

Novel homozygous ataxia-telangiectasia (A-T) mutated gene mutation identified in a Chinese pedigree with A-T

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Abstract. Ataxia-telangiectasia (A-T) syndrome is a rare autosomal recessive disorder mainly caused by mutations in the A-T mutated (ATM) gene. However, the genomic abnormalities and their consequences associated with the pathogenesis of A-T syndrome remain to be fully elucidated. In the present study, a whole-exome sequencing analysis of a family with A-T syndrome was performed, revealing a novel homozygous deletion mutation [namely, NM_000051.3:c.50_72+7del,p.Asp18_Lys24delins(23)] in ATM in three affected siblings, which was inherited from their carrier parents who exhibited a normal phenotype in this pedigree. The identified mutation spans the exon 2 and intron 2 regions of the ATM gene, causing a splicing aberration that resulted in a 30-bp deletion in exon 2 and intron 2, as well as a 71-bp insertion in intron 2 in the splicing process, which was confirmed by reverse transcription-polymerase chain reaction and sequencing analysis. The change in the three-dimensional structure of the protein caused by the mutation in ATM may affect the functions associated with telomere length maintenance and DNA damage repair. Taken together, the present study reported a novel homozygous deletion mutation in the ATM gene resulting in A-T syndrome in a Chinese pedigree and expanded on the spectrum of known causative mutations of the ATM gene.

Introduction

Ataxia-telangiectasia (A-T; Online Mendelian Inheritance in Man entry no. 208900) is a rare progressive neurological

disease mainly caused by mutations in the A-T mutated (ATM) gene. A-T patients initially present with the typical symptoms of cerebellar degeneration, telangiectasia, sensitivity to ionizing radiation and immune defects. With time, the patients display premature aging and stunting, while certain neurological symptoms appear, including oculomotor apraxia, neuropathy and nystagmus (1-4). Certain patients also exhibit gonad retardation and growth factor deficiency (5,6). An increased risk of malignancy and recurrent sinopulmonary infections are two major causes of mortality (7). Of note, certain patients may exhibit atypical symptoms, including generalized dystonia and other unassociated cerebellar disorders (8,9). To date, no effective treatments exist to delay or stop neurodegeneration. However, other manifestations of A-T, such as immunodeficiency, lung disease, hypoplasia and diabetes, can be effectively treated (10).

ATM, as a causative gene for the A-T disorder, is located in the autosomal region 11q22.3 containing 66 exons (11) and encodes the ATM protein, which has a critical role in controlling cell cycle checkpoints and responses to DNA damage after DNA double-strand breaks caused by oxidative damage, endogenous sources or ionizing radiation (12). In addition, activated ATM regulates numerous downstream proteins, including the tumor suppressor proteins p53 and BRCA1, checkpoint kinase 2, the checkpoint protein RAD17 and the DNA repair protein NBS1 (13). Therefore, the ATM protein is necessary for cells to maintain genomic stability (10). To date, >600 pathogenic variants have been reported for the ATM gene (14).

However, few studies have assessed ATM mutations in Chinese populations, and even fewer have examined their association with a family history of A-T (15,16). In the present study, a Chinese pedigree affected by A-T was subjected to genetic testing by whole-exome sequencing (WES), and a novel ATM mutation was identified, which is likely to be associated with the A-T syndrome.

Patients and methods

Subjects and clinical evaluation. The proband was an 11-year-old Chinese male, who was hospitalized at the

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Department of Cardiothoracic Surgery of the Children's Hospital of Fudan University (Shanghai, China) due to pyothorax along with typical symptoms of limb and truncal ataxia in October 2016. The proband's 10-year-old brother and his 9-year-old sister exhibited the same symptoms of limb and truncal ataxia. Their parents were healthy and non-consanguineous. Analysis of the medical family history indicated that no other members of the pedigree had presented with any A-T-like symptoms. A total of 3 healthy volunteers, including 2 25-year-old women and a 26-year-old man, were recruited as control. Blood samples of the proband, his two siblings and parents and controls were collected for further analysis.

The proband and his two siblings underwent a series of clinical examinations to evaluate the presentation of A-T, including neurological examination, eye checks, biochemical blood analysis [including detection of immunoglobulin, α -fetoprotein (AFP) and carcinoembryonic antigen (CEA) levels] and brain magnetic resonance imaging (MRI) examination.

The present study was approved by the local Ethics Committee of Fudan University (Shanghai, China). Written informed consent was obtained from the parents of proband and the controls of the study subjects.

Genetic testing. Genomic DNA was extracted from the blood samples using the TIANamp Genomic DNA Kit (DP304-03; Tiangen Biotech Co., Ltd., Beijing, China) according to the product protocol. Initially, gene chip analysis of the DNA from the proband was performed by Gemple Biotech Co., Ltd. (Shanghai, China), and subsequently, WES was performed for the two siblings and parents of the proband, also conducted by Gemple Biotech Co., Ltd. A whole-exome library was constructed on the KAPA platform using the KAPA Hyper Prep kit (KK8514 and KK8515; Roche, Basel, Switzerland). All of the exomes were captured by a custom-designed SeqCap EZ MedExome kit (7681330001; NimbleGen; Roche, Madison, WI, USA) and sequenced with a HiSeq X Ten instrument (Illumina, Inc., San Diego, CA, USA). The quality of the original data was evaluated using FASTQC (version 0.11.5; Illumina, Inc.), and the linker sequences were removed by Cutadapt (version 1.10; <https://github.com/marcelm/cutadapt/>). The reads were then aligned to the genome using the software BWA (version 0.7.15) (17). SAMtools (version 1.3.1; <http://samtools.sourceforge.net/>) was used for format conversion of the comparison results, and the Picard software package (<http://broadinstitute.github.io/picard/>) was used for sorting and deduplication. Finally, mutation detection was performed with GATK Haplotype Caller (version 3.6) (18). Mutation data were annotated and categorized according to the American College of Medical Genetics and Genomics (ACMG) guidelines for analysis of phenotype-associated mutation sites (19).

Sanger sequencing. Sanger sequencing was performed for all 5 subjects with an ABI 3730 XL automated sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) to validate the candidate variants reported by WES. Primer pairs were designed with the online software Primer3 (<http://frodo.wi.mit.edu/primer3>) (forward, 5'-ACAGTGATG TGTGTTCTGAAATTGT-3', and reverse, 5'-TCTCGAATC AGGCGCTTAAA-3') to amplify fragments including the variants. The ATM gene reference sequence was obtained from the



Figure 1. Chest X-ray of the proband, revealing left empyema.

National Institutes for Biotechnology Information GenBank. The pathogenicity of candidate variants was evaluated based on the ACMG guidelines for analysis of phenotype-associated mutation sites (19).

Reverse transcription-polymerase chain reaction (RT-PCR) and complementary DNA (cDNA) sequencing. Blood was collected from all family members for mRNA extraction. Total RNA from peripheral blood mononuclear cells of the fresh blood was extracted with TRIzol reagent (Thermo Fisher Scientific, Inc.). The RNA concentration/quality was determined using a NanoDrop spectrophotometer (ND-1,000, Thermo Fisher Scientific, Inc.). cDNA synthesis was performed with PrimeScript™ RT Master Mix (RR036A-1; Takara Biotechnology Co., Ltd., Dalian, China) using 500 ng total RNA according to the manufacturer's protocol. To determine the mRNA sequence of ATM, the target fragments of the cDNA were amplified by PrimeSTAR Max DNA Polymerase (R045; Takara Biotechnology Co., Ltd.) according to the product protocol. The PCR primer pair used, which was designed with the online software Primer3, was as follows: Forward, 5'-CAGTGATGTGTGTTCTGAAA-3', and reverse, 5'-TTGTGTTGAGGCTGATACAT-3'.

The PCR thermal cycling conditions is as follows: Activation of polymerase, 98°C, 5 min; thermal cycling, 30 cycles; denaturation, 98°C, 10 sec; Annealing: 55°C, 15 sec; elongation, 72°C, 30 sec; extension, 72°C, 5 min; and storage, 4°C.

The Axygen® AxyPrep™ DNA Gel Extraction kit (AP-GX-250; Axygen; Corning Inc., Corning, NY, USA) was used to extract the target RT-PCR product separated by agarose gel electrophoresis with a gel percentage of 4%. PCR was then used to amplify the target fragments using the same primer pair as that stated earlier. Subsequently, the PCR products were subjected to direct Sanger sequencing.

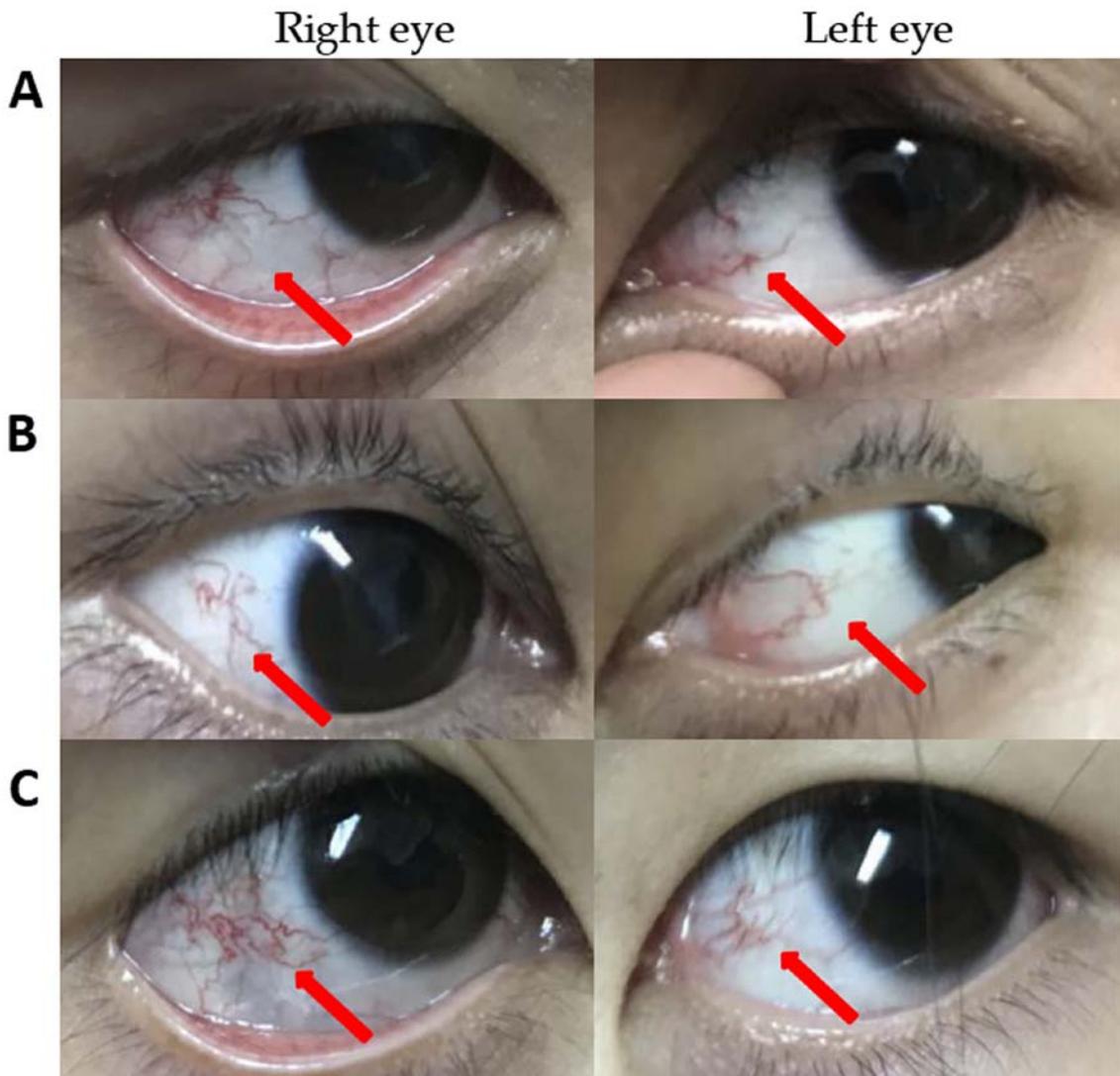


Figure 2. Images of the eyes of subjects with ataxia-telangiectasia syndrome. The right and left eyes of (A) patient II:1, (B) patient II:2 and (C) patient II:3 are shown. The red arrows indicate ocular telangiectasia in the three patients.

The cDNA nucleotide sequence analysis was based on the GenBank sequence for wild-type ATM (NM_000051.3). The novel variant was named following the recommendations of the Human Genome Variation Society (<http://varnomen.hgvs.org/>).

Three-dimensional (3D) structure of the ATM protein. The 3D structure of the protein was predicted by Swiss Model (<https://www.swissmodel.expasy.org/>), and that of its mutated form was predicted based on the cDNA nucleotide sequences of the patients. The wild-type and mutated form were compared to determine the effect of the mutation on its structure.

Results

Clinical features. The proband (patient II:1) was diagnosed with pneumonia and pyothorax diagnosed by chest X-ray (Fig. 1). Neurological examination revealed that the proband exhibited progressive limb and truncal ataxia, diagnosed based on the symptoms of difficulties in walking and gasping, choking frequently while swallowing, delay in language development,

diminished limb strength and decrease of deep tendon reflexes. The proband's brother (patient II:2) and sister (patient II:3) exhibited the same symptoms of limb and truncal ataxia, but to a less severe extent compared with the proband. The proband initially developed symptoms of ataxia at the age of 2 years, manifesting as gait instability and frequent falls. His two siblings both had developed similar symptoms when they were 6.5 years old. Their parents were healthy and exhibited a normal phenotype.

Physical examination, laboratory tests and imaging tests were performed on the proband and his siblings, and all three patients were found to exhibit the typical symptoms of A-T (20). Ocular telangiectasia was observed in all three patients (Fig. 2). The laboratory test results are summarized in Table I. The serum AFP levels were significantly increased in all three patients, whereas the serum CEA levels were within the normal levels. All three patients exhibited normal or slightly increased serum levels of immunoglobulin (Ig)A, IgE and IgM. Biochemical tests indicated no evident abnormalities, including the triglyceride, creatinine, alkaline phosphatase and lactate dehydrogenase levels. Furthermore,

Table I. Clinical and laboratory features of three ataxia-telangiectasia patients.

Patient ID	Sex	Age (years)	Age at onset (years)	Cerebellar atrophy	α -fetoprotein (ng/ml)	Carcinoembryonic antigen (ng/ml)	Immunoglobulins				Biochemical examination				
							IgA (g/l)	IgG (g/l)	IgM (g/l)	IgE (kU/l)	TG (mmol/l)	Cr (μ mol/l)	ALP (U/l)	LDH (U/l)	
Patient (II:1)	M	13	2.5	Atrophied	\uparrow 1,836.00	2.8	1.01	8.4	\uparrow 2.69	<2.0	0.92	34	286	229	
Patient (II:2)	M	12	6.5	Atrophied	\uparrow 1,034.00	1.87	\downarrow 0.17	\uparrow 14.90	\uparrow 14.90	6.62	1.14	34	260	256	
Patient (II:3)	F	11	6.5	Atrophied	\uparrow 629.90	1.86	\downarrow 0.08	\uparrow 14.10	\uparrow 3.57	<2.0	0.82	34	242	255	
Normal value					<3.7	<5.0	0.52-2.16	6.09-12.85	0.67-2.01	<100	0.56-1.7	21-65	42-383	110-290	

TG, triglyceride; ALP, alkaline phosphatase; Cr, creatinine; ALP, alkaline phosphatase; LDH, lactate dehydrogenase.

cerebellar atrophy was detected in all three patients by cranial MRI (Fig. 3).

Genetic analysis. WES was performed for the proband, his two siblings and parents against the exons and exon-intron boundaries of the ATM gene. In total, >95% of the ATM gene was examined on the test platform with a sensitivity of >99%. Point mutations and deletions were detected simultaneously. A 30-bp homozygous deletion mutation, namely NM_000051.3:c.50_72+7del.p.Asp18_Lys24delins(23), was detected in the three affected siblings, which was inherited from their carrier parents, who both had a normal phenotype. This mutation spans the exon 2 and intron 2 regions of the ATM gene, which is predicted to cause a splicing aberration, resulting in a 30-bp deletion of exon 2 and intron 2, as well as a 71-bp insertion of intron 2 in the splicing (Fig. 4). According to the ACMG guidelines, the detected mutation [NM_000051.3:c.50_72+7del.p.Asp18_Lys24delins(23)] is a suspected pathogenic mutation. However, the mutation was not included in the 1000 Genomes Project (<http://www.internationalgenome.org/>), the Exome Aggregation Consortium (<http://exac.broadinstitute.org/>), the Human Gene Mutation Database (<http://www.hgmd.cf.ac.uk/ac/index.php>) or the ClinVar database (<https://www.clinicalgenome.org/data-sharing/clinvar/>), thereby confirming its novelty.

Mutation leading to splicing abnormality. To verify the influence of the deletion variant in the ATM gene on the splicing, cDNA was synthesized by RT-PCR using total RNA as the template, followed by amplification of the 290-bp region spanning exons 2 and 3 using the cDNA as template. As expected, the controls exhibited a 290-bp band in the gel, while the three affected siblings had a 338-bp band, and the parents had two bands in the gel, including a prominent one at 290 bp and a faint one at 338 bp (Fig. 5). The DNA of the 290- and 338-bp bands in the gel was extracted and subjected to direct Sanger sequencing. As indicated in Fig. 6A, the normal controls exhibited normal precursor mRNA (pre-mRNA) splicing; however, all the patients exhibited splicing abnormality. Between exons 2 and 3, a 23-bp fragment of exon 2 and 7-bp fragment of intron 2 were deleted, while a 71-bp fragment of intron 2 was inserted in the cDNA sequence (Fig. 6B). These results indicated that the deletion mutation in the ATM gene influenced the pre-mRNA splicing.

3D structure of ATM and its variant form. Based on the cDNA sequence of patient, in which the first two bases are identical to the normal sequence, it was found that the deletion variant eventually leads to the insertion of 69 bases, replacing the 21 bp of the normal sequence. However, no new termination codon was formed, which resulted in a normal sequence of the first 17 amino acids, and an insertion of a new 23 amino acid sequence (YLMFNFLKCVISNPLLFPFYFQ), replacing the 7 normal amino acids. The sequence returned to normal from the 34th amino acid and remained normal from exon 3 onwards. The structure of the protein predicted by Swiss Model indicated that the mutation resulted in extra secondary structures and a negative effect on the normal formation of tertiary structures (Fig. 7). According to the Smart (smart.

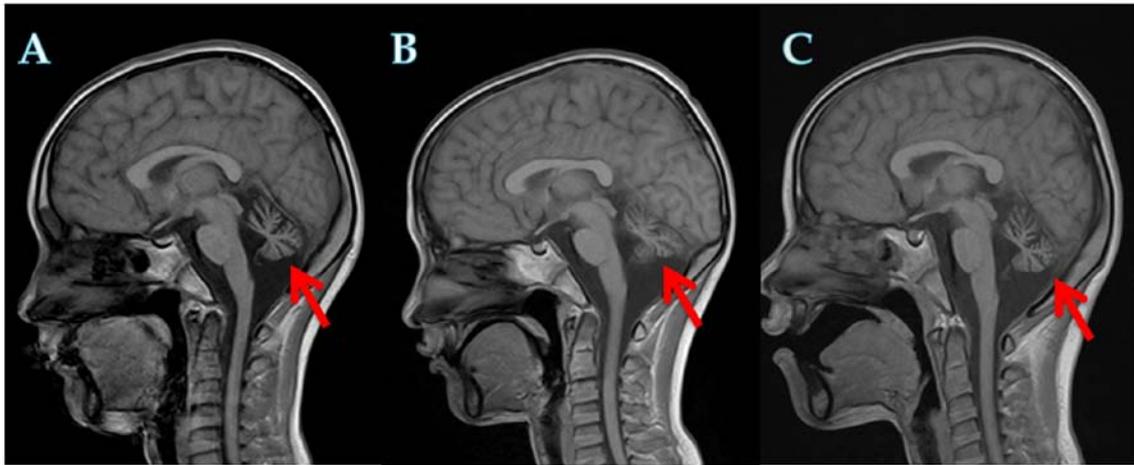
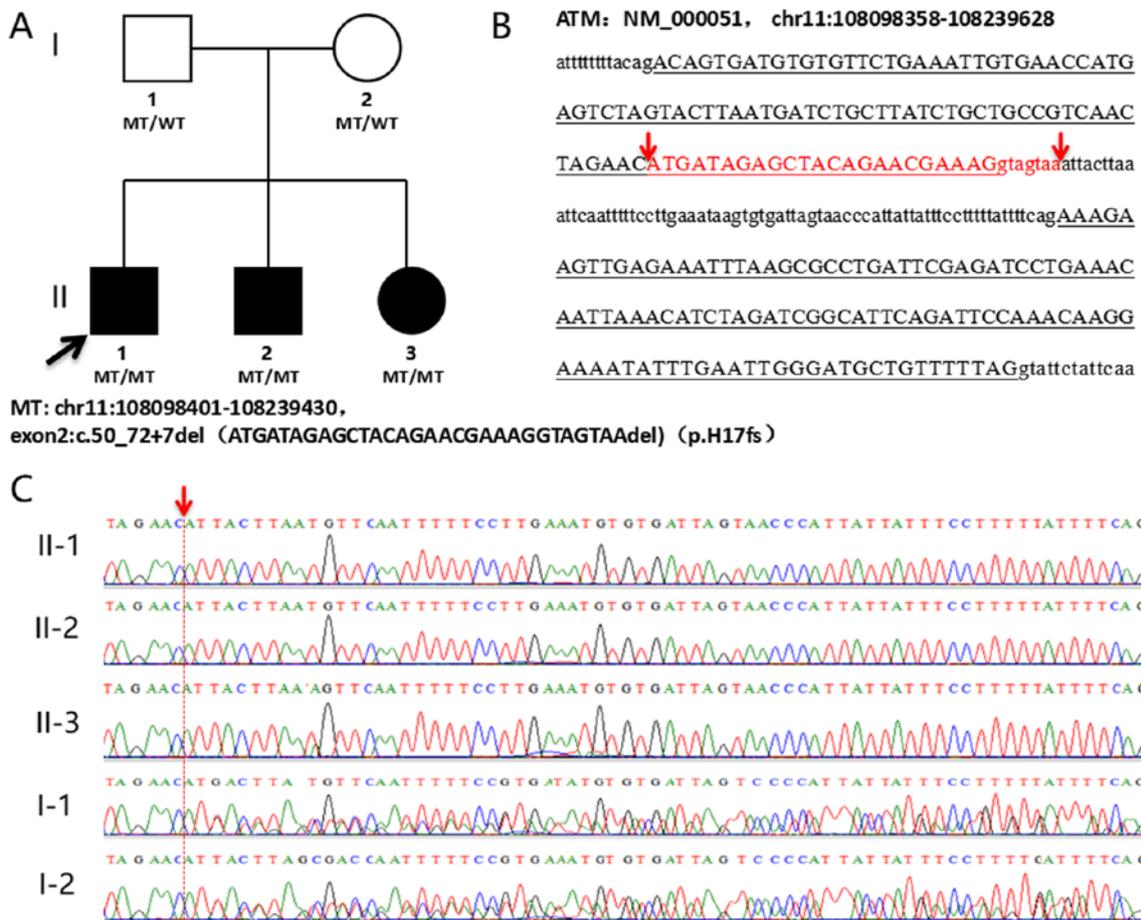


Figure 3. Magnetic resonance images of the brains of the three patients. Brain of (A) patient II:1, (B) patient II:2 and (C) patient II:3. The red arrows indicate cerebellar atrophy.



MT: chr11:108098401-108239430.

exon2:c.50_72+7del (ATGATAGAGCTACAGAACGAAAGGTAGTAA~~del~~) (p.H17fs)

Figure 4. Pedigree chart of the family with ataxia-telangiectasia and genomic DNA sequences. (A) Family pedigree, including the three affected siblings (II:1, II:2 and II:3) and their parents (I:1 and I:2). (B) Genomic DNA sequences for the ataxia-telangiectasia mutated gene region NM_000051, chr11:108098358-108239628, including exon 2 (top underlined upper-case letters), intron 2 (middle lower-case letters) and exon 3 (below underlined upper-case letters). The red region between the two red arrows was deleted in the three patients. (C) Sanger sequencing was performed to analyze the genomic DNA sequences of all family members. All three affected members (II:1, II:2 and II:3) are homozygous for the mutation, while their parents (I:1 and I:2) are heterozygous. The mutation site is indicated by a red arrow. WT, wild-type; MT, mutated type.

embl-heidelberg.de/) and Pfam (<http://pfam.xfam.org/>) databases, the function of this area is associated with telomere length maintenance and DNA damage repair, which may be impaired in the deletion variant.

Discussion

The estimated incidence of A-T is between 1 in 40,000 and 1 in 100,000 live births (21). At birth, affected individuals

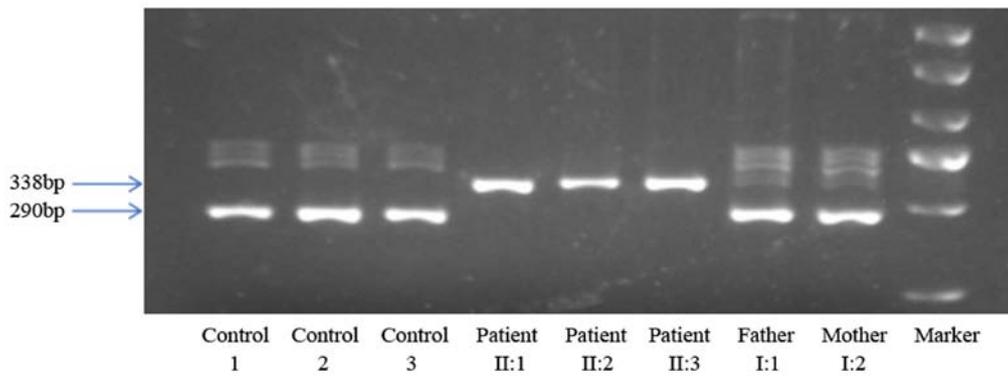


Figure 5. Electrophoresis of reverse transcription-quantitative polymerase chain reaction products. The bands of the patients (II:1, II:2 and II:3) were longer in comparison with those of the controls (338 vs. 290 bp, respectively). Two bands were observed in the parents (father, I:1 and mother, I:2): The bright one was similar to the control groups and the faint one was similar to the patients.

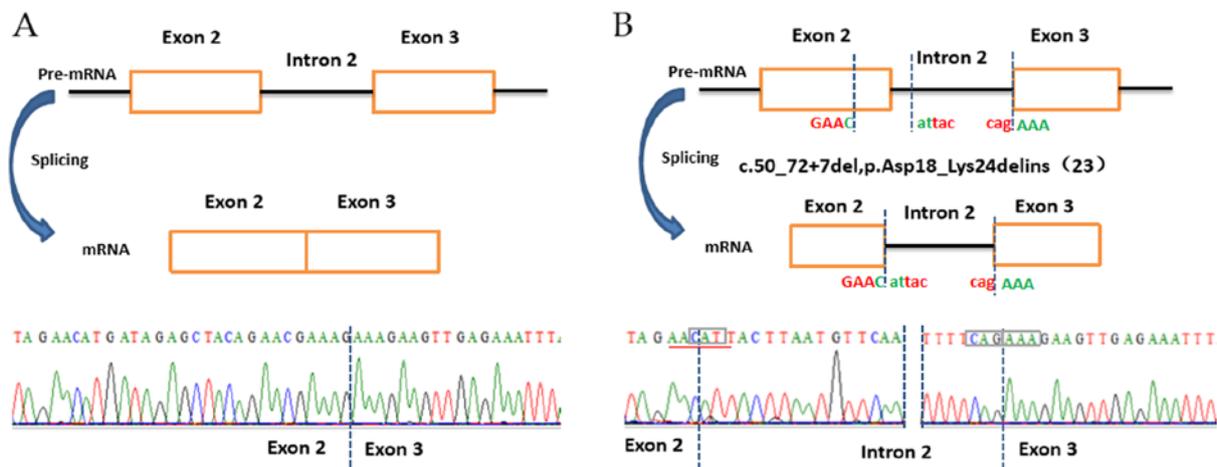


Figure 6. Schematic representation of ATM gene splicing. (A) Schematic illustrating the normal splice of the ATM gene; there are no intron sequences between exons 2 and 3. (B) Schematic illustrating the effect of the ATM gene mutation on the splice, causing a deletion of 23 bp of exon 2 and 7 bp of intron 2, and an insertion of 71 bp of intron 2 in the complementary DNA sequence. The gray boxes indicate the codon. ATM, ataxia-telangiectasia mutated.

have no evident symptoms; however, serious symptoms usually arise within the next years and deteriorate rapidly. The majority of A-T patients die of malignancy or recurrent sinopulmonary infections. Prior to the appearance of typical symptoms, the patients appear as healthy, which often results in missed diagnosis and treatment delay. Furthermore, when symptoms appear, it is difficult to diagnose A-T syndrome due to insufficient knowledge and awareness of clinicians regarding this condition, and variable clinical manifestations. In this light, genetic testing may be a valuable tool to improve the diagnostic efficacy for A-T.

As reported previously, A-T is caused by loss of function of the ATM gene in nearly 75% of cases (22,23). In the present study, a novel deletion variant was identified by WES in a family with A-T syndrome. All three patients were homozygous for the deletion mutation, while the two parents were heterozygous. cDNA sequence analysis by RT-PCR and Sanger sequencing indicated that the deletion mutation in ATM gene causes the production of pre-mRNA, which contains parts of exon 2 and intron 2 in between the exon 2 and 3 regions. Of the parents, as heterozygous carriers of the mutations, exhibited two bands with uniformity in brightness in the gel of RT-PCR products, including a normal band (290 bp) and a mutant band (338 bp); however, the actual

mutant band was faint. As intron retention in mRNAs may be regarded as a consequence of mis-splicing (24,25), transcripts retaining introns are often removed by nonsense-mediated decay (NMD), or removed by nuclear retention and exosome degradation, which may prevent the translation of intron-retaining transcripts into potentially harmful proteins (25,26). In the present study, the structure of the mutant mRNA may have been unstable due to intron retention, which may have induced an NMD-like mRNA regulation mechanism, leading to the degradation of mutant mRNA and resulting in the 338-bp fragment being faint. Furthermore, the 3D structure of the protein predicted by Swiss Model indicated that the likely cause of the disease may be that the mutation affected the protein structure, impairing its function.

In the present study, a systematic analysis of a Chinese family including three A-T patients was performed, and a novel pathogenic deletion variant in the ATM gene was identified that contributes to the spectrum of known causative mutations and phenotypes of A-T. Furthermore, the three pediatric patients exhibited almost the same symptoms, which points to a familial inherited disease. The parents of the patients were from two adjacent counties in Chongqing, China, and were not consanguineous according to their narrative. Although the parents had taken their children to several hospitals to obtain a definite

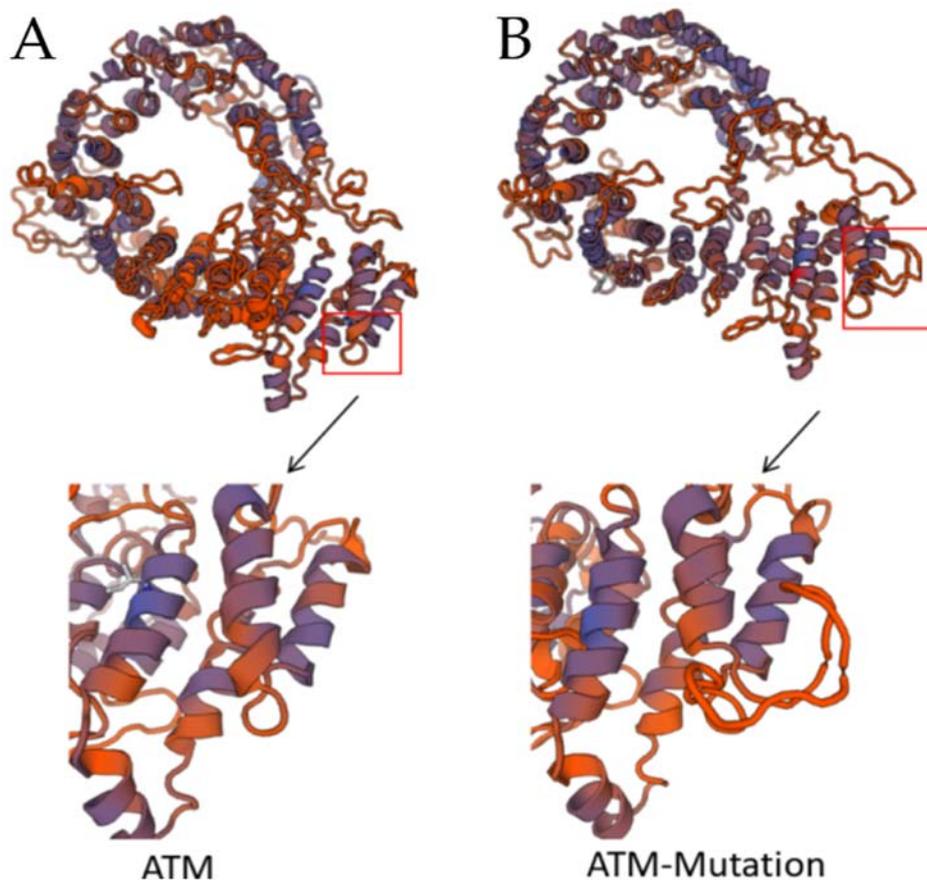


Figure 7. Three-dimensional structure of the ATM protein. Structure of the (A) wild-type and (B) mutated ATM protein. A partial magnified structure of the wild-type or mutated ATM protein region is included in the red squares. ATM, ataxia-telangiectasia mutated.

diagnosis, A-T was not confirmed until the proband was 13 years old. A-T is so rare that its current understanding is insufficient, while ocular telangiectasia and cerebellar ataxia, which are the most common clinical manifestations of this disease (27), are not exclusive clinical features based on which A-T may be suspected. Furthermore, the variable clinical manifestation of the disease increases the difficulty of recognition, which emphasizes the importance of genetic testing in the diagnosis of A-T, particularly in those cases where the clinical symptoms are not typical. Although A-T cannot be currently cured with any of the available treatment methods, the rapid development of mutation-targeted therapeutic approaches may bring hope for potential treatments for A-T patients (16). In addition, parents with a family history of A-T wishing to conceive may be subjected to genetic testing and prenatal genetic counseling to contribute to eugenics.

In conclusion, a Chinese pedigree affected by A-T was subjected to WES in the present study, revealing a novel homozygous deletion mutation in ATM [namely, NM_000051.3:c.50_72+7del, p.Asp18_Lys24delins (23)] in three affected siblings, which was inherited from their carrier parents who exhibited a normal phenotype. The deletion mutation in the ATM gene affected pre-mRNA splicing and resulted in a change of the 3D structure of the protein, which may have impaired the functions associated with telomere-length maintenance and DNA damage repair. In the Chinese non-consanguineous family, the three affected children all carried the same homozygous mutation and the parents were heterozygous, which is rare and has never been reported in China previously. Furthermore, the present study contributed to

the spectrum of known pathogenic variants of ATM, expanded the current understanding on the A-T syndrome and highlighted the importance of genetic testing in the diagnosis of this disease.

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Availability of data and materials

All data generated and analyzed during the present study are included in this published article.

Authors' contributions

GH and WS conceived and designed the study. WC, GC, SZ and BJ were responsible for the acquisition of patient information and communication with the patients' families. SL collected clinical data from the A-T family. HH performed

molecular genetics experiments on the family and the controls. HH drafted the manuscript, which was edited and revised by WC and WS.

Ethics approval and consent to participate

Informed consent for this investigation was obtained from all participating A-T patients and parents, and the principles outlined in the Declaration of Helsinki were followed. The study was conducted in agreement with the Ethical Committee of the Center for Medical Genetics, Children's Hospital of Fudan University (Shanghai, China).

Patient consent for publication

The publication of information from these three A-T patients have their support and informed consent.

Competing interests

The authors declare that they have no competing interests.

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