



CLINICAL REPORT

Identification of a novel ANK1 mutation in hereditary spherocytosis co-existing with BWS

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Abstract

Background: Beckwith–Wiedemann syndrome (BWS) is an inherited disorder affecting 1 in 10,500 to 13,700 newborns worldwide. The disease is caused in a vast majority of patients by a molecular defect in the imprinted chromosome 11p15.5. Hereditary spherocytosis (HS) is a form of hemolytic anemia associated with a variety of mutations leading to congenital red blood cell (RBC) membrane defects. The prevalence of HS varies by geographic regions around the world, ranging from 1.2 in 100,000 in Asia to 1 in 2000 in Northern Europe.

Methods and Results: Herein, we report for the first time a rare case diagnosed with co-existing BWS and HS. Based on the classical presentations, including macroglossia, hepatosplenomegaly, and macrosomia, the patient was first suspected with BWS. MS-MLPA confirmed the BWS diagnosis based on hypomethylation of maternal 11p15.5 (*KCNQ1OT1*), but no copy number variations in chromosome 11 was detected by CNV-seq. Nevertheless, to scrutinize molecular causes of other symptoms of the patient, including anemia, hyperbilirubinemia, and jaundice, a whole exome sequencing (WES) was performed. We identified a novel and de novo mutation in *ANK1* gene (c.520delC). This frameshift mutation of *ANK1* gene results in a truncated protein without important functional domains and impaired membrane stability and structure of the resultant red blood cells (RBCs), leading to a definitive diagnosis of HS.

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Conclusion: The present case demonstrated that multiple genetic and epigenetic aberrations might co-exist in the complex genetic diseases. For such kind of complicated cases, the different types of molecular tests, such as WES and MS-MLPA, should be utilized in combination to reveal independent causal molecular events. The identifications from this study added new insights into the understanding of molecular mechanisms underlying the co-existing HS and BWS.

KEYWORDS

ANK1 mutation, Beckwith–Wiedemann syndrome, hereditary spherocytosis, *KCNQ1OT1* methylation, MS-MLPA, whole exome sequencing

1 | INTRODUCTION

Beckwith–Wiedemann syndrome (BWS, OMIM: 130650) is a rare genomic imprinting disorder which affects multiple systems. Patients with BWS can present pediatric overgrowth, macroglossia, macrosomia, hemihyperplasia (lateralized overgrowth), and visceromegaly (enlarged abdominal organs) (Weksberg et al., 2010). However, the clinical presentation is highly variable between affected patients, and other symptoms might include omphalocele, creases or pits in the ear skin, hypoglycemia due to congenital hyperinsulinism in infancy, and kidney abnormalities. Molecular defects affecting two imprinted centers (IC1 and IC2) in the chromosomal region 11p15 can be detected in over 80% of BWS patients. And DNA methylation abnormalities are the most prevailing defects, with loss of methylation (LOM) at the maternal IC2 allele found in ~50% of patients.

In addition to diverse clinical presentations, BWS exhibits a high degree of heterogeneity in molecular etiology. A variety of genetic and epigenetic alterations in growth regulatory genes in the chromosomal region 11p15.5 are associated with this disorder, including the LOM of *KCNQ1OT1* (OMIM:604115), gain of methylation in the region containing *H19* (OMIM:103280) and *IGF2* (OMIM:147470). About 20% of BWS cases are caused by paternal uniparental disomy (UPD), leading to an unbalanced expression of the genes on chromosome 11 between the paternal and maternal copies which underlies the signs and symptoms of the disorder. Less commonly, BWS patients harbor mutations in *CDKN1C* (OMIM:600856). Very occasionally a chromosomal abnormality such as rearrangement and duplication of genetic material from chromosome 11 can be identified in BWS patients (Weksberg et al., 2010).

Hereditary spherocytosis (HS, OMIM: 182900) is a hereditary disease of hemolytic disorder associated with a variety of mutations in the genes encoding erythrocyte membrane proteins and leading to erythrocyte membrane defects. The clinical characteristics of HS include

hemolysis, anemia, jaundice, splenomegaly, etc. The morphologic hallmarks of erythrocyte in affected patients are the microspherocyte and loss of RBC membrane surface area, leading to severe jaundice caused by abnormal osmotic fragility of RBC (Iolascon et al., 2019).

We recently diagnosed and treated a case of co-existing BWS and HS who had severe jaundice and moderate anemia intensified by the infection of the upper respiratory tract. The patient presented typical signs of BWS including macroglossia, macrosomia, and visceromegaly, as well as symptoms of HS, such as severe hyperbilirubinemia, neonatal anemia, and jaundice. We applied MS-MLPA and next-generation sequencing (NGS) and identified the LOM at maternal *KCNQ1OT1* as well as a novel mutation in *ANK1* gene (OMIM: 612641). A definite diagnosis of co-existing BWS and HS was reached which is rarely reported. The case is described as follows.

2 | METHOD & RESULTS

2.1 | Clinical observation

A 7-day-old male infant was referred to our hospital for further evaluation. He was previously diagnosed with “jaundiced skin for 3 days.” The patient was a cesarean section born infant with a birth weight of 4.4 kg. The patient was the first full-term baby from a consanguineous marriage. The infant patient presented with symptoms of neonatal jaundice on day 1 and was administered on day 3. At administration, an anemic face, yellow skin and mucous, macroglossia, hepatosplenomegaly, and macrosomia were observed during physical examination (Figure 1). No other significant phenotypical oddities were noticed; supplementary examination revealed that the total bilirubin of 365 $\mu\text{mol/L}$ (normal 5.1–25 $\mu\text{mol/L}$) and the indirect bilirubin of 359 $\mu\text{mol/L}$ (normal 2–14 $\mu\text{mol/L}$) were elevated at administration. In addition, a low hemoglobin level (65 g/L) and normal results of neonate hemolysis test (Table 1) were obtained from the hematological tests. Bone

marrow and whole blood smear both showed decreased mature erythrocytes varied in size and morphology, with the presence of microspherocytes and erythrocytes with loss of membrane area. Erythrocyte osmotic fragility test was positive and suggested fragile erythrocytes. The



FIGURE 1 Clinical photograph of the patient at birth, showing macroglossia and typical facial appearance of BWS

patient was diagnosed with (a) suspected BWS; (b) neonatal hyperbilirubinemia; (c) Neonatal anemia; (d) suspected HS; (e) macrosomia; (f) postnatal overgrowth.

2.2 | Clinical management

The patient was given symptomatic treatments immediately on administration, including phototherapy and erythrocyte transfusion. Nine days after the initiation of the therapy, the levels of bilirubin and erythrocyte were both normalized, and the patient was discharged accordingly.

2.3 | Genetic evaluation

CNV-seq and MS-MLPA were performed to detect suspected CNVs and aberrant methylations previously known in BWS. However, no copy number variation was detected, but an LOM at *KCNQ1OT1* transcription start site differentially methylated region, TSS DMR (IC2), supported the diagnosis of BWS (Figure 2a).

TABLE 1 Clinical features of the patient

Features of the BWS case		
Clinical features		
Gender	Onset age at birth	Follow-up at 3 m
Male	Postnatal overgrowth (birth weight 4.4 kg), macrosomia, Macroglossia	Lateralized overgrowth (weight 8.5 kg)
	Hepatosplenomegaly	More severe macroglossia
	Jaundiced, anemia	Persistent hepatosplenomegaly and exomphalos
		Alleviated jaundiced and anemia
Supplementary examination		
Blood routine examination	Before treatment	After treatment(9 days)
HGB (g/L)	65	130
RBC($\times 10^{12}/L$)	2.1	4.4
TBIL ($\mu\text{mol}/L$)	236.6	194
DBIL ($\mu\text{mol}/L$)	0.5	5.1
IBIL ($\mu\text{mol}/L$)	236.1	180
Neonate hemolysis test	Negative	
Erythrocyte osmotic fragility test	Positive	
Bone marrow smear	Erythrocytes varied in size with spherical RBC	
Molecular genetic diagnosis		
CNV-seq	Negative	
MS-MLPA	Loss of maternal methylation at <i>KCNQ1OT1</i> TSS (IC2 LOM)	
Trio-WES	A novel and de novo heterozygous mutation of the <i>ANK1</i> gene (NM_000037.4: c.520delC, p.L174Sfs*79)	

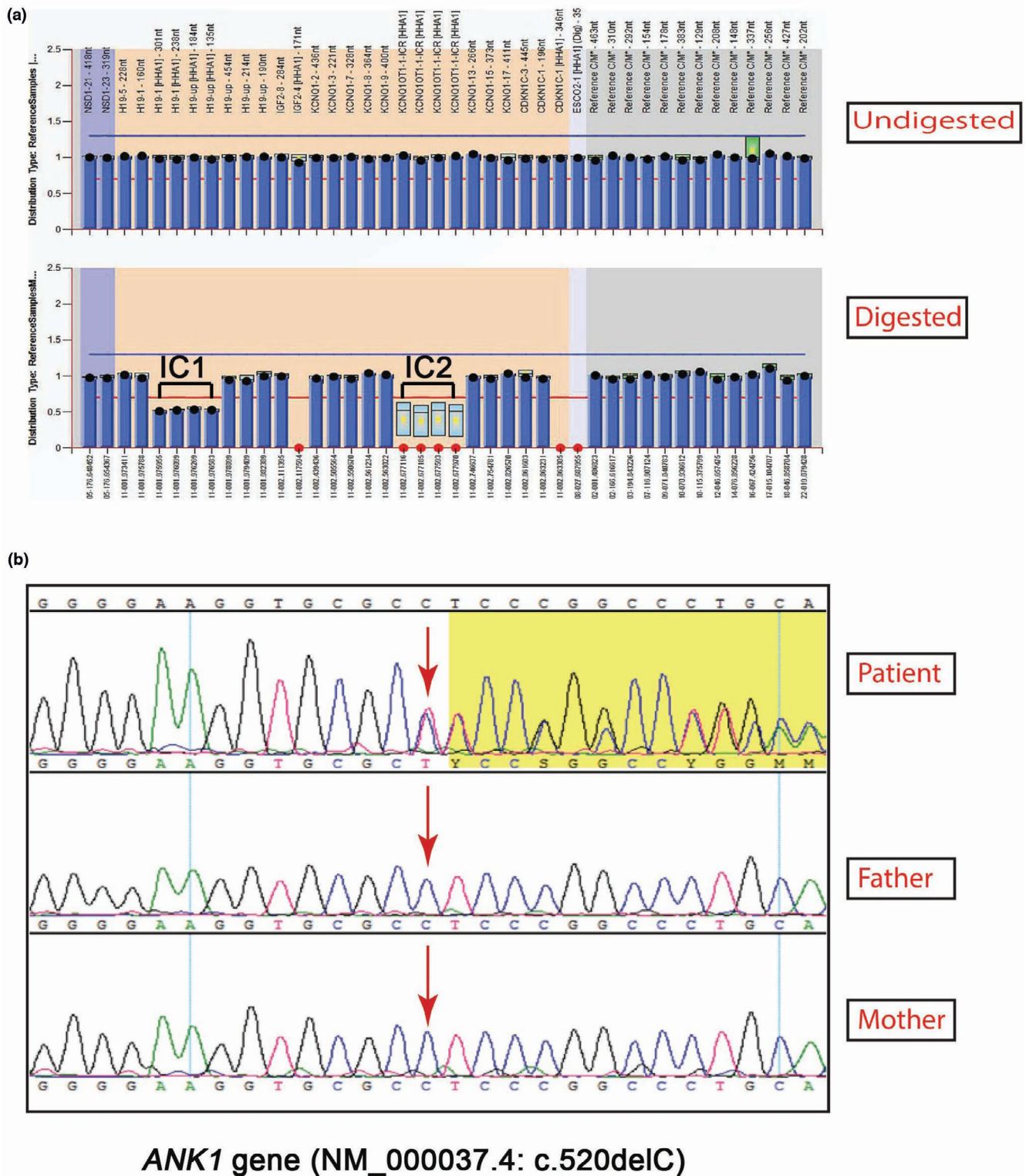


FIGURE 2 The results of molecular genetic tests. (a) MS-MLPA test result of the patient identified a loss of maternal 11p15.5 methylation at *KCNQ1OT1*:TSS-DMR (IC2 LOM). (b) WES and Sanger sequencing detected in the patient (the top panel) a novel heterozygous deletion in exon 6 of *ANK1* gene (NM_000037.4: C.520delC, p.L174Sfs*79). The gene sequences of his father and mother were normal

2.4 | Mutation identification

Trio-WES was conducted to further screen additional alternations existed in DNA sequences. The variant calling

identified a novel heterozygous mutation in *ANK1* gene (NM_000037.4: c.520delC, p.L174Sfs*79) in the patient but not the parents (Figure 2b), which is a frame-shift mutation and results in a premature stop codon leading

to a truncated product of 408 amino acids predicted by ExPASy, compared to 1881 amino acids in wild-type ANK1 (P16157). The wild-type ANK1 has a binding domain of erythroid beta spectrin, repeats 13–15 (Figure 3a), while the truncated product was unable to form repeats and terminated prematurely. The consequence of the mutation was the loss-of-function alleles probably through the lack of functional binding domains or the degradation by non-sense-mediated decay (NMD) (Figure 3b).

This mutation was defined as a pathogenic (PVS1 + PS2 + PM2_Supporting) based on the standards and guidelines for interpretation of sequence variants developed by the American College of Medical Genetics and Genomics (ACGM) (Wang et al., 2019). This mutation has not been reported by single nucleotide polymorphism database (dbSNP), and was not included in the 158 *ANK1* gene mutations reported by HGMD databases (Stenson et al., 2014). The product of this mutation is a variant with p.L174Sfs*79 in amino acid sequence (Figure 3C). This *ANK1* gene mutation was linked to the clinic feature of neonatal anemia, hemolysis, jaundice, and hepatosplenomegaly, confirming the diagnosis of HS. And pedigree analysis of patient was additionally performed using Sanger sequencing. The results were again confirmed that this mutation is spontaneously developed in the patient.

2.5 | Patient outcome and follow-up

During the follow-up period, the patient was weighed 8.5 kg at 3 months of age and the lateralized overgrowth of the patient was apparent with a 0.5 cm difference between two lower limbs' length. Other observations included more severe protrusion of inner tongue, persistent hepatosplenomegaly, and more severe macroglossia (Figure 4). Anemia and jaundice were alleviated. The patient was initially on regularly follow-up and symptomatic treatment such as regular blood transfusion, but finally lost follow-up during the preparation of the manuscript.

3 | DISCUSSION AND CONCLUSIONS

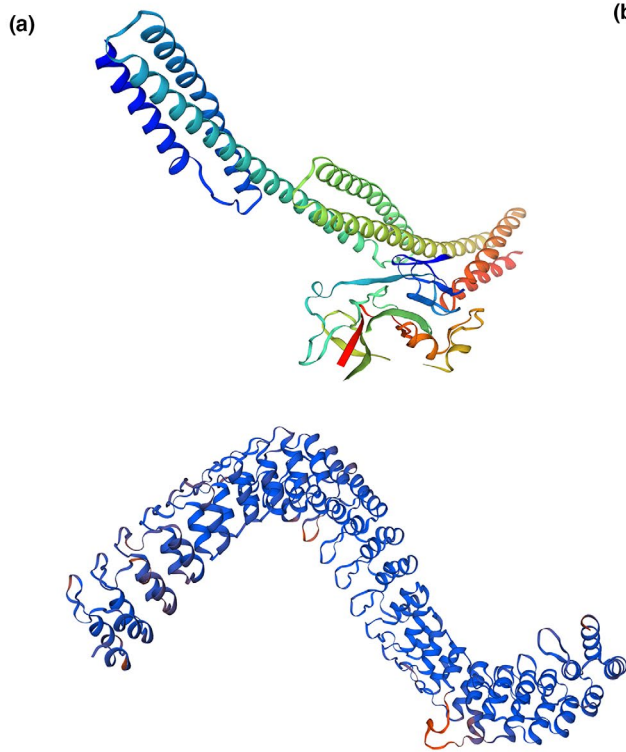
HS is a hereditary disease of hemolytic anemia whose diagnosis is straightforward (Ciepiela, 2018). On the other hand, based on molecular testing, including chromosomal CNV-seq, MS-MLPA, and mutation analysis, a complete diagnosis of BWS can be made. The estimated population incidence of BWS and HS is 1/13700 and 1/2000, respectively (Gulbis et al., 2010). However, the co-existence of HS and BWS has not yet been previously reported.

We recently diagnosed and treated a case with co-existing BWS and HS.

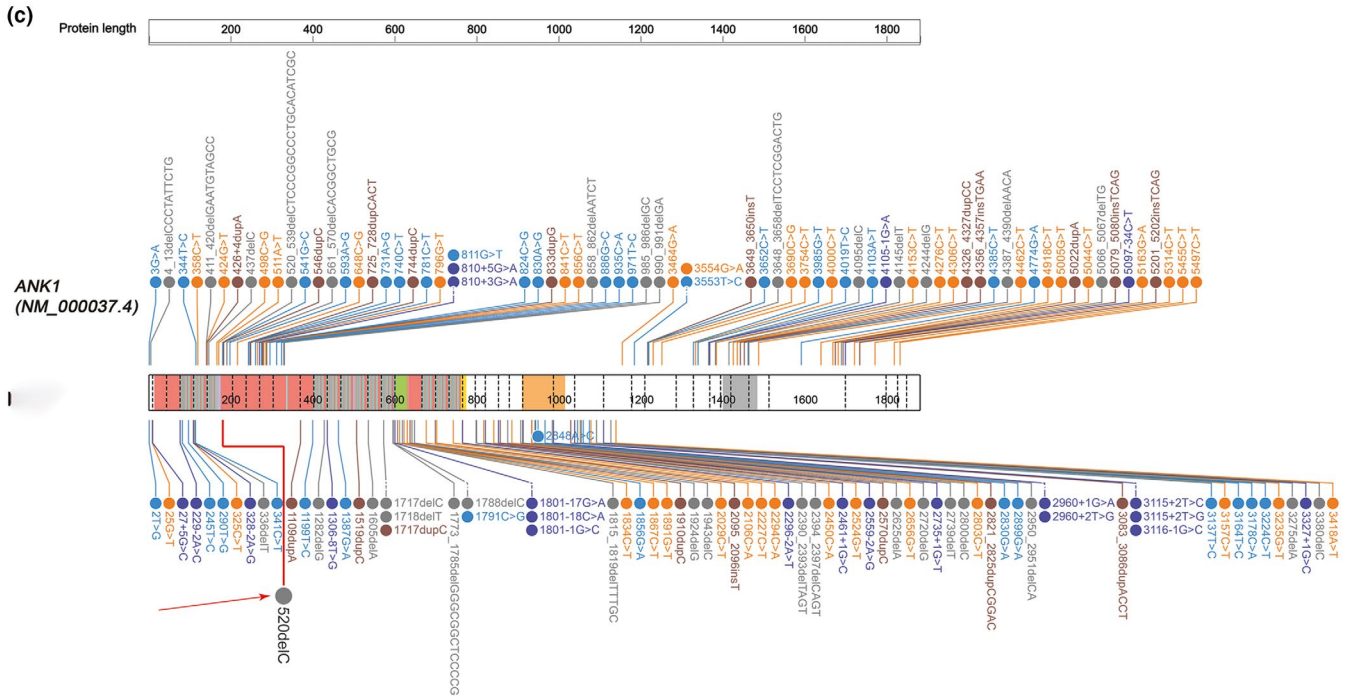
This patient was recognized with typical BWS features that included abnormally large tongue, large abdominal organs (liver and spleen), and exomphalos. However, the CNV-seq analysis failed to find apparent copy number variation, and this technology is unable to detect chromosome instabilities in genes or loci with established correlation to BWS, such as rearrangement in chromosome 11p15.5 (Wang et al., 2019). Hence, MS-MLPA was performed to further screen the existence of abnormally imprinted genes. The MS-MLPA results indeed disclosed a genetic cause of BWS, that is, the LOM at maternal *KCNQ1OT1*.

Nonetheless, the patient presented additional signs and symptoms which are considered as the characters of HS, such as hyperbilirubinemia, anemia, and jaundice. To verify the underlying causal alterations for HS, Trio-WES and hematological tests were conducted.

Very interestingly, a novel mutation was identified in *ANK1* (Gene ID: 286, NCBI, July 2020) in this patient. Previous publications have associated multiple *ANK1* mutations ($n = 158$) with HS, as archived in the repertoire in HGMD database. In this patient, a deletion c.520delC, located at chr8-41583371-41,583,371, exon 6 of *ANK1* gene was confirmed which has never been reported in HS patients. Short deletions or insertions in the coding part of an mRNA usually result in frameshifting changes, which could lead to premature stop codon and subsequently a truncated protein product. This identified mutation is categorized as frameshifting and the predicted product of this altered gene is an amino acid peptide with a p.L174Sfs*79 in sequence which makes up a truncated protein product of 408 amino acid, much shorter than the wild-type ANK1 protein of 1880 amino acids (Chai et al., 2020; Xie et al., 2021). This mutation is defined as pathogenic based on ACMG classification guideline (Wang et al., 2019). The erythrocytic ANK1 protein contains three structural domains, an amino-terminal membrane-binding domain, a central spectrin-binding domain, and a carboxy-terminal regulatory domain, of which the amino-terminal domain is highly conserved and contains multiple ankyrin repeats. In this patient, the mutation was located in exonic sequence within the membrane-binding domain. The mutant transcript is predicted to introduce a truncated aberrant protein followed by an in-frame stop codon after residue 408, lacking one-third of the ankyrin repeats, the spectrin-binding domain, and the C-terminal regulatory domain. In erythrocytes, ANK1 functions as a linker that attach the spectrin-based membrane cytoskeleton to band 3 and protein 4.2 to maintain membrane surface area and to stabilize the red cell membrane, while the truncated ANK1 proteins have disrupted functions



Accession	Gene	Position	Sequence	Position
P16157	ANK1_HUMAN	1	MPYSVGFREDAATSFLAARSGLNLDKALDHLRNGVDINTCNQGLNGLHLSKEGKGVN	60
Mutant		1	MPYSVGFREDAATSFLAARSGLNLDKALDHLRNGVDINTCNQGLNGLHLSKEGKGVN	60
P16157	ANK1_HUMAN	61	WVLRKREITLETTRKGNLHIAALAGQDEVRSLNLYGNANVQSGKGFPLVMAAG	120
Mutant		61	WVLRKREITLETTRKGNLHIAALAGQDEVRSLNLYGNANVQSGKGFPLVMAAG	120
P16157	ANK1_HUMAN	121	ENHLEVVKFLLENGANQVATEDEGTFPLAVALQGHENVAHLINVTGKGVRSRFTSR	180
Mutant		121	ENHLEVVKFLLENGANQVATEDEGTFPLAVALQGHENVAHLINVTGKGVRSRFTSR	180
P16157	ANK1_HUMAN	181	RANDPRTAAVLLQDPNFDLQKGFPELHIAA---YENI---WVQLLNRRG S	231
Mutant		181	PATTRRALRCCCRITTFRTCFRRDGRPCRLTRITRTSPWCSSTEPASLSHRRTAS	240
P16157	ANK1_HUMAN	232	WVLRKREITLETTRKGNLHIAALAGQDEVRSLNLYGNANVQSGKGFPLVMAAG	290
Mutant		232	RHTSPFAGASG---CQCQMLV---EPR-KFRPRT---N---R---LSTLV	275
P16157	ANK1_HUMAN	291	LDGKGFYQKTKNGLSPLFMQAQGHLLCQVRLILLYDAIDITL---RHLTFL	342
Mutant		276	OLEMGTCESSR--SCWITGHGSKRPRKPCQFPTWRLREITTSVSGSCNNTQR-TISFW	332
P16157	ANK1_HUMAN	343	WVLRKREITLETTRKGNLHIAALAGQDEVRSLNLYGNANVQSGKGFPLVMAAG	396
Mutant		333	TT-FHSTWLPDVTGDMGLKSLFKIKGRVWVYFEP-NALLPPTSPAKTTISVNSCC-R---	385
P16157	ANK1_HUMAN	397	ENHLEVVKFLLENGANQVATEDEGTFPLAVALQGHENVAHLINVTGKGVRSRFTSR	456
Mutant		386	---RPRSTRSPSLA-HLS---	400
P16157	ANK1_HUMAN	457	FANKARVAKKDDQFLACARIGHTNVKLENNANPNLATTSGHTPLHIAAREGLV	516
Mutant		401		400
P16157	ANK1_HUMAN	517	EVVLALEKRSQMGKTRKGFPLRWAKYKRVRSVAILLEDAHPNAGKGLTFLRW	576
Mutant		401		400
P16157	ANK1_HUMAN	577	VHNNIDLYVLLPFGSGFSSPAMNYYTPHITAAKQNVSVARSILLYGSSANRFSVGV	636
Mutant		401		400
P16157	ANK1_HUMAN	637	FELHAAQGEHAEHVALLSKQANGNENKRGTLPHIWAQGEHVPVADVLKRWVWV	696
Mutant		401		400
P16157	ANK1_HUMAN	697	TPR-VYTFPLHASYHCNKLWVFLFQDADNAXTKLQVSPHQMGCHDITVPLHLL	756
Mutant		401		400
P16157	ANK1_HUMAN	757	GASPLVSSDGTFLAIAKRLGVISYVDVIAVVTGTSVLSVSDRHRMSPFETVDEILDV	816
Mutant		401		400
P16157	ANK1_HUMAN	817	SEDEGSELISFKAERDRSDVDEKELLDPVFKLDQVSVPAIPRIPCAMPETVIRSEE	876
Mutant		401		400
P16157	ANK1_HUMAN	877	QEQASKEYDEDSLIPSPATETSINISPVASPVHTGLVFSVMDARGSGMGSRRNGLAV	936
Mutant		401		400
P16157	ANK1_HUMAN	937	VIPPTCAAPTRITCRILVFKPQLSTPPLAEEGLASRIALJLGAQFLSPVIVEIPHF	996
Mutant		401		400
P16157	ANK1_HUMAN	997	ASNGCDNELVPLASENSGVKHEWNSVCEVYLDQINDGDELSLELEKRWKRIAT	1056
Mutant		401		400



Protein domains

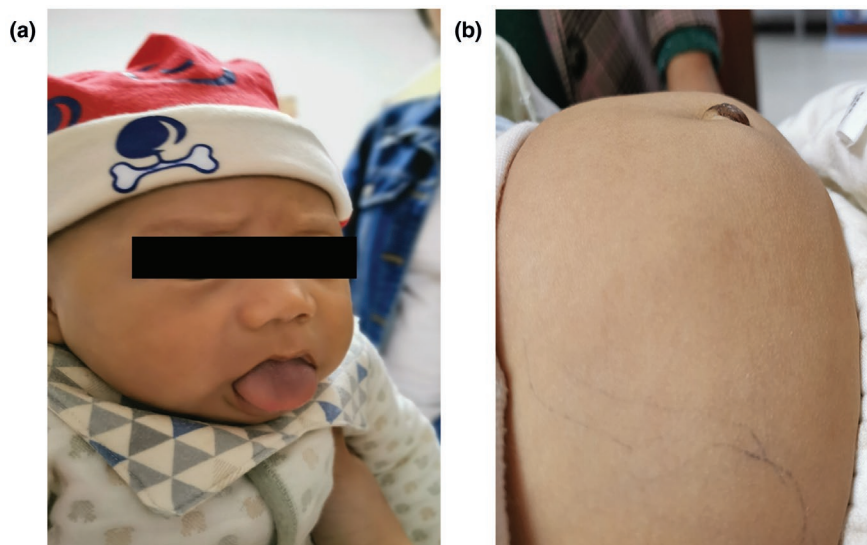
- Ank_4 Ankyrin repeats (many copies)
- ANK repeat ANK repeat [structural motif]
- ANK ankyrin repeats; ankyrin repeats mediate protein-protein interactions
- Ank_2 Ankyrin repeats (3 copies)
- other oligomer interface [polypeptide binding]
- Ank Ankyrin repeat
- ANKYR Ankyrin repeat [Signal transduction mechanisms]
- ANK ankyrin repeats
- Ank_5 Ankyrin repeats (many copies)
- ZU5 Domain present in ZO-1 and Unc5-like netrin receptors
- Death_ank1 Death domain of Ankyrin-1

Mutation classes

- MISSENSE, n=35
- NONSENSE, n=40
- PROTEINDEL, n=34
- PROTEININS, n=19
- SPLICE, n=24
- Somatic, n=152

FIGURE 3 *ANK1* mutation profile in the spherocytosis. (a) Structure analysis of *ANK1* (P16157) (upper panel) and predicted product of the p.L174Sfs*79 variant (lower panel), with repeats colored differently to illustrate the packing interactions. (b) The alignment of *ANK1* (P16157) and the p.L174Sfs*79 variant showed that the truncated product of the p.L174Sfs*79 variant was unable to form repeats (purple) and functional binding domains (yellow). (c) Each distinct mutation is represented by a disc and filled with the color representing its mutation class. Mutations are positioned by their amino acid coordinates, and those that affect the same amino acid residue are presented by a “skewer graph” with discs stacked on the same “stem.” The corresponding amino acid changes are labeled to the side of the discs. The manually curated disc shows the novel deletion variant, c.520delC, identified in this study. Inside the protein sequence frame, the dotted vertical lines delineate the boundaries of coding exons and the distinct colors represent different types of domains

FIGURE 4 Clinical photographs of the patient at 3 months, showing typical characteristics of BWS, including (a) macroglossia and facial appearance, and (b) exomphalos



due to the lack of most functional domains, causing impaired red cell membrane as presented by this patient. More functional studies on the mutation should be performed to illustrate the alternative expression and translation mechanisms adopted by this mutant transcript. The aberrant products might be either extremely or less deleterious, and be the direct reason for highly variable degrees of clinical symptoms of the relevant disorders.

The results of erythrocyte osmotic fragility test and blood tests were all in agreement with suspected diagnosis of HS, evidenced by the presence of hyperbilirubinemia and microspherocytes in both peripheral blood and bone marrow.

More interestingly, the co-manifestation of BWS and HS in an individual patient has never been identified. The diagnosis of co-existing HS and BWS is straightforward and based on genetic and molecular tests with a little chance of misdiagnosis. Moreover, the aberrant genotypes are in line with observed clinical phenotypes. Therefore, we believe that the diagnosis of co-existing BWS and HS is accurate.

WES, an effective approach to obtaining gene variation information in protein-coding regions, has been widely used for the diagnosis of human inherited diseases. WES can identify multiple variants simultaneously and discover potential novel variants associated with a

genetic condition or disorder. In this specific case, WES is proved to be an efficient way to identify causative pathogenic genes and to improve diagnostic accuracy. In routine clinic, the diagnosis of BWS requires the molecular tests, including mainly DNA methylation test and CNV test. However, it is not uncommon that these two types of diagnostics are not able to fully identify causative genetic or epigenetic alterations of the presenting diseases, especially when rare aberrations are present or complex conditions are co-existed, as demonstrated by this case which harbors non-typical CNVs and co-existing HS caused by *ANK1* gene mutation. The investigation of this BWS-HS case here highlights the importance of performing WES in addition to MS-MLPA and CNV analysis for complicated cases to provide a complete diagnosis and accurate genetic counseling. For such kind of complicated cases, the different types of molecular tests, such as WES and MS-MLPA, should be utilized in combination to complement each other in order to reveal the hidden associations between novel molecular alterations and disease characteristics, or to re-evaluate the previously under-appreciated molecular events.

This study has several limitations. Firstly, and the most important, the validation experiments, including the expression of *ANK1* variant and *ANK1* protein in patient, were not conducted due to the lack of patient samples in

this retrospective study. Secondly, the reported patient is the only case harboring this *ANK1* novel mutation and having dual diagnosis of BWS and HS. Hence, the observed associations between the detected genetic alteration and clinical phenotypes have not been validated in other independent cases.

In conclusion, we reported for the first time an extremely rare case with co-existing BWS and HS based on molecular genetic tests including CNV-seq, MS-MLPA, and Trio-WES. We identified the loss of maternal methylation at *KCNQ1OT1* TSS (IC2 LOM) which is the causal alteration for BWS. Moreover, we identified a spontaneous, novel, nonsense c.520delC in *ANK1* gene in this BWS-HS patient. The *ANK1* mutation produces a truncated ANK1 protein lacking important functional domains which leads to the production of RBCs with impaired membrane and other related clinical presentation of HS. Considering the inheritance pattern of this disease, it is very important for these parents to perform prenatal diagnosis and genetic variation detection in subsequent pregnancies in order to prevent the recurrence of this disease. Last, we advocate the adoption of WES in genetic clinics for the cases with difficulties in diagnosis.

ACKNOWLEDGMENTS

We are grateful to the patient and his parents who contributed to this work.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTIONS

Clinical data were collected and collated by ZYM and JYS, while BBZ provided the assistance with genomic DNA extraction. XF, XW, XC, FRL and LZ, YPW performed genetic testing (CNV-Seq, MS-MLPA, WES, Sanger sequencing). CZ and FPZ analyzed and interpreted the patient data and provide genetic counseling. XTL and LYD performed all bioinformatics analyses and illustration, while QHZ and JH drafted the manuscript. The study was supervised by and SJH. All authors read, reviewed, and approved the manuscript.

ETHICAL COMPLIANCE

This study was reviewed and approved by the ethical committee of Gansu Provincial Maternity and Child-care Hospital. The parents of the patient provided the written informed consent to participate in this study. Written informed consent was obtained from the patient's legal guardian for the publication of any potentially identifiable images or data included in this article.

DATA AVAILABILITY STATEMENT

All data are available on request from the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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