Rainis et al.[13] Exon 3 segments were amplified using primers (forward 5′-GCACTCAGCCAATGCCAAGA-3′ and reverse 5′-GAGCTAGGCTCAGCTCAGCTTTAC-3′) and PCR cycling conditions reported by Nichols et al.[14] All PCR reactions were carried out in 25 µL volumes using the HotStarTaq DNA Polymerase (QIAGEN, Hilden, Germany). PCR products were processed using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) to remove reaction residuals and subjected to automated nucleotide sequencing (Applied Biosystems ABI 3730XL; Thermo Fisher Scientific, Waltham, Massachusetts, USA). PCR amplification was tested with an adequate number of controls. Sequence analysis was performed using the online open-source BioEdit sequence alignment tool (version 7.0.1); the NCBI *GATA1* sequence NG_008846.1 served as a reference sequence. All sequence variations and predicted amino acid changes were categorised according to nomenclature recommendations from the Human Genome Variation Society (HGVS).[15]

RESULTS

A total of 33 patients with DS-associated myeloid disorders were encountered over the duration of the study, consisting of nine patients with TAM and 24 patients with ML-DS. Their clinical characteristics and laboratory data are summarised in Tables I and II. TAM was diagnosed at a median age of one day; five patients were diagnosed at birth. Detection of TAM was incidental in the majority (n = 7) and occurred in the course of blood testing for other conditions (jaundice, respiratory distress and bowel obstruction). One newborn was ill with hydrops fetalis, while the other developed self-limited pericardial effusion *in utero*. Three of the nine newborns required medical intervention, including intravenous cytarabine in one newborn to treat significant splenomegaly, exchange transfusion for another newborn with hydrops fetalis, and intermittent transfusion for another with symptomatic anaemia and thrombocytopenia. The newborn with hydrops fetalis developed refractory hepatic failure and died on Day 9 of life. Median time to TAM resolution was

Table III. Characteristics of *GATA1* mutations.

Patient	White blood cell ($\times 10^9$/L)	DNA source (blast [%])	DNA sequence change	Amino acid change	Type of mutation	Predicted consequence	Outcome (follow-up [mth])	Remark
TAM1	14.3	PB (22)	c. 220G > T	p.Val74Phe	Missense at exon 2 exon/ intron boundary	Splicing error	ML-DS (19)	Kanezaki et al[18]
TAM3	13.0	PB (26)	c. 105_106insC	p.Ser36Leufs*4	Frameshift insertion	Premature termination codon	CCR (6)	Novel mutation
TAM4	30.2	PB (1)	c. 150delG	p.Ser51Alafs*86	Frameshift deletion	Premature termination codon	CCR (19)	Novel mutation
TAM5	308.8	PB (79)	c. 5_14delAGTTCCCTGC	p.Glu2Ala	Frameshift deletion	Splicing error	Died of hepatic failure (0.3)	Novel mutation
TAM6	65.6	PB (NA)	c. 183_187delCTACT	p.Tyr62Glnfs*4	Frameshift deletion	Premature termination codon	CCR (31)	Novel mutation
TAM7	66.7	PB (55)	c. 216_219delCCCAinsTG	p.Pro73Gly	Sequence replacement	Splicing error	CCR (6)	Novel mutation
ML-DS4	6.4	BM (38)	c. 93_94delGG	p.Val33Phefs*7	Frameshift deletion	Premature termination codon	CCR (48)	Novel mutation
ML-DS5	107.7	BM (17)	c. 176_177insCAGGTGCG	p.Ala56Glnfs*81	Frameshift insertion	Premature termination codon	Relapse but CCR after salvage therapy (52)	Novel mutation
ML-DS7	26.6	BM (35)	c. 76_77delTC	p.Ser26Hisfs*13	Frameshift deletion	Premature termination codon	CCR (42)	Novel mutation
ML-DS9	12.9	BM (60)	c.-20G > A	Splicing error	Missense	Splicing error	Died of early marrow relapse (5)	Novel mutation
ML-DS17	200	BM (NA)	c. 222+3_ + 4insT	Splicing error	Frameshift insertion	Splicing error	Died of resistant disease (2)	Novel mutation
ML-DS19	9.3	BM (11)	c. 208_ + 9del	p.Arg70Ile	Deletion	Splicing error	Died of sepsis (2)	Novel mutation
ML-DS22	8.64	BM (15)	c. 187_188insT	p.Tyr63Leufs*5	Frameshift insertion	Premature termination codon	Died of early marrow relapse (6)	Xu et al[25]
ML-DS23	24.8	BM (75)	c. 89_149dup71	p.Ser89Trp	Duplication	Splicing error	Died of resistant disease (5)	Novel mutation
ML-DS24	5.0	BM (54)	c. 220G > A	p.Val74Ile	Missense	Splicing error	CCR (26)	Kanezaki et al[18]

Reference sequence: NG_008846.1. The first adenine of the initiation codon was assigned nucleotide + 1 (i.e. start site). BM: bone marrow; CCR: complete clinical remission; ML-DS: myeloid leukaemia of Down syndrome; NA: not available; PB: peripheral blood; TAM: transient abnormal myelopoiesis

46 (range 21–188) days. One child progressed to develop ML-DS 19 months after apparent remission at two months of age. The remaining seven children have maintained complete remission.

ML-DS was diagnosed at a median of 24 months of age. History of a preceding haematological disorder (two with TAM and six with myelodysplastic phase) was noted in 8 (33.3%) children with ML-DS. Common presenting features included fever, pallor and bleeding. Two children presented with very high leucocyte counts ($\geq 100 \times 10^9$/L); none had central nervous system leukaemia. 22 (91.7%) children with ML-DS received cytotoxic therapy. The estimated overall five-year survival rate of children with ML-DS was 58.3%; nine children died, including three due to treatment toxicity (i.e. sepsis).

A total of 29 samples were analysed for *GATA1* mutations; four samples did not have adequate material for analysis. *GATA1* exon 2 mutations were detected in 6 (75.0%) of the eight TAM patients and 9 (42.9%) of the 21 ML-DS patients (Table III). Wild-type exon 2 and 3 sequences were detected in the remainder. The bulk of the exon 2 mutations (66.7%, n = 10) were sequence deletions and insertions. The remaining five mutations were: missense mutations in three patients (1 TAM, 2 ML-DS), sequence replacement in one TAM patient and sequence duplication in one ML-DS. The majority of mutations resulted in premature sequence termination while the rest were associated with splicing errors during transcription. A majority of mutations (12 of 15, 80.0%) had not previously been reported.

Table IV. Comparison of *GATA1* mutation detection rate.

Study	PCR amplification exon	No. of patients	No. of mutations	Mutation (%)
Wechsler et al[21]	2, 3, 4, 5, 6	6 ML-DS	6	100.0
Hitzler et al[22]	2	12 TAM	9	75.0
		3 ML-DS	3	100.0
Rainis et al[13]	2	18 ML-DS	17	94.4
		17 TAM	16	94.1
Groet et al[23]	2	10 TAM	7	70.0
		6 ML-DS	4	66.7
Mundschau et al[24]	2, 3	7 TAM	7	100.0
Xu et al[25]	2, 3, 4, 5, 6	22 TAM	21	95.5
		18 ML-DS	12	66.7
Ahmed et al[26]	2	4 TAM	4	100.0
		12 ML-DS	12	100.0
Magalhães et al[27]	2, 3	6 TAM	4	66.7
		8 ML-DS	6	75.0
Cabelof et al[10]	2	5 TAM	5	100.0
		14 ML-DS	14	100.0
Kanezaki et al[18]	2, 3, 4, 5, 6	78 TAM	72	92.3
Alford et al[16]	–	134 TAM	118	88.1
		103 ML-DS	88	85.4
Present study	2, 3	8 TAM	6	75.0
		21 ML-DS	9	42.9

ML-DS: myeloid leukaemia of Down syndrome; PCR: polymerase chain reaction; TAM: transient abnormal myelopoiesis

DISCUSSION

This is the first report of *GATA1* mutations in a cohort of Malaysian children with DS-associated myeloid disorders. Although the sample is small, truncating exon 2 *GATA1* mutations were encountered in 75.0% of DS neonates who had TAM, a finding that mirrors published observations (Table IV). In contrast, *GATA1* mutations were observed in less than half (42.9%) of the children who had ML-DS. This low mutation detection rate is not readily attributable to poor PCR sensitivity, since the marrow blast burden (median 42%, range 10%–75%) was adequate in all cases.[16] It is possible that a larger proportion of mutations in the ML-DS cohort occurred at genomic loci outside the primer binding sequence or at sites affecting primer annealing; gene sequencing might have uncovered these mutations.

All sequence variations in this study were described in accordance with the nomenclature recommendations of the HGVS.[17] The majority of the exon 2 *GATA1* mutations in our cohort were sequence deletions and insertions (66.7%), resulting in frameshift errors and premature termination codons. No exon 3 mutations were identified. 80.0% (n = 12) of observed mutations had not previously been reported and are likely to be novel. However, sequence variations in *GATA1* have been inconsistently reported, making it difficult to evaluate *GATA1* mutations in our series against those in the published literature. For instance, despite using the same *GATA1* reference sequence, Alford et al, who conducted the largest study of *GATA1* mutations in DS patients to date, unusually designated the first nucleotide of exon 1 as an indication of the start of the *GATA1* sequence.[16] Uniform reporting of sequence variations, in line with standard HGVS terminology, is expected to eliminate such discrepancies in future studies.

Lower relative quantitative expression of the truncated GATA1s protein has been reported to be associated with higher risk of progression to ML-DS.[18] By contrast, the majority of mutations in our series are predicted to be associated with high protein expression of the truncated GATA1s protein (66.7%). In the one patient with TAM who progressed to ML-DS, the mutation resulted in a splicing error predicted to be associated with high expression of GATA1s. High GATA1s expression is also predicted in six of nine mutation-positive ML-DS patients (66.7%). These observations indicate continued uncertainty about the link between the type of *GATA1* mutation in TAM and the risk of progression to ML-DS.[16]

Children with ML-DS have been shown to have a superior outcome when compared with children without DS who developed acute myeloid leukaemia (AML). Children with ML-DS experience event-free survival rates of more than 80% as compared with 30%–40% in non-DS children, particularly those with FAB-M7 subtype AML.[19] It was proposed that *GATA1* mutations may be important prognostic factors. Acquired *GATA1* mutations are postulated to cause decreased transcription and expression of cytidine deaminase (*CDA*) gene. CDA enzymes catalyse the deamination of cytosine arabinoside (ara-C) to its inactive derivative, ara-U. In the presence of *GATA1* mutations, there is a higher concentration of intracellular ara-C, leadings to an increase in cytotoxic efficacy. In our ML-DS cohort, the presence of *GATA1* mutations may not necessarily confer a good prognosis. Four out of nine patients with *GATA1* mutations developed relapse or resistant disease; one patient from the cohort with negative *GATA1* mutation had relapse disease (Fig. 1). The patients with *GATA1* mutations had higher disease-related mortality compared to those without (80.0% vs. 50.0%). *CDA* mRNA expression was measured using real-time quantitative PCR in two patients (ML-DS5 and ML-DS9) who had early bone marrow relapse, in order to study the relationship between *GATA1* mutations and *CDA* mRNA. It was found that *CDA* mRNA was upregulated in these two patients and was 40-fold higher compared to that of the other ML-DS cases, suggesting greater capability to metabolise ara-C. *CDA* mRNA expression in these two patients was, however, still lower than that in non-DS AML patients.[20] Therefore, in the presence of *GATA1* mutations, *CDA* gene expression may be heterogeneous. Apart from the *CDA* gene, *GATA1* mutations may be associated with changes in the transactivational activity of many genes that influence treatment response.

Despite the limitations of sample size, our findings reiterate the observation of recurring *GATA1* mutations in children with DS-related myeloid disorders. The high incidence of potentially novel *GATA1* mutations and the low frequency of such mutations in exons 2 and 3 raise the prospect of distinctive genomic events in our patient cohort. In our study, the mere presence

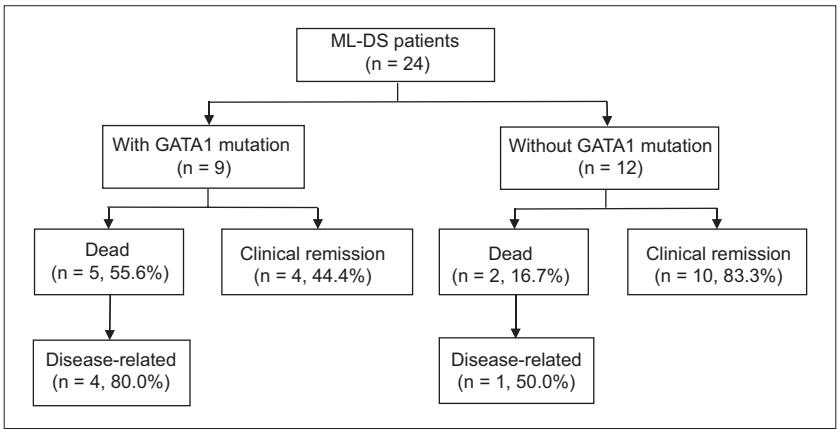

Fig. 1 Flowchart shows clinical outcome of children with myeloid leukaemia of Down syndrome (ML-DS). Of the 24 ML-DS patients, 21 had *GATA1* analysis performed. 2 patients who died did not have *GATA1* analysis performed.

of *GATA1* mutations in ML-DS does not necessarily confer a good prognosis.

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