ORIGINAL RESEARCH

Clinical Characteristics and Molecular Genetic Analysis of a Pedigree with Glanzmann's Thrombasthenia

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ABSTRACT

Objective • The objective of this study was to investigate the clinical phenotype and genetic etiology of Glanzmann's thrombasthenia in a consanguineous pedigree.

Methods • Clinical data and ancillary test results were collected from pedigrees with Glanzmann's thrombasthenia. High-throughput sequencing was used to detect variants in the proband. Candidate variants were verified by Sanger sequencing.

Results • Two patients in the pedigree were homozygous for the c.2248C>T (p. Arg750Ter) variant of the ITGB3 gene. The parents and maternal grandmother, who didn't have any recurrent haemorrhage, were found to carry a heterozygous c.2248C>T variant of the ITGB3 gene, which was absent in the aunt and paternal grandmother.

Conclusion • The homozygous variant c.2248C>T (p. Arg750Ter) in the ITGB3 gene underlies the disease in this pedigree. This diagnosis will facilitate genetic counselling in this pedigree for better patient management and life guidance. (*Altern Ther Health Med.* [E-pub ahead of print.])

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INTRODUCTION

Glanzmann's thrombasthenia (GT) is a rare inherited bleeding disorder characterised by abnormal platelet function and it was first reported by Glanzmann in 1918.[1] The worldwide prevalence of GT in the general population is estimated to be about 1 per 1 million and it is more common in consanguineous marriages.[2] Clinically, early childhood manifestations of bleeding symptoms of varying degrees, mild skin purpura, epistaxis, bleeding gums or bleeding after trauma.[1] Female patients may show excessive menstrual flow, and in severe cases, fatal bleeding such as gastrointestinal hemorrhage or intracranial hemorrhage may occur.[3] The disease is attributed to mutations in the 17q21-23 region of ITGA2B or ITGB3 genes, resulting in reduced expression or impaired function of aI5bβ3 on the platelet surface. Consequently, platelet aggregation disorders occur.[4]

There are over 256 registered GP IIb gene mutations in this disease and over 164 variations in the GP IIIa gene. (http://sinaicentral.mssm.edu/intranet/research/ glanzmann/search). Qualitative or quantitative changes in the αIIbβ3 receptor on the platelet surface can be categorized into three types based on their expression levels: type I (GPIIb/IIIa quantity on the platelet surface < 5%), type II (GPIIb/IIIa quantity on the platelet surface 5-20%), and type III (GPIIb/IIIa quantity on the platelet surface 20-50%).[5] Type III is also known as variant (normal expression but functional defect). Therefore, the study on the relationship between clinical phenotype and genotype of patients with GT is not only helpful in assessing the risk of clinical bleeding and guide treatment, but also helpful to guide Genetic counseling and gene therapy.

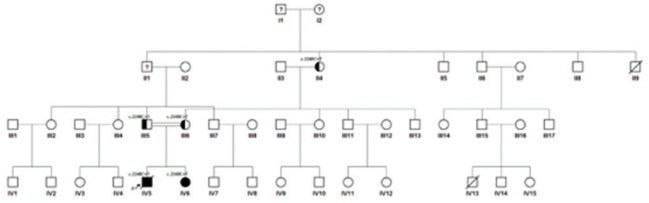
We used second-generation sequencing technologies in this investigation to examine the clinical symptoms and genotypes of a GT family. Understanding changed diseases at the clinical and molecular levels can provide not only a more comprehensive approach to disease diagnosis, but also a theoretical foundation for gene therapy research.

PATIENTS AND METHODS

Subjects

A family with Glanzmann's thrombasthenia from Guizhou (Figure 1). The proband (IV5) is a male with recurrent epistaxis and a history of "difficult to stop bleeding". A small amount of bleeding can take several to ten hours to stop bleeding, and the effectiveness of medication in stopping bleeding is poor. Due to multiple bleeding and obvious anemia, blood transfusion treatment was given. Multiple monitoring of platelets,

Figure 1. Family diagram of Glanzmann's thrombasthenia



Note:

Male died	Normal male	Male GT	Male carriers	? Male carriers may
Male proband died	Normal female	Female GT	Female carriers	? Female carriers may

coagulation function, and normal range of coagulation factors, blood smear: poor platelet aggregation. Bone marrow report: The production plate function is good, and the bone marrow picture of proliferative anemia is good. At 12 months old, he died due to "gastrointestinal bleeding, hemorrhagic shock". Gene sequencing report: ITGB3 biallelic variation (homozygous variation).

Family history: Parents were consanguineous (aunt's cousin). The sister of the proband (IV6) was born naturally at 40+4 weeks of pregnancy and weighed 3510 g. History of asphyxia resuscitation was denied. After birth, ecchymosis and ecchymosis are found on the skin of the entire body, with dense areas on the upper arms, buttocks, back, and groin. The sister of the proband (IV6) without hematuria or hematochezia, without screaming and convulsions, and without provocation. The child's blood routine hematocrit was normal, and platelets were > 150×10^9/L. The result of the blood smear showed that there were large platelets, micro aggregation, heterozygosity of 3%, and a small number of neutrophil nuclei shifted to the right. Coagulation function and coagulation factor (II, V, VII, VIII, IX, X, D, E) activity measurements were all in the normal range. Platelet function testing showed poor platelet aggregation induced by adenosine diphosphate and collagen, while the platelet aggregation rate was normally induced by ristomycin. The flow cytometry results showed that the percentages of CD41 and CD61 were 2.9% and 5.1%, respectively. The second aunt of the proband (III5) had recurrent petechiae on the skin for the past 5 years, which could subside on their own, but her menstrual flow was normal, and she did not have recurrent gingival bleeding. The proband brother-in-law (II9) died of cirrhosis of the liver and gastric hemorrhage one year ago. There was no history of recurrent epistaxis or bleeding that could not be easily stopped. The cousin of the proband (IV13) had recurrent epistaxis and bleeding in childhood and died of hemorrhage at the age of 2 years, with very similar symptoms to that of the proband.

Method

Case Collection. After obtaining the consent of some family members, sign an informed consent form. Approved by the hospital ethics committee, samples of 7 patients from this family were collected and sent to Hangzhou ZhongHanJinNuo medical laboratory for testing.

Targeted capture high-throughput sequencing. The second-generation sequencing technology is used for detection. First, select the appropriate method for DNA extraction according to the sample type and detect the DNA quality. The samples qualified in quality control will be analyzed in the next step. Next, high-throughput sequencing is performed to sequence and process DNA samples. Including target Gene trapping and library construction, Illumina HiSeqX system sequencing, sequencing data processing, gene annotation, etc. Analysis software and data screening database include Burrows-Wheeler Aligner (BWA) software (v0.7.15-r1140), Genome Analysis Toolkit (GATK) software, VarScan software, Annovar software, EXAC database, 1000 genome database, dbSNP database, PolyPhen 2 software, SIFT software, Mutation Taser software, HGMD database and Clinvar database. Analyze the pathogenicity of the variation according to the relevant guidelines issued by the American Society of Medical Genetics and Genomics.

Sanger sequencing. Sanger sequencing was used to validate the variants of the forewitnesses and family members. According to NCBI GenBank sequences, specific primers were designed for the above mutation sites using Primer 5.0 software and primer sequences (Table 1). After confirming the PCR reaction by electrophoresis, the fragments were subjected to Sanger sequencing using an ABI 3500Dx sequencer. The data were analyzed by sequencing analysis software and compared with the sequence of ITGB3 gene transcript (GRCh37/hg19).

RESULTS

Clinical characterization

The proband (IV5), a male, was diagnosed as "anemic" at birth, with a hematocrit of 90 g/L (table II). There is a history of recurrent epistaxis and difficulty in stopping bleeding. A small amount of bleeding can take several to more than ten hours to stop bleeding, and medication and other hemostatic effects are poor. He had been treated with blood transfusions because of repeated bleeding and obvious anemia. The lowest hematocrit was 50 g/L. Platelets, coagulation function, and coagulation factors were monitored for several times and were in the normal range. Blood smear: Poor platelet aggregation. Bone marrow report: The production plate function is good, and the bone marrow picture of proliferative anemia is good. At 12 months old, he died of "gastrointestinal bleeding, hemorrhagic shock". The proband sister (IV6) was born at full term with a birth weight of 3510 g. She denied any history of suffocation rescue. After birth, skin ecchymosis and ecchymosis are found throughout the body, with dense distribution in the upper arms, buttocks, back, and groin areas (Figure 2). The child's blood routine showed normal hemoglobin levels, and multiple platelet tests showed>150×10^9/L. Blood smear: occasional large platelets, microaggregation, 3% heterozygosity, and a small number of neutrophils with rightward shifted nuclei were seen microscopically. Coagulation function and coagulation factor (II, V, VII, VIII, IX, X, D, E) activity were measured within

Figure 2. Clinical signs of the sister of the proband: ecchymosis and petechiae all over the body, obvious on the buttocks

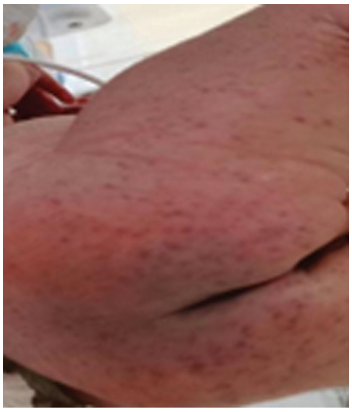
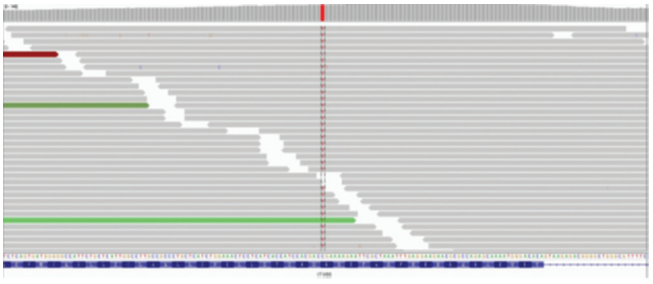


Figure 3. Schematic diagram of IGV results of ITGB3 gene variant c.C2248T sequencing results



the normal range. Platelet function testing showed poor platelet aggregation induced by adenosine diphosphate and collagen, while the platelet aggregation rate was normally induced by restomycin. The percentages of CD41 and CD61 shown by flow cytometry were 2.9% and 5.1%, respectively, suggesting that the platelet surface membrane glycoprotein GPIIb/IIIa was diminished. The cousin of the proband (IV13) experienced recurrent epistaxis during childhood, with continuous bleeding. He died of massive bleeding at the age of over 2 years, and his medical history is very similar to that of the proband. Draw a genetic family diagram, as shown in Figure 1.

Targeted capture high-throughput sequencing results

Sequencing analysis revealed a c.2248C>T pure mutation in the ITGB3 gene (Figure 3). p.R750X amino acid change. This variant causes the codon that compiles amino acid 750, Arg, to change to a termination codon (p. Arg750Ter), leading to the early termination of peptide chain synthesis, which is a nonsense variation.

Sanger sequencing validation results

Two patients in the family have a homozygous variant (homozygous variant) of the ITGB3 allele, as shown in Table III. His parents (III5, III6) and grandmother (II4) are both carriers of the ITGB3 gene c.2248C>T, and there is no evidence of recurrent bleeding. His grandfather (II1) has not returned since he went out 18 years ago, lost contact and did not complete Genetic testing. Neither his grandmother (II2) nor his second aunt (III5) was found to carry the ITGB3 gene c.2248C>T. It is speculated that his grandfather (II1) carries this gene, while the possibility of genetic variation in the father of the child (III5) is unknown. His brother-in-law (II9) died one year ago due to "cirrhosis of the liver and gastric hemorrhage", and there was no history of recurrent epistaxis or unstoppable bleeding. The proband had a cousin (IV 13) who suffered from repeated epistaxis in childhood and died of massive bleeding when he was more than 2 years old. The medical history was very similar to that of the proband. It was speculated that he might also be a patient with GT. However, their parents (III15, III16, not consanguineous) and their surviving younger siblings (IV14) and younger siblings (IV15) did not have bleeding-related symptoms and refused further gene sequencing. Due to the geographical dispersal and work commitments, other family members declined further genetic sequencing as it would be inconvenient for them to visit the hospital for testing in their respective hometowns.

DISCUSSION

GT is a rare autosomal recessive hemorrhagic disease.[6] The incidence reported abroad is 1/1 000 000, which is due to the mutation of the integrin gene ITGA2B or ITGB3 in the region of the long arm 21-23 of chromosome 17, which leads to decreased expression of αIIbβ3 on the platelet surface or functional defects, and further causes platelet aggregation disorders.[4] The gene ITGA2B is located on chromosome 17q21.31 and encodes platelet GP αIIβ. Whereas the gene ITGB3 encodes glycoprotein subunit β3 and is located on chromosome 17q21.32.[2.] Pathogenic variants in either subunit can result in quantitative or qualitative defects in αIIbβ3, leading to GT. Based on the surface of platelets α IIb β 3, qualitative or quantitative changes are divided into type I, type II, and type III (also known as variant). The variation of ITGA2B and ITGB3 genes, including missense variation, stop codon, small deletion, insertion or duplication, and splicing defect, usually with code shifting.[2] Pathogenic nonsense, missense, and splice site mutations are common, while large deletions and duplications are rare.[7,8] Pathogenic missense variants impair subunit biosynthesis in megakaryocytes or inhibit the translocation of the pro-αIIb/β3 complex from the endoplasmic reticulum (ER) to the Golgi or the export of the mature complex to the cell surface. Most of the mutations affect the β-propeller region of αIIb and the epithelial growth factor structural domain of β3.[9] In addition, it has been shown that patients with a definite diagnosis of GT have cases with undetected variants in the ITGA2B and ITGB3 genes.[10] It is also suggested that specific deletion/duplication analysis should be considered when sequencing fails to identify both pathogenic variants in GT patients.[2] The possibility of new genes associated with defective αIIbβ3 function should also be further explored, and insights into pathogenesis are needed. Recent haplotype

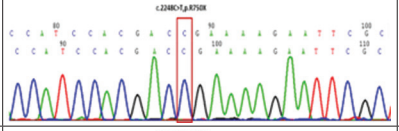
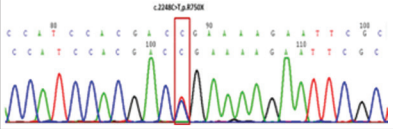
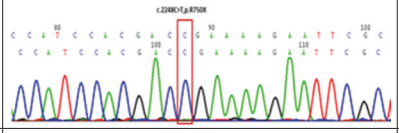
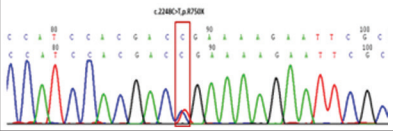
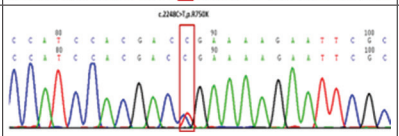
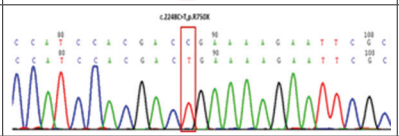
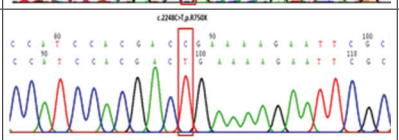
Table 1. The mutant site of the primer sequence

mutant site	primer sequence
ITGB3 c.2248C>T	F: GTGCTTCTTCCTCACAGAGT R: GAGGATAATGGAAAGACGATGGT

Table 2. The clinical characterization of research population

Research population	Clinical characterization
IV5	Anemic at birth, hard to stop the bleeding, Died of gastrointestinal haemorrhage, haemorrhagic shock at the age of 12 months.
IV6	Postnatal generalised skin erythema with reduced platelet membrane glycoprotein GPIIb/IIIa
IV13	Repeated epistaxis and bleeding in childhood, died of haemorrhage at the age of 2 years

Table 3. The results of Sanger sequencing peaks

Family line	Sanger sequencing peaks	Description of the mutation	note
II2		/	
II4		*ITGB3* NM_000212.2: c.2248C>T / p.Arg750Ter variation of heterozygosity	
III4		/	
III5		*ITGB3* NM_000212.2: c.2248C>T / p.Arg750Ter variation of heterozygosity	
III6		*ITGB3* NM_000212.2: c.2248C>T / p.Arg750Ter variation of heterozygosity	
IV5		*ITGB3* NM_000212.2: c.2248C>T / p.Arg750Ter pure mutation	
IV6		*ITGB3* NM_000212.2: c.2248C>T/ p.Arg750Ter variation of heterozygosity	

analyses have also shown that the mutation profile of GT is constantly being updated.[11]

The ITGA2B and ITGB3 genes encode GP IIb/IIIa. Compared with ITGB3, ITGA2B gene variation is more common,[4] which is related to the fact that the ITGA2B gene has 30 exons, significantly more than ITGB3. However, the diversity of gene variants caused by the large number of exons is also the main reason for the predominance of compound heterozygous variants in nonconsanguineous marriages. This was further exemplified by Zhou et al.[12] in 97 cases of platelet apraxia in Han Chinese. This study showed that both children were associated with the c.2248C>T homozygous mutation in the ITGB3 gene. Due to consanguineous marriages and both parents being carriers of the c.2248C>T mutation, the homozygous rate increased.[6,12]

Since integrins αIIb and β3 are involved in primary hemostasis for ITGA2B and ITGB3 defects, the bleeding phenotype predominates. It usually manifests as purpura, epistaxis (60-80%), gingival bleeding (20-60%), and menorrhagia (60-90%). 10-20% will have gastrointestinal bleeding in the form of black stools or blood in the stool, and 1-2% will have intracranial hemorrhage.[13] Heavy bleeding at menarche or menarche may also be the only manifestation. In contrast, gastrointestinal bleeding and hematuria may be seen in a minority of patients, and very few patients present with heavy bleeding.[3] Epistaxis is the most common cause of severe bleeding, especially in the pediatric population.[2] It has been found that the severity of bleeding is not related to the platelet surface GP IIb and GP IIIa levels. Therefore it is not possible to determine the association with either genos on the basis of the GT clinical phenotype.[13,14] There is heterogeneity in bleeding manifestations. This suggests that genotype is not the only determinant of bleeding heterogeneity and further suggests that heterogeneity is a multifactorial symptom. In general, the severity of bleeding (excluding excessive menstruation and pregnancy-related bleeding) decreases with age.

At present, the basic diagnostic features of patients are normal platelet count and morphology, while platelet aggregation function tests and platelet GPIIb/IIIa complex numbers or quality abnormalities are the most diagnostically significant. However, the testing technology of primary hospitals is limited, and initial screening tests are feasible for suspicious patients, mainly including blood smear, bleeding time (BT) and clot contraction tests. Further evaluation includes platelet function testing, including platelet aggregation test, flow cytometry or/and protein blotting to detect the expression level of GP IIb (CD41), GP IIIa (CD61), or molecular genetics.GT is an autosomal recessive genetic disease, and symptomatic treatment is the mainstay of treatment.[15] As molecular medicine continues to progress, precision medicine has become possible. Genetic testing can help diagnose the disease, and early diagnosis and early intervention can reduce the risk of severe bleeding.

Due to an insufficient understanding of this disease and the heterogeneity of bleeding manifestations, the initial diagnosis and onset time are often delayed significantly. Therefore, for patients with repeated clinical bleeding and normal monitoring of platelets, coagulation function, and coagulation factors, we need to be highly alert to the possibility of this disease, and platelet function and genetic molecular biology testing should be performed. Both children in this report were detected with a pure heterozygous variant of the ITGB3 gene c.2248C>T, with normal blood counts, normal coagulation function, and poor platelet aggregation on blood smears. The preexisting patient presented with recurrent epistaxis, difficulty in stopping bleeding,

gastrointestinal bleeding, and severe anemia, and eventually died of gastrointestinal hemorrhage and hemorrhagic shock. The sister of the proband shows only skin petechiae ecchymosis at birth. She was born for 4 months old with no hematuria, hematochezia or neurologic symptoms and satisfactory weight gain.

The parents of this child are carriers of the ITGB3 gene c.2248C>T and are closely related (aunt-cousin) by marriage. Both of their children have a pure variant of the ITGB3 gene c.2248C>T. Today, after many years of promoting eugenics, there are still consanguineous marriages. When the diagnosis of the first child is clear and the second child is born again, there is not enough attention to do prenatal genetic counseling. This reminds us of the importance of multidisciplinary cooperation in prenatal diagnosis, genetic counseling, and health promotion, especially for parents in their childbearing years who are not well educated. Multidisciplinary assistance in the management of patients with GT is advocated[16] for better patient management and life guidance. The diagnosis of this case may assist the parents in further genetic counseling.

ONLINE RESOURCE

EXAC database (http://exac.broadinstitute.org)
Thousand Genomes Database (http://www.internationalgenome.org/data)
dbSNP database (https://www.ncbi.nlm.nih.gov/snp/)
HGMD database (http://www.hgmd.cf.ac.uk/ac)
Clinvar database (https://www.ncbi.nlm.nih.gov/clinvar/)

ETHICAL COMPLIANCE

The ethics committee of Jinhua Maternal & Child Health Care Hospital approved this study. Signed written informed consents were obtained from the patients and/or guardians.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to report relevant to this article.

AUTHOR CONTRIBUTIONS

QZ and KJ designed the study and performed the experiments, CF, ZC and WF collected the data, HL, HW and YG analyzed the data, QZ and KJ prepared the manuscript. All authors read and approved the final manuscript.

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