をチェックしておくことが重要であると考えられる。

好酸球性食道炎の標準的な治療はフルチカゾンなどの局所作用型の吸入ステロイドを口腔内に噴霧してそれを30分ぐらいかけてゆっくりと嚥下し食道粘膜に接触させる治療である。フルチカゾンを用いる場合には1日2回に分けて投薬することが多く行われている。局所作用ステロイドが有効でない場合にはプレドニゾロンの経口投薬がおこなわれることもある。

おわりに

好酸球性食道炎は欧米においては急速にその有病率が増加している疾患であり日本においても増加していると考えられる。またヘリコバクター・ピロリの感染者の減少に伴って今後も増加し続ける可能性もある。GERDと好酸球性食道炎は症状に類似点があるためGERDと考えてPPIを投薬したにもかかわらず有効な治療効果が得られない場合には好酸球性食道炎を念頭に置いた内視鏡検査と生検診断を行い局所作用ステロイドを用いた治療を行うことが重要であると考えられる。

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症 例

クローン病に合併した好酸球性食道炎の1例

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要 旨

症例は29歳男性. 2001年に小腸型クローン病と診断されたが, 治療自己中断. 2012年にクローン病再燃で入院した際の上部消化管内視鏡で中部食道中心に白斑の付着と発赤した縦走溝所見を, 生検で扁平上皮層内に高倍率視野1視野内に20個以上の好酸球浸潤を認めた. 無症状のため, 好酸球性食道炎疑診例とし, メサラジンと成分栄養食でクローン病治療を開始し寛解導入できた. 約4カ月後に胸部つかえ感, 胸やけ症状が出現し, 再検した上部消化管内視鏡ならびに生検所見とあわせて, 好酸球性食道炎と確定診断した. フルチカゾンプロピオン酸エステルの嚥下療法を開始し, 症状は改善した. 上記2疾患の合併は稀であり報告した.

Key words 好酸球性食道炎／クローン病／フルチカゾンプロピオン酸エステル嚥下療法

I 緒 言

好酸球性食道炎 (eosinophilic esophagitis ; EoE) は食道粘膜上皮層内のみに好酸球が多数浸潤し慢性炎症を起こす結果, 粘膜障害, 運動異常, 知覚障害がおこる病態である[1]. 一方, クローン病 (Crohn's disease ; CD) は非連続性に分布する全層性肉芽腫性炎症や瘻孔を特徴とする消化管の慢性炎症性疾患である. この2つの慢性炎症性疾患の合併例は本邦ではまだない. 今回, われわれはCDに合併したEoEの1例を経験し, 内視鏡的経過も含め, 報告する.

II 症 例

症例：29歳, 男性.

主訴：下痢, 粘血便, 腹痛.

既往歴：2001年に小腸型CDと診断された. 気管支喘息やアレルギー歴はなし.

生活歴：喫煙：4～5本/日, 飲酒：ビール

Table 1 入院時検査所見.

Biochemistry		CBC		
TP	8.4 g/dl	WBC	10,000	/μl
Alb	5.0 g/dl	Neutro	83.8	%
T-Bil	1.1 mg/dl	Mono	2.4	%
ALP	195 U/L	Eosino	0.2	%
AST	19 U/L	Baso	0.1	%
ALT	26 U/L	Lymphocyte	13.5	%
LDH	202 U/L	RBC	540	×10⁴/μl
γGTP	22 U/L	Hb	16.0	g/dl
ChE	316 U/L	Ht	45.2	%
UN	7.0 mg/dl	Plt	28.7	×10⁴/μl
Cre	0.67 mg/dl			
Amy	53 U/L	**ESR**		
T-Cho	195 mg/dl	60min	4	mm
TG	42 mg/dl	120min	14	mm
FBS	108 mg/dl			
Na	138.7 mmol/l	**CEA**	<0.5	ng/ml
K	3.4 mmol/l	*H.pylori*-Ab	(−)	
Cl	100.7 mmol/l	IgE	86	IU/ml
CRP	0.19 mg/dl			

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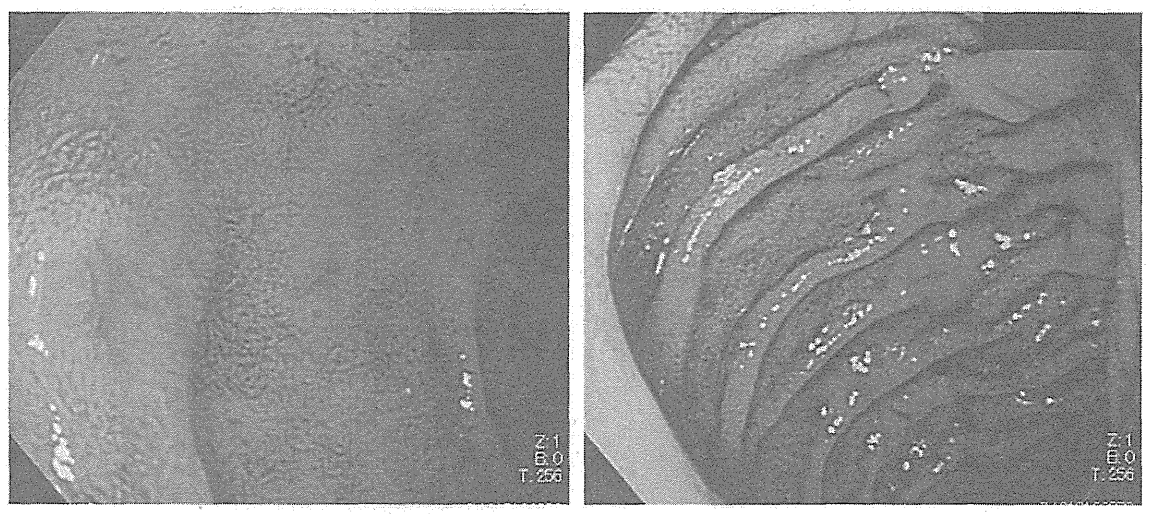


a　　b

Figure 1　2001年CD診断時上部消化管内視鏡所見：上十二指腸角（a）から下行部（b）にかけて縦走するびらんを認めた．

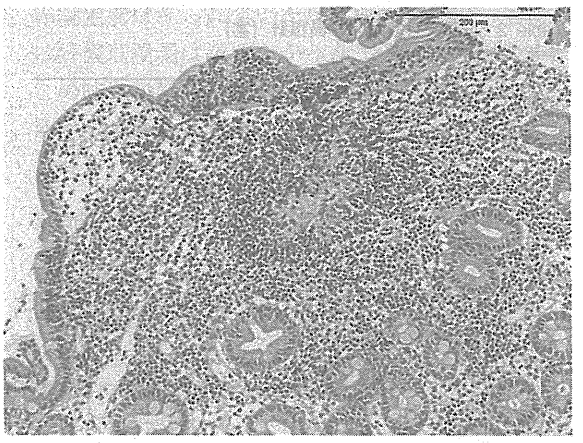


Figure 2　十二指腸びらん部の生検で非乾酪性類上皮細胞肉芽腫を認めた（HE染色200倍）．

350ml/日．

家族歴：特記事項なし．

現病歴：2001年に小腸型CDと診断されたが，2003年以降は治療自己中断していた．2012年11月中旬に1日10行の下痢，粘血便，腹痛を認めたため，当院受診．CD悪化を疑い，入院となった．入院時のCD Activity Index（CDAI）は150．嚥下障害や胸部つかえ感，胸焼け症状は認めなかった．

2012年の入院時現症：身長165cm，体重58.0kg，体温36.9℃，脈拍90/分整，血圧100/60．眼瞼結膜に貧血なし，眼球結膜に黄疸なし．体表リンパ節触知なし，胸部は異常所見認めず．腹部は平坦，軟で下腹部に軽度の圧痛を認めた．

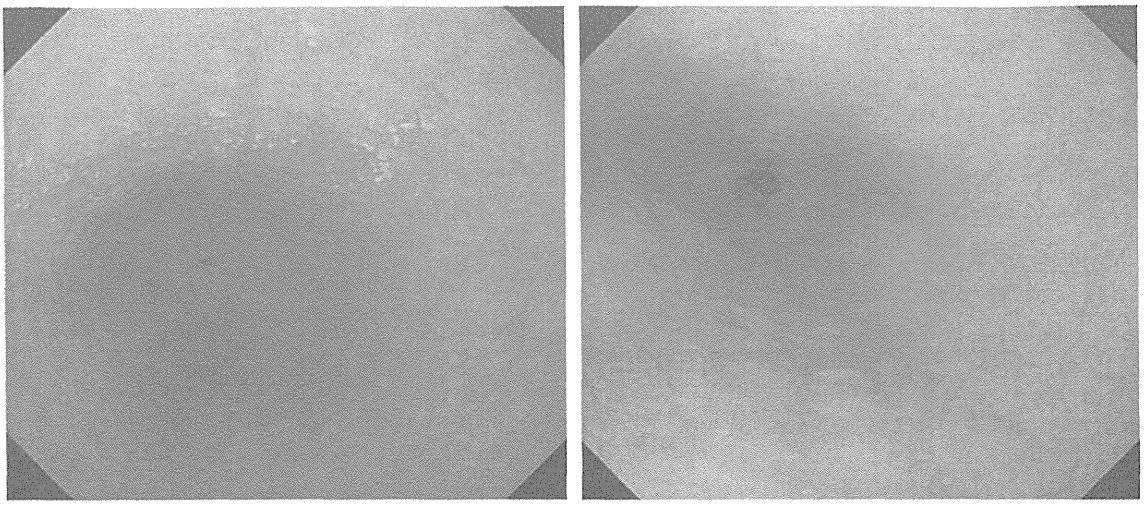
2012年の入院時検査所見（Table 1）：末梢血検査では軽度の白血球上昇を認めたものの好酸球の増加はなく，CRPや血沈も正常であった．総IgEも正常範囲内であった．

2001年のCD診断時の上部消化管内視鏡（以下EGD）（Figure 1）：上十二指腸角から下行部に縦走するびらんを認めた．同部位の生検から非乾酪性類上皮細胞肉芽腫を認め，CDと診断するのに矛盾しない所見であった（Figure 2）．なお，同時期の食道には特に異常所見は認めなかった．

2012年入院時EGD（Figure 3）：中部食道中心に白斑付着，発赤調の縦走溝を認めた．同部位からの生検で扁平上皮層内に高倍率視野1視野内に約30個の好酸球浸潤が内腔側から基底層側まで認められ（Figure 4），EoEを疑った．逆流性食道炎を疑うmucosal breaksは認められず，ロサンゼルス分類grade Nと診断した．また，食道裂孔で内腔が狭小となった部位より上方に一部胃粘膜が認められる程度の食道裂孔ヘルニアを認めた．胃には竹の節状所見などはみられなかったが，十二指腸には縦走傾向の発赤粘膜を認めた．胃十二指腸生検では，粘膜内には異常な好酸球浸潤はみられなかったが，十二指腸からは非乾酪性類上皮細胞肉芽種が認められ，CDに矛盾しない所見であった．

下部消化管内視鏡では，回腸末端から大腸内では活動性CDを示唆する所見は認めず，生検でも炎症所見や好酸球浸潤の増加はみられなかった．

小腸透視では明らかな縦走潰瘍や瘻孔は認めな



a b

Figure 3 上部消化管内視鏡所見：中部食道中心に白斑の付着（a），発赤調の縦走溝（a，b）を認めた．

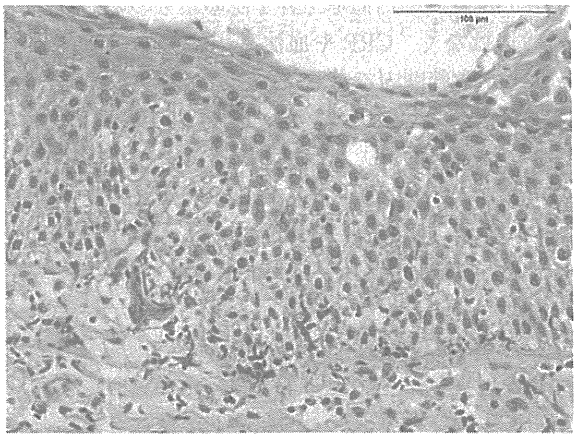


Figure 4 食道生検：扁平上皮内に高倍率視野1視野内に約30個の好酸球浸潤が内腔側から基底層側まで浸潤していた（HE染色400倍）．

Table 2 好酸球性食道炎診断指針（案）．
参考文献5）より引用．

①症状（嚥下障害，つかえ感等）を有する．
②食道粘膜の生検で上皮内に20/HPF以上の好酸球が存在している．
（生検は食道内の数カ所を行うことが望ましい）
③内視鏡検査で食道内に白斑，縦走溝，気管様狭窄を認める．
④CTスキャンまたは超音波内視鏡検査で食道壁の肥厚を認める．
⑤末梢血中に好酸球増多を認める．
⑥男性．
⑦プロトンポンプ阻害薬は無効でグルココルチコイド製剤が有効である．

①と②は必須 これら以外の他の項目も満たせば可能性が高くなる．

かった．

胸部造影CTでは食道壁の肥厚はみられず，腹部造影CTについても腸管壁の肥厚や瘻孔所見は認めなかった．

以上からまずCDに対して，メサラジン内服と成分栄養療法で治療を開始した．食道については内視鏡，病理所見からはEoEを強く示唆する所見であったが，食道に起因すると考えられる自覚症状がなく，EoEの診断基準案（Table 2）からも疑診とした．約4カ月後，CDAIは30で寛解状態となったが，胸部つかえ感，胸焼け症状が出現したため，EGDを再検した．

2013年3月EGD：食道の白斑は消失したが，発赤した縦走溝は残存しており，生検でも前回同様，食道粘膜上皮層内に高倍率視野1視野内に約30個程度の異常な好酸球浸潤が認められた．なお，逆流性食道炎の悪化はみられなかった．

今回は，EoEの診断基準案の4項目を満たすことになり，EoEと診断し，フルチカゾンプロピオン酸エステルの嚥下療法を行った．具体的には，気管支喘息治療薬のフルタイド200ディスカス®を1回400μgで1日2回，息止めの後口腔内に噴霧し直ちに嚥下し，口腔内のうがいを施行という方法で8週間投与した．開始後2週間程度で胸部つかえ感，胸焼け症状は消失した．投与開始8週間後のEGD所見では食道にはまだわずかに発

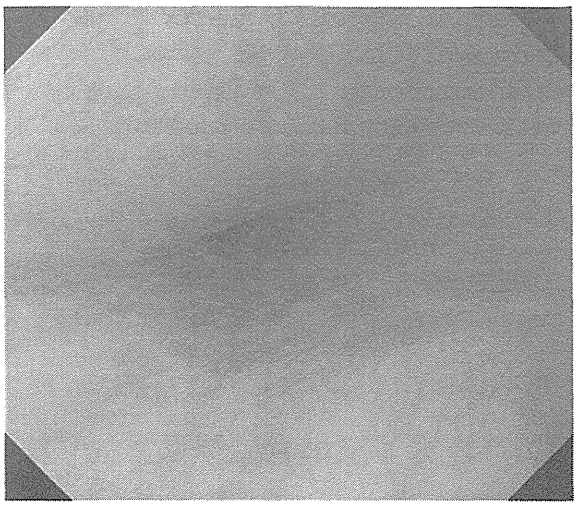


Figure 5　上部消化管内視鏡所見：軽度の発赤縦走溝は残存していたが，前回より幾分改善傾向と考えた．

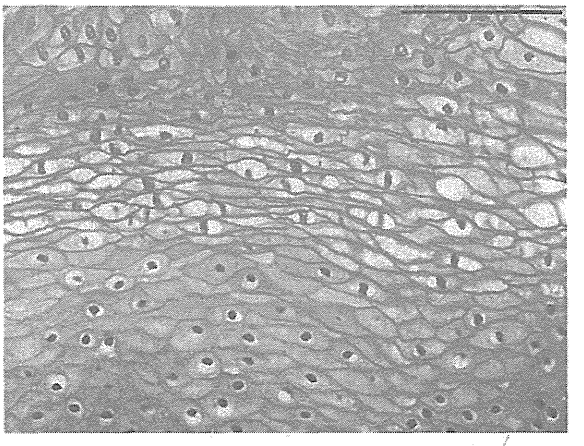


Figure 6　治療後食道生検：扁平上皮内には好酸球浸潤は認めなかった（HE染色400倍）．

赤縦走溝はみられていたが（Figure 5），生検では好酸球浸潤は認めなかった（Figure 6）ため，この時点で投与終了とした．以後経過観察中であるが，症状の再燃はみられず，CDも寛解維持中である．

Ⅲ 考　察

EoEは嚥下障害，食道つかえ感，胸やけ症状などを呈し，食道上皮層中に多数の好酸球浸潤が慢性的に持続するアレルギー性疾患である[1),2)]．本邦ではFurutaら[3)]が初めて報告し，以後，増加傾向であり，上部消化管内視鏡で調査された日本人でのEoE有病率は17.1人/100,000と報告されている[4)]．現在，厚生労働省研究班からTable 2の如く，診断指針案が示されている[5)]．本症例では，初回時は症状がなく，診断基準案からは②③⑥が該当するのみであり，確定に至らなかったが，4カ月後には①②③⑥を満たすことになり，EoEと診断した．

EoEの内視鏡所見としては，白色点状浸出物の付着，縦走する溝状裂孔，発赤，輪状狭窄，局在性の狭窄，しわ状パターンを示す食道粘膜，cobble stone-likeのパターンを示す食道粘膜，肉芽，なだらかな凹凸を有する粘膜，血管透見の不良，長い狭窄，瘢痕など多彩である[6)]．本邦では，縦走する溝状裂溝や白斑所見が多い[7)]．本症例でもこれらの所見が見られた．しかし，異常を認めない症例も42%に認め[7)]，Horiらは縦走溝や気管支輪状所見が白斑所見よりも有益ではあるものの，EoEには特異的内視鏡所見はないと述べており[8)]，生検は必須と考える．

CDの食道病変については諸所の報告があるが，縦走潰瘍，敷石像，広範囲に認める不整形から類円形潰瘍またはアフタ，縦走傾向が明らかなびらん，アフタというような他部位の消化管と同様の所見を呈することが多く[9)]，本症例のような白斑や発赤縦走溝を呈する例は報告がない．CDでも食道粘膜に好酸球浸潤を認めることはあるが，EoEのように高度な浸潤は認めない．以上から今回の食道病変はCDの食道病変ではなくEoEと考えた．さらに，EoEにおいては食道粘膜内でのIL-13，IL-15，Eotaxin-3，periostinなどのサイトカインの産生の亢進が特徴的な所見として報告されている[10),11)]．本症例でもフルチカゾンプロピオン酸エステルを用いた嚥下療法後に採取された検体で，治療の影響を受け異常が軽度となっている可能性が考えられるが，それでも末梢血中のperiostinが上昇していた．

本症例は，CDにEoEが合併していた．PubMedで"Eosinophilic esophagitis, Crohn's disease（検索期間2004年から2013年の10年間）"で検索したところ，これら2疾患の合併例の報告は2例のみであった[12),13)]．1例目[12)]は11歳男性でEoEが先行しその寛解後にCDが発症した症例，2例目[13)]は25歳女性でCD寛解後にEoEが発症した症例であり，両者とも一方の寛解後にもう一方が発症していた．CDは主にTh1やTh17がその病

態に関与しているといわれている．一方，EoEはTh2免疫反応の異常亢進が主な機序と推察されている[12]．一般的にTh1免疫系とTh2免疫系は相反的な関係にあると考えられている[14]．今回，CDが再燃しその活動性が高い時期には内視鏡検査や病理組織所見ではEoEに矛盾しない異常像が認められたが，食道病変に起因すると考えられる自覚症状はなかった．ところが，CDの寛解導入後にEoEの内視鏡所見は軽微にはなったものの組織学的には依然好酸球浸潤は残存し，EoEに起因する自覚症状が出現してきたことからEoEの病勢が相対的に強くなった可能性も示唆される．EoEは内視鏡的所見が正常である例も存在する[7]ことから，症状と内視鏡所見が合致しないこともおこりうるのではないかと推測した．既報と同様，本例のこのような経過はTh1免疫系とTh2免疫系の相反的な関係を考えると大変興味深く，今後CDとEoEの発症リスクの関係に関して検討をしていくことが必要であると考えられる．本例はEoEの治療にフルチカゾンプロピオン酸エステルの嚥下療法[15]を選択し，奏効した．近年，プロトンポンプ阻害剤（PPI）投与で症状や好酸球浸潤が改善する病態をPPI-responsive esophageal eosinophilia（PPI-R EE）とし，EoEはPPIが効果のないものとする考え方も報告されている[16),17]．ただPPI-R EEに関してはその概念を否定する考え方も多く提示されており[18]，今後検討と議論が進んでいくものと考えられる．本例にはPPIの投与は行っておらず，PPI-R EEとの区別は厳密にはできていない．しかし，好酸球浸潤が扁平上皮の内腔側にもみられており，基底層側への浸潤が多いとされる胃食道逆流症[19]とは病理組織所見は全く異なっていた．内視鏡検査でもmucosal breaksは認められておらず胃食道逆流による食道の異常が存在した可能性は低いと考えられる．以上のように本例ではCDを合併していた点，組織的に好酸球浸潤が内腔側にも認めた点，内視鏡的に逆流性食道炎を認めなかった点を鑑み，フルチカゾンプロピオン酸エステルの嚥下療法を第一選択とした．しかし，厳密にはまずPPIを投与して効果判定ならびにPPI-R EEとの区別を行うべき手段もあったと考える．本例では，現在，CD，EoEともに安定した状態で維持できているが，今後，一方が悪化した場合，その治療強化により，もう一方が悪化する懸念もある．ステロイド治療や抗TNF-α抗体，さらに開発中ではあるが抗IL-5中和抗体製剤のような両者に効果が期待できる薬剤の選択も今後視野に入れる必要があると考えられる．

Ⅳ 結　論

CDに合併したEoEの1例を報告した．

本要旨は第110回日本消化器内視鏡学会中国地方会にて発表した．

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A CASE OF EOSINOPHILIC ESOPHAGITIS COMPLICATED WITH CROHN'S DISEASE

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A 29-year-old man who had received a diagnosis of Crohn's disease (CD) in 2001 was referred to our department because of relapse of CD. Upper gastrointestinal endoscopy revealed white stipple-like exudates and red linear furrows in the esophagus, and pathological examination of biopsied specimens showed infiltration of eosinophils which was confirmed by the presence of more than 20 eosinophils in every high-power field. Eosinophilic esophagitis (EoE) was suspected; however, only treatment for CD was started, because the patient was asymptomatic for EoE with no dysphagia, food impaction nor heartburn. After induction of remission of CD, the patient developed food impaction and heartburn without a change in endoscopic and pathological findings. Therefore, the diagnosis of EoE was confirmed. Swallowed fluticasone therapy was provided for 8 weeks. The patient's symptoms improved immediately, and the endoscopic and histologic findings improved as well. EoE in patients with CD is very rare.

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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

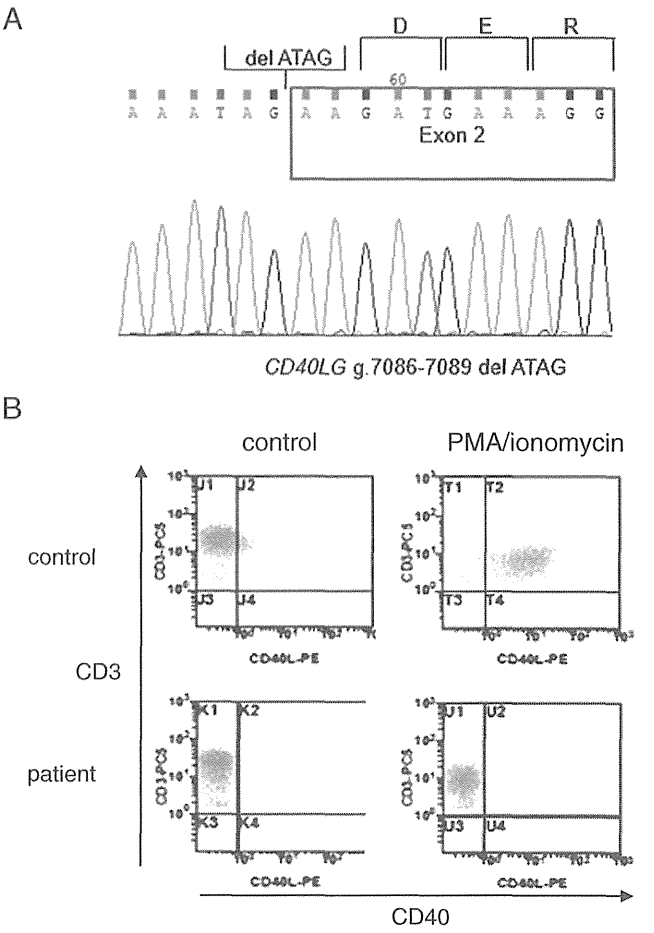

Fig. 2. A, Sequence electropherogram of *CD40LG*. A hemizygous frameshift mutation was identified. B, PMA/ionomycin treatment induced CD40LG expression. 88.68% of $CD3^+CD8^-$ cells were positive for CD40LG in healthy controls. On the other hand, only 0.82% were positive in patient-derived activated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

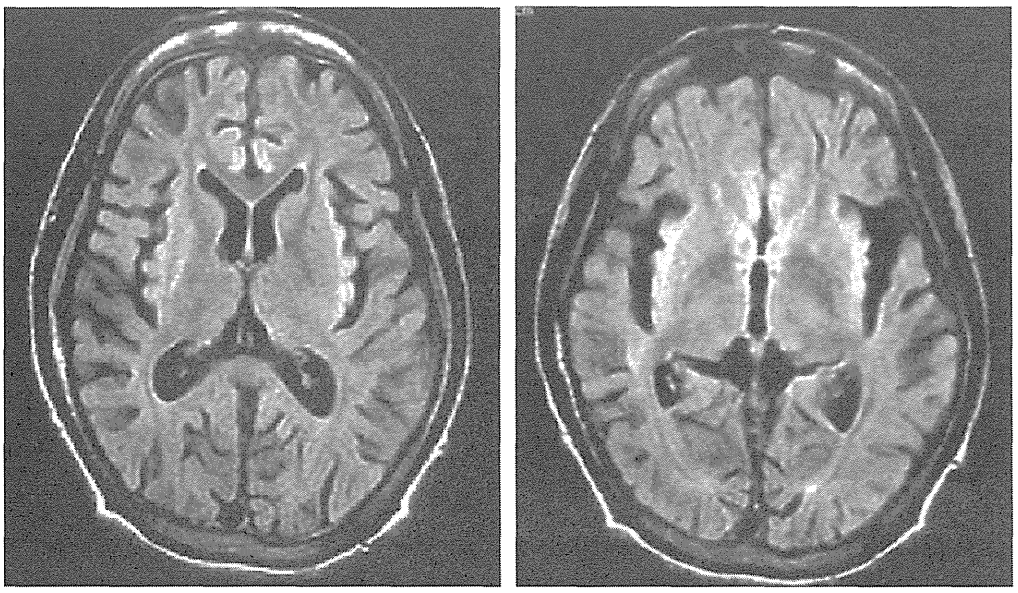

Fig. 1. Fluid attenuated inversion recovery axial images of patient 1, demonstrating cerebral cortical atrophy.

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

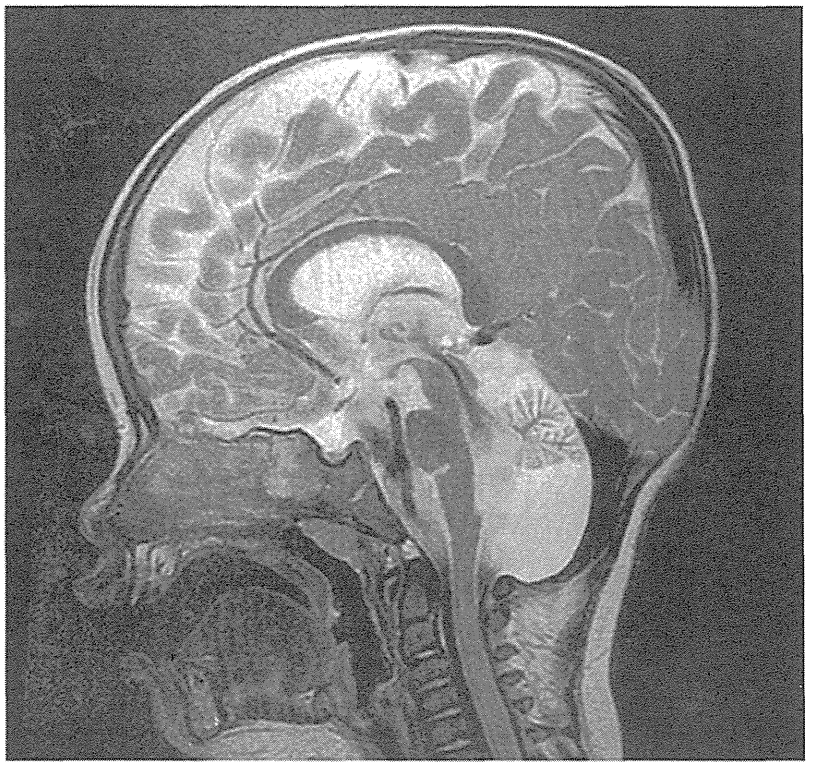

Fig. 3. Sagittal midline T2-weighted MR image of patient 5, demonstrating cerebellar atrophy of the vermis.

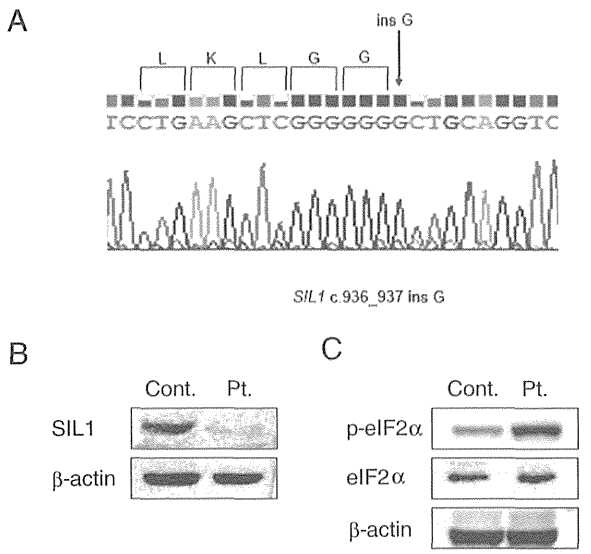

Fig. 4. A, Sequence electropherogram of *SIL1*. A homozygous frameshift mutation was identified. B, Western blotting analysis of SIL1 expression. C, eIF2α and phosphorylated eIF2α (p-eIF2α) levels. Cont: protein extract from EBV-transformed lymphoblastoid cell line derived from a healthy volunteer. Pt: 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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Aldehyde dehydrogenase-2 polymorphism contributes to the progression of bone marrow failure in children with idiopathic aplastic anaemia

Aplastic anaemia is a syndrome of bone marrow failure (BMF) that is characterized by peripheral pancytopenia and marrow hypoplasia. Injury to haematopoietic stem cells (HSCs), such as immune-mediated cytotoxicity, can cause aplastic anaemia; the successful treatment of aplastic anaemia using immunosuppressive therapy (IST) supports this hypothesis (Kulagin *et al*, 2014). Another proposed mechanism is an intrinsic defect of HSCs, which is the presumed major cause of congenital BMF. However, this has not been definitively established in idiopathic aplastic anaemia.

The aldehyde dehydrogenases (ALDHs) are a group of enzymes that are involved in critically important biological processes, such as detoxification of exogenous and endogenous aldehydes. ALDH2 deficiency, resulting from a Glu504Lys substitution (rs671, c.1510G>A) in the *ALDH2* gene, is prevalent in the Japanese population. AA homozygotes show very low catalysis of aldehydes, and GA heterozygotes display strongly reduced catalysis compared with GG homozygotes. Recently, ALDH2 deficiency was shown to be associated with accelerated progression of BMF in Japanese patients with Fanconi anaemia (FA), the most frequent inherited cause of BMF (Hira *et al*, 2013).

We hypothesized that ALDH2 deficiency underlies the progression of BMF in patients with idiopathic aplastic anaemia as well as in FA patients.

Seventy-nine Japanese children aged ≤15 years, referred to our institution between January 1990 and April 2011, were included in this study. Patients were excluded if they had paroxysmal nocturnal haemoglobinuria (PNH), exposure to toxic chemicals, chromosomal fragility, or mutations in *TERC*, *TERT*, *SBDS* or *SH2D1A* (Liang *et al*, 2006; Wang *et al*, 2007). Disease severity was classified based on the criteria of the International Aplastic Anaemia Study Group (Camitta *et al*, 1975; Bacigalupo *et al*, 1988). The *ALDH2* Glu504Lys polymorphism was genotyped using a duplex polymerase chain reaction with confronting two-pair primers (Tamakoshi *et al*, 2003). Statistical analysis was performed using the Fisher's exact test and the Kruskal-Wallis test. Failure-free survival (defined by survival in the absence of relapse, additional therapy, PNH, or secondary malignancy)

was analysed with the Kaplan-Meier method. All statistical analyses were conducted using JMP Pro 10·0·2 software (SAS Institute Inc., Cary, NC, USA). The study was approved by the ethics committee of Nagoya University Graduate School of Medicine.

The study included children whose disease type was classified as very severe (n = 10), severe (n = 40) and not severe (n = 29). Regarding the *ALDH2* Glu504Lys polymorphism, 40 children were genotyped as GG, 29 as GA, and 10 as AA (Table I). This distribution of the *ALDH2* polymorphism was not significantly different from the reported allele frequencies in the healthy Japanese population (Matsuo *et al*, 2006) (GG = 1141, GA = 941, AA = 217; P = 0·5015). However, the age at diagnosis was significantly younger in children harbouring AA (median 2 years, range 0·83–6 years) compared with children harbouring GG (median 9·5 years, range 1·6–15 years) and GA (median 9 years, range 1–14 years) (P = 0·0094; Table I, Fig 1A). In contrast, the severity of the disease and peripheral blood cell counts were not significantly different among the *ALDH2* groups (Table I). Of the 56 children who received IST as the initial treatment, 14 of 34 in the GG group, 10 of 16 in the GA group, and 4 of 6 in the AA group underwent an HSC transplant later in the disease course. The failure-free survival rate at 10 years from IST was significantly lower in the GA/AA group (0·162, 95% confidence interval, 0·043–0·454) than in the GG group (0·467, 95% confidence interval, 0·282–0·661; P = 0·0465; Fig 1B).

ALDH2 preferentially catalyses the breakdown of acetaldehydes and other aldehydes, such as 4-hydroxynonenal and malondialdehyde, that can be genotoxic due to DNA-protein crosslinking. Recent studies have revealed that ALDH2 dysfunction may contribute to a variety of diseases and biological processes. However, to date, no study has defined the

Table I. Patient characteristics.

	ALDH2 genotype			
	GG	GA	AA	*P*
Patients (*N*)	40	29	10	0·0094*
Age at Diagnosis, years, median (range)	9·5 (1·6–15)	9 (1–14)	2 (0·83–6)	
Gender (*N*)				
Male	23	16	8	0·8738
Female	17	13	2	
Disease Severity (*N*)				
Very severe	4	5	1	0·5453
Severe	22	12	6	
Non-severe	14	12	3	
CBC at Diagnosis				
Median WBC (x 10^9/l)	2·6	2·54	3·5	0·0587
Median ANC (x 10^9/l)	0·7	0·667	1·197	0·1879
Median Hb (g/l)	73	61	69	0·7765
Median Ret (‰)	8	16	34	0·4654
Median PLT (x 10^9/l)	11	17·5	10·5	0·3184

ALDH2, aldehyde dehydrogenase 2; CBC, complete blood cell count; WBC, white blood cell count; ANC, absolute neutrophil count; Hb, haemoglobin; Ret, reticulocyte count; PLT, platelet count.
*P < 0·01.

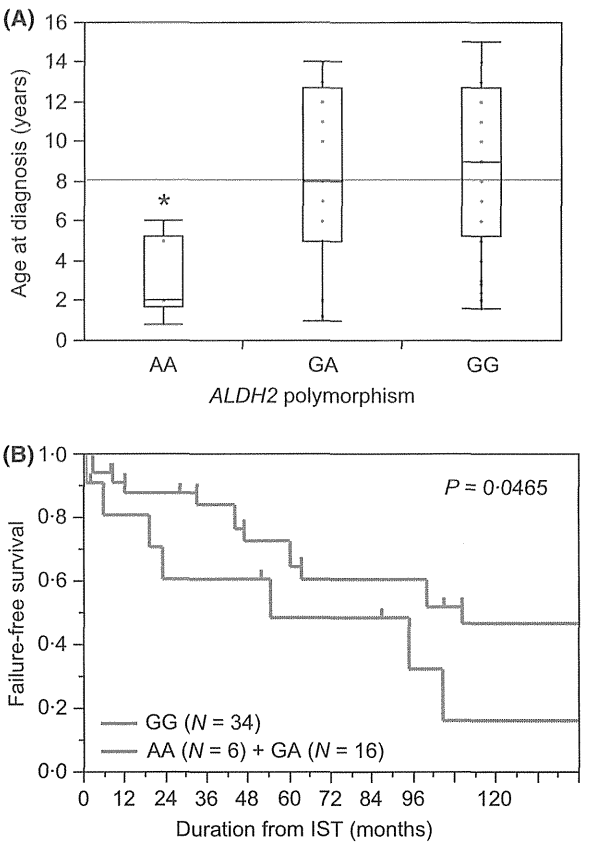

Fig 1. (A) Age at the diagnosis of idiopathic aplastic anaemia among *ALDH2* polymorphism groups. Age at diagnosis was significantly lower in the AA group harbouring a null capacity of ALDH2 (median 2 years, range 0·83–6 years) than in the GG group harbouring full capacity (median 9·5 years, range 1·6–15 years) and the GA group harbouring approximately 1/16 capacity (median 9 years, range 1–14 years). *P = 0·0094 with Kruskal-Wallis one-way analysis of variance. (B) Kaplan-Meier curve showing the failure-free survival (defined by survival in the absence of relapse, additional therapy, paroxysmal nocturnal haemoglobinuria, or secondary malignancy) of a cohort of 56 children who received immunosuppressive therapy (IST) for initial treatment of aplastic anaemia. The failure-free survival rate 10 years after beginning the treatment was significantly lower in the GA/AA group (0·162, 95% confidence interval, 0·043–0·454) than in the GG group (0·467, 95% confidence interval, 0·282–0·661; P = 0·0465, log-rank test).

role of ALDH2 deficiency in human haematopoietic diseases in the general population.

The accumulation of DNA damage partially explains the declining function of HSCs. The type of *ALDH2* variant is associated with accelerated progression of BMF in Japanese patients with FA, and patients carrying an AA allele develop myelodysplastic syndrome with BMF at a very young age (Hira *et al*, 2013), suggesting that aldehydes are an important source of genotoxicity in the human haematopoietic system. In an FA murine model, $Aldh2^{-/-}$ $Fancd2^{-/-}$ double mutant mice spontaneously develop aplastic anaemia due to disruption of DNA repair pathways that are required for HSC homeostasis. $Aldh2^{-/-}$ alone leads to a reduction in the HSC pool (Garaycoechea *et al*, 2012), indicating that endogenous aldehydes may cause an intrinsic defect in HSCs. In that study, aged $Aldh2^{-/-}$ $Fancd2^{-/-}$ double mutant mice that developed aplastic anaemia showed accumulation of damaged DNA within the HSC pool. Interestingly, *Aldh2* is dispensable for counteracting aldehydes in more mature haematopoietic precursors, suggesting that the emergence of BMF is possibly due to aldehyde-mediated genotoxicity that is restricted to HSCs.

Given that our cohort included only children (alcohol intake is not a factor), intrinsic aldehydes may play a role in accelerating BMF in children with idiopathic aplastic anaemia without other apparent genetic backgrounds that may cause intrinsic defects in HSCs. An intrinsic defect in HSCs, which is probably caused by endogenous aldehyde toxicity, may negatively affect the failure-free survival after IST in children whose aldehyde metabolism is suppressed. This intrinsic defect in HSCs is salvaged by additional treatment, most likely, HSC transplant. Our data suggest that patients with ALDH2 deficiency who are diagnosed with aplastic anaemia may need therapy in addition to conventional IST. ALDH inhibitors, such as disulfiram, may need to be avoided to retain residual ALDH2 activity in patients with low or absent ALDH2 activity. Importantly, a therapeutic approach with ALDH2 activators such as Alda-1 (Chen *et al*, 2014) may be beneficial for preventing a functional decline in HSCs.

In conclusion, endogenous aldehydes may damage HSCs, resulting in early-onset BMF in children with idiopathic aplastic anaemia. Our data suggest a novel therapeutic target for rescuing HSCs from genetic injury for the treatment of idiopathic aplastic anaemia and BMF.

Author contributions

NK designed the research, analysed the data and wrote the paper; AN, XW, YX, HS and SD collected specimens and patient data; HM, AH, KN and YT participated in the analysis and coordinated the research; SK led the entire project, designed the research and wrote the paper.

Conflict of interest

There are no relevant conflicts of interest to disclose.

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Allogeneic haematopoietic cell transplantation with reduced-intensity conditioning for elderly patients with advanced myelodysplastic syndromes: a nationwide study

The role of allogeneic haematopoietic cell transplantation (Allo-HCT) in the treatment of younger patients with myelodysplastic syndromes (MDS) is well established (de Witte *et al*, 2000; Sierra *et al*, 2002). However, early registry studies showed an adverse association between advanced age and increased non-relapse mortality (NRM) (Arnold *et al*, 1998). The introduction of reduced-intensity conditioning (RIC) has drastically reduced NRM, leading to a significant increase in the number of elderly patients with haematopoietic malignancy being referred for Allo-HCT. We conducted a nationwide retrospective study to clarify whether Allo-HCT with RIC actually improves overall survival (OS) for elderly patients with advanced MDS and sought to identify other variables that significantly influenced the outcome of Allo-HCT for these patients.

Data for patients who fulfilled the following criteria were obtained from the Transplant Registry Unified Management Program (TRUMP) (Atsuta *et al*, 2007): (i) aged 50–69 years, (ii) presence of French-American-British (FAB) class of refractory anaemia (RA) with excess blasts (RAEB) or RAEB in transformation (RAEBt) at any time between diagnosis and HCT, and (iii) received the first allogeneic bone marrow (BM) or peripheral blood stem cell transplantation from a human leucocyte antigen (HLA)-matched-related donor or a HLA-matched or -unmatched unrelated donor (URD) (Weisdorf *et al*, 2008) (Table SI) in Japan between 1 January 1 2001 and 31 December 2010. Patients with RA, RA with ringed sideroblasts or acute myeloid leukaemia evolving from MDS were excluded; such tight restriction of the disease stage meant that the study could deliver practical and useful information to clinical physicians. The conditioning regimen was classified as myeloablative conditioning (MAC) if it included total body irradiation (TBI) >8 Gy, oral busulfan (Bu) ≥9 mg/kg, intravenous Bu ≥7·2 mg/kg, or melphalan (Mel) >140 mg/m^2; otherwise, it was classified as RIC (Giralt *et al*, 2009). Data on the International Prognostic Scoring System (IPSS) components at HCT (Greenberg *et al*, 1997) were missing in TRUMP. Therefore, disease risk stratification used FAB class of RAEBt at any time between diagnosis and HCT, BM blasts ≥5% at HCT, and poor cytogenetics according to IPSS. Further details are provided in the Supplementary Methods.

Patient characteristics are shown in Tables I and SII. Of the 448 patients, 197 (44%) received MAC [cyclophosphamide (Cy)-TBI-based (n = 63), Bu-Cy-based (n = 58), fludarabine (Flu)-Bu-based (n = 42), Flu-Mel-based (n = 22) and other MAC (n = 12)]. The remaining 251 (56%) patients received RIC [Flu-Bu-based (n = 132), Flu-Mel-based (n = 80), Flu-Cy-based (n = 18) and other RIC (n = 21)]. Comparison of the patients who received MAC and RIC revealed that the RIC patients were significantly more likely to be 60–69 years of age (16% vs. 47%; P = 0·001), and less likely to receive a URD transplant (70% vs. 54%; P = 0·001) (Table SII).

The 3-year OS rates of patients receiving MAC and RIC were comparable (42·7% vs. 44·1%; P = 0·330; Fig 1A, Table SIII). The early mortality ratios were also similar between patients receiving MAC and RIC (5·6% vs. 3·6% at 30 days [P = 0·309]; 21·3% vs. 15·4% at 100 days [P = 0·106]). The multivariate analysis (Table I) revealed that the patients receiving MAC and RIC were comparable in terms of OS (RIC: relative risk [RR] 0·85; 95% confidence interval [CI] 0·65–1·13; P = 0·262). Other variables that were significantly and independently associated with OS in the whole cohort were HCT-comorbidity index >2 (RR 1·87; 95% CI 1·15–3·06; P = 0·012) (Sorror *et al*, 2005), BM blasts ≥5% at HCT (RR 1·46; 95% CI 1·02–2·10; P = 0·041), poor cytogenetics (RR 1·78; 95% CI 1·37–2·32; P < 0·001), time from diagnosis to HCT ≥6 months (RR 0·73; 95% CI 0·54–0·99; P = 0·042), partially matched (PM)-URD (RR 1·95; 95% CI 1·31–2·91; P = 0·001) and mismatched (MM)-URD (RR 2·47; 95% CI 1·61–3·79; P < 0·001).

Patients receiving RIC had a significantly lower 3-year cumulative incidence of NRM than patients receiving MAC (25·6% vs. 37·9%; P = 0·002, Fig 1B, Table SIII). The 100-day cumulative incidence of grade II-IV acute graft-versus-host disease (GVHD) and the 1-year cumulative

Peripheral blood lymphocyte telomere length as a predictor of response to immunosuppressive therapy in childhood aplastic anemia

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ABSTRACT

Predicting the response to immunosuppressive therapy could provide useful information to help the clinician define treatment strategies for patients with aplastic anemia. In our current study, we evaluated the relationship between telomere length of lymphocytes at diagnosis and the response to immunosuppressive therapy in 64 children with aplastic anemia, using flow fluorescence *in situ* hybridization. Median age of patients was ten years (range 1.5-16.2 years). Severity of the disease was classified as very severe in 23, severe in 21, and moderate in 20 patients. All patients were enrolled in multicenter studies using antithymocyte globulin and cyclosporine. The response rate to immunosuppressive therapy at six months was 52% (33 of 64). The probability of 5-year failure-free survival and overall survival were 56% (95% confidence interval (CI): 41-69%) and 97% (95%CI: 87-99%), respectively. Median telomere length in responders was -0.4 standard deviation (SD) (-2.7 to +3.0 SD) and -1.5 SD (-4.0 to +1.6 (SD)) in non-responders (P<0.001). Multivariate analysis showed that telomere length shorter than -1.0 SD (hazard ratio (HR): 22.0; 95%CI: 4.19-115; P<0.001), platelet count at diagnosis less than 25x10^9/L (HR: 13.9; 95%CI: 2.00-96.1; P=0.008), and interval from diagnosis to immunosuppressive therapy longer than 25 days (HR: 4.81; 95%CI: 1.15-20.1; P=0.031) were the significant variables for poor response to immunosuppressive therapy. Conversely to what has been found in adult patients, measurement of the telomere length of lymphocytes at diagnosis is a promising assay in predicting the response to immunosuppressive therapy in children with aplastic anemia.

Introduction

Aplastic anemia (AA) is defined as bone marrow aplasia and peripheral blood pancytopenia; disease pathogenesis is thought to involve immune-mediated processes. The first choice of treatment for severe AA in children is hematopoietic stem cell transplantation from a human leukocyte antigen (HLA)-matched sibling donor.[1,2] However, 60-70% of children with severe AA have no matched sibling donor and receive immunosuppressive therapy (IST), consisting of antithymocyte globulin (ATG) and cyclosporine (CyA). According to previous studies in children, the response rate to IST at six months was 60-70%, with the probability of survival at five years being over 90%. On the other hand, relapses occur in 10-30% of patients who responded to IST and, overall, clonal evolution develops in 10-15% patients.[3-5] In adults, several pre-treatment biomarkers have been proposed as promising tests for predicting favorable response to IST, including the presence of either human leukocyte antigen (HLA)-DR15 or a minor population of paroxysmal nocturnal hemoglobinuria (PNH)-type cells.[6-9] However, we previously reported that neither test was useful to predict response to IST and that lower white blood cell count and shorter interval from diagnosis to IST were significant predictive markers of better response,[10] on the other hand, a National Institutes of Health (NIH) study showed that higher base-line absolute reticulocyte and lymphocyte counts were highly predictive of response to IST in adult patients.[11] These results suggest a difference in etiology of AA between adults and children.[10]

Dyskeratosis congenita (DC) is a rare inherited disease characterized by the classical mucocutaneous triad of abnormal skin pigmentation, nail dystrophy, and mucosal leukoplakia.[12] Patients with DC are unable to maintain the telomere complex that are protein-DNA structures at the end of eukaryotic chromosomes that prevent degradation and aberrant recombination of the chromosome ends,[13,14] and consequently have very short telomeres.[15] Shortened telomeres can cause a wide variety of clinical features consisting not only of

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mucocutaneous abnormalities, but also other symptoms, including bone marrow failure, pulmonary fibrosis, hepatic fibrosis, and predisposition to malignancy.[16] Several recent studies revealed cryptic forms of DC among patients with seemingly acquired AA who did not have apparent physical abnormalities.[17,18] Failure of AA patients to respond to IST may be explained by the presence of cryptic inherited bone marrow failure syndromes (IBMFSs).

Several investigators have demonstrated that telomere lengths of leukocytes in patients with AA vary widely, with an increased proportion of the patients having shorter telomeres than healthy individuals.[19,20] It is known that not only patients with typical DC, but also those with cryptic DC have very short telomeres.[21] Moreover, the telomere length in leukocytes is decreased in subsets of patients with other IBMFSs including Fanconi anemia,[22] Diamond-Blackfan anemia,[23] and Schwachman-Diamond syndrome.[24] Therefore, measuring telomere length of patients with AA at diagnosis may be useful in detecting patients with cryptic type of IBMFSs.

Recently, Scheinberg *et al.* reported that the telomere length of peripheral blood leukocytes was associated with risk of hematologic relapse, clonal evolution to myelodysplastic syndrome, and overall survival (OS), but not related to hematologic response to IST in patients with severe AA.[25] Because there was no study to validate their observation, we evaluated the relationship between telomere length in hematopoietic cells before IST and the response to IST in children with AA.

Methods

Patients

Peripheral blood samples at diagnosis and clinical records were obtained from 64 children who fulfilled entry criteria and enrolled in two prospective studies conducted by the Japan Childhood Aplastic Anemia Study Group.[26,27] Patients with acquired AA were eligible if selection criteria were satisfied (see *Online Supplementary Appendix* for details). Thirty-eight patients received horse ATG (Lymphoglobulin; Genzyme, Cambridge, MA, USA) at 15 mg/kg/day for five days and 26 received rabbit ATG (Thymoglobulin, Genzyme, Cambridge, MA, USA) at 3.75 mg/kg/day for five days. CyA (6 mg/kg/day, orally) was started on Day 1 and continued to at least Day 180. The dose was adjusted to achieve a whole blood trough level of 100-200 ng/mL. Standard supportive care was supplied in each institute. Response to IST was evaluated according to previously described criteria.[3] We defined patients with complete response or partial response at six months after IST as responders, and the other patients as non-responders. Relapse was defined by conversion to no response from a partial or complete response and/or the requirement for blood transfusions.

All samples and clinical records were collected after written informed consent had been obtained according to protocols approved by the Ethics Review Committee, Nagoya University Graduate School of Medicine (Research n. 732).

Measurements of telomere length and population of PNH clones

The average relative telomere length (RTL) of peripheral lymphocytes was measured by flow fluorescence *in situ* hybridization (flow-FISH), using a Telomere PNA kit (Dako Cytomation, Glostrup, Denmark).[28] Lymphocytes were derived from fresh peripheral blood in 38 cases and from frozen stored peripheral blood in 26 cases. We used delta RTL to compare patients' telomere length with that of age-matched healthy controls. Details of methods for measuring telomere length and definition of delta RTL are described in the *Online Supplementary Methods.* A minor population of paroxysmal nocturnal hemoglobinuria (PNH)-type granulocytes and red blood cells were also evaluated by flow cytometry according to a previously described method.[10]

Statistical analysis

We analyzed predictive variables associated with response to IST, failure-free survival (FFS; in which relapse, clonal evolution, second IST, HSCT, and death were censored), transplantation-free survival (TFS; in which HSCT and death were censored), and OS. Pre-treatment variables included patient's sex, age, etiology, disease severity, interval from diagnosis to IST, leukocyte count, lymphocyte count, neutrophil count, hemoglobin (Hb) level, platelet count, reticulocyte count, presence of HLA-DR15, presence of minor PNH clone, and delta RTL. Differences in these variables between responders and non-responders were assessed using the Mann-Whitney U-test and Fisher's exact probability test. Predictive factors with $P<0.10$ in the univariate analyses were set in the multivariate analysis (logistic regression modeling). $P<0.05$ was considered statistically significant. Measures of association were expressed as hazard ratios (HR) with 95% confidence intervals (CI). All tests were two-tailed with a type I error of less than 0.05 considered as statistically significant. All analyses were performed using STATA12.0 software (STATA, College Station, TX, USA).

Results

Pre-treatment patients' characteristics and clinical outcomes

A total of 64 patients with AA were included in this study. Patients' characteristics are shown in Table 1. The median age at IST was 10.0 years (range 1.5-16.2 years). Disease severity was assessed as very severe in 23 patients, severe in 21 patients, and moderate in 20 patients. Causes of AA were idiopathic in 60 patients and hepatitis in 4 patients. Median follow-up time from the time of IST was 35 months (range 6-132 months).

Overall, 33 of 64 patients (52%) responded to IST at six months after administration of ATG. Of the 33 responders, 4 children relapsed at 6, 34, 66, and 91 months after IST, respectively. The probability of 5-year cumulative incidence of relapse was 8% (95%CI: 2-28%). Nineteen transplantations were carried out for non-responders or patients with relapse. Of 64 children with AA, only one patient developed clonal evolution at 23 months after IST. During the observation period, 2 patients died; both of them had shown no response to IST, one suffered from lethal cerebral hemorrhage at six months, and the other underwent bone marrow transplantation from an HLA-matched unrelated donor at 12 months after IST and died of transplantation-related hepatic failure. The probability of 5-year FFS, TFS, and OS were 56% (95%CI: 41-69%), 63% (95%CI: 48-75%), and 97% (95%CI: 87-99%), respectively.

Telomere length of children with AA

Comparing SD calculated in 71 healthy individuals, median telomere length was -0.9SD (range -4.0 to +3.0SD) in all patients (n=64), -0.4SD (range -2.7 to +3.0SD) in

Table 1. Patients' characteristics.

Variables		Total	Responder	Non-responder	P
N		64	33	31	
Sex	M/F	38 / 26	22 / 11	16 / 15	NS
Age at diagnosis (range)	median	10.0 (1.5-16.2)	10.0 (1.5-16.2)	9.7 (2.6-15.1)	NS
Severity	VSAA/ SAA/MAA	23/21/20	12/10/11	11/11/9	NS
Etiology	Idiopathic/hepatitis	60/4	31/2	29/2	NS
ATG	Horse/rabbit	38/26	23/10	15/16	0.08
Interval from diagnosis to IST	median (range)	22 (1-341)	18 (1-85)	28 (4-341)	0.02
WBC at diagnosis (x10⁹/L)	median (range)	2300 (20-8700)	2300 (20-8700)	2400 (300-5000)	NS
NEU at diagnosis (x10⁹/L)	median (range)	300 (0-3130)	380 (0-3130)	260 (0-1140)	NS
LYM at diagnosis (x10⁹/L)	median (range)	1900 (20-5600)	1800 (20-5600)	2000 (200-4300)	NS
Hb at diagnosis (g/dL)	median (range)	7.3 (2.7-11.4)	7.2 (2.8-11.0)	7.4 (2.7-11.4)	NS
PLT at diagnosis (x10⁹/L)	median (range)	1.6 (0.3-5.4)	2.1 (0.4-5.2)	1.6 (0.3-5.4)	0.04
RET at diagnosis (x10⁹/L)	median (range)	27 (0-96)	27 (3-96)	27 (0-75)	NS
PNH clone	Positive/negative	11 / 53	7 / 26	4 / 27	NS
HLA-DR15	Positive/negative	20 / 44	13 / 20	7 / 24	NS
delta RTL (SD)	median (range)	-0.9 (-4.0 - +3.0)	-0.4 (-2.8 - +3.0)	-1.5 (-4.0 - +1.6)	<0.001

ATG: antithymocyte globulin; F: female; Hb: hemoglobin; HLA: human leukocyte antigen; IST: immune suppressive therapy; LYM: lymphocyte count; M: male; MAA: moderate aplastic anemia; NEU: neutrophil count; NS: not significant; PLT: platelet count; PNH: paroxysmal nocturnal hemoglobinuria; RET: reticulocyte count; RTL: relative telomere length; SAA: severe aplastic anemia; SD: standard deviation; VSAA: very severe aplastic anemia; WBC: white blood cell count.

Table 2. Multivariate analyses for poor response to IST, failure-free survival, and transplantation-free survival.

	HR	95% CI	P
Multivariate analysis for response to IST			
Interval from diagnosis to IST >25 days	4.81	1.15-20.1	0.031
IST with rabbit ATG	0.79	0.16-3.96	0.77
PLT <25x10⁹/L	13.9	2.00-96.1	0.008
RTL <-1SD	22	4.19-115	<0.001
Multivariate analysis for FFS			
IST with rabbit ATG	1.27	0.47-3.48	0.64
LYM >2.0x10⁹/L	2.32	1.02-5.24	0.044
PLT <25x10⁹/L	4.11	1.17-14.5	0.028
RTL <-1SD	2.01	0.83-4.89	0.12
Multivariate analysis for TFS			
IST with rabbit ATG	1.32	0.45-3.86	0.61
LYM >2.0x10⁹/L	3.42	1.32-8.81	0.011
PLT <25x10⁹/L	4.64	1.00-21.6	0.051
RTL <-1SD	2.84	1.01-7.97	0.048

ATG: antithymocyte globulin; CI: confidence interval; FFS: failure-free survival; HR: hazard ratio; IST: immunosuppressive therapy; LYM: lymphocyte count; PLT: platelet count; RTL: relative telomere length; SD: standard deviation; TFS: transplantation-free survival; MAA: moderate aplastic anemia; NEU: neutrophil count; NS: not significant; PLT: platelet count; PNH: paroxysmal nocturnal hemoglobinuria; RET: reticulocyte count; RTL: relative telomere length; SAA: severe aplastic anemia; SD: standard deviation; VSAA: very severe aplastic anemia; WBC: white blood cell count.

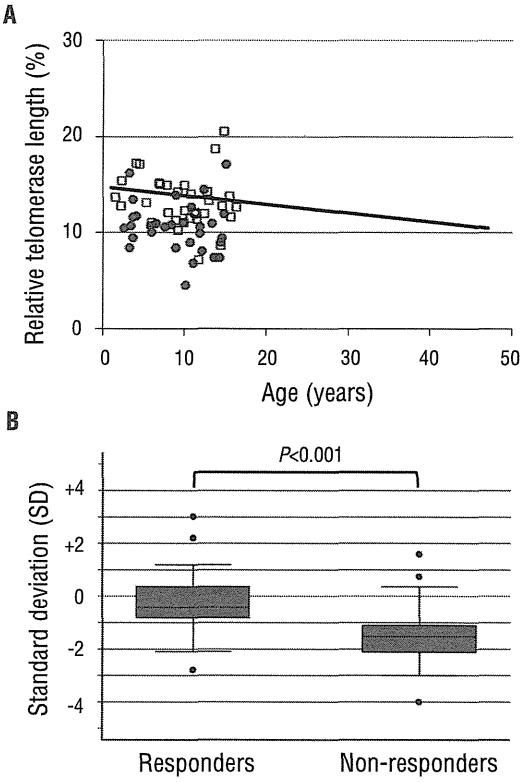

Figure 1. Relative telomere length in responders and non-responders. (A) Scatter plot of relative telomere length (RTL) *versus* age in patients with aplastic anemia (AA). The regression line for healthy individuals is shown as a solid line (Y= -0.0907X + 14.751). Results for AA patients were shown for responders (n=33; open squares) and non-responders (n=31; closed circles). (B) Comparison for telomere length between responders and non-responders. Box plots representing the distribution of telomere length in responders (n=33) and non-responders (n=31). The upper and lower limits of the boxes represent the 75th and 25th percentiles, respectively; the horizontal bar across the box indicates the median and the ends of the vertical lines indicate the minimum and maximum data values. Dots indicate outliers.

responders (n=33), and -1.5SD (range -4.0 to +1.6SD) in non-responders (n=31) (Figure 1A and B). There was a significant difference in telomere length between responders and non-responders (P<0.001). We evaluated the effects of age-adjusted telomere length quartiles on the response rate. There was a significant relationship between hematologic response and telomere length. The response rates at six months were 12.5% in the first (the shortest), 37.5% in the second, 75% in the third, and 81.3% in the fourth (the longest) quartiles of telomere length (Figure 2). The most powerful cut-off point for dividing responders and non-responders by telomere length was -1.0 SD (P=6.9x10^{-6}). There was no statistical tendency between relapse rate / clonal evolution / overall survival and telomere length.

We evaluated the pre-treatment variables for predicting response to IST in 64 children with AA (Table 1). Univariate analysis showed that interval from diagnosis to IST longer than 25 days (P=0.01), platelet count at diagnosis less than 25x10⁹/L (P=0.01), and telomere length shorter than -1SD

(P<0.001) were the variables statistically significant for poor response to IST, while there were no significant differences between responders and non-responders in terms of patient age, sex, disease severity, WBC count, neutrophil count, lymphocyte count, reticulocyte count, presence of HLA-DR15, and presence of minor PNH clones. Patients with rabbit ATG showed a tendency of poorer response to IST than patients with horse ATG (P=0.08).

Multivariate analysis confirmed that telomere length shorter than -1.0SD (HR 22.0; 95%CI: 4.19-115; P<0.001), platelet count at diagnosis less than $25x10^9$/L (HR 13.9; 95%CI: 2.00-96.1; P=0.008), and interval from diagnosis to IST longer than 25 days (HR 4.81; 95%CI: 1.15-20.1; P=0.031) were the significant predictive variables for poor response to IST (Table 2).

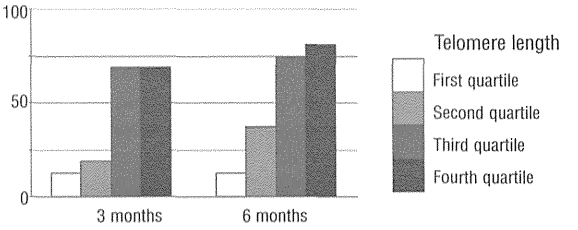

Figure 2. Response rates for immune suppressive therapy at three and six months according to telomere length. A poorer response rate was observed with each quartile as the telomere length shortened from the fourth to the first quartile.

Discussion

Our study demonstrated that the measurement of telomere length of lymphocytes is useful for predicting the response to IST in patients with AA. Recently, the NIH group reported that the telomere length of peripheral blood leukocytes was not related to hematologic response to IST, but was associated with the high risk of hematologic relapse, clonal evolution to myelodysplastic syndrome, and OS.[25] Several reasons may explain the conflicting results of the two studies. To begin with, there are several differences between the current study and the NIH study, including the methods of telomere length measurement and patients' characteristics. In the NIH study, the telomere length of pre-treatment total leukocytes was assessed by quantitative polymerase chain reaction (PCR). We measured the telomere length of lymphocytes using flow-FISH, which enabled us to measure median telomere length in the subpopulations of blood cells. Alter *et al.* compared the diagnostic sensitivity and specificity of short telomeres in different subpopulations of blood cells.[29] Their results indicated that lymphocytes were more suitable for diagnosis of DC than total leukocytes, which were a heterogeneous mixture of cell populations. The proportions of each cell population were different in each patient. The use of total leukocytes is suspected to provide less consistent results than analyses of defined leukocyte subpopulations.

Another difference between the two studies was the distribution of patients' age. Patients in our study were much younger (mean age 10 years) than those in the NIH study (mean age 35 years). Because telomeres shorten with age,[30] the differences in telomere length between patients and healthy individuals may become smaller in adults than in children. Moreover, in the NIH study, the cohort was restricted to patients with severe AA, and patients with moderate AA were not included. In contrast, 20 of 64 AA patients in our study had moderate disease. We could not estimate the frequency of clonal evolution since in our cohort there was only one patient who evolved into myelodysplastic syndrome during the observation period.

The causes of the difference in telomere length between responders and non-responders remain unknown. The short telomere length in non-responders may be ascribed to the presence of cryptic forms of IBMFS in the study cohort. Alter *et al.* reported that nearly all of the patients with both typical and cryptic DC have very short telomeres, as low as the first percentile of normal controls.[25] In our previous study, the RTLs of lymphocytes were below the 5% of normal controls in all of 6 DC patients and 2 AA patients harboring TERT mutation.[31] In the current study, there were 10 AA patients with shorter telomere length than the -2.0 SD of the cohort of healthy controls, but none of them showed clinical features of DC or had any mutation in *DKC1, TERC, TERT, NOP10, TINF2,* and *TCAB1*. It is unlikely that short telomeres in non-responders are to be ascribed to the presence of a cryptic form of DC.

Another possibility is that short telomere length may be a surrogate marker for longer disease duration that damages the hematopoietic stem cells and causes a higher number of compensatory stem cell divisions. We recently reported a significant inverse correlation between response rate to IST and interval between diagnosis and treatment in a large cohort of 312 children with newly diagnosed AA.[32] It is often difficult to determine the exact date of onset of the disease in patients with AA, especially in patients with moderate AA. The shorter telomere length may simply reflect longer duration of the disease in non-responders.

However, our study has several limitations, including a heterogeneous study population, a relatively small number of patients and a short follow-up period. To validate the results, we are conducting a prospective study to determine the optimal use of rabbit ATG for severe AA, in which we evaluate the relationship between telomere length of lymphocytes at diagnosis and the response to IST.

In conclusion, measurement of the telomere length in lymphocytes by flow-FISH is a promising assay, not only for identifying cryptic DC, but also for predicting the response to IST of patients with AA.

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Authorship and Disclosures

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