Novel hereditary spherocytosis-associated splice site mutation in the *ANK1* gene caused by parental gonosomal mosaicism

Hereditary spherocytosis (HS) is a heterogeneous condition of inherited hemolytic anemia characterized by anemia, jaundice, cholelithiasis and splenomegaly with a prevalence of 1 in 10,000 in China.[1] Diagnosis of HS is mainly based on a positive familial history, clinical features and laboratory data, and observation of spherocytes in a peripheral blood smear. Additional tests include the eosin-5′-maleimide (EMA) binding test, the osmotic fragility test, and the acidified glycerol lysis test (AGLT).[2,3] Splenectomy is an effective surgical treatment for adult patients with HS in moderate and severe forms.[4]

A couple planning for a second child came for a genetic consultation since their 3-year-old daughter, who presented with anemia, jaundice and splenomegaly, had been diagnosed with HS. The red cell count was reduced to 2.35×10^{12}/L (ref: 3.80 - 5.10 $\times 10^{12}$/L), and hemoglobin was only 6.3 g/dL (ref: 11.5 - 15 g/dL). Glucose-6-phosphate dehydrogenase (G6PD) activity, an autoimmune antibody test, hemoglobin electrophoresis, and the α- and β-thalassemia genetic mutation screen found no abnormality. Osmotic fragility was increased, and spherocytes were observed in a peripheral blood smear. Both her 28-year-old father and 27-year-old mother were asymptomatic. The available laboratory data are summarized in Table 1.

Targeted NGS was performed for DNA extracted from peripheral blood mononuclear cells (PBMCs) of the proband using a panel targeting all exons and adjacent introns of *ANK1, EPB42, SLC4A1, SPTA1*, and *SPTB* genes. For the proband, the mean depth was 2,096-fold; 99.00% of the mapped reads were on target, and 98.41% of the target bases were covered at least 20 times. The mean uniformity of base coverage was 95.07%.

A total of 97 variants were identified in the proband, distributed in exons, introns, 3′-UTR, 5′-UTR and splice site. These variants were filtered according to mutation type, amino acid alteration, effect on reading frame, minor allele frequency in 1000G, ExAC, dbSNP, ClinVar, and gnomAD databases, records in the HGMD database, and mutation functional prediction. After filtration, a novel heterozygous *ANK1* c.3084-2A>G (NM_001142446.1) splice site mutation (covered by 1286-fold) was selected for further validation by Sanger sequencing, and the *ANK1* c.3084-2A>G mutation was not found in the 1000G, dbSNP, ClinVar, ExAC, gnomAD, or HGMD databases. Sanger sequencing confirmed the heterozygous ANK1 c.3084-2A>G mutation in the proband. Genetic screening within the family members revealed that this mutation was absent from

Table 1. Laboratory test results.

Test	Proband Result	Father Result	Reference
RBC	2.35×10^{12}/L (↓)	5.01×10^{12}/L	3.8-5.1 $\times 10^{12}$/L
PCV	20.1 (↓)	42.2	35-45%
Hb	6.3 (↓)	14.6	11.5-15.0 g/dL
MCV	85.5 fL	84.2 fL	82-100 fL
MCH	26.8 pg (↓)	29.1 pg	27-34 pg
MCHC	31.3 g/dL (↓)	34.6	31.6-35.4 g/dL
TBIL	52.1 mol/L (↑)	NA	3.4-20.5 μmol/L
BRD	15.0 mol/L (↑)	NA	0-6.8 mol/L
IBIL	37.1 mol/L (↑)	NA	≤ 13.3 μmol/L
G6PD	4254 U/L	NA	> 1100 U/L
ALT	9	NA	≤ 33 U/L
AST	25	NA	≤ 32 U/L
LDH	285	NA	120-300 U/L
D-Dimer	1.03 (↑)	NA	< 0.5 μg/mL
ACA IgG	4.29	NA	0-12 GPL/mL
ACA IgM	0.55	NA	0-12 GPL/mL
ANA	Negative	NA	Negative
Anti-LKM antibody	Negative	NA	Negative
AMA	Negative	NA	Negative
Anti-SLA	Negative	NA	Negative
Anti-GP210	Negative	NA	Negative
Anti-SP100	Negative	NA	Negative
Anti-Ro-52	Negative	NA	Negative
Anti-SMA	Negative	NA	Negative

RBC: red blood cell; PCV: packed cell volume; Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; TBIL: total bilirubin; BRD: bilirubin direct; IBIL: indirect bilirubin; G6PD: glucose-6-phosphate dehydrogenase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; LDH: lactate dehydrogenase; ACA: anti cardiolipin antibody; ANA: antinuclear antibody, Anti-LKM antibody: anti-liver-kidney microsomal antibody; AMA: anti-mitochondrial antibody, SLA: soluble liver antigen antibody; SMA: smooth muscle antibody. NA indicates not available.

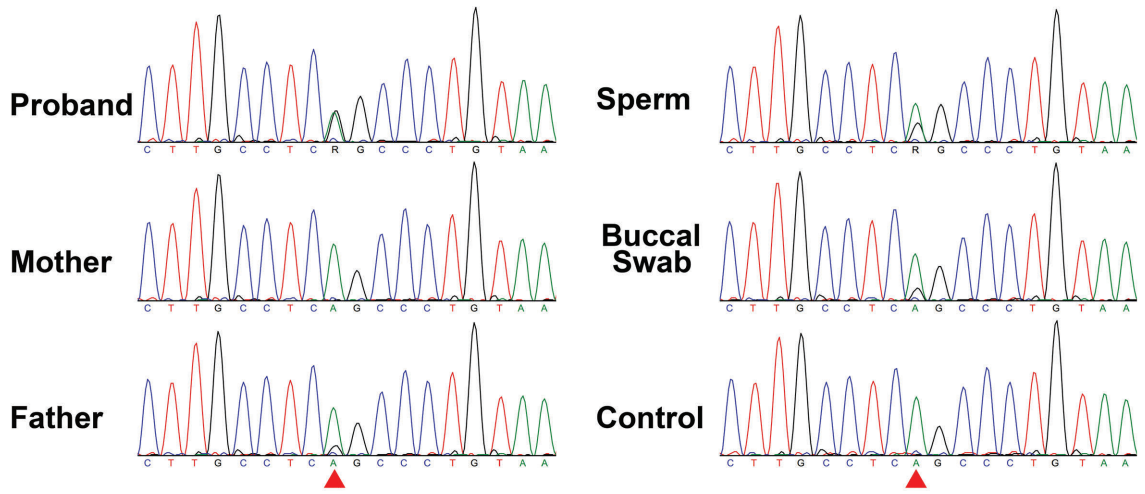

Figure 1. Sanger sequencing validation of the *ANK1* c.3084-2A>G mutation. DNA was extracted from PBMCs from the family members and a healthy control. Moreover, DNA from a buccal swab and sperm was also extracted and sequenced. The equilateral triangle indicated the mutation site.

her mother. However, her father's chromatogram showed a small peak of G within the A reference nucleotide at the *ANK1* c.3084-2 position, which was repeated in a buccal swab sample and was more obvious in his sperm (Figure 1). These results suggest the probability of germline and somatic mosaicism for the proband's father. To evaluate this hypothesis, NGS was further performed for DNA from PBMCs, the buccal swab, and the sperm of her father. The *ANK1* c.3084-2A>G mutation (chr8:41552851) was present at 16%, 15%, and 29% with 735-, 745-, and 901-fold sequence coverage for PBMCs, the buccal swab and sperm, respectively, providing evidence of germline and somatic mosaicism. Buccal mucosa may contain variable contamination of leukocytes. Taken together, her father was a combination of germline and somatic mosaicism, and the proband's constitutional *ANK1* c.3084-2A>G mutation was caused by her father's mutant germline cells.

To explore the splice site effect of the *ANK1* c.3084-2A>G mutation (located in intron 27), mRNA of PBMCs was isolated from the family members and reverse transcribed into cDNA. Primers covering exon 27 to exon 29 to generate a 624 nucleotide PCR product were used. An additional, 100 nucleotide larger PCR product was observed in the proband cDNA sample (Figure 2A). This band was not observed in the other family members or the healthy control. The absence of this larger band in the father's sample may be due to the low frequency of the mutation in the father's PBMCs (16%) and instability of the alternatively spliced mRNA product. This result was also consistent with the clinical symptoms in this family, as spherocytes were observed in the film of the proband and absent in the father's blood smear (Figure 2C). Sanger sequencing validated that the last 126 nucleotides of intron 27 were inserted between exon 27 and exon 28 (Figure 2B), resulting in an in-frame insertion of a 42 amino acid sequence. Several splice site prediction programs also predicted the presumptive effect of this splice site mutation. Scores of 7.4 and -3.5 in GENIE, 7.82 and -0.13 in MES (First-order Markov Model), 0.32 and 0 in NetGene2, were predicted for the wild type and mutant, respectively. The high values meant a high possibility of being splice sites, and the change in value (from high to low) represented the loss of a splice site by the programs. Therefore, the *ANK1* c.3084-2A>G mutation disrupted the normal splice site of *ANK1* mRNA.

The *ANK1* c.3084-2A>G mutation induced amino acid insertion (p.Leu1027_1028Serins42), and this insertion maintained the reading frame but codon 1028 which split between exon 27 and 28 was altered. One base of the new sequence fulfilled codon 1028, then the new sequence was inserted (41 codons + 2 bases), and the last base of codon 1028 in exon 28 fulfilled the inserted sequence in frame and codon 1029 continued unchanged. ZU5-1 domain is comprised of codon 954~1109, spanning exon 27 (954~1028) and 28 (1029~1079). The UPA (codon: 1275~1403) and the two ZU5 domains (ZU5-2 codon: 1111~1257) form a structural supramodule named ZZU to bind to spectrin, especially β-spectrin. The ZU5-1 domain is required for this binding, while mutations in ZU5-2 and UPA showed no impact on the binding to spectrin, indicating that the ZU5-2 domain and the UPA are involved in ankyrin's functions other than binding to spectrin.[5] The wild-type and mutant structure of ZU5-1 was predicted by SWISS-MODEL.[6] This insertion of this family occurred in the center (Figure 2D), and it may interrupt ZU5-1 domain, therefore impairing ankyrin's binding to spectrin.

In most cases, HS is caused by heterozygous mutations in the ANK1 gene located on 8p11.21, encoding ankyrin, which constitutes the major component of the red cell membrane skeleton, with a 24 homologous repeat N-terminal membrane-binding domain (MBD) involved in the binding of band 3 protein, spectrin-binding central domain (SBD), and the least conserved regulatory C-terminal, which is subject to extensive.[7] Ankyrin interacts with band 3 protein, band 4.2 protein, α- and β-spectrin, and deficiency of ankyrin leads to decreased incorporation of spectrins.[8] The majority of HS cases carrying *ANK1* mutations were inherited in an autosomal dominant pattern, although autosomal recessive inheritance has also been observed.[9]

Genetic mosaicism refers to the presence of two or more genetically distinct cells within an organism, which

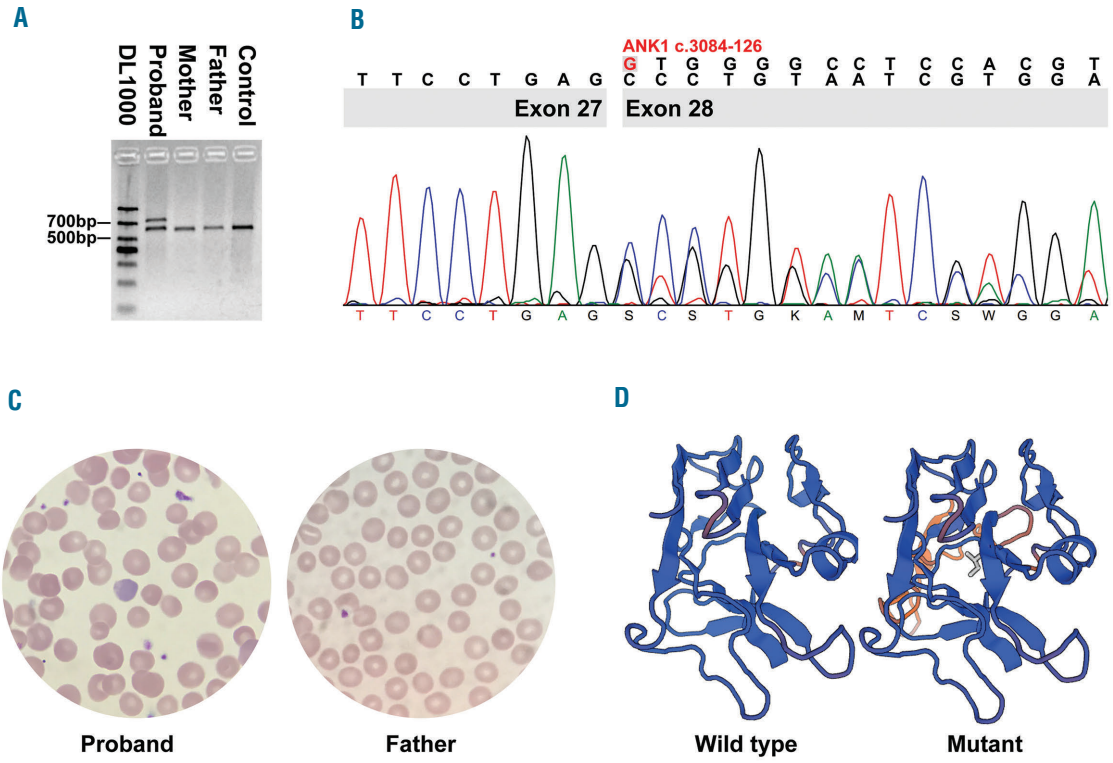

Figure 2. Sanger sequencing validation of the *ANK1* c.3084-2A>G mutation at an mRNA level. A, Exon 27 to exon 29 was amplified from mRNA reverse transcribed cDNA isolated from PBMCs. A DNA gel identified an additional band in the proband that was approximately 100 bp larger. B, Sanger sequencing validated that the last 126 nucleotides of intron 27 were inserted between exon 27 and exon 28. C, Spherocytes were observed in the film of the proband and were absent in the father's blood smear. D, The wild-type and mutant structure of the ZU5-1 domain were predicted by SWISS-MODEL, and an insertion occurred in the center of the ZU5-1 domain.

results from postzygotic mutational events. There are several types of mosaicisms categorized by the tissue distribution of the mutant cells, including somatic, germline, and gonosomal mosaicisms. Somatic mosaicism restricts mutations to somatic cells and consequently the proband, precluding mutation in gonadal tissues. Germline mosaicism describes genetic heterogeneity within the gonadal tissue, permitting mutations to be inherited and constitutionally expressed by subsequent offspring. The labelling of an individual with germline mosaicism is typically based on the presence of mutation in some proportion of germ cells (typically sperm), and the absence of the mutation in PBMCs and/or skin fibroblasts. Gonosomal mosaicism is a combination of both somatic and germline mosaicisms, where mutation is present in both somatic cells and the gametes. Similar to germline mosaicism, gonosomal mosaicism is also transmissible.[10] Mosaicism can lead to a less severe phenotype compared to mutations expressed in a constitutional state, indicating that more widespread mosaicism may have a more severe phenotype.[11] Diverse molecular types of genetic lesions, ranging from a single nucleotide change to large scale chromosomal alteration, can be present in mosaic forms. Germline mosaicism showed an overall risk of 1-2% for point mutations, and it may rise up to 4% for chromosomal rearrangements.[12,13]

NGS has been widely used to validate mosaicism, which might be missed by Sanger sequencing due to its detection limit of approximately 20%.[14] In this family, the proband's father harbored 16%, 15% and 29% with the *ANK1* c.3084-2A>G mutation in the PBMCs, buccal swab, and sperm, respectively, and it was obviously observed only in the sperm. Therefore, this mutation may be transmitted to the second child they are planning to have.

This is the first report of genetic mosaicism of HS caused by an asymptomatic father who has gonosomal mosaicism. Bassères DS *et al.* reported a *de novo* frameshift mutation in the SPTB gene, and they supposed that the mutation might be caused by parental germline mosaicism without further confirmation.[15]

In conclusion, mosaicisms pose challenging dilemmas for the diagnosis, prognosis and reproductive counseling of families with individuals affected by mosaic diseases. When parents have a child with constitutional, de novo occurrence of a disorder inherited in autosomal dominant pattern, parental germline or gonosomal mosaicisms must be considered, and they are at risk of passing on the same mutation to future children.

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