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Original Article

A novel mutation in the *ANK1* gene causes hereditary spherocytosis in a Chinese patient

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Abstract: Background: Hereditary spherocytosis (HS) is the highest incidence disease of hemolytic anemia and is characterized by the production of spherocytes red blood cells. To date, a number of mutations in 5 genes have been identified in patients with HS and the mutations in *ANK1* gene account for 75% patients. Methods: Whole exome sequencing (WES) was performed in a Chinese HS patient with his parents to identify mutation genes that were responsible for the disease. Prioritized candidate genes were screened based on clinics, pedigree, and mutation characters, and were validated by Sanger sequencing. The crystal structures determined previously were downloaded from PDB and further analyzed. Sequence conservation together with mutation characteristics of ANK1 were studied. Results: The proband suffered from severe HS that requiring blood transfusion. WES revealed a heterozygous c.3398 (exon29) delA deletion in *ANK1* gene in the proband. The 1 bp-deletion causes a frameshift mutation and is absent from the parents. Structure analyzation shows that the mutation found in this study is possible to induce the instability of ZU5B which in turn lead to HS. Conclusions: Our study detected a new de novo ankyrin gene mutation c.3398 (exon29) delA that could lead to severe HS.

Keywords: Hereditary spherocytosis, *ANK1*, frameshift, whole exome sequencing

Introduction

Hereditary spherocytosis (HS) is the most common hemolytic anemia induced by abnormal red blood cell membrane and is characterized by the production of spherocytes red blood cells, behaving as anemia, jaundice, and splenomegaly. HS is a heterogeneous disorder, which means various clinical phenotypes, variable severity and different inheritance patterns in individual patients [1]. The common complications of HS comprise cholelithiasis, hemolytic episodes, and aplastic crises [1]. HS occurs in about 1 in 2,000 individuals in northern Europe and North America, and it has been found in other populations such as Japan with a lower incidence rate [1, 2].

To date, a number of mutations in 5 genes, namely *ANK1*, SPTA1, SPTB, SLC4A1 and EPB4, have been identified in patients with HS, and mutations in *ANK1* gene account for about 50% patients in northern Europe and North America but as low as 5%-10% in Japan [1]. It is reported that the disruptions of the *ANK1* translation caused by frameshift and nonsense mutations are the main reason for HS [3, 4]. For HS patients, autosomal dominant pattern, which means a heterozygous mutation is enough to cause disease, makes up 75% of the cases [5]. In addition, novel mutations of other forms causing HS in *ANK1* gene have been constantly found around the world.

The decreases of cost and time make whole exome sequencing (WES) proper for detecting novel mutations that cause Mendelian diseases [6]. WES authorizes a specific enrichment step and sequences exons of all protein-coding genes, including the HS genes and other genes related to blood disorders [7, 8]. WES has been suggested as a first-tier molecular test for suspected monogenic disorders [9]. In this study, we identified a de novo *ANK1* 1-bp deletion via WES in a Chinese patient who suffered from severe dominant HS. Our study confirmed that WES is effective for discovering de novo causal mutations in patients with HS.

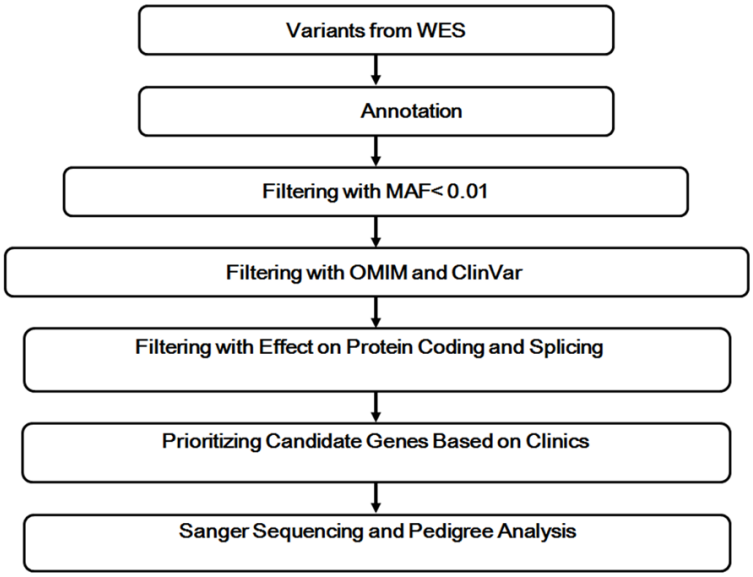

Figure 1. Workflow to find disease caused genes from whole exome sequencing data.

Patients and methods

Patient's data

The proband was a 2 months old male child and was admitted to hospital because of pallor and tachypnoea. He was pale after birth and blood routine examination showed that he was with mild anemia and neonatal hyperbilirubinemia. Cured with blue light irradiation, he got better and discharged from hospital. One month earlier, he was diagnosed as severe anemia, hemolytic anemia, and urinary tract infections (E. coli infection) and discharged after treated with anti-infection, iron supplementation, blood transfusion, and anemia correction. Sallow complexion gradually appeared after his discharge and repeatedly blood routine test showed that he was with low hemoglobin level (hemoglobin 103-70 g/L), however, no special treatment was applied. This study was approved by the ethics committee of Hunan Provincial People's Hospital, and informed consents were obtained from parents of the participant.

WES analysis

The patient's exome DNA was captured using the Roche-NimbleGen Sequence Capture EZ Exome v2 kit (Roche NimbleGen, Madison, WI) and sequenced paired-end 150 bp sequencing on the Illumina HiSeq 2500 platform (Illumina Inc.). Sequence reads were aligned to human reference genome 19 (hg19) using BWA [10]. Duplicates were removed using Picard (v1.67, (http://broadinstitute.github.io/picard/) tool. And GATK Unified Genotyper (v2.3.6) was used to call variant [11]. ANNOVAR tools were applied in annotating Single nucleotide variants (SNVs) and small indels [12]. Then we constructed a filtering pipeline to select candidate variants. Variants with frequency more than 1% in the population were removed. Genes with pathological variants that have been described in OMIM (http://www.omim.org/) and ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/) and that leading to loss function of proteins were given higher priority. In addition, the pathogenicity of variants was assessed by three protein prediction algorithms SIFT [13], PolyPhen2 [14], MutationTaster2 [15]. Finally, variants in candidate genes that are known to be involved in HS and other blood disorders were selected for Sanger sequencing.

Sanger sequencing

Sanger sequencing was performed on all family members. Genomic DNA was extracted from each of the 3 family members using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). We designed primers with PerlPrimer software (http://perlprimer.sourceforge.net/) [16]. Primers were designed as following: forward, ANK1-F: 5'-AGAGAAGAAGAGGGTGTGCC-3', reverse, ANK1-R: 5'-TGTGGCATTTCAAAGCACCA-3'.

Structure investigation and conservative analysis

ANK1 gene mutational situation that was responsible for HS occurrence was studied by inquiry of HGMD (http://www.hgmd.cf.ac.uk/ac/index.php) [17]. The structures of ANK1 revealed previously were downloaded from PDB (http://www.rcsb.org/pdb/home/home.do) and further analyzed using PyMOL (http://www.pymol.org/). Protein sequence conservation was detected by using the online server of AL2CO [18].

Table 1. One-bp deletion c.3398 (exon29) delA was found in the proband

Gene	Position	Mutation	Protein effect	Type
ANK1	chr8:41552162-41552162	c.3398 (exon29) delA	p.Q1133Rfs*13	Het

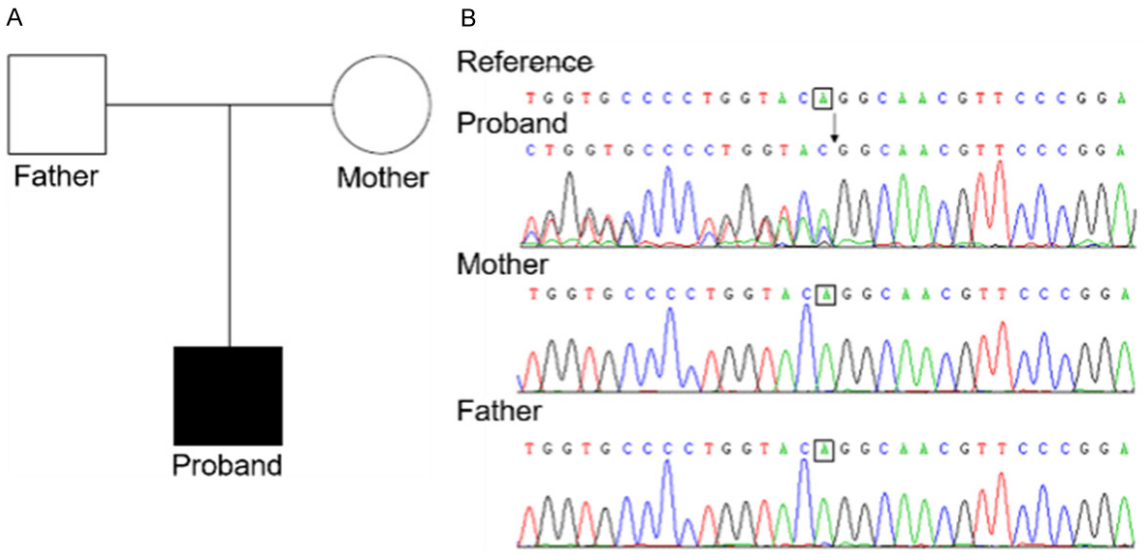

Figure 2. Pedigree of the family affected by hereditary spherocytosis (HS) and the mutations identified. A. Individuals affected with HS are indicated by a black filled circle (females) or square (males). B. Electropherograms indicate the *ANK1* mutation identified in the patient.

Results

Case presentation

Two month old boy developed normally, with good nutrition. Physical examination showed that he had a sallow complexion, mild systemic yellow stain and *yellow conjunctiva*. No abnormal was found beside that the liver was 2 cm below costal arch. Accessory examination showed that WBC was 8.55 × 10^9/L, NEUT was 25.6%, HGB was 62 g/L, PLT was 311 10^9/L, MCV was 83.4 fl, and MCHC was 348 g/L. Reticulocytes percentage was 10.9%, with the absolute value of 0.224. Mature red blood cells varied in size. Abdominal ultrasound showed hepatosplenomegaly and liver function showed that total bilirubin was 39.57 μmol/L, direct bilirubin was 11.29 mmol/L, and indirect bilirubin was 28.28 mmol/L. Bone marrow cytological examination indicated proliferation of anemia. Iron protein was 282 ng/ml, vitamin B_{12} was 699 pg/ml B12, zinc was 34.34 μmol/L, and iron was 4.22 mmol/L. No abnormalities were found in genetic metabolic disease screening, thalassemia gene, and Coombs' test. HbF was 63% and HBA2 was 1.29%. Isopropanol precipitation test was negative. Red cell osmotic fragility test showed that G-6-PD gene was normal. No abnormal was found in red cell H inclusion body test and Heinz body rest was normal. No abnormal region was found by hemoglobin electrophoresis.

Clinical manifestations of anemia, jaundice, splenomegaly, increased peripheral blood shaped red cells, and positive family history, are commonly used for the diagnosis of HS. As for this child, anemia, jaundice, hepatosplenomegaly, and neonatal hyperbilirubinemia were found, however, no significantly increased peripheral red blood cells were observed. In addition, no red cell osmotic fragility increase was observed and he had no positive family history, as a result the clinical diagnosis was difficult.

Characterization of ANK1 mutation

To identify the gene mutation that induced HS in the patient, WES was performed. By WES, data with an average depth of 88.4X were achieved. A total of 10,667 variants were iden-

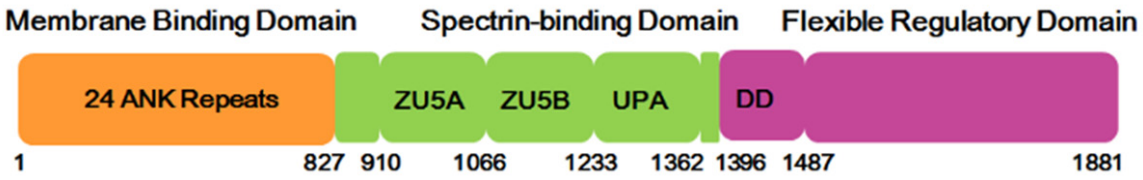

Figure 3. Diagrammatic sketch of domain organization of human ANK1 protein.

tified in the proband and the pipeline as shown in **Figure 1** was applied to select pathogenic mutations. After filtering, we identified one-bp *ANK1* deletion (c.3398 (exon29) delA) that might be responsible for the patient's clinic phenotypes. The 1-bp deletion was at heterozygous state in the exon 29 and has not yet been reported previously (**Table 1**).

Sanger sequencing validation

To confirm the mutation identified by WES, Sanger sequencing analysis of the mutation was performed on all available family members. The results confirmed that the proband was heterozygous for the 1-bp *ANK1* deletion, while the 'his parents had wild type ANK1 gene (**Figure 2**). The 1-bp deletion was not found in the ExAC database (http://exac.broadinstitute.org/), dbSNP database (http://www.ncbi.nlm.nih.gov/snp), 1000 Genome Project (http://www.1000genomes.org/), OMIM, and ClinVar. These findings suggested that we found a novel de novo *ANK1* mutation that might be responsible for dominant HS.

Distribution of mutational sites determined among ANK1 gene

Human ANK1 protein is composed of 1881 amino acids and consists of membrane binding domain, spectrin-binding domain, and a C-terminal flexible regulatory domain (**Figure 3**). Membrane binding domain consists of 24 tandem ankyrin repeats, spectrin-binding domain is further divided into ZU5A, ZU5B, and UPA domains, and a DD domain is found in regulatory domain [19]. To assess the functional relevance of the mutation identified in this study with the occurrence of HS, mutational situation of *ANK1* gene was studied using the online server of HGMD. As listed in **Table 2**, a total of 45 mutations in the exons were identified so far in *ANK1* with the mutation types of missense/nonsense, small deletions, and small insertions. Generally, Ankyrin repeats domain has the most mutations, as many as 24 points. In addition, mutations in ZU5A are found in all mutational types, with a total of 6 mutations. Notwithstanding the Ankyrin repeats domain has the most mutations, the mutational ratio of ZU5A is the highest. The result indicated that mutation in each domain of *ANK1* gene could lead to HS and ZU5A is prone to mutate and is extremely diver.

Characteristics of mutational sites of ANK1

In order to further unravel the cellular basis of the effect of our finding on the occurrence of HS, crystal structures involving spectrin-binding domain of Ankyrin repeats proteins determined so far were analyzed. Altogether, the crystal structures of ANK1 (PDB 3 kbt, and 3ud1) and ANK2 (PDB 4d8o) were applied. Jonathan J. Ipsaro and Alfonso Mondragon have solved the structure of human I-spectrin repeats 13 to 15 in complex with the ZU5-ANK domain of human ANK1 (3 kbt). As illustrated in **Figure 4A**, the result shows that ZU5A domain itself is capable of binding with spectrin without the involvement of ZU5B. Structure-based sequence alignment of ZU5A and ZU5B demonstrated that they are highly conserved (**Figure 4B**), therefore, it is natural to imagine what role ZU5B plays in the interaction when ANK1 interacts with other proteins. And then, Alfonso Mondragon and coworkers have determined the structure of spectrin-binding domain of Ankyrin (3ud1), and additionally, they performed binding affinity detection. The result argued that ZU5B domain does not affect spectrin/ankyrin binding although the sequences of ZU5A and ZU5B domains are highly conserved [20].

There is no structure of ANK1 which could directly points out the role that ZU5B domain plays and the sequence of Ankyrin repeats are highly conserver [21], therefor, the structure of

Table 2. Missense, nonsense, small deletion and small insertion in the exons of ANK1 gene

Exon	Codon number	Mutation DNA	Protein	Type of variant	Domain	Reference*
1	1	ATG-ATA	Met-Ile	Missense	MBD	[26]
1	9	GAA-TAA	Glu-Term	Nonsense	MBD	[27]
6	277	CAC-CGC	His-Arg	Missense	MBD	[28]
12	463	GTC-ATC	Val-Ile	Missense	MBD	[29]
16	612	CAG-TAG	Gln-Term	Nonsense	MBD	[30]
17	631	GAG-TAG	Glu-Term	Nonsense	MBD	[31]
20	765	TCG-TAG	Ser--Term	Nonsense	MBD	[31]
27	1046	CTA-CCA	Leu-Pro	Missense	SBD-ZU5A	[32]
27	1053	CGA-TGA	Arg-Term	Nonsense	SBD-ZU5A	[31]
27	1055	ATC-ACC	Ile-Thr	Missense	SBD-ZU5A	[28]
28	1075	ATC-ACC	Ile-Thr	Missense	SBD-ZU5B	[32]
30	1230	TAC-TAG	Tyr-Term	Nonsense	SBD-ZU5B	[30]
31	1252	CGA-TGA	Arg-Term	Nonsense	SBD-UPA	[30]
35	1436	CGA-TGA	Arg-Term	Nonsense	SBD-DD	[29]
36	1488	CGA-TGA	Arg-Term	Nonsense	RD	[31]
38	1592	GAC-AAC	Asp-Asn	Missense	RD	[32]
38	1640	CAG-TAG	Gln-Term	Nonsense	RD	[30]
38	1669	GAA-TAA	Glu-Term	Nonsense	RD	[33]
38	1721	TGG-TGA	Trp-Term	Nonsense	RD	[34]
40	1833	CGA-TGA	Arg-Term	Nonsense	RD	[34]
1	1	ATG ccc tat tct gTG GGC	Frameshift	Deletion	MBD	[30]
4	111	GGT TtR ACA	Frameshift	Deletion	MBD	[30]
5	145	TTC AcG CCT	Frameshift	Deletion	MBD	[35]
6	173	CGC ctc ccg gcc ctg cac atc gcG GCC	Frameshift	Deletion	MBD	[32]
6	186	ACG CGc acg gct gcg GTG	Frameshift	Deletion	MBD	[30]
9	328	GAC gcA GAG	Frameshift	Deletion	MBD	[36]
11	426	CGG gGG GCG	Frameshift	Deletion	MBD	[29]
14	536	AaA GGA	Frameshift	Deletion	MBD	[32]
15	571	ACC CcC CTG	Frameshift	Deletion	MBD	[30]
15	572	CCC CtG CAC	Frameshift	Deletion	MBD	[37]
16	595	CCG Cac AGC	Frameshift	Deletion	MBD	[32]
21	797	TTA GTc agt GAT	Frameshift	Deletion	MBD	[30]
21	799	tta gTC AGT	Frameshift	Deletion	MBD	[29]
24	906	GCC AgC CCG	Frameshift	Deletion	SBD	[31]
25	932	AAC GGc CTG	Frameshift	Deletion	SBD-ZU5A	[36]
26	982	GGG Gca CAG	Frameshift	Deletion	SBD-ZU5A	[36]
28	1126	GCC AcA TTC	Frameshift	Deletion	SBD-ZU5B	[29]
33	1381	CCC CtG GCC	Frameshift	Deletion	SBD	[31]
5	142	GAA_E515_GTA aAG	Frameshift	Insertion	MBD	[30]
14	505	ACC CcC CCT	Frameshift	Insertion	MBD	[38]
15	571	ACC CcC CCT	Frameshift	Insertion	MBD	[29]
17	636	GTG Acc GCC	Frameshift	Insertion	MBD	[30]
26	941	CGG Acc gga cGT	Frameshift	Insertion	SBD-ZU5A	[31]

MBD: Membrane Binding Domain; Spectrin-binding domain: SBD; Regulatory domain: RD. *References for the table.

ANK2 (4d8o) is applied for analysis. Zhang et al have determined the structure of ANK2 including the domains of ZU5A, ZU5B, UPA, and DD. The structure showed that there ZU5A/UPA

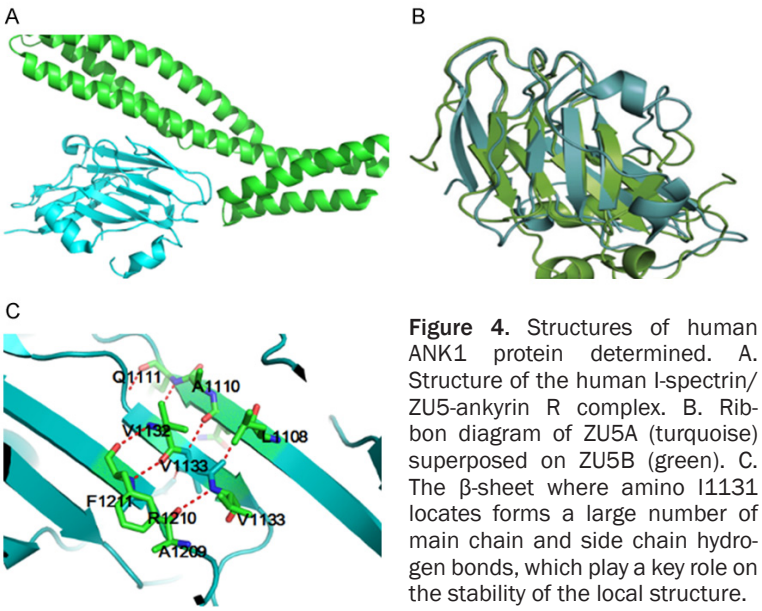

Figure 4. Structures of human ANK1 protein determined. A. Structure of the human I-spectrin/ZU5-ankyrin R complex. B. Ribbon diagram of ZU5A (turquoise) superposed on ZU5B (green). C. The β-sheet where amino I1131 locates forms a large number of main chain and side chain hydrogen bonds, which play a key role on the stability of the local structure.

interaction is required for ankyrins's function other than binding to spectrin. In addition, the structure indicated that ZU5B does not affect the binding of ZU5A to spectrin, however, its interaction to ZU5A could stabilize the ZU tandem, which might be responsible for binding to most of its partners. As for this case, the c.3398 (exon29) delA deletion found in this study is located in the highly conserved ZU5A-ZU5B-UPA-DD region of ANK1 and alters the translation reading frame after 3398 site of the Ankyrin 1 gene. The mutational sites started from 1131, which locates in a β-sheet. This sheet together with another two antiparallel β-sheets interact by forming a large number of main chain and side chain hydrogen bonds, which play a key role on the stability of the local structure (**Figure 4C**). Altogether, by analyzing the structures determined previously it is possible to assume that the de novo mutation found in this study is possible to induce structural instability of ZU5B. This could in turn lead to the instability of ZZU tandem and hence result in impaired interaction of ANK1 to its interacting proteins, which brings about HS in the final.

Discussion

In the present study, we reported the treatment procedure of a 2 month old child admitted because of sallow complexion, mild systemic yellow stain and *yellow conjunctiva*. Physical examination showed no abnormal except that the liver was 2 cm below costal arch. Clinically, diagnosis of HS depends on the manifestations of anemia, jaundice, splenomegaly, increased peripheral blood shaped red cells, and positive family history. For this patient, he had anemia, jaundice, hepatosplenomegaly, and neonatal hyperbilirubinemia but without significantly increased peripheral red blood cells. Beyond that, no red cell osmotic fragility increase was observed and he was without positive family history, which, made the clinical diagnosis rather difficult.

DNA sequencing has been greatly applied both for research and for clinical diagnosis since the discovery of DNA double helix structure. WES is a powerful tool to find new pathological mutations, especially when there is limited clinical information, no familiar history and disease heterogeneity. As the clinical diagnosis was quite difficult, we performed WES so as to identify whether the child was caused by mutations in genes. WES of the proband revealed a c.3398 (exon29) delA 1-bp deletion in *ANK1* gene. With the WES result the child was easily diagnosed as HS.

HS is a heterogeneous disorder with various clinical phenotypes, variable severity and different inheritance patterns, which bring about difficulty to the diagnosis. Moreover, because of no family history of the phenotype, the de novo mutations and possible novel disease caused genes should be taken into account. For the proband reported in this study, WES was the most useful tool because it investigated all possible coding DNA sequence and had potentials to clarify his clinic phenotypes. Sanger sequencing analysis of all the proband's family members found that both the parents were absent for the c.3398 (exon29) delA mutation and had no signs of HS. It means we found a de novo mutation in *ANK1* gene caused dominant HS. And we found the mutation was also absent from the existing SNP database, which confirmed that this mutation is quite

rare. Our report is the first work which correlating the mutation c.3398 (exon29) delA in *ANK1* to HS around the world.

To further unravel the molecular mechanism of the mutation for the onset of HS in the proband, domain composition together with crystal structures of ANK1 and ANK2 were studied. The integrity of the metazoan cell membrane is maintained by extensive protein network and spectrin plays an important role in membrane skeleton scaffold via attaching to adaptor protein Ankyrin [22]. In humans, Ankyrin is divided into 3 types namely 1, 2, and 3 (or R, B, and G) [23], which, all are capable of interacting with multiple binding partners. Protein ANK1 has three domains: membrane binding domain, spectrin-binding domain and regulatory domain [20]. And spectrin-binding domain includes the ZU5A, ZU5B, and UPA domains [20]. The ZU5B domain resembles ZU5A domain. However, unlike ZU5A motif which is the β-spectrin-binding domain of ankyrins, the ZU5B domain does not affect binding to spectrin and extends away from the ZU5A spectrin-binding site revealed by the structure [24]. Instead, ZU5B is interacted with DD domain mediated by UPA domain [20, 21]. Structural investigation result suggested that ZU5B domain does not affect spectrin/ankyrin binding even if the sequences of ZU5A and ZU5B domains are highly conserved [20]. Notwithstanding that ZU5B does not affect the binding of ZU5A to spectrin, its interaction to ZU5A could stabilize the ZU tandem, which might be responsible for binding to most of its partners. For this patient, the c.3398 (exon29) delA deletion found in this study locates in the highly conserved ZU5A-ZU5B-UPA-DD region of ANK1 and could alter the translation reading frame after 3398 site of the Ankyrin 1 gene. The first mutation amino 1131 is located in a β-sheet, which, could form a large number of main chain and side chain hydrogen bonds with its adjacent two antiparallel β-sheets. This interaction is responsible for the maintenance of local structure. To date, four HS-caused points mutations have been detected in the highly conserved ZU5B of the ZU5A-ZU5B-UPA-DD domain (p.lys1140term, p.tyr1230ter, p.arg1340ter and c.3364delC) of the ANK1 protein [3, 25]. Like the above three mutations, c.3398 (exon29) delA deletion in the ZU5B domain reported in this study is quite possible to cause dominant HS. In a summary, a novel 1-bp deletion in *ANK1* (c.3398 (exon29) delA) was identified in a Chinese family affected by HS. It was the fifth mutation location in the ZU5B domain to cause dominant HS. We also showed that WES is of great value in finding de novo disease-caused mutations in monogenic diseases.

Acknowledgements

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Disclosure of conflict of interest

None.

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