MYO5B Novel Homozygous Variant c.2090+3A>T Causes Intron Retention and is Related To Severe Cholestasis and Intractable Diarrhea

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Research

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Abstract

Background

Myosin Vb (*MYO5B*) plays an important role in protein trafficking and recycling. Biallelic mutated *MYO5B* has been found related to microvillus inclusion disease (MVID) or predominant cholestatic liver disease or mixed the two diseases. This study aims to clarify the splicing alteration and associated clinical or pathological features of a newly identified homozygous variant, and thus to provide supporting information for disease mechanism and clinical treatment.

Results

Here we identified a novel homozygous variant c.2090 + 3A > T by exome sequencing in a female patient. Minigene assay using recombined vectors and cell transfection found 185bp intention of intron 17 in the mRNA, which was predicted a pre-termination of myoVb (p.Arg697fs*47) at the end of the head motor domain. Further bowel biopsies via immunohistochemistry and the electron microscope detected decreased microvillus and local lesion of microvillus inclusion in the mucosa of duodena. The patient presented intractable diarrhea alleviated from severe in early life to mild later. She also had congenital cholestasis, liver cirrhosis, cholelithiasis, hepatic cyst, corneal opacity, and failure to thrive. Medicines were supplied to alleviate symptoms, maintain her intestines and nutrition. Liver transplantation would be the final choice.

Conclusions

Our study reported a novel homozygous variant that altered splicing and further supported the underlying mechanism that led to complicated enterohepatic phenotypes. The results expanded clinical practice in understanding the genotype-phenotype correlation of *MYO5B* and associated disease management.

Background

MYO5B encodes the myosin Vb protein, which works as an actin-based molecular motor and plays an important role in intracellular trafficking and plasma membrane recycling. It is widely expressed in multi-tissues of the liver, intestine, brain, etc. Disfunction of *MYO5B* was firstly reported causing autosomal recessive microvillus inclusion disease (MVID, MIM: 251850) in 2008 (1, 2). MVID was recognized as intractable diarrhea of infancy, with enterocyte brush border defects, or congenital microvillus atrophy (3, 4). Presently it was also called Diarrhea 2 and characterized by the onset of intractable life-threatening watery diarrhea during infancy. Knockdown of *MYO5B* reproduced the disease phenotypes in both intestine cell lines and animal models, which confirmed that loss function of *MYO5B* would cause MVID (5–9). Later in 2017, *MYO5B* deficiency was found associated with previously undiagnosed predominant cholestatic liver disease (also named Progressive Familial Intrahepatic Cholestasis, PFIC) but no MVID

(10, 11). The international MYO5B website (http://www.MVID-central.org) collected the patients reported with *MYO5B* mutations and their phenotype information (12, 13). To date, about 421 variants of *MYO5B* have been reported, and 86 variants were associated with MVID, PFIC, or mixed MVID-PFIC (14). It is important to clarify the relationship between the genotype to phenotype.

MYO5B defects can cause various phenotypes from enterocytes to hepatocytes. The disease-causing genotypes involve different variant types and protein domains. The myoVb protein has five domains successively: motor, Isoleucine-glutamine calmodulin-binding (IQ) 1–6, coiled-coil, and globular tail. Biallelic mutations in the motor domain were found causing aberrant localization of bile canalicular proteins and subapical accumulation of bile salt export pump (BSEP) in hepatocytes (15, 16). However, neither the complete deficiency nor the truncated myoVb without the globular tail domain resulted in the mislocalization of canalicular proteins in hepatocytes (15). Complete deficiency or truncating myoVb led to microvillus inclusion and subapical clusters of aberrant recycling endosomes in enterocytes (16). Aldrian et al. recently summarized the phenotypic spectrum related to *MYO5B* mutations and speculated: the lack of myoVb protein caused by loss of function (LoF) mutation could lead to MVID, while missense or other variant types disrupting the expression of *MYO5B* would lead to PFIC or mixed MVID + FPIC (14). If the mutation disturbed protein-protein interactions and the full-length myoVb expressed with residual function in hepatocytes, the FPIC would happen. If the mutation caused the expression of myoVb without residual function, mixed MVID + FPIC would occur (14).

Here we reported a novel homozygous variant c.2090 + 3A > T which is located in the N-terminal of the motor domain of *MYO5B*. We transferred the variation to MCF-7 and HEK293T cells and sequenced the RNA transcripts to validate the modification of function and expression. We also performed immunohistochemistry and electron microscope to detect the histology changes in the mucosa tissue of the patient's bowel. By genotype-phenotype analysis, whether the novel splicing variant would produce an aberrant transcript and disrupt *MYO5B* expression and interaction with other proteins will be investigated.

Results

Biallelic c.2090 + 3A > T identified

Whole exome sequencing identified a biallelic variation NM_001080467.2:c.2090 + 3A > T (NP_001073936.1:p.?) at *MYO5B*. Her father and mother carried a monoallelic variation c.2090 + 3A > T respectively. This variant was absent from the gnomAD database (17) and not recorded in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/). It is located in the motor domain of *MYO5B*, which is a mutational hot spot region. Bioinformatics software dbscSNV (Ada score = 1, RF score = 0.9) (18), SpliceAI (score = 0.85) (19) and Trap (version 3.0, score = 0.895) (20) predicted this variant was splice altering and deleterious. In addition, the patient's phenotype was highly specific for MVID-PFIC which had a single genetic etiology. According to the ACMG criteria (21), this variant was classified as likely pathogenic (PM1, PM2, PP3, PP4). Her parents are carriers and their marriage is not consanguineous.

Intron 17 intention of 185bp leading to truncated myoVb at motor domain

In the minigene splicing assay, RT-PCR detected a longer product of pEGFP-C1-MYO5B-mut than the wild type pEGFP-C1-MYO5B-wt (slightly over 500 bp vs. 335 bp) in the transfected MCF-7 cells (Fig. 2B). The consistent result was generated in HEK293T cells with over 500 bp of pcDNA3.1-MYO5B-mut vs. 403 bp of pcDNA3.1-MYO5B-wt (Fig. 2B). Sanger sequencing demonstrated that the splicing model of the wild type (pEGFP-C1-MYO5B-wt and pcDNA3.1-MYO5B-wt) was: Exon17(87bp)-Exon18(112bp), while in the mutant type (pEGFP-C1-MYO5B-mut and pcDNA3.1-MYO5B-mut) the splicing model was: Exon17(87bp)-▽intron17(185bp)-Exon18(112bp) (Fig. 2D). Thus, the minigene splicing assay revealed an intention of 185 bp from the 5’ strand of intron 17 in the expressed transcripts in the transfected cells.

Basing on the mutant sequence of the transcripts, the intention of the 185bp of the intron sequence would be translated into 44 aa and then terminated. Therefore, the variant MYO5B:c.2090 + 3A > T was predicted to generate protein p.Arg697fs*47. The translation stopped near the C terminal in the motor domain. In the wild type of myoVb, the motor domain is normally stopped at 764 aa. Supplementary Fig. 2 shows the structure of the motor domain of wild myosin V and the predicted structure of the mutant myoVb. This variant was located upstream of converter subdomains (from 703 to 713 aa and 734 to 749 aa, Supplementary Fig. 2). The role of the converter is to amplify the conformational changes of the motor domain (22).

Pathology result and phenotype confirmed mixed MVID-PFIC

As for the pathology result, both Immunohistochemistry and electron microscope revealed decreased microvillus and local lesion of microvillus inclusion in the mucosa of her duodenum (Fig. 3). No abnormal microvillus was found in her small bowel, large bowel, and colon specimens. As no liver biopsy was performed, the pathology modifications of her liver had not been detected. Nevertheless, the patient had a history of disrupted liver function with prolonged jaundice and progressive cholestasis, and her liver cirrhosis was developed to end-stage liver disease. She suffered chronic malnutrition and development delay. Therefore, her phenotype of mixed MVID-PFIC was explicit, which was related to the genotype of *MYO5B* c.2090 + 3A > T. With the additional *in vitro* functional study, the c.2090 + 3A > T variant in *MYO5B* was likely pathogenic (criteria matched PS3, PM1, PM2, PP3, PP4).

Patient phenotypes and maintenance

The female patient had chronic diarrhea with intermittent severe diarrhea in her early life. She manifested progressive cholestasis from birth. Up to 7-year-old, she manifested decompensated cirrhosis, portal hypertension, cholelithiasis, hepatic cysts, splenomegaly, encephaledema, corneal opacity, malnutrition, anemia, growth delay, and short stature. Cholestyramine and ursodeoxycholic acid were supplied daily to alleviate liver cirrhosis and pruritus. Bifid tetragenous viable bacteria tablets, vitamin A and D were supplied routinely to maintain her intestines and provide nutritional supplements. Cefdinir dispersible

tablets were also used to control bacterial infections and prevent sepsis. This patient refused to do liver transplantation. By the time of this report, this patient was still alive.

Discussion

This study identified a novel homozygous variation c.2090 + 3A > T in *MYO5B* in a female patient with mixed MVID-PFIC. She presented severe cholestasis and varying diarrhea alleviated from severe occasionally in early life to mild later. Further minigene splicing assay revealed this variant caused 185 bp retention of intron 17 in the transcripts of *MYO5B*. The retention was predicted to generate amino acid sequence p.Arg697fs*47 and the translation pre-terminated near the end of the head motor domain of myoVb. In addition, the duodenum biopsy of this patient confirmed the decrease of microvillus and local lesion of microvillus inclusion. Combining the clinical features, the genotype-phenotype relationship of this newly identified splicing variant was confirmed.

This variant would cause the loss of downstream two converter subdomains, six IQ light chains, the PEST site rod region (coiled-coil), and the globular tail domain. The remaining head motor domain acts as binding actin and utilizing ATP hydrolysis to generate directed movement toward the plus end along actin filaments (23). According to a previous study, the truncated myoVb mutants p.Arg363* with incomplete motor domain were found expressed in hepatocytes with normal polarity and canalicular protein localization (15). However, the apical bile transporters ABCC2/MRP2 were mislocated intracellularly (14, 15), which led to cholestasis in hepatocytes. In enterocytes, the expressed truncated myoVb mutants were observed aberrant localization, and the protein Rab11 and syntaxin aberrantly bound to myoVb (16). Generally shorter microvilli but deeper rootlets than controls were observed and few local areas had no brush border. Microvillus inclusions were found sporadically in the epithelium of the duodenal biopsies (16). Aldrian et al. proposed that this kind of truncated mutant would cause decreased apical brush border, subapical secretory granules, and mislocated apical proteins (14). In this study, our patient presented persistent cholestasis and moderate to mild diarrhea, and her phenotype was consistent with the previous studies and confirmed the hypothesis.

A variation c.2090_2090delG very close to our reported variation c.2090 + 3A > T was reported before (11). c.2090_2090delG was predicted yield p.Arg697Glyfs*74. The patient had this variant together with c.4852 + 11A > G being formed of compound heterozygous variants, whose phenotype was isolated cholestasis and was classified as PFIC. Compared with the homozygous variant located in the motor domain in our study, c.2090_2090delG was in the motor domain and the other was located in the globular tail domain near the N-terminal myoVb. The compound variants were proposed to produce a myoVb mutant with residual function causing PFIC but without the intestinal disorder.

In this study, previous gene-based or panel-based gene testing returned negative results, and exome sequencing revealed *MYO5B* as a disease-causing gene. Interestingly, cholestasis was a lately identified phenotype relating to *MYO5B* which was previously relating to infantile diarrhea only (11). This patient did not obtain a precise diagnosis until 6-year-old. The congenital disorder led to severe malnutrition,

severe growth delay, and almost blind eyes in her late life. An early diagnosis and intervention could improve her life quality.

This patient has refused to do liver transplantation so far. Since no liver biopsies were available, the alterations caused by the identified variant in liver ultrastructural pathology cannot be further examined. Other limitations of this study include no detection of the mutant protein regarding weight and structure. The damaging effect of this variant on the interaction with other proteins could also be further studied.

Conclusions

This study identified a novel homozygous variant c.2090 + 3A > T altering splicing in *MYO5B* and leading to intron retention and translation pre-termination. The patient who expressed the truncated myoVb with only the incomplete head motor domain presented a mixed phenotype with both intrahepatic cholestasis and MVID. Pathology detection further confirmed the decreased microvillus and local lesion of microvillus inclusion in the mucosa of the duodenum. Our study provided new evidence that confirmed the previous hypothesis of the genotype-phenotypic spectrum of *MYO5B* and further clinical significance in associated disease diagnosis and management.

Methods

Aim of the study

This study aims to clarify the genotype-phenotype relationship based on the novel variant in *MYO5B*. By confirming the splicing alteration of the newly identified homozygous variant and summarizing the associated clinical, pathological features and clinical treatment of the affected patient would make us deeper understand the mechanism and management of *MYO5B* associated diseases.

Patient characteristics and interventions

A five-year-old girl was admitted to our hospital with severe liver cirrhosis, chronic diarrhea, corneal opacity, and short stature. The patient had prolonged jaundice in the first two months of her life. Her stools were always loose from birth. When she was six-month-old, she presented severe diarrhea and was admitted to Pediatric Intensive Care Unit (PICU). When she was two and a half years old, she was admitted to PICU again because of bloody stool. Later, she was admitted to PICU twice due to severe diarrhea when she was 34-month-old and 36-month-old respectively. Her liver function was abnormal from birth and was found progressive cholestasis with increased directorial bilirubin (DBIL, 13.4–29 umol/L), aspartate aminotransferase (AST, ~ 105.1 U/l), and total bile acid (TBA, normal in 6-month-old and gradually increased to 181.2 umol/L in 6-year-old). Growth delay presented from 6-month-old. Pruritus and yellow skin manifested from 2-year-old. Her left eye manifested corneal opacity at 5-year-old and was blind later. Her right eye manifested corneal opacity at 6-year-old. When she was 7-year-old, she manifested decompensated cirrhosis, portal hypertension, cholelithiasis, hepatic cysts,

Table 1
Clinical diagnosed phenotypes and interventions of the patient.

Age	New phenotype	Hospital turn-around days	PICU Turn-around days	Gene testing
0–2 months	Prolonged jaundice			
6 months	Diarrhea, Infantile Rotavirus enteritis, Metabolic acidosis, Cholelithiasis	10	7	Inborn metabolism: -
1 year	Mild diarrhea, Hepatic cysts of left lobe, Enlarged liver			
1.5 year	Fracture of femur			ATP8B: - ABCB11: -
2 years	Pruritus, Cholestasis, Yellow skin, Hypocalcaemia			
2.5 years	Bloody stool, Malnutrition, anemia, Abnormality of coagulation		4	
2.8 years	Watery stool		8	
3 years	Watery stool	5	4	
5.5 years	Corneal opacity of left eye, Chronic mild diarrhea			
6 years	Left eye blindness, Corneal opacity of right eye, Growth delay			Hepatic panel: -
6.8 years	Liver cirrhosis, Portal hypertension, Splenomegaly, Encephaledema, Proteinuria, Short stature			ES: + (MVID diagnosed)
7 years	Sepsis, Decompensated cirrhosis, Abnormality of coagulation, Anemia	10		
9 years	Atopic dermatitis, Hypokalemia, Bronchopneumonia	7		

splenomegaly, encephaledema, proteinuria, malnutrition, anemia, and short stature. Up to this report, her diarrhea was mild with unshaped stools at least three times per day. The history of her clinically diagnosed phenotypes and interventions was listed in Table 1.

Cholestyramine and ursodeoxycholic acid were supplied daily to alleviate liver cirrhosis and pruritus. Bifid tetragenous viable bacteria tablets, vitamin A and D were supplied routinely to maintain her intestines and provide nutritional supplements. Cefdinir dispersible tablets were also used to control bacterial

infections and prevent sepsis. This patient refused to do liver transplantation. By the time of this report, this patient was still alive.

Exome sequencing and bioinformatics analysis

Since hepatopathy-associated gene panel testing returned a negative result before 2018, trios-based exome sequencing was subsequently performed. Genomic DNA was extracted from the peripheral blood of the patient and her parents. DNA libraries were prepared to capture the coding sequences and known pathogenic non-coding regions of over 4,000 human coding genes recorded in OMIM (24) and ClinVar (25) databases. The captured libraries were then sequenced by the Illumina HiSeq X Ten system (Illumina, San Diego, California, USA) to generate 2×150 bp reads. The average coverage of the three samples was about 343X. 99.7% of the target regions were covered above 20X. NextGENe (version: 2.4.1.2, SoftGenetics, LLC., USA) software was employed to polish reads, align to the human reference genome (version: GRCh37), call SNPs, InDels, and CNVs, and annotate variants. All the detected variants were interpreted and classified according to the American College of Medical Genetics and Genomics (ACMG) guidelines (21). The Human Phenotype Ontology (HPO) keywords were used to describe the patient's phenotypes. Pathogenic, likely pathogenic, and variants with uncertain significance in suspect genes relating to the patient's HPO words were prioritized. The phenotype-matched variant with the highest priority was verified by Sanger sequencing in the patient and her parents.

Minigene splicing assay

To validate whether this variant has splice alteration in mRNA transcripts in vitro, the minigene splicing assay was performed. The minigene pEGFP-C1-MYO5B-wt/mut was constructed by inserting the whole genomic sequence from Exon 17 to Exon 18 (including the inter-intron sequence) into vector pEGFP-C1 (Fig. 1) and pcDNA3.1 respectively. We designed two pairs of primers: 282104-MYO5B-F and 283709-MYO5B-R,282511-MYO5B-F and 283355-MYO5B-R. The sequences of the PCR primers used in this minigene assay were detailed in Supplementary Table 1. Nested-PCR was performed using the patient's gDNA 0.5 ug and 57 ℃ as the flame temperature. Using the second-round product of nested-PCR as a template, wild-type or mutational (wt/mut) segments were generated following the subsequent steps: (1) primers GFP-MYO5B-HindIII-F and MYO5B-intron17-R were designed to do PCR to obtain the segment 1 of pEGFP-C1 with a length of 629bp; (2) primers MYO5B-intron17-F and GFP-MYO5B-BamHI-R were used to do PCR to obtain the segment 2 of pEGFP-C1 with a length of 290bp; (3) segment 1 and segment 2 were recycled and mixed with 1:1 to do PCR using primers GFP-MYO5B-HindIII-F and GFP-MYO5B-BamHI-R. We then obtained the final segment of pEGFP-C1 with a length of 883bp. Vector pEGFP-C1 and DNA fragment were subsequently performed enzyme digestion, recovery, ligation, transformation, and colony PCR identification (the reaction conditions and results of gel electrophoresis were provided in Supplementary Fig. 1). Sanger sequencing was performed to validate the wt/mut sequences. Vector pcDNA3.1 was constructed similar to pEGFP-C1.

Subsequently, the constructed vector pEGFP-C1-MYO5B-wt/mut (sequences shown in Fig. 2A) were transiently transfected into MCF-7 and HEK293T cells respectively using low toxicity Lipofectamine

following the manufacturer's instructions (Life Technologies, Carlsbad, CA, United States). The product was recycled after 36 hours. Total RNA was isolated using TaKaRa Trizol (RNAiso PLUS) and then synthesized to cDNA. cDNA was performed RT-PCR and then bidirectional sequencing using an ABI 3130 DNA analyzer (Applied Biosystems, Foster City, CA, USA). pcDNA3.1-MYO5B-wt/mut experimented following similar steps. Figure 2C shows the scheme of sequencing the splicing region in the minigene splicing assay.

Protein sequence and structure prediction

According to the result of the minigene splicing assay, the generated mutant cDNA sequence due to the splicing alteration was used to do protein sequence prediction. Lasergene EditSeq Pro software (version 7.1.0, DNASTAR, Inc., USA) was employed to translate the mutant cDNA sequence into the amino acid sequence. The 3D crystal structure of the motor domain of wild myosin-Vb (PDB ID: 1OE9) was displayed using Cn3D (version: 4.3.1). The mutant sequence was also loaded to SWISS-MODEL (http://swissmodel.expasy.org/) for homology modeling. The structural modification of the mutant myoVb protein was predicted by Swiss-Pdb Viewer software.

Immunohistochemistry and electron microscope

Biopsies of her duodenum, small intestine, large intestine, and colon were performed. Specimens of the tissue were fixed in 4% acetic formalin, embedded in paraffin, stained with Periodic Acid-Schiff (PAS), and immunostained with antibodies against CD10 (ZSGB-BIO, Zhongshan Jinqiao Biotechnology Co. Ltd, Beijing, China). Duodenum specimens were fixed in glutaraldehyde and cut into 0.2 cm^3 sections, and then scanned using the electron microscope.

List Of Abbreviations

MVID
microvillus inclusion disease
FPIC
Progressive Familial Intrahepatic Cholestasis
BSEP
bile salt export pump
LoF
loss of function

Declarations

Ethics approval and consent to participate

This study was approved by Hunan Children's Hospital Medical Ethics Committee (Approval No. HCHLL-2018-58). The samples were obtained appropriate informed consent from all participants.

Consent for publication

All authors read and approved the final manuscript for publication.

Availability of data and materials

The reported novel variant has been submitted to ClinVar (accession: SCV001761606). Other datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

QY: supervision and resources acquisition. YZ: original manuscript writing and editing, data analysis. YMP: sample collection, methodology, clinical data curation, and validation. HMZ and WJC: pathology analysis and clinical data collection and validation, QY, YMP, and YZ: manuscript review and editing. The remaining authors contributed to ideas refining, additional analyses, and finalization.

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WEB resources used

UCSC, https://genome.ucsc.edu/

OMIM, https://www.omim.org/

Varsome, https://varsome.com/

Trap, http://trap-score.org/

GnomAD, https://gnomad.broadinstitute.org/

ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/

Primer-BLAST, https://www.ncbi.nlm.nih.gov/tools/primer-blast/

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Supplementary Files

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