A Novel Homozygous *TTC7A* Missense Mutation Results In Familial Multiple Intestinal Atresia And Combined Immunodeficiency

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Abstract

Multiple intestinal atresia with combined immune deficiency (MIA-CID) are autosomal recessive disorders characterized by intestinal obstructions and profound immune defects. The study of patients with MIA and related disorders has established that tetratricopeptide repeat domain 7A (*TTC7A*) plays a critical role in intestinal and immune homeostasis and it is now shown that biallelic missense mutations have better survival outcomes. However, clues to related underlying molecular dysfunction remains elusive. In this study, we reported a patient with the diagnosis of severe CID and MIA that involved the pyloric diaphragm, ileum atresia, and colon stenosis, and the clinical course was complicated by multiple episodes of sepsis. In spite of multiple surgeries and supportive treatment, the patient died of severe sepsis and multiple organ failure at 3 months of age. The whole exome sequencing (WES) identified a novel homozygous *TTC7A* missense mutation (c.206T>C, p. L69P). The structural analysis showed that a hydrogen bond present between Gly65 and Leu69 in the wild-type *TTC7A* was broken by the Leu69Pro mutation. Moreover, this homozygous missense mutation led to a severely reduced *TTC7A* expression in lymphocytes and intestinal tissues, accompanied by prohibited lymphocyte development. Further studies demonstrated that the PI4K-FAM126A-EFR3A pathway was impaired in intestinal tissues. Our results strongly suggest that the missense mutation in *TTC7A* gene causes severe MIA-CID. More knowledge of the *TTC7A* protein functions will have important therapeutic implications for patients with MIA-CID.

Introduction

Multiple intestinal atresia (MIA) is an extremely rare and typically lethal congenital disorder involving multiple lesions at various levels throughout the intestine and colon. Associated combined immunodeficiency (CID) is also commonly present, allegedly caused by deficient thymopoiesis and lymphocyte differentiation, proliferation, and survival (1–5). Unlike classic CID in which opportunistic infections are common, patients with MIA-CID usually suffer from recurrent sepsis caused by intestinal bacteria (6, 7).

Recently, MIA-CID association was linked to mutations in tetratricopeptide repeat domain 7A (*TTC7A*) gene. *TTC7A* plays a vital role in epithelial cell growth, polarity, and differentiation in the gastrointestinal tract and thymus gland (2, 8). Since 2013, there have been more than 50 patients with *TTC7A* mutations reported in the literature (9, 10). Loss-of-function mutations in *TTC7A* have been shown to cause immune and intestinal disorders of variable severity (11).

The composition of the plasma membrane in particular levels of phosphatidylinositol 4-phosphate (PI4P) are crucial for preserving epithelial cell polarity and survival. It has been confirmed that *TTC7A* acts as a scaffolding protein, where it binds and recruits phosphatidyl inositol 4 phosphatidylinositol 4-kinase III alpha (PI4KIII α) to the cytoplasmic membrane, facilitating the synthesis of PI4P (1, 10). *TTC7A* has also been reportedly interacts with the protein EFR3 homolog B (EFR3), which may serve as a membrane anchor for PI4K. Furthermore, *TTC7A* downregulates the RhoA-ROCK signaling pathway and the latter's downstream targets involved in actin dynamics (10).

Although the causal nature of TTC7A to MIA-CID has been established through these findings and functional studies, questions remain on the exact correlation between mutations in this gene and phenotypic variations. The majority of the reported missense mutations are hypomorphic and had better survival outcomes. However, we report a patient of MIA-CID with a previously unreported homozygous missense mutation in *TTC7A* gene. This report adds to the ever-growing knowledge on MIA-CID that can contribute to the better understanding of these challenging disorders.

Materials And Methods

Human sample collection

Clinical information (including the demographic information, details of surgery, complications, clinical course, imaging examination results) and blood samples were collected from the patient, the parents, and controls, all of whom had given their prior informed consent to participation in the study. Genetic studies and data collection procedures involving human subjects conducted in the study were approved by the ethics committee of Beijing Children's Hospital. Ethics was approved by the Medical Ethical Committee of Beijing Children's Hospital which acted in compliance with ethical standards defined by the Declaration of Helsinki.

Preparation of peripheral Blood Mononuclear Cells (PBMCs)

Peripheral blood from the patient, the parents, and age- and sex-matched healthy controls were collected into EDTA-coated tubes. The PBMCs were isolated from peripheral blood by density gradient centrifugation using Ficoll-Hypaque. Briefly, 5 ml freshly isolated EDTA anticoagulated blood was diluted with 5 ml RPMI-1640 medium (HyClone) and layered onto Ficoll-Hypaque (Sigma) in a 3:1 ratio. After centrifuged at 1000 g for 20 minutes at room temperature, the buffy layer containing PBMCs was obtained, washed and re-suspended in RPMI-1640 medium supplemented with FBS and antibiotics cocktail (streptomycin and penicillin). Cell viability was determined by trypan-blue dye (Sigma) and cells were counted on an EVETM automated cell counter (NanoEnTek).

Histology and Immunohistochemistry

Samples from biopsies were fixed in 10% buffered formalin, embedded in paraffin, and sections stained with hematoxylin and eosin according to standard protocols. Tissues from age-matched children were used as control tissues.

Immunohistochemistry was performed on 3 mm paraffine imbedded sections. Intestine sections were deparaffinized and rehydrated with successive baths of xylene and ethanol. Antigen retrieval was performed in pH 6.0 citrate buffer for 20 minutes at 100°C. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide (v/v in methanol). Sections were blocked for 30 minutes at room temperature in 1% BSA PBS, then labeled using a rabbit anti-human TTC7A antibody (Proteintech, 21600-1-AP, 1:60 dilution) that recognized the sequence corresponding to amino acids 149-465 at the N-terminal of human

TTC7A for 15 minutes. Anti-Rabbit HRP secondary antibody was applied at room temperature for 8 minutes. Signal amplification was performed using the DAB detection kit for 5 minutes and the cell nuclei were stained with hematoxylin. Sections were finally dehydrated and mounted, and then observed under a light microscope. Images were acquired with a bright-field light microscope at a magnification $\times 50$ to $\times 100$.

Whole exome sequencing (WES) and variants analysis

Genomic DNA was extracted from peripheral blood samples obtained from the proband and his parents using a QIAamp DNA blood midi kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Whole exome library was captured by a SureSelect Human All Exon Kit v6.0 (Agilent Technologies, Santa Clara, CA, USA). Targeted regions were sequenced on NovaSeq 6000 (Illumina, San Diego, USA) with 150bp pair-end runs. Sequencing reads were aligned to the GRCh37/hg19 human reference sequence. The mean coverage was $> 100\times$. Single nucleotide variants and indels were called using the *SAMtools* pipeline and annotated using ANNOVAR. Variant frequency was analyzed using 4 SNP databases (dbSNP 147, gnomAD, ExAC, and 1000 Genomes Project Database) and 2 disease databases (ClinVar and Human Gene Mutation Database). We filtered all common variants with minor allele frequency > 0.05 . For the candidate gene variants, we used the following 4 applications to predict their potential impact on protein function: SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html), Polyphen-2 (<http://genetics.bwh.harvard.edu/pph2/>), Mutation Taster (<http://www.mutationtaster.org/>), and CADD (<http://cadd.gs.washington.edu/>). All candidate variants were classified and interpreted according to the American College of Medical Genetics and Genomics (ACMG) criteria (12). Putative pathogenic variants detected by WES were confirmed by Sanger sequencing. Primers were designed online using Primer 3.0.

Conservation analysis of amino acid sequences of *TTC7A*

Amino acid conservation was evaluated using the UGENE database with the sequences of *Homo sapiens*, *Pan troglodytes*, *Macaca mulatta*, *Bos taurus*, *Mus musculus*, *Rattus norvegicus*, *Gallus gallus*, *Danio rerio* and *Xenopus (Silurana) tropicalis*.

Structural analysis of *TTC7A*

The sequences of *TTC7A* were obtained from the UniProtKB (UniProtKB: Q9ULT0). The crystal structure of *TTC7A* (SMTL ID: 5dse.2.A) was used as the template, which was determined by X-ray diffraction at a resolution of 2.9 Å. The amino acid residue at coordinate 69 was mutated for variant Leu69Pro. The structures were analyzed with PyMOL software. The residue 69, together with certain nearby ones within 3 Å, was illustrated. For clear demonstration of interresidue relationship, some residues were highlighted in the indicated colors with the computed hydrogen bonds being labeled.

Western blot analysis

Total protein was extracted from PBMCs of the patient, and age- and sex-matched healthy controls using RIPA buffer supplemented with 100 mM PMSF and complete protease inhibitor cocktail (Roche Molecular Biochemicals). Prepared samples were electrophoresed in SDS-PAGE gels following protein quantitation by BCA protein assay (Pierce, Thermo fisher scientific) according to the manufacturer's protocol and equivalent protein amounts were loaded. Proteins were then blotted onto PVDF membranes. The membranes were probed with appropriate primary and secondary antibodies, developed using ECL substrate, and images were acquired using a chemiluminescence imaging system. The following primary antibodies were used: TTC7A (Proteintech, 21600-1-AP, 1:300 dilution), b-actin (Cell Signaling, 4970, 1:2000 dilution).

q-PCR analysis

Total RNA was extracted from intestinal specimens by using a RNeasy Kit (Qiagen, Hilden, Germany). Quantitative PCR (qPCR) was performed in an ABI Prism 7300 sequence detecting system (Applied Biosystems). A list of primers used is included in Table 1.

Table 1. Primer list and probe sequences

Primer	Sequence (5'-3')
18S rRNA-F	GTAACCCGTTGAACCCATT
18S rRNA-R	CCATCCAATCGGTAGTAGCG
TTC7A-F	GCACCTCAAGGAAGCAGGTT
TTC7A-R	GCTATGCATGATGCGCACG
PI4KA-F	CCATGAAGTGGGCACCTACC
PI4KA-R	CCATACACCCCAAGAGTTGTG
PI4KB-F	CCCCCAGAGCCTGTTTCGAC
PI4KB-R	AGTGGGCAGCCAGACTCG
FAM126A-F	GTTGTGGAGGAATGGTTGTCA
FAM126A-R	GACAGGTTCTAGCAACTCACTT
FAM126B-F	CAGAGTAATGGTTGCATTGAAGC
FAM126B-R	AGGGGATAGTGAAAGACAGAACT
EFR3A-F	GGATCGAATTGGTTCTTACCTGG
EFR3A-R	GGCTTAATGCTTTGAGAATGGCA
EFR3B-F	GGTACGTGTGCATTGCTATGG
EFR3B-R	TGGCAAACCTTCACAAACGAGT

Flow cytometry

Red blood cells-removed peripheral blood was incubated with the fluorochrome-conjugated monoclonal antibodies for 20 minutes at room temperature in the presence of Fc blocking (BD Biosciences) reagents. All the antibodies used were from BD Pharmingen (BD Biosciences, US) and listed below: CD3 (OKT3), CD19 (4G7), CD4 (OKT4), CD8 (RPA-T8), CD45RA (HI100), CD24 (ML5), CD38 (HIT2), CD16 (3G8), and CD56 (B159). Samples were acquired on a BD LSRFortessa flow cytometer (BD Biosciences), with FlowJo (Tree Star, Ashland, Oregon, USA) as the analysis software.

RNA extraction, sequencing and analysis

Total RNAs were extracted from blood samples and purified Oligo (dT) beads. The RNA libraries were constructed by poly(A) protocol. Fragmentation was performed using Fragmentation Buffer. First-strand cDNA was synthesized using a random hexamer primer and M-MuLV Reverse Transcriptase (RNase H Minus). Second-strand cDNA synthesis was subsequently performed using DNA polymerase I and RNase H. The cDNA libraries were sequenced by Illumina X-ten platform using paired-end 150 bp reads.

The paired-end reads were mapped to human reference genome and Ensembl gene annotation (GRCh37/hg19 assembly) using hisat v2.2.1 (13). The mapped reads were then processed by Stringtie v2.1.4 to quantitate the gene expression against the Ensembl transcript annotation (14). The gene expression data was normalized to fragments per kilobase of transcript per million mapped reads (FPKM). The differentially expressed genes (DEGs) were identified based on the fold change between the proband and his parents (fold change > 2 or < 0.5). The gene set enrichment analysis (GSEA) was conducted to identify the Gene Ontology (GO) terms or pathways overrepresented by the DEGs. The pathways used for GSEA were curated by KEGG (Kyoto Encyclopedia of Genes and Genomes) (15), Reactome (16), and WikiPathway (17), and collected from MSigDB database. The GSEA was implemented by R enricher function in clusterProfiler package (18).

Statistical analysis

Differences between groups were calculated using unpaired Student *t*-test with GraphPad Prism 9. Data were presented as the mean $\pm$ standard error of the mean (SEM). Statistical significance was defined as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Results

Identification and intestinal histopathology of the MIA patient

The proband was a boy born to 3-generation consanguineous parents of Chinese origin, as shown in Fig. 1A. The proband was the fifth of 5 children of the couple. The first child of the parents was diagnosed with MIA and died in the first month of life because of post-operation complications consisting of severe septicemia and multiple organ failure. The second and fourth child could not eat after birth and died in the first months of life without going to the hospital. The third child was 11 years old and asymptomatic.

His mother also had a history of previous abortion (fifth pregnancy, Fig. 1A). The proband's uncle (father's younger brother) has congenital absence of the left forearm, and other relatives did not have other congenital malformations and diseases.

The proband was delivered by vaginal delivery at 34 weeks estimated gestational age. At birth, neonatal intestinal perforation and meconium peritonitis secondary to intestinal obstruction was suspected. The patient was transferred to our hospital and underwent surgery on his third day, during which the ileum atresia, and pyloric diaphragm were removed, and an ileum ostomy was performed because of distal colon obstruction. The proband subsequently suffered from severe sepsis and persistent watery diarrhea (through the distal ileum ostomy) with no relief to aggressive antibiotic and parenteral nutrition. The proband underwent multiple surgeries and was finally given a diagnosis of MIA that involved the pyloric diaphragm, ileum atresia, and colon stenosis.

During hospitalization, the patient's clinical course was complicated by multiple episodes of sepsis. The patient had seven episodes of hyperpyrexia associated with elevated C-reactive protein (CRP), white blood cell (WBC) and neutrophils. *Klebsiella oxytoca* and *Methicillin-Resistant Staphylococcus epidermidis* were detected by separate bilateral blood culture. Recurrent and refractory sepsis was diagnosed with poor respond to antibiotics of Aztreonam, Amikacin, Meropenem, Fluconazole. In spite of three times of surgeries and supportive treatment, the patient died of severe sepsis and multiple organ failure at 3 months of age.

In Fig. 1B, pathology results of the ileum atresia showed mucosal erosion, epithelial depolarized, diffused apoptotic-like changes, and dense mixed inflammatory infiltrated within the lamina propria with a majority of lymphocytes. Inherent glands are dilated and twisted, with diffused submucosal patchy lymphoid hyperplasia.

A novel homozygous missense mutation was identified in exon 2 of TTC7A gene through WES

To dissect the genetic cause that could possibly associate with the disease, we carried out the WES using the patient's and his parents' genomic DNA. We identified a novel homozygous missense mutation c.206T > C in exon 2 of *TTC7A* gene in the patient that had not been reported in the Clinvar and the Human Gene Mutation Database (HGMD). The unaffected parents were heterozygous for *TTC7A* missense mutation c.206T > C. Sanger sequencing of *TTC7A* gene confirmed this variant of the patient and suggested one allele was inherited from his father, and the other from mother (Fig. 1C). Missense mutation pathogenicity prediction was performed using MutationTaster software and the results showed that the mutation was predicted to be damaging.

Phylogenetic analysis indicated that the position surrounding the proline 69 residue were highly conserved across different species by UGENE software, Fig. 2A. As shown in Fig. 2B, this site-mutation could cause a leucine to proline change at position 69 of the peptide which located in exon 2 (p. Leu69Pro). Structural analysis showed that the hydrogen bond present between Gly65 and Leu69 in the wild-type TTC7A was broken by the Leu69Pro mutation, Fig. 2C.

Taken together, we concluded that the homozygous missense mutation c.206T > C was the causal mutation for the patient.

The homozygous TTC7A missense mutation leads to a T^{low}B⁻NK^{low} immunotype

In a subset of *TTC7A* mutated patients, MIA is associated with CID (3). Thus, we examined whether the immunotype of the patient was affected. Immunologic profiling of the patient’s PBMCs revealed significantly low T (both CD4 and CD8) and NK cells (CD3, 184 cells/μl, reference 2179–4424 cells/μl; CD4, 47 cells/μl, reference 1461–3018 cells/μl; CD8, 123 cells/μl, reference 556–1687 cells/μl; NK cells, 155 cells/μl, reference 290–780 cells/μl, Table 2). In addition, majority of T cells in the patient’s CD4 and CD8 T cells were of memory phenotype without CD45RA expression, Table 2. The patient showed markedly elevated double negative T (DNT, CD3⁺CD4⁻CD8⁻) cells, which suggested that T cell differentiation might be changed, Fig. 3A. Taken together, these results indicated that the homozygous *TTC7A* missense mutation led to an abnormal T-cell and NK-cell development.

Table 2
Immunological phenotypes of the TTC7A patient.

Patient			Control value range	
	%	Absolute (cells/μl)	%	Absolute (cells/μl)
T cells				
CD3+	6.09%	184	54.28-71.67 ^a	2179-4424 ^a
CD3 + CD4+	1.54%	47	33.72-52.43 ^a	1461-3018 ^a
CD3 + CD8+	4.06%	123	14.08-24.70 ^a	556-1687 ^a
CD4 + CD45RA+	6.87%	13	69.15-88.10 ^a	1170-2595 ^a
CD8 + CD45RA+	16.61%	31	68.90-94.60 ^a	503-1276 ^a
B cells				
CD19+	0.53%	16	17.34-36.03 ^a	734-2265 ^a
NK cells				
CD3-CD16 + CD56+	5.13%	155	5.89-14.85 ^a	290-780 ^a
^a 1–6 months (1).				

Reference:

1. Ding Y, Zhou L, Xia Y, Wang W, Wang Y, Li L, et al. Reference values for peripheral blood lymphocyte subsets of healthy children in China. J Allergy Clin Immunol. 2018;142(3):970-3 e8.

The phenotype of B cells was also analyzed. The patient exhibited a significantly decreased number of peripheral B cells (16 cells/ μ l, reference 734–2265 cells/ μ l, Table 2). The proportion of CD19⁺ B cells in lymphocytes were markedly reduced (0.5%, reference 17.3–36.0%, Table 2). We then analyzed the subpopulation of B cells. The B cells were predominantly transitional B cells (CD24^{bright}CD38^{bright}) and virtually no CD24⁺CD38^{dim} naive B cells or CD24⁺CD38⁻ memory B cells were shown in the patients (naive B cell, 60.5% versus 11.4%; memory B cells, 14.2% versus 1.49%; transitional B cells, 13.6% versus 77.2%, Fig. 3B). Taken together, these results indicated that the homozygous *TTC7A* missense mutation led to a compromised B-cell development.

Collectively, these results demonstrated that the homozygous *TTC7A* missense mutation led to a compromised lymphocyte development.

The homozygous *TTC7A* missense mutation leads to diminished *TTC7A* expression

We speculated if this novel homozygous missense mutation might affect the *TTC7A* expression. We found that patient's PBMCs expressed very low level of *TTC7A* compared with healthy controls by Western blot, Fig. 4A. We then tested the *TTC7A* expression in mRNA level in patient's intestinal tissue, qPCR analysis indicated that the expression level of *TTC7A* was lower in the proband compared with patients with Hirschsprung's disease (HD), Fig. 4B. Immunohistochemical staining of patient's ileum tissue confirmed a reduced level of *TTC7A* expression compared with the meconium ileus patient, Fig. 4C. Taken together, these results indicated that this homozygous *TTC7A* missense mutation could lead to reduced *TTC7A* expression in lymphocytes and intestinal tissues.

Loss of *TTC7A* expression results in a prohibited lymphocyte development

To shed light on the functional impact of *TTC7A* variants on the gene expression, we compared the gene expression profile of the patient with those of his parents, and identified a total of 4,852 differentially expressed genes (1,826 upregulated and 3,026 downregulated) between the proband and the parents (fold change > 2 or < 0.5).

Subsequently, the upregulated and downregulated genes were subjected to GSEA analysis, respectively. Worth to be noted, the downregulated genes were significantly enriched in GO terms and pathways related to T- and B-cell proliferation, differentiation and activation, and NK-cell mediated cytotoxicity (Fig. 5A, B), which might account for the T^{low}B⁻NK^{low} immunotype observed. Taken together, these results demonstrated that the loss of *TTC7A* of the current patient resulted in a prohibited lymphocyte development.

Loss of *TTC7A* expression results in impaired PI4K-FAM126A-EFR3A pathway in intestinal tissues

Avitzur et al. have shown that *TTC7A* mutations directly reduced the transport of PI4KIII α , a major *TTC7A*-interacting protein via coimmunoprecipitation experiments, into plasma membrane for membranous stabilization (1). We next examined the expression of *PI4KA* and *PI4KB* through qPCR experiments in

intestinal tissue and found that *PI4KA* and *PI4KB* expression were significantly reduced in patient's intestinal tissue compared with that of patients with HD, Fig. 6A, 6B. As the TTC7A-PI4K complex is stabilized by an adaptor protein, FAM126A, and once at the plasma membrane, tethered by EFR3, a membrane-bound protein (10, 19). We next confirmed *FAM126A/B* expression through qPCR experiments in intestinal tissue of the patient. The results showed that the intestinal tissue of patient had possessed much lower levels of *FAM126A* and *FAM126B* mRNA compared with that of patients with HD, Fig. 6C, 6D. We also observed reduced expression levels of *EFR3A* in the patients' intestinal tissue, Fig. 6E. However, the intestinal tissue of patient showed comparable *EFR3B* expression compared with that of patients with HD, Fig. 6F.

Previous studies reported that TTC7A protein was supposed to act as a repressor of RhoA signaling pathway, crucial to normal ROCK function (4, 7). As shown in Fig. 6G, 6H, reduced levels of *ROCK1* and *ROCK2* expression were detected in the intestinal tissue between the patients and controls.

Taken together, these results indicated that loss of TTC7A of the current patient resulted in the dysregulation of the PI4K-FAM126A-EFR3A pathway, rather than the ROCK pathway, in intestinal tissues.

Discussion

TTC7A deficiency is an autosomal-recessively inherited disease. Multiple mutations in the *TTC7A* gene, combined with heterogenous disease presentation involving inflammatory, gastrointestinal and immunological manifestations (10), has made it difficult to correlate phenotype and genotype (20). In this study, we presented a new case of familial MIA-CID with a novel homozygous *TTC7A* missense mutation.

There exists a strong correlation between genetic mutations and the survival of patients and their clinical manifestations. The majority of the reported missense mutations were hypomorphic and had better survival outcomes. These patients were likely to have missense mutations without TPR domain involvement, which mediating protein-protein interaction. Under these circumstances, it is possible that residue function of the protein may be retained, which may correspond to a relatively better chance of survival. The patient in current study died even after aggressive treatment, unlike most other patients with *TTC7A* missense mutations. This might be related to the missense mutation he carried, and resulted in disruption of these TPR domains. The reduced TTC7A expression was also consistent with this. The diminished TTC7A expression in PBMCs and intestinal tissues of the current patient, as confirmed by western blot, qPCR and immunohistochemical experiments, suggested the loss of TTC7A protein function, consistent with the severity of the clinical manifestations.

Less than 20% of *TTC7A*-deficiency patients presents with MIA alone (20). The incidence of MIA accompanying CID is higher, approximately 75%, in *TTC7A*-deficiency patients (5, 20). Intestinal disease manifestations can present as early in utero, whereas CID may be difficult to recognize before birth (10). The patient in current study had severe T and B cell and mild NK cell lymphopenia, which might be caused by a prohibited lymphocyte development. Indeed, our GSEA analysis showed that the downregulated genes were significantly enriched in GO terms and pathways related to lymphocyte

development. Besides, the results of this patient's chest X-ray examination showed that the thymus was smaller than aged-matched healthy control. Accordingly, our GSEA analysis also showed that the downregulated genes were significantly enriched in GO terms and pathways related to T cell differentiation in thymus. These results indicated the abnormalities in thymus development. However, it is still unclear whether the T cell immunodeficiency of the disease results primarily from defects of thymic epithelial cells, or from abnormalities intrinsic to the T cell lineage, or both.

Here we show that the homozygous *TTC7A* missense mutation c.206T > C impairs the PI4KA-FAM126A-EFR3A signaling pathway in a way that impairs lymphocyte homeostasis (Supplementary Fig. 1) and intestinal epithelial depolarization. *TTC7A* is known to regulate cell adhesion, polarization, and migration by inducing the phosphorylation of ROCK effectors. Avitzur et al. have shown impaired cell adhesion to collagen and fibronectin in *TTC7A*-depleted cells (1). Accordingly, our GSEA analysis showed that the downregulated genes were also significantly enriched in GO terms and pathways related to cell adhesion.

Unfortunately, there is no standard therapy for *TTC7A*-deficiency and traditional therapies do little to treat the intestinal disease (MIA and very early onset inflammatory bowel disease, VEOIBD). Former studies showed that surgical resections for MIA did not prevent the formation of new atresias (21). Intestinal epithelial cell apoptosis and chronic diarrhea are refractory to traditional IBD therapies including immunosuppressive treatments (1, 22). Although many new treatment strategies, including early total enterectomy combined with parenteral nutrition, prophylaxis immunoglobulin, antibiotic, and small-bowel-liver transplant, have been reported successfully, their safety and limitations remain to be evaluated in the future (23, 24). Hematopoietic stem cell transplantation (HSCT) has been reported to restore immunologic defects to overcome thymus dysfunction, however, the epithelial phenotype is only marginally attenuated, suggesting that intestinal disease is independent of the immune deficiency (9, 20). Also, early recognition of the immunodeficiency and early intervention with IgG replacement and prophylactic antimicrobials may provide time to better assess and monitor the immune system in order to make long-term therapeutic decisions for these patients.

The PI4KA-*TTC7A*-EFR3 or ROCK-*TTC7A* signaling pathways may become therapeutic targets. *TTC7A* protein, through defective control of RhoA signaling pathway activation, interferes with key cellular functions in epithelial and lymphocyte homeostasis (10). The administration of ROCK inhibitors is thought to ameliorate proliferative activity and epithelial architecture of the lumen and intestinal crypts. In particular, disturbed intestinal epithelia and abnormalities of thymic thymocytes associated with excessive RhoA signaling can be reversed in 3D organoids cultures in vitro by treatment of Y-27632 (2). This opens the perspective of future therapeutic option based on use of ROCK inhibitors.

In conclusion, *TTC7A* mutations should be investigated in patients with MIA with or without CID. Our study expands the spectrum of *TTC7A* mutations in MIA-CID. Characterization of the role of *TTC7A* protein in the immune system and intestinal development, as well as in thymic epithelial cells, might have important therapeutic implications in MIA-CID patients in the future. On the other hand, further studies are

needed to determine and recognize whether modified mechanisms can compensate defective TTC7A signaling to open alternative therapeutic avenues.

Declarations

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Conflicts of interest

The authors declare that they have no conflict of interest.

Availability of data and materials

Not applicable.

Code availability

Not applicable.

Authors' contributions

WM, SY, JG and SJH designed most of the studies. WM and SY carried out much of the work together with RG, LF, LZ, QR, WG, JD, CH and YP. SY, WM, XJH, JG and SJH analyzed the data. WM wrote up the manuscript with input from SY, JG, JH, LZ and RG. All authors read and approved the final manuscript.

Ethics Approval

The study was approved by the Ethics Committee of Beijing Children's Hospital, Capital Medical University, and performed according to the Declaration of Helsinki. The written informed consents for publication were obtained from the parents of the patient.

Consent to participate

Clinical information and blood samples were collected from the patient, the parents, and controls, all of whom had given their prior informed consent to participation in the study.

Consent for publication

All authors consent to publish the manuscript.

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Figures

Figure 1

A novel homozygous TTC7A missense mutation was identified in the patient with familial MIA. A. Pedigree chart of the proband's family. Consanguinity was indicated with a double horizontal line. Males were depicted as squares, females as circles, deceased individuals with a diagonal line. Abortion was depicted as triangle with a diagonal line. Black filled symbols represented individuals who were clinically affected, while grey filled symbols indicated individuals who did not receive medical testing. The proband who underwent WES were indicated by an arrow. B. H&E photomicrographs of patient's small bowel of

these atretic areas showed mucosal erosion, diffused apoptotic-like changes, and dense mixed inflammatory infiltrate within the lamina propria with marked eosinophilia. C. Sanger sequencing chromatogram depicted the 206T>C missense mutation of TTC7A.

Figure 2

Structure analysis of the mutant TTC7A and Amino acid conservation for the p. L69P variants. A. Protein alignment indicated that the position of L69P mutation was highly conserved across different species. Residue L69 was shown in red arrow. B. The schematic structure of TTC7A showed the location of the

variant identified in this study. Illustration of TTC7A protein with TPR domains was in red and the identified mutation were highlighted. C. The hydrogen bond presenting between Gly65 and Leu69 in the wild-type TTC7A was broken by the L69P mutation. The residue 69 together with certain nearby residues within 3Å was illustrated in the wild-type (WT) and the mutant by PyMOL. Computed hydrogen bonds were shown as yellow dashed lines. Residues Leu69/Pro69 were highlighted in blue.

Figure 3

Increased frequencies of DNT and transitional B cells. A. Representative dot plots showed markedly elevated frequencies of DNT compared with age- and sex-matched healthy control (HC), as measured by flow cytometry. B. Representative dot plots showed significantly increased frequencies of CD24^{bright}CD38^{bright} transitional B cells, as measured by flow cytometry.

Figure 4

Diminished TTC7A expression in lymphocytes and intestinal tissues. A. Western blot of patient-derived PBMCs showed a severely impairment of TTC7A protein expression compared with healthy controls (HC). β-actin was used as an internal loading control in the western blot. The result was a representative of 3 independent experiments. B. The protein band intensity was quantified and analyzed by ImageJ software. C. The qPCR results demonstrated that patient’s intestinal tissue had a significant reduction in the amount of TTC7A expression compared with Hirschsprung’s disease (HD). TTC7A expression was normalized to 18S rRNA, and results were represented as relative expression normalized to the control. **P < 0.01, ***P < 0.001. D. In the patient’s intestinal tissue, immunohistochemistry demonstrated that TTC7A protein expression was diminished compared with the meconium ileus patient (control). Magnification for left panel was ×50, magnification for right panel was ×100.

Figure 5

GO and pathway analysis indicated a prohibited lymphocyte development and function. GO terms (A) and pathways (B) enriched by the downregulated genes in the patient. Enriched pathways with an adjusted P value of less than 0.05 were considered significantly enriched.

Figure 6

Impaired PI4K-FAM126A-EFR3A pathway in patient's intestinal tissues. (A-B). qPCR analysis of patient-derived intestinal biopsy specimens showed reduced PI4KA and PI4KB expression compared with Hirschsprung's disease (HD). (C-E, G, H). qPCR analysis of patient-derived intestinal biopsy specimens showed reduced FAM126A (C), FAM126B (D), EFR3A (E), ROCK1 (G), and ROCK2 (H) expression compared with HD. (F). qPCR analysis of patient-derived intestinal biopsy specimens showed comparable EFR3B expression compared with HD.

Supplementary Files

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