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GATA1 Gene Polymorphisms in Down Syndrome Patients

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Objectives: Down syndrome (DS) patients have a 500 fold higher possibility of developing acute megakaryoblastic leukemia (AMKL), compared with the general population. *GATA1* mutations, acquired in the early prenatal stages, contributes to leukemogenesis in AMKL and has been the explanation for the cause of early hematopoietic disorders. The aim of this study was to investigate the influence of *GATA1* gene polymorphisms in patients with DS. **Methods:** Thirty-nine DS patients, aged ≤ 4 years, were recruited into the study. *GATA1* gene polymorphisms were identified by unidirectional deep sequencing. **Results:** *GATA1* gene polymorphisms were identified in four patients: proband-11 had *GATA1* gene polymorphism, NP_002040.1:p.His71Arg (rs374300356); proband-17 had NP_002040.1:p.Tyr69Cys; proband-19 had NP_002040.1:p.Lys100Arg; and proband-20 had NP_002040.1:p.Tyr69Cys. Analyzing these *GATA1* gene polymorphisms with 14 different software programs to evaluate its pathogenicity showed that NP_002040.1:p.His71Arg had damaging effects on *GATA1* gene function. **Conclusion:** We identified four *GATA1* gene polymorphisms in this cohort of 39 patients. The polymorphism identified in proband-11 (NP_002040.1:p.His71Arg) has possible damaging effects on gene regulation, and thus, we recommend routine clinical examination in this patient.

Keywords: Down Syndrome, *GATA1* Transcription Factor, Acute Megakaryoblastic Leukemia, Single Nucleotide Polymorphism

Introduction

Down syndrome (DS) is one of the most common aneuploidy associated with mental retardation, occurring in 1 of 700 babies [1]. More than 95% of DS patients have complete trisomy 21, 4% of DS patients have translocation trisomy 21, and about 1% of DS patients have mosaic trisomy 21 [2]. In a 2005 survey, the birth incidence of DS was estimated to be about 1.6 in 10,000 live births, which is 30-50 new cases every year [3]. DS patients demonstrate multisystemic manifestations, including short stature, mental retardation, dysmorphism, congenital heart disease, congenital anomaly of gastrointestinal and

genitourinary tract, abnormal endocrine function, leukemia, and leukemoid reactions [4]. Aside from mental retardation, other anomalies, such as leukemia in early childhood cases and dementia in adults, can be treated or controlled with currently available medical care.

The diagnosis of DS is often suspected by clinical facial appearance, survey of congenital malformations, and is further confirmed by karyotyping. In Mongolia, due to the lack of standard laboratory equipment, the diagnosis of DS is made only by clinical manifestations, which hampers identification and thus, the management of this disease.

DS patients have 10-20 fold higher rates of leukemia and a 500 fold higher possibility of developing acute megakaryoblastic leukemia (AMKL), compared to the healthy population [5, 6]. It has been estimated that 1 in 100-200 DS patients carry leukemia [5]. Sixty percent of DS acute leukemia (DS-AL) is myeloid (ML-DS), and of that, AMKL accounts for at least 50% [5].

In DS patients, abnormalities due to hematopoietic disorders arise early in life. Around 10% of the newborns with DS develop transient myeloproliferative disorder (TMD) in their early childhood, and of them, 20% progress to AMKL within 4 years of age [7, 8]. The cause of early hematopoietic disorders has been explained by *GATA1* (*GATA1* transcription factor) mutations, acquired in the early prenatal stage, which contributes to the leukemogenesis within TMD and AMKL [9, 10].

GATA1 encodes a zinc finger transcription factor and is essential for normal erythroid development and megakaryocytic differentiation [11]. It has been reported that almost all DS-AMKL and TMD patients carried mutations in exon 2 of *GATA1* gene, resulting in a premature stop codon within the N-terminal activation domain [9, 10]. These mutations prevent the synthesis of a full-length *GATA-1*, but preserve the translation of *GATA-1*s, a truncated form of *GATA-1* lacking the N terminal activation domain [12, 13]. Both *GATA-1*s and *GATA1* show comparable DNA binding abilities and interact with partner proteins, i.e., "Friend of *GATA1*" (FOG1), though *GATA-1*s exhibits perturbed transactivation capacity due to the loss of the N-terminal activation domain [14]. This contributes to the uncontrolled proliferation of the poorly differentiated megakaryocytic precursors [15].

It has been noted that DS patients with positive *GATA1* mutations should be screened for leukemia regularly [16]. Monitoring *GATA1* mutations in DS patients with leukemia may help hematologists in classifying and designing the management of DS patients with leukemia.

Additionally, because of the somatic nature of these mutations, a sensitive method is necessary to detect low percentages of mosaicism and to help with clinical diagnosis. When using a sensitive method, sequencing the *GATA1* mutation by deep sequencing is a good choice. The recent progress in developing high throughput methods for deep sequencing of DNA from dried blood spots and highly sensitive sequencing methods to detect mosaicism makes these the method of choice when detecting *GATA1* mutations in DS patients [17,18].

In addition to *GATA1*, there are other signaling pathways related to megakaryopoiesis and promoting AMKL, such as PI3K/AKT (Phosphoinositide 3-kinase/ AKT) constant signaling [19]. In this study, we aimed to identify *GATA1* gene polymorphisms in patients with DS to evaluate the pathogenicity of alleles with regards to leukemia.

Materials and Methods

Patients (n=39) clinically suspected to be DS positive were enrolled after confirming diagnosis with karyotyping at the Laboratory in the Department of Molecular Biology and Genetics, School of Pharmacy and Biomedicine, Mongolian National University of Medical Sciences (MNUMS). An inclusion criterion of the study was that the DS patient was aged less than 4 years old. Informed consent was obtained from the patients and their parents. The study was approved by the Institutional Review Board of MNUMS.

We observed B. Hall's ten clinical signs in this cohort of 39 patients. The ten clinical signs were single palmar crease; upward-slanting palpebral fissures (eyelid openings); flat facial profile; morphologically simple, small round ears; hypoplasia of fifth finger middle phalanx; hyper-extensible large joint; hypotonia; poor moro reflex; redundant loose neck skin; and abnormal pelvis.

To analyze *GATA1* gene polymorphisms in the patients, DNA was extracted at the Department of Molecular Biology and Genetics, School of Pharmacy and Biomedicine, MNUMS, and *GATA1* gene analysis was performed at the Department of Medical Genetics, National Taiwan University.

1. DNA extraction from peripheral blood mononuclear cells

Whole blood (5 ml) was drawn from the patients and were quickly transferred into a tube containing ethylene diamine-tetra-aceticacid (EDTA). Leukocytes were separated from the plasma as the buffy coat layer and were immediately subjected to DNA extraction. Total cellular DNA of leukocytes were extracted using methods described by J. Sambrook [21].

2. *GATA1* mutation analysis by deep sequencing

The deep sequencing of *GATA1* mutations from dried blood spots was carried out in the following manner [17]. Genomic DNA (10 µl) isolated from dried blood spots were applied for *GATA1* mutation analysis. *GATA1* coding regions and their flanking intronic sequences were amplified by polymerase chain reaction (PCR) using the primers (Table 2.) Following PCR, we inserted the barcode for each patient's DNA sample according to user's manual [17]. PCR amplicons were purified and quantified, and the amplicons from patients were pooled at equimolar concentrations with a Beckman Biomex FX system (Beckman, USA) equipped with a DTX880 Multimode Detector (Beckman, USA). The pooled amplicons were then clonally amplified on beads using emulsion PCR, loaded into two lanes of the picotitre plate (24/lane), fitted with a 16-lane gasket, and were unidirectionally pyrosequenced using a GSFLX Titanium pyrosequencing kit (Roche, Canada). Data was analyzed by Roche Amplicon Variant Analyzer software. The cDNA of *GATA1* was numbered starting from the translation initiation site (Accession number NM_000023.10).

For comparison, a whole *GATA1* gene sequencing was also performed using the traditional Sanger sequencing method. The PCR products were purified using Gel-M™ Gel Extraction system (Viogene, USA) and were analyzed by direct sequencing using the ABI Prism Big Dye dideoxy chain terminator Cycle sequencing kit and the ABI Prism 310 genetic analyzer (Applied Biosystem, USA).

The *GATA1* gene polymorphisms were analyzed by the following 14 different software programs to evaluate its pathogenicity: SIFT, Polyphen2_HDIV, LRT, Mutation Tester, FATHMM, Radial SVM, VEST3, CADD, phyloP100way, SIPhy_29way, Polyphen2_HVAR, Mutation Assessor, LR and GERP++.

Table 1. Socioeconomic data of DS patients (age ≤ 4 years) and their caregivers

Number of children	n (%)	Socio-economic class	n (%)	Mother's age	n (%)
One	3 (7.6)	Very poor	2(5.1)	20-30	5(12.8)
Two	10 (25.6)	Poor	2(5.1)	30-40	11(28.2)
Three	18 (46.2)	Average	13(33.3)	40-50	18(46.1)
Four	5 (12.8)	Wealthy	15(38.4)	50-60	5(12.8)
Five	3 (7.7)	Very wealthy	7(3.2)	60-70	(0)

Table 2. Clinical symptoms of patients (n=39) (B. Hall's criteria)

No	Clinical Signs	Frequency (%)
1	Single palmar crease	87
2	Upward-slanting palpebral fissures (eyelid openings)	81
3	Flat facial profile	78
4	Morphologically simple, small round ears	67
5	Hypoplasia of fifth finger middle phalanx	67
6	Hyper-extensible large joint	61
7	Hypotonia	56
8	Poor Moro reflex	28
9	Redundant loose neck skin	14
10	Abnormal pelvis	10

Table 3. Primer sequence of GATA1 gene

Exon	Amplified length (bp)	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
1	397	CAGGGAGGGTGGAAGAGG	GTGGGTTGGAGGAGCTGTAA
2	454	AAGGAGGAAGAGGAGCAGGT	AGTGGTCGGCACATCCAT
3	687	TGTTCTGGTAGCCTGTGGAA	CATGAGAACAGCGTTCCTATATAATG
4	382	CAAGCTTCGTGGAACTCTCC	GAGGTAGAACAGGAACAGAGTGG
5	355	CAGAATCCTCAGGCATCACC	GAGGTAGAACAGGAACAGAGTGG
6	779	CAGCTCCTATCAGCCTTGGA	TGTCATCAGAGAGCCACAGG

Results

In our cohort of 39 patients (Table 1), we observed B. Hall's ten clinical signs. The most frequent signs were single palmar creases (87%) and upward-slanting palpebral fissures (81%) (Table 2).

Compared to the primer sequencing for the *GATA1* gene using the traditional Sanger sequencing method (Table 3), the following four *GATA1* gene polymorphisms were identified:

A polymorphism in exon 2 of the *GATA1* gene, NM_002049.3:c.212A>G; (NP_002040.1:p.His71Arg), was detected in mosaic form (99%) in proband-11. The pathogenicity of NP_002040.1:p.His71Arg was calculated by 14 softwares, including SIFT and Polyphen, and they identified pathogenic effects on gene function (Table 4).

A polymorphism also detected in exon 2 of the *GATA1* gene, NM_002049.3:c.206A>G, (NP_002040.1:p.Tyr69Cys), was in mosaic form (1.3%) in proband-17.

A polymorphism, NM_002049.3:c.299A>G (NP_002040.1:p.Lys100Arg), was detected in exon 3 of the *GATA1* gene in mosaic form (1.1%) in proband-19.

Lastly, a polymorphism, NM_002049.3:c.206A>G (NP_002040.1:p.Tyr69Cys), was detected also in exon 2 of the *GATA1* gene in mosaic form (1.1%) in proband-20.

Discussion

Children with DS have a significantly higher risk of developing childhood leukemia in comparison to children without DS, although interestingly, they have a lower risk of developing solid tumors [22, 23]. DS is defined by constitutive trisomy in chromosome 21 and is the most common cytogenetic abnormality seen during births, at a rate of 1/700- 1/1,000 newborns [24].

The effect of trisomy 21 and *GATA1* mutations in promoting abnormal megakaryopoiesis has recently been understood. The acquisition of trisomy 21 is the first target, as trisomy 21 without *GATA1* mutations leads to altered myeloid progenitor self-renewal, altered lineage development, and increased clonogenicity of MKPs in human fetal livers [25- 27]. Somatic *GATA1* mutations have been identified as a secondary target, and it is thought to block megakaryocyte (MK) differentiation [28]. The *GATA1* gene mutations have been identified in almost all the cases of transient TMD and ML-DS.

GATA1 somatic mutations were first identified from a small series of ML-DS samples [28]. *GATA1* is located on the X chromosome, which encodes a zinc finger-containing protein that is important for normal erythropoiesis and megakaryopoiesis [28, 29]. *GATA1* gene mutations lead to the production of a

Table 4. Effect of NP_002040.1:p.His71Arg on GATA1 gene function

Software program	Effect on GATA1 gene function	Software program	Effect on GATA1 gene function
SIFT	0.17(Tolerable)	Polyphen2_HDIV	0.871(Possible_damaging)
LRT	0.901 (Unknown)	Mutation Taster	0.998 (Disease_causing)
FATHMM	-5.12 (Damaging)	RadialSVM	0.729 (Damaging)
VEST3	0.603(Damaging)	CADD	12.77 (Tolerable)
phyloP100way	4.784 (Nonconserved)	SIPhy_29way	7.813 (Nonconserved)
Polyphen2_HVAR	0.178 (Benign)	Mutation Assessor	1.83 (Low)
LR	0.901(Damaging)	GERP++	3.3 (conserved)

truncated *GATA1* protein, *GATA1*s. *GATA1*s lack an amino-transactivation domain but retains both DNA-binding zinc fingers [28]. The majority of mutations have been described in exon 2, with a minority in exon 3 or at the intronic boundary of exon 1 and 2. In 75% of cases, these were insertions, deletions, or duplications. The presence of *GATA1*s is thought to impair *GATA1*-mediated regulation of other transcription factors, including GATA2, MYB, MYC, and IKAROS family zinc finger 1 (IKZF1) in fetal MKs [30].

GATA1 mutations, in the absence of trisomy 21, have not been associated with leukemia. Instead, specific hematopoietic alterations due to *GATA1* mutations alone include cytopenias, Diamond-Blackfan anaemia, and trilineage bone marrow dysplasia in germline *GATA1* mutations [28, 31, 32]. Thus, with regard to abnormal megakaryopoiesis, *GATA1* mutations are considered a secondary target, only influential in the presence of trisomy 21.

An analysis of paired samples from the same patient has found identical *GATA1* gene mutations in both the preleukemia (TMD) and leukemia (ML-DS) stages [29, 33]. It is highly likely that additional transforming events, or "targets", are involved in this leukemogenic process [32, 33].

In another study, 44% of ML-DS samples demonstrated additional genetic mutations, aside from trisomy 21 and *GATA1* mutations [33]. These additional "targets" may contribute to the survival of pre-leukemic cells in the postnatal environment and play a role in subsequent risk of leukemic transformation within the bone marrow compartment. In our study, we identified a rare polymorphism (rs374300356) with a frequency of 0.0002649 in a DS patient (proband-11).

In silico predicting software has been widely used in last decades, and the most common ones have been SIFT and PolyPhen. SIFT (Sorting Intolerant From Tolerant) is an algorithm which predicts whether an amino acid substitution will affect protein function based on sequence homology and the physical properties of amino acids. A substitution with a SIFT score of less than 0.05 is predicted to be deleterious, and a score greater than or equal to 0.05 is predicted to be tolerated. Poly Phen is a tool that predicts possible impact of an amino acid substitution on the structure and function of a human protein using physical and comparative considerations. Structural features, such as amino acid atomic contacts and solvent accessibility, are also assessed, and empirically determined cut-offs used to predict if the substitution is 'probably damaging', 'possibly damaging' or 'benign'. 14 softwares have been developed by different research institutions, but thus far, none of them can fully replace a functional analysis of the pathogenic alleles. In our study, we integrated these computational analyses for the *GATA1* alleles to get more confident data.

Our rare polymorphism, rs374300356, is suspected to negatively impact *GATA1* gene function, and thus, routine clinical and laboratory examinations for our patient (proband-11) is necessary to prevent late diagnosis of blood leukemia. *GATA1* gene polymorphisms may regulate gene expression in DS children since these children harbor extra copies of the genes located in the 21st chromosome. Thus far, *GATA1* gene polymorphisms have not been reported in the literature, but we believe that specific SNPs found on the *GATA1* gene may be pathogenic, and further investigations are required for confirmation. Pathological effect of this extraordinary polymorphism warrants elucidation, and thus, functional analysis is highly recommended in future studies.

In conclusion, we identified four *GATA1* gene polymorphisms in this cohort of 39 patients, allowing us the opportunity to conduct *GATA1* gene analysis in DS patient screening. The polymorphism identified in proband-11 (NP_002040.1:p. His71Arg) has possible damaging effects on gene regulation, as indicated by Gel Extraction system In Silico predictions, and thus, we recommend routine clinical examination of our patient and other patients with this polymorphism.

Conflict of Interest

The authors state no conflict of interest.

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