厚生労働科学研究委託費（難治性疾患等実用化研究事業）
委託業務成果報告（業務項目）

毛細血管拡張性小脳失調症およびDNA損傷修復異常を基盤とするその類縁疾患の病態解明・診断法の確立及び治療法の開発に関する研究

DNA損傷に対する自然免疫応答に関する研究

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公益財団法人東京都医学総合研究所　脳発達・神経再生研究分野　主席研究員

研究要旨
DNA損傷修復応答障害の一つである毛細血管拡張性小脳失調症において小脳が変性する機序は十分明らかにされていない. 自然免疫系は自己由来の核酸であってもその存在部位や構造によっては免疫応答を誘導する そこで二本鎖切断等の損傷を受けたDNAに対して過剰な免疫応答が誘導され，これが細胞傷害性に作用して神経変性をもたらすのではないかという仮説を立てて検討した. マウス神経芽細胞腫由来のNB2a細胞に抗がん剤temozolomideを投与するとphospho-H2A.X陽性細胞の割合が増加し，DNA二本鎖切断が生じたと考えられた．またNB2a細胞より抽出したDNAをLipofectamineと共にMG6細胞に導入すると細胞生存率が低下し，DNA認識の結果として何らかの機序により細胞死を誘導することが推定された.

A．研究目的

毛細血管拡張性小脳失調症 Ataxia telangiectasia（AT）はDNA損傷修復応答の障害により進行性小脳失調等を呈する疾患である. 責任遺伝子としてATM(Ataxia telangiectasia mutated)が同定されているが，これがどのような機序で小脳の変性をもたらすかについては十分明らかにされていない.

自然免疫系は抗原非特異的な免疫応答であり，獲得免疫系とは異なり基本的にあらゆる体細胞にその能力が備わっている．自然免疫のセンサーが認識する分子の一つにRNAやDNAなどの核酸が挙げられ，これらはその存在部位や構造によってウイルスであるか自己由来の核酸であるか識別される．しかし時に何らかの原因のよりこの識別機構が正常に機能しなくなると，自己由来の核酸が異物と誤認されて過剰な免疫応答を誘導し，これは全身性紅斑性狼瘡等の自己免疫疾患の一因となると推定されている.

自然免疫系がDNAのどのような構造の違いを認識するのかについては少しずつ知見が得られつつあるが，我々は二本鎖切断等の損傷を受けたDNAに対して過剰な免疫応答が誘導され，これが細胞傷害性に作用して神経変性をもたらすのではないかという仮説を立てた．そこで本年度の研究では，まず自己の核酸に対する自然免疫系の応答機序を明らかにすることを目的とした.

B．研究方法

1) 細胞の培養　①マウス神経芽細胞腫由来細胞株NB2a，②マウス不死化ミクログリア細胞株MG6[1]を使用し，これらはいずれも理研バイオリソースセンターより購入した．NB2aはRPMI1640培地，MG6はインスリン. 2-mercaptoethanolを添加したDMEM培地により培養した.

2) NB2a細胞のtemozolomideによるDNA損傷誘導とDNA抽出　NB2a細胞をアルキル化／メチル化抗がん剤であるtemozolomide (TMZ) で刺激し，一部はATM kines阻害剤であるKU-55933を添加して24時間培養した. その後DNeasy blood&tissue kit (QIAGEN)を用いて総DNAを抽出した．DNAは-20℃で凍結保存紙，熱変性等の処置を加えることなくトランスフェクションに用いた．またTMZ/KU-55933刺激後のDNA二本鎖切断を抗phospho-H2A.X抗体によるフローサイトメトリー解析により測定した.

3) MG6細胞へのDNAトランスフェクションとMTTアッセイ　NB2a細胞から抽出したDNAをLipofectamine 2000 (Invitrogen) によりMG6細胞に導入し，24時間後にMTTアッセイ（CellTiter 96 AQ One, Promega）により性細胞数を相対定量した.

（倫理面への配慮）
本研究ではマウス由来の細胞株のみを実験に用いているため倫理面への特段の配慮は必要としなかった.

C．研究結果

1) NB2a 細胞の temozolomide による DNA 損傷誘導

Temozolomide 投与により phospho-H2A.X 陽性細胞の割合は用量依存的に増加した．KU-55933 は陽性細胞の割合を減少させた．

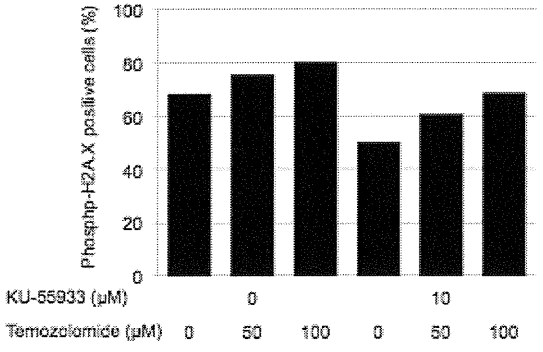

図 2

NB2a 細胞の temozolomide による DNA 損傷誘導

2) DNA トランスフェクションが MG6 細胞の生存へ及ぼす効果

Lipofectamine を使用せず DNA を単独で投与した場合には細胞の生存に変化はなかったが，Lipofectamine と共に導入した場合には DNA の投与量依存的に MG6 細胞の生存割合が低下した（図１）．

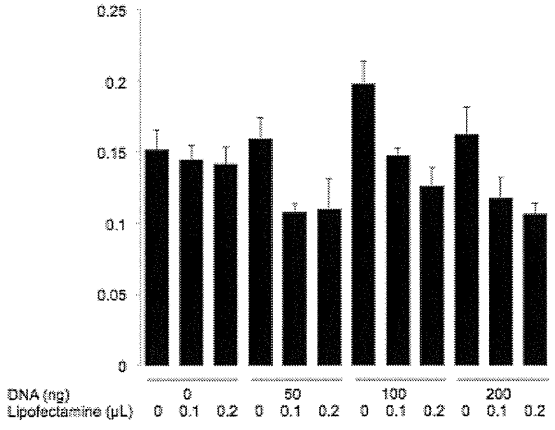

図１　DNA トランスフェクションが MG6 細胞の生存へ及ぼす効果（MTT アッセイ）

D．考察

Phospho-H2A.X 抗体による解析結果より，temozolomide は NB2a 細胞に DNA 二本鎖切断を引き起こすことが示唆された．腫瘍細胞の中には temozolomide に対して感受性を示す細胞と示さない細胞があるが，NB2a は感受性があると考えられ，薬剤による DNA 二本鎖切断誘導のモデルとして適切と考えられた．

NB2a 細胞より抽出した DNA を Lipofectamine 2000 により MG6 細胞に導入すると DNA の投与量依存的に MG6 細胞生存率の低下が認められた．この原因として，細胞質に導入された DNA がホストの自然免疫系により認識され，その結果何らかの機序により細胞死が誘導された可能性が考えられる．

導入された DNA を認識した細胞質内センサーは不明であるが，細胞内二本鎖 DNA は STING を介して認識されることから，今後は STING のノックダウン等を行って確認する．また temozolomide±KU-55933 により二本鎖切断を誘導した DNA に対してどのような変化が起こるかを検討する．

E．結論

抗がん剤 temozolomide を NB2a 細胞に投与すると phospho-H2A.X 陽性細胞の割合が増加し，この実験系は薬剤による DNA 二本鎖切断モデルとして有用と考えられた．また NB2a 細胞より抽出した DNA を Lipofectamine と共に MG6 細胞に導入すると細胞生存率が低下し，DNA 認識の結果として何らかの機序により細胞死を誘導することが推定された．

F．健康危険情報

委託業務成果報告（総括）に記入

G．研究発表

1.　論文発表

なし

2.　学会発表

なし

H．知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3.その他

なし

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AT遺伝子治療の基礎研究
担当責任者　岡戸 晴生 公益財団法人東京都医学総合研究所 脳発達・神経再生研究分野 プロジェクトリーダー

研究要旨
毛細血管拡張性小脳失調症(AT)の原因遺伝子であるATMの遺伝子治療に、ガットレスアデノウイルスとpiggybacの系を利用することを提案した。また、in silico解析からATMのターゲット候補として転写抑制因子RP58が同定され、それを検証するために、RP58の特異抗体を作製した。

A．研究目的

毛細血管拡張性小脳失調症(AT)の遺伝子治療のために、その原因遺伝子ATMの補充を目的に、子宮内エレクトロポレーション法、ウイルスベクターの利用を検討する。それとともに、ATMのターゲットとして、転写抑制因子RP58が機能している可能性を検討する。

B．研究方法

マウスを用いて、ATMを子宮内エレクトロポレーション法、ウイルスベクターを用いて検討する。また、薬物処理によりDNA損傷を惹起し、その後RP58の特異抗体でRP58を回収し、ATMによるリン酸化の有無を検討する。

（倫理面への配慮）
必要としない。

C．研究結果

①ATMの遺伝子治療のための子宮内エレクトロポレーションを用いた予備実験の経験から、ATMの遺伝子治療に、ガットレスアデノウイルスとpiggybacの系を利用することを提案した。

②RP58を免疫沈降するために、RP58のリコンビナント蛋白を作製し、ウサギにて抗体を作製した。作製したリコンビナントを用いて抗体を精製し、RP58欠損マウス脳を用いて、特異性を確認した。

D．考察

RP58にはヒトとマウスで保存されているLSQが1カ所、ヒト、マウス、ゼノパスで保存されているSQ配列が2カ所、TQ配列が2カ所あり、ATMによってリン酸化される可能性がある。

E．結論

RP58がATMのターゲットである可能性を検証するために、RP58のポリクローナル抗体の作製を試み、特異性の高い抗体作製に成功した。

F．健康危険情報

委託業務成果報告（総括）に記入

G．研究発表

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2．学会発表

なし

H．知的財産権の出願・登録状況

1．特許取得
なし
2．実用新案登録
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なし

厚生労働科学研究委託費（難治性疾患等実用化研究事業）
委託業務成果報告（業務項目）

毛細血管拡張性小脳失調症およびDNA損傷修復異常を基盤とする その類縁疾患の病態解明・診断法の確立及び治療法の開発に関する研究

毛細血管拡張性運動失調症由来iPS細胞の基礎解析

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研究要旨
近年、iPS細胞（多能性幹細胞： induced pluripotent stem cell）の研究が著しく発展する中、数多く存在している未だに治療法のない疾患に対する病態解明に向けた研究に期待が高まっている。毛細血管拡張性運動失調症（Ataxia Telangiectasia：AT）もその一つである。そこで、AT患者由来繊維芽細胞よりiPS細胞 (AT-iPS) を作製し、疾患モデルとして病態発症の解明を目指した研究を行った。得られたiPS細胞はいずれも未分化性・多分化能性を有し、放射線感受性も保持されていた。一方で長期培養下においても非常に安定であり、染色体の異常も認められなかった。

A．研究目的

毛細血管拡張性運動失調症はDNA修復にかかわるATM遺伝子の変異で発症する遺伝性小児疾患である。主な症状として、毛細血管拡張、体液性免疫不全、神経変性として小脳失調があり、また、不妊症や放射線感受性が強く癌になりやすい。40万人に1人の割合で発症し、日本における平均寿命は12.1歳と非常に短命である。しかしながら、未だにこの疾患の発症メカニズムや治療法は発見されていない。また、*ATM*遺伝子がどのような分子機構により疾患を発症するのかはまだ十分に分かっておらず、他の遺伝的バックグラウンドによる影響も考えられている。

毛細血管拡張性運動失調症は現時点で根治的な治療法のない、予後不良な難病であり、再生医療を含めた新規治療法の開発が望まれている。将来的な再生医療への道筋をつけるため、本研究では、毛細血管拡張性運動失調症由来細胞よりiPS細胞を樹立し、その細胞生物学的な特性について検討を行う。

B．研究方法

iPS細胞はOCT4、 SOX2、KLF4、c-MYCをレトロウイルスベクターを用いて感染させ樹立した。

AT由来iPS細胞を用いて増殖能と長期培養における核型の安定性について検討した。増殖曲線は無フィーダー細胞培養系で行い、播種してから10日程度計測を行った。長期培養したiPS細胞での核型解析を行った。

AT由来iPS細胞のゲノム変化は、通常のG分染核型解析、コピーナンバー解析（CNV）はIllumina HumanCytoSNP-12 v2.1 DNA Analysis BeadChip Kitを用いて、SNVはAgilent SureSelect Human All Exon V4 +UTRs+lincRNAを用いた次世代シークエンサーを用いた解析により検討した。

AT-iPS細胞に0Gy、0.5Gy、1.0Gy、2.0Gy、3.0Gyの条件でX線照射を行い、その後の細胞数の測定によりX線感受性を検定した。たま核型異常が起こるかどうかについても検討した。

（倫理面への配慮）
本研究では公的細胞バンクにあるAT患者細胞株を用い、国立成育医療研究センター倫理委員会の承認を得て行われた。

C．研究結果

1、毛細血管拡張性運動失調症由来細胞よりiPS細胞を樹立した。

2、樹立に用いたAT患者細胞株は解析した10/20染色体で異常核型を持ち、正常の物は約半数低ウドであった。それにもかかわらず、樹立されたAT由来iPS細胞は13-15継代で2/4細胞株、41継代で4/4細胞株、76-85継代で4/4細胞株で正常核型を有していた。

3、CNV解析ではAT由来iPS細胞4クローンを解析し、親細胞株に比較し、合計12のユニークな構造以上異常を持っていた。

4、SNV解析ではAT由来iPS細胞4クローンを解析し、親細胞株に比較し、合計212のユニークなSNVを持っていた。

5、X線0.5Gy照射条件下でAT由来iPS細胞の放射線感受性が顕著に高いことが示された。またX線照射(0.5Gy、1.0Gy)によりDNA損傷を起こし、突然変異を誘発して核型解析を行った。その結果、正常由来iPS細胞に比し多くの核型異常が検出された。

D．考察

AT 由来 iPS 細胞を樹立することができた。この iPS 細胞は DNA 損傷応答機構の基礎研究に、また AT 患者の再生医療の開発に有用なツールとなることが想定される。

AT 由来は染色体不安定性を持つことが知られ、iPS 細胞に用いた培養線維芽細胞も様々な核型異常を持っていた。しかし驚くべきことに、樹立された iPS 細胞は核型異常が継代とともに減少することが明らかとなった。この現象は核型異常を持つ細胞が、細胞増殖が遅い、生存に不利などの状況があり、より正常に増殖できる、正常核型を持った iPS 細胞が選択的に増殖した結果と推測される。

また CNV 解析、SNV 解析によって樹立された iPS 細胞はいくつかのゲノム変化を抱えていることが明らかとなった。しかし、この数は従来から報告されている、正常細胞由来 iPS 細胞にみられるゲノム変化の数と大きな違いはなく、AT 由来 iPS 細胞がよりゲノム不安定性を示しているとは考え難い。

これら観察結果は AT 由来 iPS 細胞は再生医療を目指す段階で染色体不安定性によって引き起こされる発がんのリスクの上昇があるのではないかと従来から危惧されていたが、逆に iPS 化によってある程度そのリスク回避することができる可能性すら示唆している。

E．結論

AT 由来 iPS 細胞を樹立し、AT の形質を保持しているが、観察されるゲノム不安定性は正常細胞と同程度であることがあきらかとなった。

F．健康危険情報

委託業務成果報告（総括）に記入

G．研究発表

1．論文発表

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2．学会発表

なし

H．知的財産権の出願・登録状況

1．特許取得

なし

2．実用新案登録

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3．その他

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III. 学会等発表実績

学 会 等 発 表 実 績

委託業務題目「毛細血管拡張性小脳失調症およびDNA損傷修復異常を基盤とする
その類縁疾患の病態解明・診断法の確立及び治療法の開発に関する研究」

機関名　国立大学法人東京医科歯科大学・国立成育医療研究センター・鳥取大学・公益財団法人
東京都医学総合研究所

1. 学会等における口頭・ポスター発表

発表した成果 （発表題目、口頭・ポスター発表の別）	発表者氏名	発表した所 （学会等名）	発表した時期	国内・外の別
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2.学会誌・雑誌等における論文掲載

掲載表した論文（発表題目）	発表者氏名	発表した場所 (学会誌・雑誌等名)	発表した時期	国内・外の別
Whole-exome sequence analysis of ataxia telangiectasia-like phenotype.	Hasegawa S, Imai K, Yoshida K, Okuno Y, Muramatsu H, Shiraishi Y, Chiba K, Tanaka H, Miyano S, Kojima S, Ogawa S, Morio T, Mizutani S, Takagi M	J Neurol Sci	2014	国外

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VII 先天性代謝異常. DNA 修復障害 色素性乾皮症	林雅晴	別冊日本臨床新領域別症候群シリーズ No.28 神経症候群（第 2 版）III	2014	国内

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Whole-exome sequence analysis of ataxia telangiectasia-like phenotype

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ABSTRACT

A number of diseases exhibit neurodegeneration with/without additional symptoms such as immunodeficiency, increased cancer risk, and microcephalus. Ataxia telangiectasia and Nijmegen breakage syndrome, for example, develop as a result of mutations in genes involved in the DNA damage response. However, such diseases can be difficult to diagnose as they are only rarely encountered by physicians. To overcome this challenge, nine patients with symptoms that resemble those of ataxia telangiectasia, including neurodegeneration, hypogammaglobulinemia, telangiectasia, and/or elevated serum α-fetoprotein, were subjected to whole-exome sequencing (WES) to identify the causative mutations. Molecular diagnosis was achieved in two patients: one displayed CD40 ligand (CD40LG) deficiency, while a second showed a homozygous *SIL1* mutation, which has been linked to Marinesco-Sjögren syndrome (MSS). Typical features of CD40LG deficiency and MSS are distinct from the symptoms usually seen in ataxia telangiectasia. These dissociations between phenotype and genotype make it difficult to achieve molecular diagnosis of orphan diseases. Whole-exome sequencing analyses will assist in the molecular diagnosis of such cases and allow the identification of genotypes that would not be expected from the phenotype.

1. Introduction

Neurodegenerative disease is characterized by progressive nervous system dysfunction. Primary immunodeficiency is a disorder of immune regulation. Occasionally, progressive nervous system dysfunction and primary immunodeficiency can occur together within single disorders, and the genes responsible for such conditions have been identified. Ataxia telangiectasia (A-T) is one such disorder involving progressive cerebellar ataxia and immunodeficiency, as well as conjunctival telangiectasia. The gene responsible, *ATM*, plays a central role in the DNA damage response (DDR) [1]. Mutations of *NBS1* and *Mre11*, genes also involved in DDR network, can give rise to phenotypically A-T-like patients, such as those with Nijmegen breakage syndrome (NBS) and A-T-like disease (ATLD). Not only NBS and ATLD, but also a number of diseases also feature both neurological symptoms and immunodeficiency. Gatti et al. proposed a disease category named XCIND (X-ray irradiation sensitivity, Cancer susceptibility, Immunodeficiency, Neurological abnormality, Double strand DNA breakage) syndrome [2], in which failure of the DDR pathway results in genome instability and an increased risk of cancer. A number of human genetic disorders are characterized by a defective DDR pathway and feature neurodegeneration, which suggests that maintaining genome stability is also important for preserving post-mitotic neurons [3].

Pediatric neurodevelopmental disorders comprise various diseases with multi-system symptoms. Some of the features characteristic of these diseases appear only in later years, and some patients only manifest non-specific symptoms, leading to a delayed diagnosis. In certain cases, different phenotypes can arise from the same genotype. For example, mutation of SETX, which is involved in DDR, can give rise to three distinct types of disease: ataxia-ocular apraxia-2 (AOA2), autosomal recessive spinocerebellar ataxia (SCA) 1, and juvenile amyotrophic lateral sclerosis (ALS) 4. In the present study, nine patients with clinical features of neurodegeneration, hypogammaglobulinemia and/or telangiectasia were analyzed by whole-exome sequencing (WES). The

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results reveal that one patient had CD40LG deficiency and that another patient had Marinesco–Sjögren syndrome (MSS).

2. Materials and methods

2.1. Patient samples

Patients with neurological symptoms resembling an A-T-like phenotype, comprised mainly of cerebellar ataxia plus hypogammaglobulinemia, telangiectasia and/or elevated serum alpha-fetoprotein (AFP), were recruited. ATM western blotting was performed with these patients to exclude A-T. Patients with normal ATM levels were subjected to WES.

Patients provided informed written consent, and the experimental design was approved by the ethics committee at Tokyo Medical and Dental University (No. 103).

2.2. Whole-exome sequencing analysis (WES)

WES analysis was performed as previously described [4]. Briefly, genomic DNA was fragmented, and exonic sequences were enriched using SureSelect Target Enrichment with the SureSelect Human All Exon 38 Mb kit (Agilent). The captured fragments were purified and sequenced on an Illumina Hiseq2000 platform using paired-end reads. Bioinformatic analysis was performed using an in-house algorithm based on published tools. Identified single nucleotide variants (SNVs) were filtered using dbSNP version 131 and 132, the 1000 Genomes database, an in-house SNP database, and the Human Genetic Variation Database (HGVD) (http://www.genome.med.kyoto-u.ac.jp/SnpDB/).

2.3. Genome sequencing

The mutations identified by WES were confirmed by direct sequencing. Genomic DNA from peripheral blood mononuclear cells was obtained using the QIAamp DNA Mini kit (Qiagen). Exons of the respective genes were amplified by PCR. Nucleotide sequencing was performed by cycle sequencing using ABI BigDye Terminator chemistry (Applied Biosystems) followed by capillary electrophoresis on an ABI 3100 automated sequencer.

2.4. CD40LG expression analysis

CD40LG expression was measured by flow cytometry using activated T-cells [5]. Cells were treated with phosphate-buffered saline (PBS) or PMA/ionomycin, and incubated for 4 h. CD40LG expression in T-cell gates was monitored by phycoerythrin (PE)-conjugated anti-human CD40LG antibody (Beckman Coulter), combined with the T-cell marker CD3 (PC 5-conjugated anti-CD3 antibody: Beckman Coulter). Flow cytometric analysis was performed using FACS Caliber with the CellQuest program (Becton-Dickinson).

2.5. Western blotting

Cells were lysed in RIPA buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, phosphatase, and a protease inhibitor cocktail). Samples were resolved on SDS-polyacrylamide gels. The gels were transferred to nitrocellulose membranes (Millipore) and blocked with 5% nonfat milk. The membranes were incubated with the appropriate anti-SIL1 (Abcam), anti-β-actin (Sigma), anti-eIF2α, and anti-phospho-eIF2α (Cell Signaling) antibodies. Primary antibodies were detected by binding horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibody with an ECL kit (GE Healthcare).

3. Results

Patients presenting with more than two features of ataxia or other neurological degeneration symptoms and hypogammaglobulinemia, telangiectasia and/or elevated serum AFP were examined in this study (Table 1). Most of the causative ATM mutations in typical A-T patients are truncating, and ATM protein is therefore absent in these patients [6]. Western blot analysis confirmed ATM protein expression in all of the subjects in this study (data not shown). Furthermore, WES analysis failed to identify an ATM mutation, and thus A-T was ruled out in these subjects. We speculated that these patients had XCIND syndrome. WES revealed 238 non-synonymous SNV, frameshift, or splice site mutations. Although SNVs located within DDR-related genes were identified (Supplementary Table 1), no mutations were seen in the genes responsible for XCIND (data not shown). Intriguingly, a hemizygous *CD40LG* mutation and a homozygous *SIL1* mutation were identified in patients 1 and 5, respectively.

3.1. Patient 1

Patient 1 is a 21-year-old male and a child of non-consanguineous healthy Japanese parents. He has no familial history of any immunological disorders, while his grandfather suffered from Parkinson's disease. He showed normal motor development during infancy, but failed to thrive. Due to recurrent otitis media, he was presumed to have a primary immune deficiency of unknown origin, and began intravenous immunoglobulin treatment every 2 weeks at 12 months of age. In childhood, he manifested clumsiness, and an asymmetrical arm motion was identified during walking at 16 years of age. At 20 years, he developed involuntary movements that were induced by eating and that deteriorated over a few days. He was admitted with involuntary movements of the extremities; he was alert and conscious. His intelligence quotient was 58. He had mild dysarthria. Neurologic examinations

Table 1
Clinical features of patients.

patient	Sex	Age (years)	Immunodeficiency	Neurological symptoms	Telangiectasia	Serum AFP
1	M	21	Recurrent otitis media, low IgG, and elevated IgM	Choreoathetosis, dysarthria, hyperreflexia, psychomotor retardation, and cerebral cortex atrophy	−	NE
2	F	5	–	Ataxia and cerebellar atrophy	+	Normal
3	F	21	Low IgA and normal IgG and IgM	Nystagmus, dysarthria, hypotonus, myoclonus, ataxia, hyporeflexia, and cerebellar atrophy	+	Normal
4	F	2	Low IgG	Psychomotor retardation and regression	−	Elevated
5	M	1	Low IgG and IgG_2 subclass and normal IgM	Gross motor developmental delay, nystagmus, and cerebellar atrophy	−	Normal
6	F	1	–	Myoclonus, choreoathetosis, psychomotor retardation, and epilepsy	−	Elevated
7	F	11	Aspergillosis and low IgA, IgG and IgM	Psychomotor retardation and epilepsy	−	Normal
8	F	7	Oral candidiasis, *Pneumocystis carinii* pneumonia, and low IgA, IgG and IgM	Psychomotor retardation	−	NE
9	F	5	Low IgM and reduced B cell number	Ataxia, mental retardation, and microcephaly	+	NE

NE: not examined.

showed involuntary movements of the limbs, face, and trunk. This non-rhythmic involuntary movement appeared dominant in the right arm, and was induced by motor action. This movement did not occur during sleep. The deep tendon reflex was markedly hyperactive in the bilateral ankle clonus, but there was no pathological reflex.

Laboratory data showed normal complete blood cell count with no acanthocytes. Electrolyte and hepato-renal functions were within normal limits, and euthyroidism was confirmed. Serological examination showed low serum levels of IgG (687 mg/dl) and elevated serum IgM (462 mg/dl). Serum ceruloplasmin levels were normal, and the autoantibodies, anti-streptolysin O and anti-streptokinase antibodies, were negative. The cerebrospinal fluid cell count was 21 cells/mm^3 and comprised 100% mononuclear cells. Protein and glucose concentrations were 21 mg/dl and 54 mg/dl, respectively. No pathogens indicating infection were identified. There was no calcification at the time of brain computed tomography (CT). Brain magnetic resonance imaging (MRI) revealed cerebral cortex atrophy without abnormal signal intensity and atrophy of the striatum (Fig. 1). Electroencephalography demonstrated generalized intermittent slow waves and focal sharp waves over the bilateral occipital region. He had no clinical seizures. Within 6 months, he was unable to walk or sit unaided, as a consequence of choreoathetosis.

WES identified a *CD40LG* mutation in this patient, which was validated by Sanger sequencing (Fig. 2A). A functional assay for CD40LG expression confirmed that the mutation impaired CD40LG functioning (Fig. 2B).

3.2. Patient 5

Patient 5 is a 14-month-old male and a child of non-consanguineous healthy Japanese parents. He has no familial history of any immunological disorder. His sister (6 years old) and brother (3 years old) have had several febrile seizures. He was born uneventfully, and showed mild developmental delay. He was able to hold his head up at 10 months of age, rolled over at 12 months, and has yet to sit up and crawl. He showed nystagmus at 12 months and his brain MRI revealed cerebellar atrophy (Fig. 3). Serological examination showed relatively low serum levels of IgG (490 mg/dl), IgG_2 subclass (18%), and IgA (15 mg/dl) and normal serum levels of IgM (68 mg/dl). Opportunistic infections or recurrent

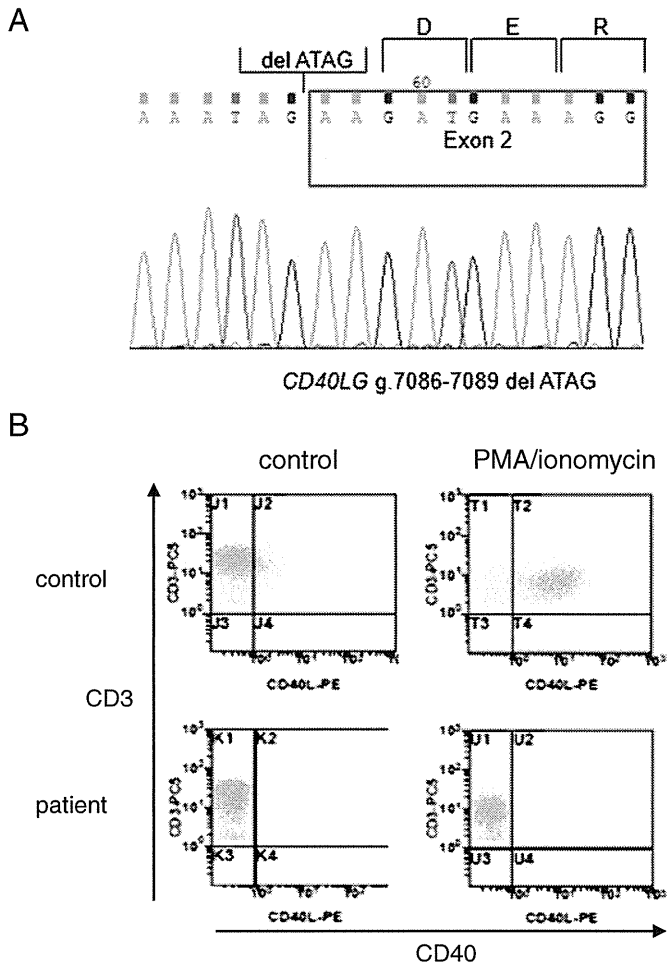

Fig. 2. A, Sequence electropherogram of *CD40LG*. A hemizygous frameshift mutation was identified. B, PMA/ionomycin treatment induced CD40LG expression. 88.68% of $CD3^+CD8^-$ cells were positive for CD40LG in healthy controls. On the other hand, only 0.82% were positive in patient-derived activated T-cells.

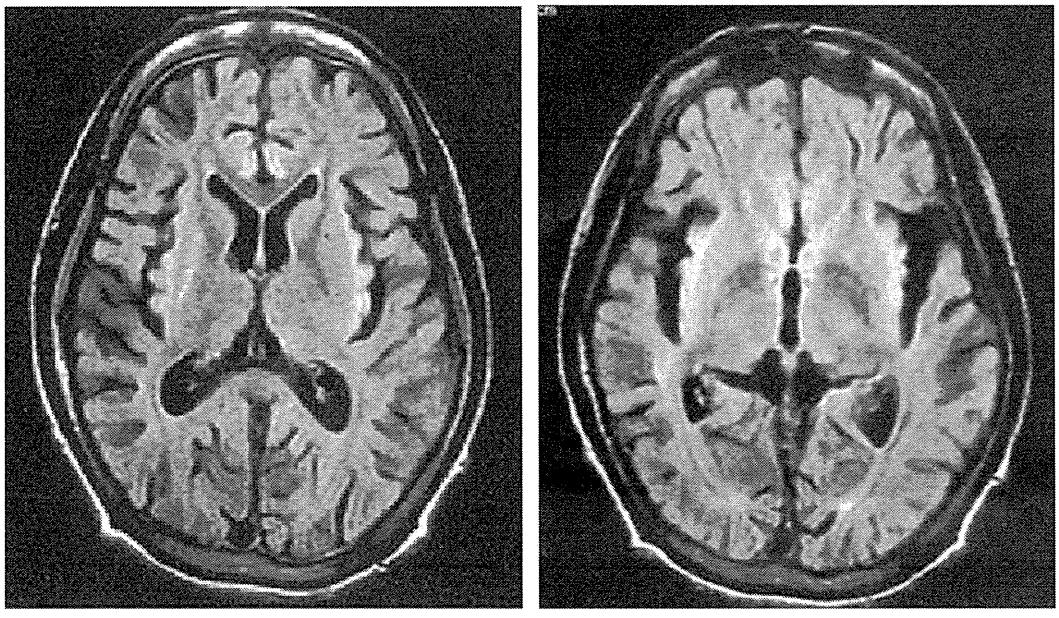

Fig. 1. Fluid attenuated inversion recovery axial images of patient 1, demonstrating cerebral cortical atrophy.

infections have not been observed in this patient. Cerebrospinal fluid analysis was normal, as were levels of pyruvate and lactate.

Although hypoglobulinemia has not been previously reported in MSS, WES identified a homozygous frameshift mutation in *SIL1*, c.936_937ins G, which was validated by Sanger sequencing (Fig. 4A). SIL1 expression was markedly decreased in a patient-derived EB virus-transformed lymphoblastoid cell line (Fig. 4B). SIL1 functions in combination with binding immunoglobulin protein (BiP) to ensure proper folding of proteins in the endoplasmic reticulum (ER) [7]. Accumulation of misfolded proteins suppresses de novo protein synthesis via translation inhibition. eIF2α is involved in this process. ER stress induces phosphorylation of eIF2α on serine 51 [8]. The patient-derived EB virus-transformed lymphoblastoid cell line exhibited increased phosphorylation of eIF2α, suggesting increased ER stress (Fig. 4C).

4. Discussion

A splice acceptor mutation of *CD40LG* was identified in patient 1, and CD40LG expression was lower in this patient (Fig. 2B). This *CD40LG* mutation has previously been reported in hyper IgM syndrome (HIGM) [9], but neurodegeneration is not a common feature of HIGM disorder. We speculated that mutations in other genes were probably the cause of the atypical symptoms seen in our patient. A heterozygous non-frameshift deletion (c.1242_1244 del) in *POLG* (DNA polymerase subunit γ gene), which has not been described before, was identified as a candidate. *POLG* is essential for mitochondrial DNA (mtDNA) replication. Mutations in *POLG* have been identified in various diseases such as progressive external ophthalmoplegia (PEO), Alpers syndrome and other infantile hepatocerebral syndromes, ataxia-neuropathy syndromes, Charcot–Marie–Tooth disease, and idiopathic parkinsonism [10]. These diseases are characterized by mtDNA depletion in symptomatic tissues. Although a *POLG* in-frame nucleotide deletion was identified in patient 1, mtDNA levels were the same as in the other patients, suggesting that this in-frame nucleotide deletion does not interfere with POLG function (data not shown). This result suggests that the neurological symptoms in this patient are very unlikely to be modified by mutation of *POLG*.

Patients with CD40LG deficiency are susceptible to central nervous system (CNS) infections. The incidence of CNS infection or progressive neurodegeneration is 12–16% among patients with CD40LG deficiency [11]. Dysfunction of CD40–CD40LG dependent T-cell immunity attenuates $CD8^+$ T-cell trafficking to the CNS in mice, and this led to elevated West Nile virus titers and resulted in neurodegeneration [12]. Immunodeficiency caused by *CD40LG* deficiency can increase susceptibility to CNS infection, or allow persistent CNS infection, and this can explain the neurodegeneration observed in patients. Bishu et al. reported five patients exhibiting neurological symptoms, including ataxia, in a cohort of 31 patients. Although an infectious etiology is the most plausible explanation, no pathogens were identified in four of the patients with neurological symptoms. This group proposed that the lack of proof of infection necessitates consideration of other etiologies [13], which may also be the case with our patient. There are several interesting previous reports suggesting a relationship between CD40–CD40LG function and neuronal function. CD40LG is critical for protection from demyelinating disease and for development of spontaneous remyelination in a mouse model of multiple sclerosis produced by infection with Theiler's murine encephalomyelitis virus [14]. CD40–CD40LG interaction enables astrocytosis and microgliosis in response to amyloid-beta peptide [15]. Although CD40LG deficiency does not lead directly to neurodegeneration, CD40 is expressed and functional on mouse and human neurons. CD40-deficient mice display neuronal dysfunction, aberrant neuronal morphology, and associated gross brain abnormalities [16]. These findings suggest that an infection-based hypothesis is not the only possibility; changes in neuronal function could also explain the neurodegeneration seen as a result of CD40LG deficiency.

Mutation in *SIL1* causes MSS [17], an autosomal recessive disorder that is principally associated with cerebellar ataxia, bilateral cataracts, myopathy and mental retardation. The mutation seen in patient 5 in

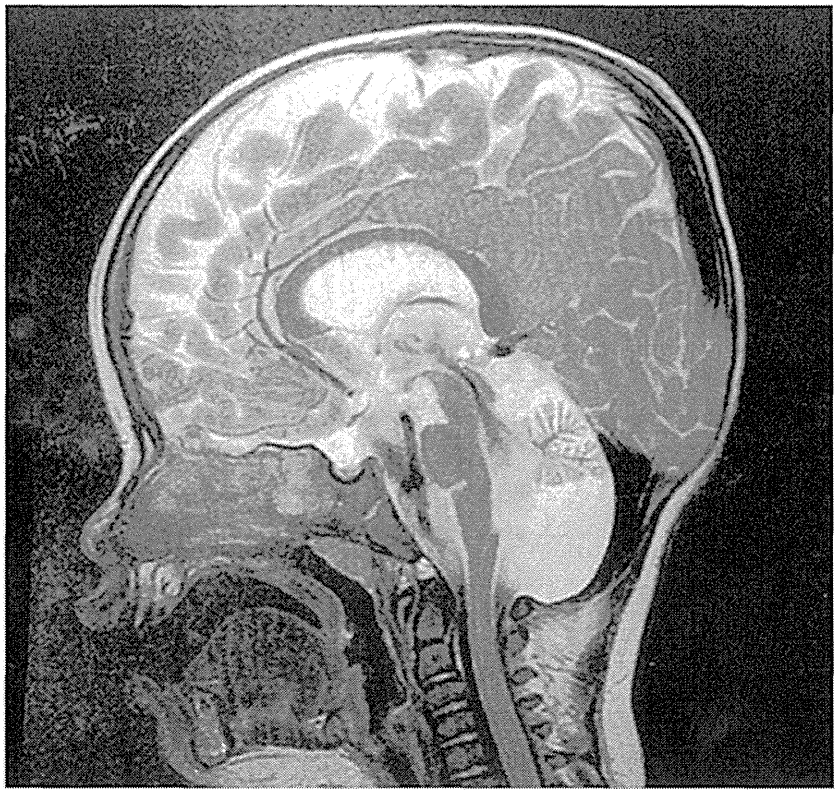

Fig. 3. Sagittal midline T2-weighted MR image of patient 5, demonstrating cerebellar atrophy of the vermis.

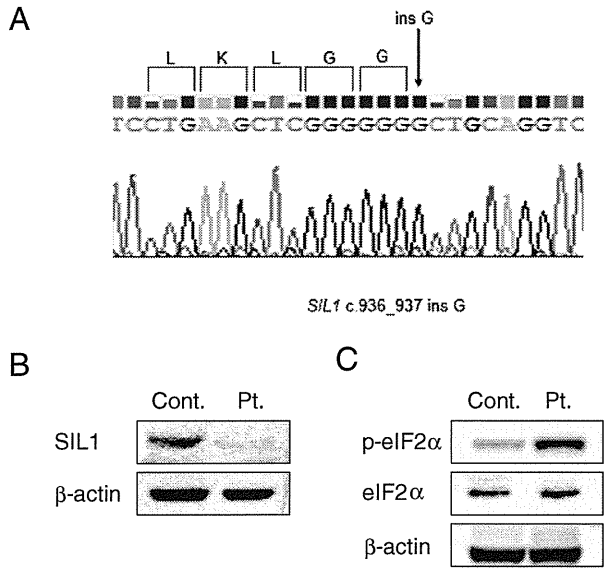

Fig. 4. A, Sequence electropherogram of *SIL1*. A homozygous frameshift mutation was identified. B, Western blotting analysis of SIL1 expression. C, eIF2α and phosphorylated eIF2α (p-eIF2α) levels. Cont: protein extract from EBV-transformed lymphoblastoid cell line derived from a healthy volunteer. Pt: protein extract from EBV-transformed lymphoblastoid cell line derived from patient 5.

this study was also reported in three unrelated Japanese patients with MSS [18]. Patient 5 did not demonstrate cataracts at 1 year of age, although cataracts are known to appear later in life [17]. Although hypogammaglobulinemia has not been previously described in MSS, patient 5 exhibited remarkably reduced levels of serum IgG_2 with a moderate decrease in total IgG and IgA levels. SIL1 functions in combination with BiP to ensure proper folding of proteins in the ER [7]. Assembly of the immunoglobulin heavy chain and light chain is performed in the ER in association with the ER chaperone, BiP [19]. In this study, we have not examined if hypogammaglobulinemia is a common feature of MSS or a specific feature of this case 5 patient. A further study of cases is therefore needed to reveal whether hypogammaglobulinemia is a common feature in MSS.

Several non-synonymous SNV, frameshift, or splice site mutations in DDR-associated genes were identified (Supplementary Table 1). Further studies are required to evaluate the functional effects of these SNVs.

Molecular genotypes are occasionally obscured by exogenous or endogenous factors, infections, treatments or the disease process. In addition, these factors can sometimes hinder the identification of causative mutations. Recent advances in genome analysis technology allow the identification of such mutations in subjects with indistinguishable phenotypes, and this can lead to an unpredictable molecular diagnosis for these patients. However, many of the well-known hereditary ataxias, including SCA, dentatorubral-pallidoluysian atrophy (DRPLA), and Friedreich's ataxia, are caused by tri-nucleotide expansions. In these cases, WES analysis may fail to identify the causative mutation. In fact, molecular diagnoses for the other seven patients in the present study remain elusive. A combination of copy number and WES analyses of family members may increase the sensitivity and accuracy of genetic diagnosis. WES analyses will help to diagnose cases in which symptoms have been altered by infections or concomitant multiple gene alterations.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jns.2014.02.033.

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Case report

Myoclonic axial jerks for diagnosing atypical evolution of ataxia telangiectasia

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Abstract

Background: Ataxia telangiectasia (A-T) is a common inherited cause of early childhood-onset ataxia, distinguished by progressive cerebellum malfunction, capillary vessel extension, and immunodeficiency. The diagnosis of A-T is sometimes difficult to establish in patients with atypical clinical evolution.

Case report: We experienced a pediatric 12-years-old female patient, who was finally diagnosed with classic A-T, demonstrating progressive dystonic-myoclonic axial jerks with ataxia as a predominant clinical feature. Oculocutaneous telangiectasias and immune status were unremarkable. Her myoclonic jerks were spontaneous or stimulus-sensitive, and partially ameliorated by levodopa treatment, but the ataxia was slowly progressive. A laboratory examination showed moderate atrophy of the vermis and cerebellum on brain magnetic resonance imaging, elevated serum alpha fetoprotein (AFP) levels, and total absence of A-T mutated (ATM) protein activity. We subsequently confirmed compound heterozygous truncating mutations of the *ATM* gene in this patient.

Conclusion: Our findings highlight the importance of recognizing dystonic-myoclonic jerks as one of the extrapyramidal signs of classic A-T. Measurement of AFP levels should be considered in patients with unexplained myoclonic jerk movements with ataxia in whom definitive diagnoses are not identified. Physicians should be aware that there are cases where typical findings of A-T may not be fulfilled.

Keywords: Ataxia telangiectasia; Myoclonic jerk; AFP; Extrapyramidal sign; *ATM* gene

1. Introduction

Ataxia telangiectasia (A-T) is an autosomal recessive disorder characterized by progressive cerebellar ataxia, oculocutaneous telangiectasia, chromosomal instability, immunodeficiency, radiosensitivity, and susceptibility to cancer [1]. The responsible A-T mutated (*ATM*) gene on chromosome 11q22-23 encodes the ATM protein,

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playing an important role in the cellular response to DNA damage [2].

Although A-T is a common inherited cause of early childhood-onset ataxia in most countries, making an early and definitive diagnosis of A-T can sometimes be challenging for clinicians. Some of the hallmarks of classic A-T, such as ataxia, oculocutaneous telangiectasia, and immunodeficiency, can appear only later in life or even remain absent in variant A-T [3]. Oculocutaneous telangiectasia usually appears between the ages of 2 and 4 years, but it may be occur as late as 14 years [1,4].

We present here a pediatric patient with classic A-T, who had progressive dystonic-myoclonic axial jerks with ataxia as a predominant clinical feature. There were no signs of oculocutaneous telangiectasias or of immunodeficiency. The clinical manifestation of extrapyramidal features, which are reminiscent of a series of spinal cerebellar ataxias or miscellaneous diseases of the basal ganglia, has clinical diagnostic value for the atypical evolution of classic A-T.

2. Case report

The patient, a 12-years-old girl, was delivered by vacuum extraction with mild asphyxia and jaundice. She developed normally until the age of 5 years when she developed sluggish movements, difficulty in hand writing, and mild dysarthria. An initial examination at 7 years old demonstrated mild spastic paraplegia with increased reflexes and tone in the lower limbs, ataxic and mild equinus gait, and developmental delay with skills equivalent to those normally observed at 4 years of age. Brain magnetic resonance imaging (MRI) showed moderate atrophy of the vermis and cerebellum (Fig. 1a and b). There were no prominent telangiectasias or oculomotor apraxia. There was no history of recurrent sinopulmonary infections. Her uncle has spastic paraplegia with relationship to the patient's phenotype unknown. Her parents and brothers are healthy. A tentative diagnosis of hereditary spastic paraplegia with mental retardation was made. During the follow-up period, she slowly developed progressive myoclonic jerks at 8 years. These movements were accompanied by spontaneous axial dystonic posture, which sometimes resulted in chronic myalgic pain. This extrapyramidal symptom was partially ameliorated by levodopa treatment with 100 mg/day (5 mg/kg/day), enabled her to perform daily activity smoother. However, her ataxia was progressive and led to an inability to ambulate without assistance from 10 years of age. Written informed consent and permission of following genetic testing and photo/video recording were obtained from the patient's parents. We obtained informed assent from the patient.

At an evaluation at 11 years old, her weight was 26 kg (−1.4 SD) and her height was 136 cm (−1.1 SD). She had no nystagmus, but had jerky pursuits and undershoot saccades. She had slurred speech with lingual dysarthria. She demonstrated spontaneous or stimulus-sensitive

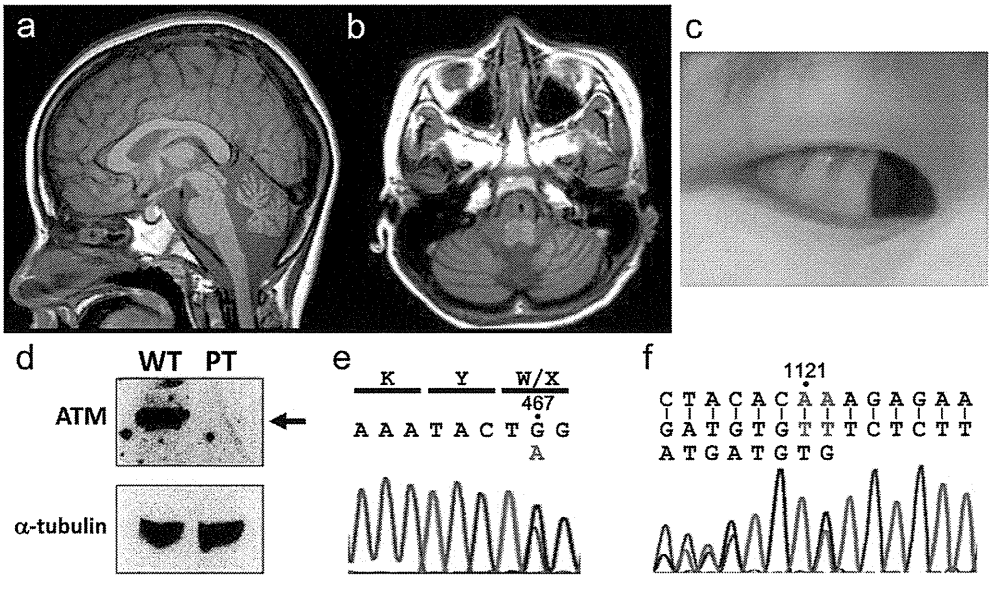

Fig. 1. Characteristics of the patient. (a, b) Magnetic resonance imaging (MRI) of the patient. Sagittal section (a) and axial section (b) of T1-weighted MRI brain images are shown. The patient showed moderate atrophy of the vermis and cerebellar hemispheres. (c) External sclera of the right eye. Her oculocutaneous telangiectasias were unremarkable. (d) Western blotting analysis of ATM protein. ATM was not detected in cell lysates. Alpha-tubulin served as a loading control. WT: wild type, PT: patient, ATM: ATM protein. (e) Electropherogram of ATM exon 5 obtained by sequencing. The red letter indicates the point mutation leading to a heterozygous nonsense mutation, named c.G467A (p.W156X). (f) Electropherogram of ATM exon 9 obtained by reverse sequencing. Red letters indicate the deleted base pairs leading to a heterozygous frameshift mutation, named c.1121_1122delAA (p.E376IfsX2). Forward sequencing results were indecipherable. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

myoclonic jerks with cervical and axial predominance, enhanced by mental or physical activation (Supplementary Movie). She had pronounced axial extension dystonia of her trunk associated with myoclonic head jerks, which were assumed to be dystonic-myoclonic on electromyogram (EMG; Supplementary Figure). Dysmetria and dysdiadochokinesis were also noted. Patellar tendon reflexes were brisk bilaterally, but we did not observe pathological reflexes. She could walk with assistance, with a wide-based and unsteady gait. Scleral telangiectasias were unremarkable (Fig. 1c). Routine laboratory and neurometabolic investigations showed normal results. Abnormal laboratory tests included elevated serum AFP (468.2 ng/ml; normal range 0–20 ng/ml) levels, and low levels of IgE (<1 mg/dl). Immunoglobulin levels (IgG, IgA, and IgM) were within the normal range (data not shown). Brain MRI showed no remarkable changes compared with that taken at 7 years of age.

The clinical phenotype of our patient and the raised serum AFP levels were reminiscent of atypical A-T or possibly ataxia with oculomotor apraxia type 2 (AOA2). Western blot analysis of cultured lymphoblast cell lysates, according to a method described elsewhere [5], showed that there was no ATM protein (Fig. 1d). Subsequent mutation analysis of the *ATM* gene showed that she was compound heterozygous for a nonsense, truncating mutation, which presumably resulted in complete loss of ATM protein (Fig. 1e and f). Independent mutation screening of *APTX* and *SETX* genes showed no significant mutations in this patient. After making the definitive diagnosis, she has been enrolled in a very-low-dose betamethasone trial for A-T, to assess its effectiveness in improving neurological signs.

3. Discussion

Progressive dystonic myoclonic jerks together with cerebellar ataxia suggested the diagnosis A-T in the present case. Extrapyramidal symptoms of myoclonic jerks and dystonia have been described in A-T [6]. Early-onset dystonia with cervical and brachial onset, and prominent cranial involvement have been recognized as major features of variant A-T [7]. Although the neuropathology underlying these movement disorders is not fully understood, several human and mice studies have indicated that biochemical dysfunction of dopaminergic nigrostriatal neurons is a contributory factor. Atm-deficient mice exhibited severe degeneration of dopaminergic nigro-striatal neurons, and their terminals in the striatum [8]. In those mice, a reduction in dopaminergic neurons was evident in the ventral tegmental area. A single photon emission computed tomography study of cerebral dopamine-D2-receptor binding showed a decreased tracer uptake in striatal regions in a patient with A-T [9]. These facts are consistent with the partial improvement of extrapyramidal symptoms with levodopa observed in our patient.

The clinical diagnosis of A-T is sometimes difficult to establish in patients who do not present with the characteristic classic phenotype. The predominance of dystonic-myoclonic jerks and ataxia, and an absence of telangiectasia and immune deficiency made diagnosis difficult in our patient. The phenotypic spectrum in A-T from severe classical child-onset AT to adult-onset variant A-T generally depends on the presence of ATM protein and kinase activity [3].The present case, which we eventually assumed to be classic A-T, included compound heterozygous truncating mutations resulting in total absence of ATM kinase activity, which is in line with previous reports [1,3]. However, it is unlikely that the absence of hallmark features of classic A-T can be attributed solely to ATM kinase activity. The reason for this could be partly explained by the fact that A-T shares many features with mitochondrial disorders. A number of reports have shown that continuous oxidative stress in A-T cells is attributed to mitochondrial dysfunction, supporting a model in which ATM plays a critical role in modulating mitochondrial homeostasis [10]. The phenotypic heterogeneity within classic A-T with null ATM kinase levels may be partly due to different levels of mutant mitochondrial DNA heteroplasmy in different tissues.

The findings in our patient highlight the importance of recognizing dystonic-myoclonic jerks as one of the extrapyramidal signs of classic A-T. Measurement of AFP levels should be considered in all patients with familial or sporadic unexplained myoclonic jerk movements with ataxia in whom definitive diagnoses are not identified. The finding of elevated serum AFP levels strongly indicates that the diagnosis was correct. Early recognition of A-T is important for avoiding diagnostic radiation exposure, preventing the initiation of cancer, and providing appropriate genetic counseling.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.braindev.2014.06.001.

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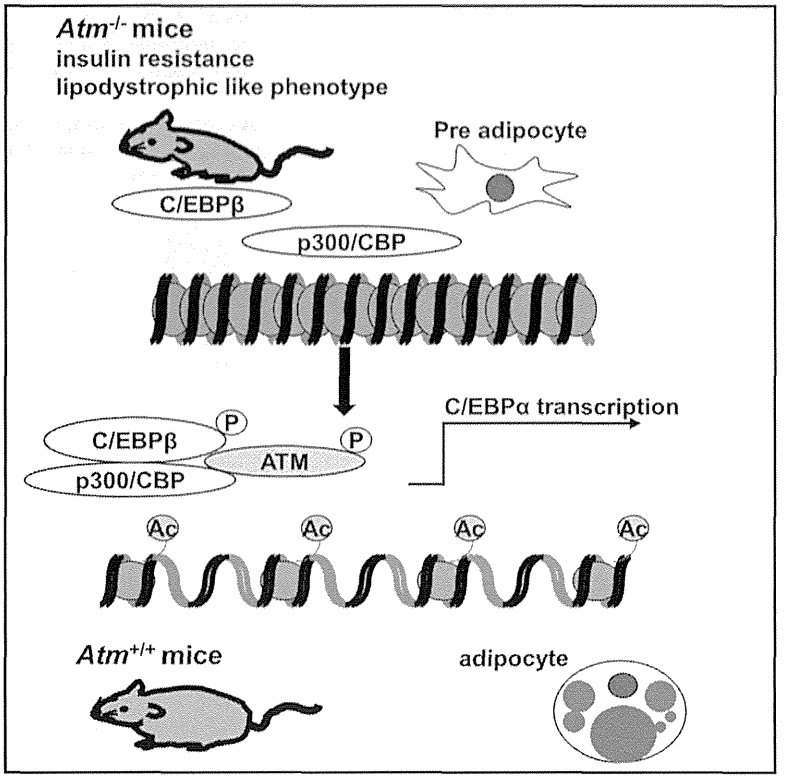
Article

Cell Reports

ATM Regulates Adipocyte Differentiation and Contributes to Glucose Homeostasis

Graphical Abstract



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In Brief

Ataxia telangiectasia (A-T) patients develop diabetes mellitus. ATM, linked to A-T, is known to be involved in the DNA damage checkpoint. Takagi et al. reveal that ATM regulates adipocyte differentiation and attenuates differentiation of adipocytes in A-T patients, contributing to glucose metabolism in vivo.

Highlights

- ATM, linked to ataxia telangiectasia, regulates adipocyte differentiation
- The adipocyte differentiation defect in A-T contributes to type 2 diabetes
- Transcriptional activation of C/EBPα and PPARγ depends on ATM
- Binding of ATM to C/EBPβ and p300 induces transcriptional regulation of C/EBPα

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