患者数は0.5～1人/100,000人であり，諸外国からの既報告例と概ね同様であった．発症者数，保因者数などについては，引き続き検討を要する．今回の調査では，SMAの確定診断には遺伝子検査を用いた例が半数を占めることが明らかとなった．最高到達運動機能を検討すると，I型では定頸不可能例が，II型では坐位保持可能例が最も多く，III型では歩行は全例で可能であった．臨床症状については多様性が認められた．罹患年齢が幅広いSMAの臨床像の分析は，複数の診療科による協力が不可欠である．診断基準を満たす例のみではなく，SMAの周縁疾患の範疇にある例も含めて，今後も臨床研究を進めていく必要がある．わが国でも統一基準をもって多施設共同研究が可能となるような基盤ができれば，医療的ケアの充実，治療法開発に向けての研究が今後も進展していくと思われる．

本研究は，平成15年度文部省科学研究費基盤研究(課題番号B12470173)の助成によって開始され，平成20年度本学女性医学研究者支援室の助成を受けて進められた．現在は，平成20年度厚生労働科学研究費補助金(難治性疾患克服研究事業)「神経変性疾患に関する調査研究班」(研究代表者　中野今治，分担研究者　斎藤加代子)，および，平成22-24年度厚生労働科学研究費補助金(難治性疾患克服研究事業)「脊髄性筋萎縮症の臨床実態の分析，遺伝子解析，治療法開発の研究」(研究代表者斎藤加代子)において継続して行われている．

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開示すべき利益相反状態はない．

文　献

1) **Osawa M, Shishikura K**: Werdnig-Hoffmann disease and variants. *In* Handbook of Clinical Neurology, vol 15 Disease of the Motor System (Virken PJ, Bruyn GW eds), pp51-80, Elsevier, Amsterdam New York (1991)
2) **Dubowitz V**: Chaos in the classification of SMA: a possible resolution. Neuromuscul Disord **5**: 3-5, 1995
3) **Munsat TL**: Workshop report. International SMA Collaboration. Neuromusc Disord **1**: 81, 1991
4) **Wirth B, Brichta L, Hahnen E**: Spinal muscular atrophy: from gene to therapy. Semin Pediatr Neurol **13**: 121-131, 2006
5) **斎藤加代子**：脊髄性筋萎縮症（SMA）とは.「脊髄性筋萎縮症診療マニュアル」(SMA診療マニュアル編集委員会編)，pp1-5, 金芳堂，東京（2012)
6) **Lefebvre S, Burglen L, Reboullet S et al**: Identification and characterization of a spinal muscular atrophy determining gene. Cell **80**: 155-165, 1995
7) **Van der Steege G, Grootscholten PM, van der Viles P et al**: PCR-based DNA test to confirm clinical diagnosis of autosomal recessive spinal muscular atrophy. Lancet **345**: 985-986, 1995
8) **福山幸夫，大澤真木子，原田淳子ほか**：小児脊髄性進行性筋萎縮症の臨床遺伝学的研究.「厚生省筋ジストロフィー症の病因に関する研究．昭和52年度研究報告書」，pp201-205（1978)
9) **橋本修二，福富和夫，永井正規ほか**：難病の全国疫学調査に基づく患者数の区間推定．日本公衛誌 **11**：880-883, 1991
10) **大川弥生，江藤文夫，上田　敏**：Werdnig-Hoffmann病（慢性経過型）の障害経過についての検討．リハ医学　**23**：115-120, 1986
11) **Ogino S, Wilson RB**: Genetic risk assessment in carrier testing for spinal muscular atrophy. Am J Med Genet **110**: 301-307, 2002
12) **Mostacciuolo ML, Daniell GA, Trevisan C et al**: Epidemiology of spinal muscular atrophies in a sample of the Italian population. Neuroepidemiology **11**: 34-38, 1992
13) **Thieme A, Mitulla B, Schulze F et al**: Epidemiological data on Werdnig -Hoffmann disease in Germany (West-Thuringen). Hum Genet **91**: 295-297, 1993
14) **Burd L, Short SK, Martsolf JT et al**: Prevalence of type 1 spinal muscular atrophy in North Dakota. Am J Med Genet **41**: 212-215, 1991
15) **Zaldivar T, Monteio Y, Acevedo AM et al**: Evidence of reduced frequency of spinal muscular atrophy in the Cuban population. Neurology **65**: 636-638, 2005
16) **Jedrzejowska M, Milewski M, Zimowski J et al**: Incidence of spinal muscular atrophy in Poland-more frequent than predicted? Neuroepidemiology **34**: 152-157, 2010
17) **Arkblad E, Tulinius M, Kroksmark AK et al**: A population-based study of genotypic and phenotypic variability in children with spinal muscular atrophy. Acta Paediatrica **98**: 865-872, 2009
18) **Mailman MD, Heinz JW, Papp AC et al**: Molecular analysis of spinal muscular atrophy and modification of the phenotype by SMN2. Genet Med **4**: 20-26, 2002
19) **斎藤加代子**：脊髄性筋萎縮症の遺伝子診断．神経内科　**69**：528-532, 2008
20) **Saito K**: Clinical features and molecular genetics in spinal muscular atrophy. J Tokyo Wom Med Univ **70**: 2-9, 2000
21) **Ito M, Saito K, Du J et al**: Phenotype-genotype correlation in Japanese spinal muscular atrophy patients: Analysis of DNA and mRNA of the *SMN* gene. J Tokyo Wom Med Univ **74**: 167-178, 2004
22) **Krosschell KJ, Maczulski JA, Crawford TO et al**: A modified Hammersmith functional motor scale

for use in multicenter research on spinal muscular atrophy. Neuromuscul Disord **16**: 417–426, 2006
23) **斎藤加代子，伊藤万由里**：成人の脊髄性筋萎縮症，脊髄性進行性筋萎縮症の臨床の分析.「厚生労働科学研究費補助金（難治性疾患克服研究事業）神経変性疾患に関する調査研究班　2008年度（分担）研究報告書」，pp39–44（2009）

成人型脊髄性筋萎縮症SMAの診断基準症例の臨床と遺伝学的背景

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成人型脊髄性筋萎縮症（spinal muscular atrophy; 以下SMA）は進行性の下位運動ニューロン（lower motor neuron: LMN）変性によって臨床的にLMN症候のみを呈し、経過が緩徐で予後が良好な疾患です。本稿では、成人型SMAについての概説と成人型SMAと考えられる自験症例で検討した臨床像と遺伝学的背景の関連について紹介します。

成人型SMAの位置づけ

進行性に筋力低下や筋萎縮、線維束性収縮、腱反射の減弱・消失といったLMN症候のみを呈する成人症例は、臨床的にはしばしば筋萎縮性側索硬化症(amyotrophic lateral sclerosis: ALS)と診断されます。しかし、中には、凡そ２～５年で呼吸筋麻痺を来すALSとは明らかに異なり、上位運動ニューロン症候を認めることなく経過し、かつ進行が非常に緩徐な症例も数多く経験されます。このような症例は従来脊髄性進行性筋萎縮症(spinal progressive muscular atrophy:SPMA)あるいは単に進行性筋萎縮症(progressive muscular atrophy:PMA)と診断されてきました。

日本ではSMN遺伝子の異常が原因である小児発症のSMAと成人発症のSPMAを合わして、「広義のSPMA」と呼んでおりましたが、海外の教科書や論文では、「広義のSPMA」という病名は用いられておらず、「広義のSMA」と呼ばれています。そこで、国際的に統一を図るために、わが国でもSPMAの病名に代わって、小児、成人発症の脊髄前角病変によって起こる進行性の筋萎縮症に対してSMAと呼ぶことになり、成人発症のSMAはⅣ型に分類されることになりました。

SMAの原因として、乳幼児期発症のI, II型については95%以上、小児期発症のⅢ型の40-50%にSMN遺伝子やNAIP遺伝子に異常が認められ、遺伝形式は常染色体劣性です。一方、成人発症のSMAでは孤発性（家族・親族内で一人のみ発病）の場合が多く、遺伝性を示す場合でも常染色体優性遺伝、常染色体劣性遺伝、伴性遺伝いずれの報告もあります. その上、成人発症SMAではSMNの遺伝子異常はまれにしか見られず、その原因は様々だと考えられております。

成人型SMAの診断基準を図１に提示しました。経過については進行性とのみ記載されていて具体的な期間は明確にされていませんが、前述しましたよ

1. 主要項目
(1)臨床所見
① 下記のような下位運動ニューロン症候を認める。
筋力低下、筋萎縮、舌・手指の線維束性収縮、腱反射は減弱から消失
② 下記のような上位運動ニューロン症候は認めない。
痙縮、腱反射亢進、病的反射陽性
③ 経過は進行性である。
(2) 臨床検査所見
筋電図で高振幅電位や多相性電位などの神経原性所見を認める。
(3) 遺伝子診断
survival motor neuron(SMN)遺伝子変異を認める。
2. 鑑別診断
(1) 筋萎縮性側索硬化症
(2) 球脊髄性筋萎縮症
(3) 脳腫瘍・脊髄疾患
(4) 頸椎症、椎間板ヘルニア、脳及び脊髄腫瘍、脊髄空洞症など
(5) 末梢神経疾患
(6) 多発性神経炎（遺伝性、非遺伝性）、多発限局性運動性末梢神経炎
(7) 筋疾患（筋ジストロフィー、多発筋炎など）
(8) 感染症に関連した下位運動ニューロン障害（ポリオ後症候群など）
(9) 傍腫瘍神経症候群
(10) 先天性多発性関節拘縮症
(11) 神経筋接合部疾患
3. 診断の判定
1. (1)①②③全てと(2)あるいは(3)を1項目以上を満たし、かつ2. のいずれでもない。

図１　脊髄性筋萎縮症（SMA）の診断基準

うに、‘極めて緩徐な’進行性の経過であることが重要です。つまり平均2～5年の経過で呼吸筋麻痺を呈する（予後の悪い）ALSと臨床上区別するためには経過が緩徐であることを示す必要がありますが、6年以上の経過を示す例があるため、‘～年以上はSMA’と断定的な定義はできないのです。今回、自験例を検討する際、この経過の期間を“5年以上経過しても極めて進行が緩徐な”と定義して検討しました。

また、SMAの鑑別疾患として重要なものに遺伝性運動ニューロパチーがあります。神経細胞から出ている軸索や軸索を包む鞘である髄鞘が障害される疾患をニューロパチーと呼びます。遺伝性を示すものは遺伝性ニューロパチーと呼ばれ、運動神経や感覚神経、自律神経など障害される神経の種類や部位（軸索か髄鞘か）によって分類がなされており、原因遺伝子も30以上見つかっています。その中で運動神経の軸索のみが障害されるタイプがあり、それを遺伝性軸索型運動ニューロパチーといいます。臨床症状の特徴は少年期～中年期に、四肢遠位筋優位の進行性の筋萎縮・筋力低下で発症する点です。図2に示しますように、その障害される解剖学的病巣部位からSMAとは臨床症候や末梢神経伝導検査・針筋電図検査など電気生理学的に区別がつかないことがあります。

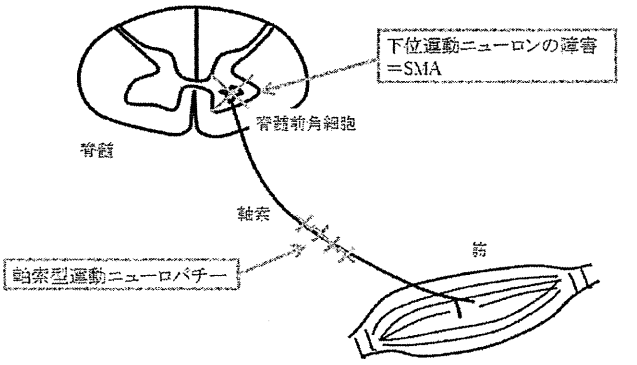

図2　下位運動ニューロンと軸索

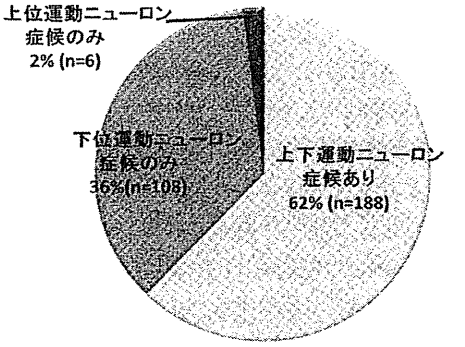

図3　自験302例（当科入院時の臨床所見）

自験例での検討

1）目　的

前項で述べてきました様に、成人型SMAの原因は様々である可能性があると考えられています。今回、実際に成人型SMAと考えられる症例の臨床像と遺伝学的背景を調べることで、成人型SMAと考えられる症例群がどういった臨床的特徴をもった集団になるのか検討しました。

2）方　法

当院において，過去15年の間に運動ニューロン疾患と診断された302例の中で、LMN症候のみ呈して、かつ発症後5年以上の極軽微な進行で推移し成人型SMAと考えられた11例を抽出し、臨床像をまとめました。そのうち同意の得られた9例については小児発症のSMAの大多数で原因遺伝子とされるSMN及びNAIP遺伝子の解析を行いました。

3）結　果

当院入院時より、上位運動ニューロン症候およびLMN症候を同時に満たす、すなわち筋萎縮性側索硬化症と確定診断できたのは188例（62%）、LMN症候のみを呈した症例は302例中108例（36%）でした（図3）。上位運動ニューロン症候のみを呈した症例も6例ありました．LMN症候のみを呈した108例についてその後の転帰を図4に示しました。108例の中で、嚥下機能が増悪し胃瘻造設を余儀なくされた15例、呼吸筋麻痺を認めた17例、死亡に至った31例、上位運動ニューロン症候が出現した2例の合計65例（61%）は臨床的にALSと診断し得る症例と判断しました。追跡が可能でLMN症候のみを呈した症例は16例（14%）であり、その中で5年以上の間、極めて軽微な進行で推移した11例を成人型SMAと臨床診断しました。その臨床像は以下の通りです。

11例のうち男性4例、女性7例、家族歴を有したのは2症例2家系（両家系とも常染色体優性遺伝形式）でした。初発症状は、1例が対称性両側下肢近位筋優位の筋力低下、7例が一側上肢遠位筋優位筋

力低下、3例が一側下肢遠位筋優位筋力低下でした。発症年齢は平均52.9歳で、男女で大きな差はなく20代から70代まで広く分布していました。罹病期間は5～9年が3例、10～14年が3例、15～19年が2例、20年以上経過した例が3例で、男女で大きな差はありませんでした。現在のADLについては7例が歩行可能、4例が不可能でした。また4例が自立生活、7例が部分介助生活であり、2例で軽度の嚥下障害を認めました.寝たきりの症例はありませんでした。

以上のような自験例11例の内、同意の得られた9例についてSMN遺伝子、NAIP遺伝子の解析を行いましたが、何れの遺伝子にも変異は認められませんでした。

4）自験例とSMN遺伝子異常を有するSMA報告例との対比から

文献上検索できたSMN遺伝子異常を有する成人型SMA報告例21例と自験例（遺伝子検索を行った9例）の臨床像を比較しました。報告例は男性13例、女性8例であり、家族歴は9例と半数で認められました。その発症年齢は平均31.5歳で20～64歳でしたが、18例が35歳以下で自験例と比べると若年発症の傾向がありました（図5）。しかし64歳と高齢発症も1例あり、SMAの診断には高齢発症であっても診断を確定するためには遺伝子検索が必要です。

報告例21例の初発症状は、19例が対称性下肢近位筋優位の筋力低下および筋萎縮であり、2例が非対称性遠位筋優位の筋力低下であるものの、症候学的にもほぼ均質な症例群といえそうです。逆に自験例で1例が対称性下肢近位筋優位の筋力低下で、その他の症例が遠位筋優位の様々な分布で発症していたことは、症候学的な多様性を示唆しています（図6）。自験例と報告例の罹病期間の分布に差は見られませんでした。21報告例中14例でADLについて記載が有り、6例が自立歩行生活可能、6例が平地歩行のみ可能、2例が歩行不可能で、自験例と類似していました。以上から、自験例を成人型SMAとして抽出した症例群は冒頭にも述べたようにやはり多様な臨床像を呈し、遺伝学的にもSMN遺伝子異常を認める均一な症例群とは異なることが考えられます。

最後に、前述したように、SMAとの鑑別が困難な遺伝性運動ニューロパチーについて、一部の例で原因遺伝子を網羅的に検索すると、1例では遺伝子異常が見つかりました。病因としての意義はまだ検討中ですが、やはり遺伝性運動ニューロパチーと考えられる症例も含まれた疾患群である可能性が示唆されます。

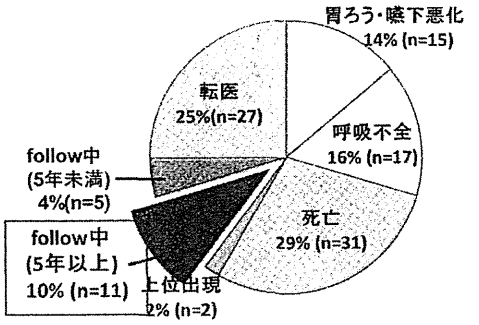

図4　下位運動ニューロン症候のみ108例の転帰

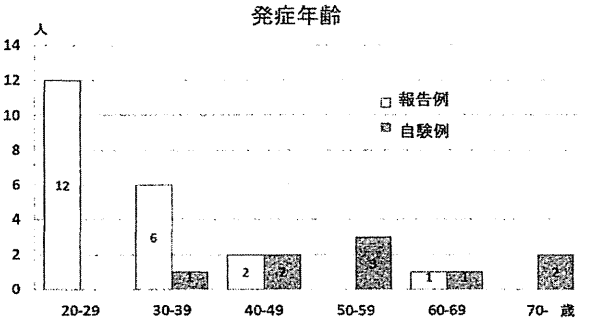

図5　自験例とSMN遺伝子異常を有する成人型SMA報告例との比較

	自験例	報告例
対称性下肢近位筋優位の筋力低下	1	19
非対称性遠位筋優位の筋力低下	8	2

図6　自験例とSMN遺伝子異常を有する成人型SMA報告例との発症部位での対比

今回の検討を通して、成人型SMAは一時点の評価だけでは診断できないことがわかり、改めて診断の難しさと遺伝子診断の重要性を実感しました。

参考文献
1）難病情報センターHP；http://www.nanbyou.or.jp/
2）中野 今治, 他: 成人型脊髄性筋萎縮症と考えられる当科症例の臨床像と遺伝学的背景の検討. 厚生労働科学研究費補助金(難治性疾患克服研究事業)神経変性疾患に関する調査研究班, 平成23(2011)年度・総括・分担研究報告書.
3）Ropper AH, etc. Adams and Victor's Principles of Neurology. 9th edition: Mc Graw Hill; 2009. p1058-1068
4）Brahe C, et al.Genetic homogeneity between childhood-onset and adult-onset autosomal recessive spinal muscular atrophy.Lancet 346: 741-742, 1995.
5）Moulard B,et al. association between centromeric deletions of the SMN Gene and sporadic adult-onset lower motor neuron disease. Ann Neurol 43: 640-644, 1998

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ORIGINAL ARTICLE

Creatinine/cystatin C ratio as a surrogate marker of residual muscle mass in amyotrophic lateral sclerosis

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Key words
amyotrophic lateral sclerosis, creatinine, cystatin C, residual muscle mass, surrogate maker.

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Abstract

Aim: Identification of sensitive surrogate markers that indicate disease progression in amyotrophic lateral sclerosis might be useful in clinical trials and clinical care. Determination of the creatinine/cystatin C (Cr/CysC) ratio eliminates the effect of renal function on serum creatinine levels; therefore, we considered that the ratio might serve as a surrogate marker of residual muscle mass. We studied the Cr/CysC ratio as a useful surrogate marker of residual muscle mass in patients with amyotrophic lateral sclerosis.
Methods: A total of 103 participants were recruited: 62 patients with amyotrophic lateral sclerosis and 41 healthy controls. Serum levels of Cr and CysC were measured in both groups. We subsequently investigated the correlation between the Cr/CysC ratio and disease severity in patients with amyotrophic lateral sclerosis.
Results: The ratio was significantly lower in the amyotrophic lateral sclerosis group than in the control group. Furthermore, the ratio decreased as the severity of amyotrophic lateral sclerosis increased. The Cr/CysC ratio might be a better and more reliable method than the serum Cr level as a means of monitoring residual muscle mass of the entire body in patients with amyotrophic lateral sclerosis.
Conclusion: The present results show that the Cr/CysC ratio might be a suitable candidate for a useful and quantitative surrogate marker for the assessment of disease severity and progression in patients with amyotrophic lateral sclerosis.

Introduction

Amyotrophic lateral sclerosis (ALS) is a uniformly fatal and debilitating disease; therefore, there is tremendous interest in developing effective therapies to slow or halt the progression of this disease. Current study designs often use a primary end-point of either death from ALS or initiation of long-term mechanical ventilation. This design requires a relatively long observation time to determine whether there is a positive treatment effect. Thus, identification of sensitive surrogate markers that indicate disease progression in ALS could be useful for the rapid identification of beneficial drugs, prompt exclusion of ineffective candidates and to determine clinical care for ALS patients. Surrogate markers of disease progression would provide a means of more rapid monitoring of drug efficacy in clinical trials.[1–5]

Muscle atrophy is a disease-defining feature of ALS, and clinical experience shows that atrophy might correlate with progressive weakness. Muscle atrophy is a qualitative marker of disease progression, but as yet, there is no clear quantitative marker of atrophy. Thus, as daily life activities decrease, the severity grade of ALS approximates to the residual skeletal muscle volume. A parameter that reflects muscle volume would enable estimation of disease severity.[6–8] The serum level of creatinine (Cr) is currently considered to be the most useful blood parameter that reflects the severity of motor dysfunction in spinal and bulbar muscular atrophy[9]; serum Cr levels were found to be correlated with the ALS Functional Rating Scale-Revised (ALSFRS-R) score in patients with spinal and bulbar muscular atrophy (correlation coefficient = 0.566, $P < 0.001$). However, because serum Cr almost exclusively originates from the skeletal muscle and its levels are dependent of renal function, we considered that the use of serum Cr levels as an accurate marker in ALS patients might be questioned.

Cystatin C (CysC), a known cysteine protease inhibitor, could potentially be used as a surrogate marker of glomerular filtration rates (GFR).[10,11] CysC is a non-glycosylated, 13.3-kDa basic protein that contains two disulfide bridges, and it is produced by all nucleated cells. It is considered to be unaffected by any factors (e.g. muscle mass, lean tissue mass, age, ambulation, circadian rhythm and sex) other than renal function status.[12–14] Furthermore, CysC is independent of the body muscle volume, and it is excreted from the kidneys in the same manner as Cr. Thus, the Cr/CysC ratio, which remains almost constant irrespective of renal function

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in individuals with neuromuscular diseases, is theoretically considered to be a good surrogate marker of muscle volume in ALS patients. In the present study, we compared the Cr-based estimated GFR (eGFR) with the CysC-based eGFR in ALS patients and healthy controls. The Cr/CysC ratio was further comparatively analyzed according to disease severity in ALS patients.

Methods

ALS patients and controls. Amyotrophic lateral sclerosis was diagnosed according to the revised El Escorial criteria.[15] A total of 62 patients serially diagnosed with definite ALS at Jichi Medical University Hospital in Shimotsuke, Japan, were enrolled in the present study. These ALS patients had no prior history of renal disease, no known concomitant disease and were not participating in any experimental treatment. A total of 41 subjects free from diseases characterized by muscle atrophy were recruited as controls in the present study. The mean age of the study participants was 62.9 ± 9.6 years for ALS patients and 61.8 ± 12.4 years for healthy controls. Clinical variables of ALS patients were analyzed for age, sex, onset site, symptom duration and grading according to the ALSFRS-R score.[16] In the current study, 10 patients experienced a bulbar onset and 52 patients experienced a limb onset of disease. The mean symptom duration was 4.4 ± 5.1 years (Table 1). Classification of disease severity based on the severity scale established by the modified Rankin scale (mRs; Table 2) revealed five patients with a disease severity of grade 1, nine with grade 2, 13 with grade 3, 13 with grade 4 and 22 with grade 5 (Table 1). Informed consent was obtained from all patients and healthy controls.

Measurements. All study participants were actively engaged in their usual daily life activities. Blood samples were collected in distinct serum-separator tubes and analyzed for serum Cr and CysC. Serum Cr levels were measured using an enzymatic method at our hospital laboratory. Serum CysC levels were measured using colloidal gold particles coated with anti-CysC antibodies at SRL Laboratory (Tokyo, Japan).[17]

eGFR (mL/min/1.73 m^2) was determined by measuring serum Cr levels using the following equation developed by the Committee on Chronic Kidney Disease of the Japanese Society of Nephrology[18]: men, Cr-based eGFR = $194 \times Cr^{-1.094} \times age^{-0.287}$; women, eGFR = $194 \times Cr^{-1.094} \times age^{-0.287} \times 0.739$. In addition, eGFR (mL/min/1.73 m^2) was determined by measuring serum CysC levels using the following equation developed by A Rule[19]: men and women, CysC-based eGFR = $66.8 \times CysC^{-1.30}$. The ratio of Cr (mg/dL) to CysC (mg/L) × 10 was defined.

Table 1 Clinical background of amyotrophic lateral sclerosis patients and control subjects

	ALS patients (n = 62)	Control subjects (n = 41)	P-value
Sex (male/female)	39/23	19/22	0.10 (χ^2-test)
Age (years)	62.9 (±9.6)	61.8 (±12.4)	0.52 (Mann–Whitney's U-test)
Symptom duration (years)	4.4 (±5.1)		
Bulbar onset/limb onset	10/52		
ALSFRS-R	25.8 (±17.3)		
Severity scale (mRs)	Grade 1 5		
	Grade 2 9		
	Grade 3 13		
	Grade 4 13		
	Grade 5 22		

Data were expressed by mean (standard deviation).
ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised; mRs, modified Rankin scale.

Table 2 Modified Rankin scale

Grade	Description
0	No symptoms at all
1	No significant disability despite symptoms; able to carry out all usual duties and activities
2	Slight disability; unable to carry out all previous activities, but able to look after own affairs without assistance
3	Moderate disability; requiring some help, but able to walk without assistance
4	Moderately severe disability; unable to walk without assistance and unable to attend to own bodily needs without assistance
5	Severe disability; bedridden, incontinent, and requiring constant nursing care and attention
6	Dead

Statistical analyses. For general statistical analyses, we used the SPSS v.11.0.1 program (Tokyo, Japan). Student's t-test was applied to the mean eGFR for serum Cr. Mann–Whitney's U-test was applied to the mean age, mean eGFR for serum CysC, and mean ratios of serum Cr to serum CysC between ALS patients and healthy controls. The χ^2-test for independent testing was applied to a two-by-two contingency table with sex between both groups. We analyzed differences in the Cr/CysC ratio between severity grades (mRs) by ANOVA followed by Tukey's honestly significant difference post-hoc test.

Correlation coefficients between serum Cr levels, Cr/CysC ratios and ALSFRS-R scores were determined. A scatter diagram between the serum levels and ALSFRS-R score was constructed. The correlation coefficient between Cr/CysC ratios and ALS duration was determined, and a scatter diagram between both sides was made. Correlations were determined using Spearman's rank-correlation coefficient. All tests were two-tailed and significance was set at $P < 0.05$.

Results

In this current study, clinical parameters (sex and age) were not significantly different between the ALS and control groups (Table 1).

The mean Cr/CysC ratios were 8.2 ± 2.2 for the control group and 5.5 ± 3.3 for the ALS group, the value being significantly lower in the ALS group (Mann–Whitney's *U*-test, $P = 0.01$; Table 3). The mean eGFR determined by serum Cr levels was 381.6 ± 486.9 mL/min/1.73 m^2 for the ALS group, which was significantly different (Mann–Whitney's *U*-test, $P < 0.001$; Table 3) from the control group value of 98.5 ± 57.9 mL/min/1.73 m^2. However, the mean eGFR determined by serum CysC was 103.3 ± 24.8 mL/min/1.73 m^2 for the ALS group, which was not significantly different from the control group value of 97.6 ± 21.6 mL/min/1.73 m^2. Thus, Cr-based eGFR in the ALS group was markedly higher than any other values. This is probably explained by reduced serum Cr levels in ALS patients on account of the reduced residual muscle mass. The mean Cr/CysC ratios were not significantly different according to sex between both groups (Table 3).

We compared the mean Cr/CysC ratios with disease severity (Fig. 1a) in ALS patients. The ratio was 10.13 ± 1.27 for grade 1 disease severity, 7.82 ± 1.1 for grade 2, 6.47 ± 1.92 for grade 3, 6.3 ± 2.78 for grade 4 and 2.37 ± 2.25 for grade 5. Significant differences were observed in the Cr/CysC ratio between severity grades 1–3, 1–4, 1–5, 2–5, 3–5 and 4–5 ($P < 0.05$). Thus, the ratio linearly decreased with an increase in disease severity (Fig. 1a). A relatively high Cr/CysC ratio observed in the patient group with grade 4 disease severity could be explained by the fact that this group included a number of patients with the bulbar-palsy type of ALS, in which the muscle mass in the four extremities is relatively well preserved. Therefore, we compared the mean Cr/CysC ratios after excluding patients with this type of ALS. This analysis revealed a ratio of 10.13 ± 1.27 for grade 1 disease severity, 7.82 ± 1.1 for grade 2, 6.47 ± 1.92 for grade 3, 4.6 ± 1.42 for grade 4 and 1.6 ± 1.58 for grade 5 (Fig. 1b). Significant differences were observed in the Cr/CysC ratio between severity grades 1–3, 1–4, 1–5, 2–4, 2–5, 3–5 and 4–5 ($P < 0.05$). A steady decrease in the ratio with an increase in disease severity became more evident when patients with the bulbar-palsy type of ALS were excluded from the analysis.

The scatter plot showed strong simple correlations between the Cr/CysC ratio and disease severity in ALS patients as determined by the ALSFRS-R score (correlation coefficient = 0.84, $P < 0.001$; Fig. 2). Furthermore, the plot showed that ALS severity increased with a decrease in the Cr/CysC ratio. A correlation between serum Cr levels and disease severity in ALS patients as determined by the ALSFRS-R score was also recognized (correlation coefficient = 0.78, $P < 0.001$; Fig. 2). The correlation coefficient of the Cr/CysC ratios was higher than that of serum Cr levels.

The relationship between the Cr/CysC ratio and ALS duration is shown on the scatter plot; the plot shows that the Cr/CysC ratio decreased with an increase in the duration of ALS (correlation coefficient = 0.75, $P < 0.001$; Fig. 3a). Figure 3b shows the percentage of patients with the Cr/CysC ratios of ≥ 5 and those with ratios of <5 as classified by disease severity. The percentage of patients with ratios of <5 was markedly higher in patients with grade 4 and grade 5 disease severity (71% and 94%, respectively).

Discussion

We believe that CysC levels are not basically affected by ALS itself. By calculating the serum Cr/CysC ratio, we eliminated the effect of renal function on serum Cr, which is dependent on the muscle mass of the entire body. We subsequently used this ratio as a surrogate marker of the residual muscle mass throughout the body. The Cr/CysC ratio was significantly lower in the ALS group, which was characterized by a decreased residual muscle mass, than that in the control group. Furthermore, the ratio decreased with an increase in ALS severity. This finding suggested that changes in the Cr/CysC ratio might reliably reflect the decrease in muscle mass throughout the body with an increase in ALS severity. According to the severity scale used in the present study, patients with the bulbar-palsy type of ALS, in whom the residual muscle mass was relatively conserved, were assigned higher grades of disease severity. Therefore, we also carried out the analysis after excluding patients with this type of ALS. With the exclusion of these patients from the analysis, it became even more evident that the Cr/CysC ratio reliably reflected the residual muscle mass of the entire body. Correlation of the Cr/CysC ratio was higher than that

Table 3 The ratios of creatinine to cystatin C, and the estimated glomerular filtration rate of creatinine and cystatin C in amyotrophic lateral sclerosis patients and controls

	ALS patients ($n = 62$)	Control subjects ($n = 41$)	*P*-value
The ratio of Cr (mg/dL) to CysC (mg/L) ×10 (Cr/CysC)	5.5 (±3.3)	8.2 (±2.2)	0.01* (Mann–Whitney *U*-test)
Cr-based eGFR (mL/min/1.73 m^2)	381.6 (±486.9)	98.5 (±57.9)	<0.001* (Mann–Whitney *U*-test)
CysC-based eGFR (mL/min/1.73 m^2)	103.3 (±28.4)	97.6 (±21.6)	0.28 (Student's *t*-test)
	Male	Female	*P*-value
ALS patients (Cr/CysC ratio)	5.2 (±3.7)	5.9 (±2.5)	0.37 (Student's *t*-test)
Control subjects (Cr/CysC ratio)	8.8 (±2.6)	7.6 (±1.8)	0.09 (Student's *t*-test)

*Data were expressed by mean (standard deviation). Significant difference.
Cr, creatinine; CysC, cystatin C; eGFR, estimated glomerular filtration rate.

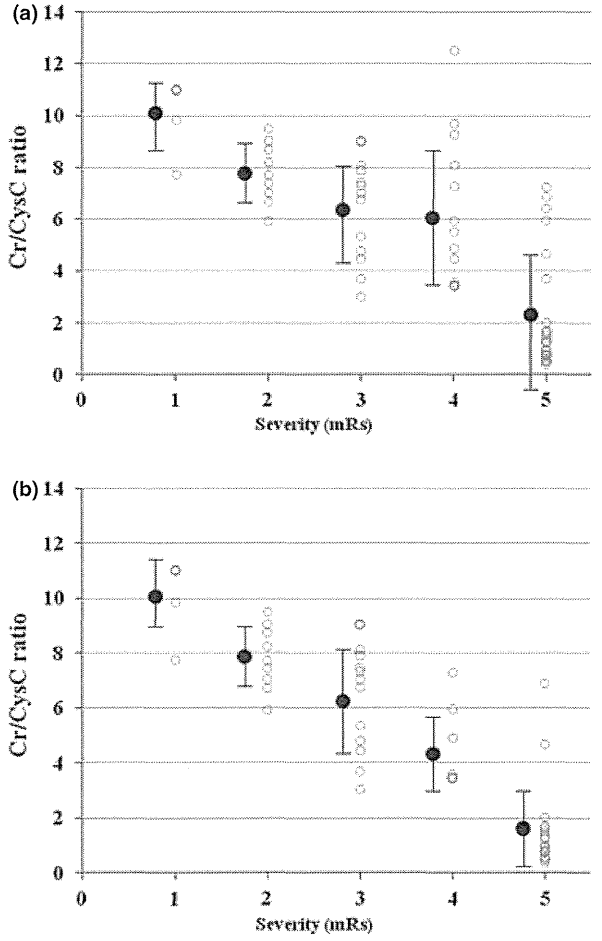

Figure 1 The mean creatinine/cystatin C (Cr/CysC) ratios were compared by disease severity (modified Rankin scale [mRs]) in amyotrophic lateral sclerosis (ALS) patients, and differences were analyzed by ANOVA followed by Tukey's honestly significant difference post-hoc test. (a) The ratio decreased linearly as disease severity increased (severity grades 1–3, 1–4, 1–5, 2–5, 3–5 and 4–5; $P < 0.05$). (b) After excluding patients with the bulbar-palsy type of ALS, the steady decrease of the ratio with increasing disease severity became more apparent (severity grades 1–3, 1–4, 1–5, 2–4, 2–5, 3–5 and 4–5; $P < 0.05$).

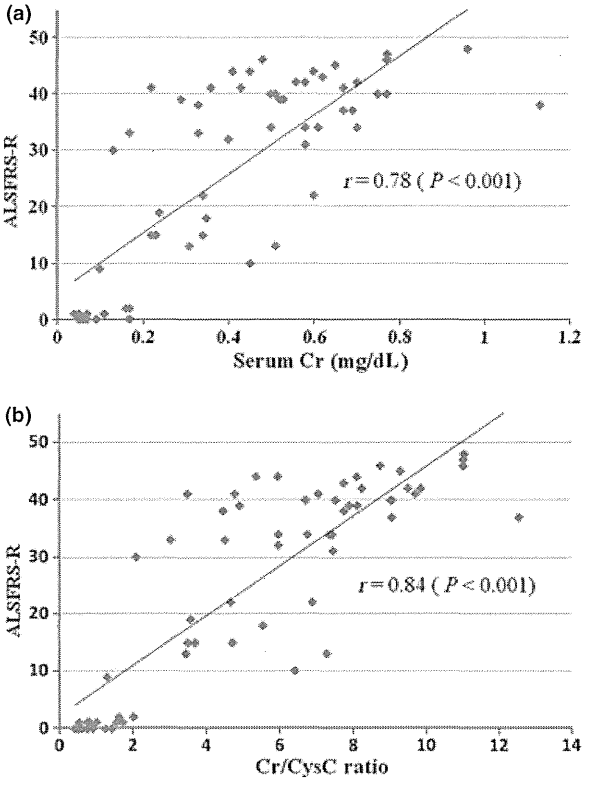

Figure 2 The scatter diagram showed strong simple correlations between (a) Cr level, (b) creatinine/cystatin C (Cr/CysC) ratio and the severity by Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised (ALSFRS-R) score in amyotrophic lateral sclerosis (ALS) patients. The plot showed that ALS severity increased with a decrease in both. The correlation coefficient of Cr/CysC ratio was higher than that of Cr level.

of serum Cr levels. The results also indicated that the Cr/CysC ratio might be better and more reliable than serum Cr levels for monitoring the residual muscle mass of the entire body in ALS patients. In addition, in ALS patients with serum Cr/CysC ratio of <5, daily life activities were markedly restricted. Therefore, this value might constitute a critical cut-off point during the clinical course of the disease.

To date, insensitive, non-parametric surrogate markers, which presume quantitative assessment of the disease severity in ALS patients, such as forced vital capacity, and the ALSFRS-R score have been used to assess disease progression.[16,20] We suggest that the Cr/CysC ratio might permit quantitative assessment of disease severity in ALS patients, assessment of therapeutic responses and determination of disease progression. Lee CD *et al.* presented an alternative approach that involved assessment of muscle thickness by muscle ultrasound (MUS) in ALS patients.[7] These authors reported that MUS is sufficiently sensitive as a potential surrogate marker to quantitatively detect changes in muscle thickness over time. Their study proposed biceps brachii as a suitable candidate for study; however, it is likely that assessment of multiple muscles would be required to account for the heterogeneity of ALS. Furthermore, muscle gene expression changes in skeletal muscle that could reliably define the degree of disease severity were reported.[21] However, we think that the current study presents a more convenient method for assessing disease status and progression than these approaches. Additional methods of measuring limb muscle mass include magnetic resonance imaging (MRI) and bioelectric impedance analysis (BIA). Applications for measuring residual muscle mass in ALS patients by MRI and BIA have been previously reported.[8,22] In particular, BIA is reported to be safe, portable, highly reliable and relatively inexpensive.[23] However, both methods can measure only a limited segment of body muscles. As a result of the heterogeneity of ALS that often affects different muscles

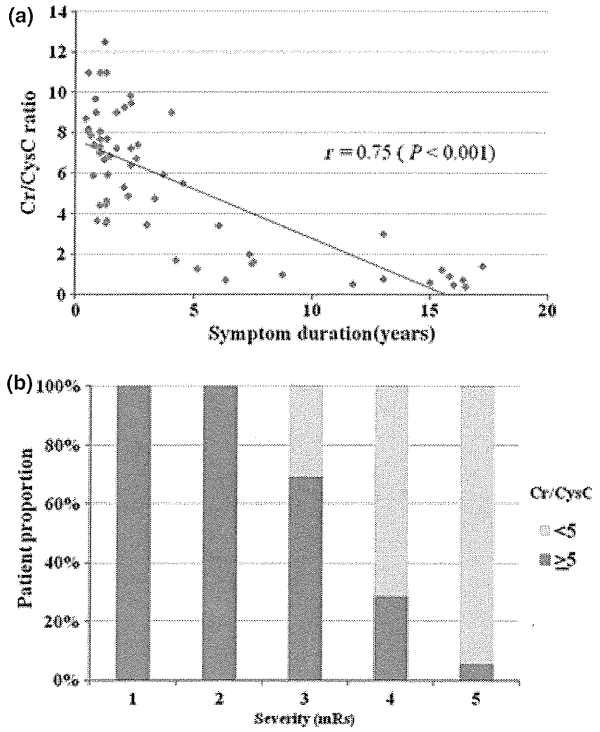

Figure 3 (a) The relationship between the creatinine/cystatin C (Cr/CysC) ratios and amyotrophic lateral sclerosis (ALS) duration is shown in the scatter plot; the plot shows that the Cr/CysC ratio decreased with an increase in the duration of ALS. (b) The percentage of patients with the Cr/CysC ratios of ≥5 and those with ratios of <5 as classified by disease severity (modified Rankin scale [mRs]) are shown. The percentage of patients with ratios of <5 was markedly higher in patients with grade 4 and grade 5 disease severity (71% and 94%, respectively).

at different rates, these methods are not considered to be capable of accurately determining residual muscle mass in ALS patients. Thus, we propose that measurement of the serum Cr/CysC ratio in ALS patients, which might account for residual muscle mass of the entire body, might be superior to these methods and eliminate the effect of renal function on serum Cr levels. However, under certain circumstances, the serum Cr/CysC ratio might not be a reliable surrogate marker of the residual muscle mass. For example, if ALS patients have a mild renal dysfunction, the ratio might be erroneously decreased. Thus, we have to interpret the data carefully, and should use only serum CysC levels for monitoring renal function accurately in ALS patients in such a case.A limitation of the present study was the lack of data measuring the actual residual muscle mass; however, as aforementioned, it is challenging to determine residual muscle mass of the entire body in ALS patients. Thus, taking previous studies together with our data and theory of the Cr/CysC ratio, it might be reasonable to suggest that serum Cr/CysC ratio might be a suitable candidate for a surrogate marker of residual muscle mass.

In addition, the use of the serum Cr level as a marker of renal function might be underestimated in diseases such as ALS that are characterized by reduced muscle mass. Thus, it is difficult to accurately assess renal function using serum Cr levels in these patients. This is clearly shown by the result shown in Table 3; eGFR determined by serum Cr was markedly higher in ALS patients than that determined by serum CysC. The latter is, therefore, considered to be a more accurate indicator for the assessment of renal function in ALS patients.

In conclusion, the present results show that the Cr/CysC ratio might be a suitable candidate for a surrogate marker of residual muscle mass of the entire body in ALS patients. Furthermore, the Cr/CysC ratio might also be a useful marker for assessing the response to therapy and determining the progression of ALS.

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References

1 Winhammar JM, Rowe DB, Henderson RD, Kiernan MC. Assessment of disease progression in motor neuron disease. *Lancet Neurol.* 2005; **4**: 229–38.
2 Turner MR, Kiernan MC, Leigh PN, Talbot K. Biomarkers in amyotrophic lateral sclerosis. *Lancet Neurol.* 2009; **8**: 94–109.
3 DiBernardo AB, Cudkowicz ME. Translating preclinical insights into effective human trials in ALS. *Biochim. Biophys. Acta* 2006; **1762**: 1139–1149.
4 Bowser R, Cudkowicz M, Kaddurah-Daouk R. Biomarkers for amyotrophic lateral sclerosis. *Expert Rev. Mol. Diagn.* 2006; **6**: 387–398.
5 Wagner KR. The need for biomarkers in amyotrophic lateral sclerosis drug development. *Neurology* 2009; **72**: 11–12.
6 Kiernan MC, Vucic S, Cheah BC *et al.* Amyotrophic lateral sclerosis. *Lancet* 2011; **377**: 942–55.
7 Lee CD, Song Y, Peltier AC, Jarquin-Valdivia AA, Donofrio PD. Muscle ultrasound quantifies the rate of reduction of muscle thickness in amyotrophic lateral sclerosis. *Muscle Nerve* 2010; **42**: 814–9.
8 Rutkove SB, Zhang H, Schoenfeld DA *et al.* Electrical impedance myography to assess outcome in amyotrophic lateral sclerosis clinical trials. *Clin. Neurophysiol.* 2007; **118**: 2413–8.
9 Hashizume A, Katsuno M, Banno H *et al.* Longitudinal changes of outcome measures in spinal and bulbar muscular atrophy. *Brain* 2012; **135**: 2838–48.
10 Abrahamson M, Olafsson I, Palsdottir A *et al.* Structure and expression of the human cystatin C gene. *Biochem. J.* 1990; **268**: 287–294.
11 Filler G, Bokenkamp A, Hofmann W, Le Bricon T, Martinez-Bru C, Grubb A. Cystatin C as a marker of GFR—history, indications, and future research. *Clin. Biochem.* 2005; **38**: 1–8.
12 Newman DJ. Cystatin C. *Ann. Clin. Biochem.* 2002; **39**: 89–104.
13 Grubb A, Lo¨fberg H. Human gamma-trace, a basic microprotein: Amino acid sequence and presence in the adenohypophysis. *Proc. Natl Acad. Sci. USA* 1982; **79**: 3024–7.

14 Vinge E, Lindergard B, Nilsson-Ehle P, Grubb A. Relationships among serum cystatin C, serum creatinine, lean tissue mass and glomerular filtration rate in healthy adults. *Scand. J. Clin. Lab. Invest.* 1999; **59**: 587–592.
15 Brooks BR, Miller RG, Swash M, Munsat TL. World federation of neurology research group on motor neuron diseases. El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. *Amyotroph. Lateral Scler. Other Motor Neuron Disord.* 2000; **1**: 293–299.
16 Cedarbaum JM, Stambler N, Malta E *et al.* The ALSFRS-R: a revised ALS functional rating scale that incorporates assessments of respiratory function. BDNFALS Study Group (Phase III). *J. Neurol. Sci.* 1999; **169**: 13–21.
17 Tanaka M, Matsuo K, Enomoto M, Mizuno K. A sol particle homogeneous immunoassay for measuring serum cystatin C. *Clin. Biochem.* 2004; **37**: 27–35.
18 Matsuo S, Imai E, Horio M *et al.* Collaborators developing the Japanese equation for estimated GFR. Revised equations for estimated GFR from serum creatinine in Japan. *Am. J. Kidney Dis.* 2009; **53**: 982–92.
19 Rule A, Bergstralh EJ, Slezak JM, Bergert J, Larson TS. Glomerular filtration rate estimated by cystatin C among different clinical presentation. *Kidney Int.* 2006; **69**: 399–405.
20 Miller RG, Rosenberg JA, Gelinas DF *et al.* Practice parameter: the care of the patient with amyotrophic lateral sclerosis (an evidence-based review): report of the Quality Standards Subcommitte of the American Academy of Neurology: ALS Practice Parameters Task Force. *Neurology* 1999; **52**: 1311–1323.
21 Pradat PF, Dubourg O, de Tapia M *et al.* Muscle gene expression is a marker of amyotrophic lateral sclerosis severity. *Neurodegener. Dis.* 2012; **9**: 38–52.
22 Bryan WW, Reisch JS, McDonald G, Herbelin LL, Barohn RJ, Fleckenstein JL. Magnetic resonance imaging of muscle in amyotrophic lateral sclerosis. *Neurology* 1998; **51**: 110–3.
23 Baumgartner RN, Ross R, Heymsfield SB. Does adipose tissue influence bioelectric impedance in obese men and women. *J. Appl. Physiol.* 1998; **84**: 257–262.

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ZNF512B gene is a prognostic factor in patients with amyotrophic lateral sclerosis

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ABSTRACT

Recently, Iida et al. discovered a new single-nucleotide polymorphism (SNP) in the ZNF512B gene associated with susceptibility to amyotrophic lateral sclerosis (ALS). The ZNF512B gene was found to be a transcription factor promoting the expression of a downstream gene in the signal transduction pathway of the transforming growth factor-β (TGF-β), which is essential for the protection and survival of neurons but the influence of the new SNP (rs2275294) in actual ALS patients remained unknown. The objective of our study was to examine whether the new SNP in the ZNF512B gene might influence the phenotype of ALS. We conducted a retrospective analysis of the ZNF512B gene in 176 patients diagnosed as having ALS at our hospital. Evaluation of the prognosis after the onset using Kaplan–Meier survival curves in patients with versus without the risk allele (C allele: CC and CT genotypes) revealed a significantly lower survival probability in those with the risk allele (log-rank test, $P<0.01$), independent of the other prognostic factors in ALS. Our study revealed the influence of the new SNP in actual ALS patients. It would be clinically reasonable to suggest that the ZNF512B gene is a new prognostic factor in ALS. This study is the first, as per our knowledge, to indicate that the association between the new susceptibility gene for ALS and its pathway could be identified.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease characterized by a loss of both upper and lower motor neurons usually leading to death due to failure of the respiratory muscles. The mean survival time is 3–5 years. Knowledge of disease progression and survival time is very important for clinical care and research. Due to the current lack of an effective disease-modifying therapy, increasing attention has recently been directed towards possible prognostic factors in order to identify additional therapeutic targets but the influence of genetics on the rate of disease progression and survival is scarcely known.

Recently, the study team led by Iida et al. conducted a large-scale genetic association study in 1305 Japanese patients with ALS, discovering a new single-nucleotide polymorphism (SNP) associated with susceptibility to ALS in the ZNF512B gene in chromosome 20q13.33 [1]. In regard to the susceptibility genes for sporadic ALS (sALS), 30 association studies based on the candidate-gene approach have been reported [2,3], and an analysis involving Japanese subjects resulted in the first discovery of the sALS susceptibility gene in East Asians [4]. The ZNF512B gene is a transcription factor with unknown function, and the mechanism underlying the association between the onset of ALS and this gene was unknown. Iida et al. revealed that the new SNP (rs2275294) located in intron 12 of the ZNF512B gene reduces expression of the gene. Moreover, the ZNF512B gene was found to be a transcription factor promoting the expression of a downstream gene in the signal transduction pathway of transforming growth factor-β (TGF-β) [5], which is essential for the protection and survival of neurons [6–8]. Based on these results, the following mechanism is proposed. In patients with the susceptibility SNP for ALS, ZNF512B gene expression is reduced, resulting in the weakening of the neuronal protection signals.

The objective of the present study was to examine whether the new SNP in the ZNF512B gene might influence the phenotype of ALS. For this purpose, we investigated the disease course and survival data in ALS patients with and without the risk allele (C allele: CC and CT genotypes).

2. Patients and methods

2.1. Patients

We analyzed the ZNF512B gene in 176 patients diagnosed as having definite ALS according to the revised El Escorial criteria [9] between April 1995 and December 2010 at our hospital, and the site of disease onset was determined by experienced neurologists on the basis of the first symptoms. Although ALS patients diagnosed as having probable or definite ALS were included in the original study of Iida et al. [1], probable ALS patients were excluded in our study. The first

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symptoms were confirmed by the family or other observers, whenever possible. All patients were screened for mutations in SOD1, TARDBP, and ANG, and none of these mutations was detected in any of the patients. Thus all patients were ones with sALS. Written informed consent was obtained from each of the patients prior to his/her participation in this study. The study was conducted with the approval of the ethics committees at the participating institutions.

2.2. SNP analysis

All of the subjects were unrelated Japanese individuals. Using standard protocols, genomic DNA was extracted from the peripheral blood leukocytes. We used a primer pair containing the new SNP (rs2275294). Each PCR was performed with 5 ng of genomic DNA derived from ALS subjects, and the amplification was performed in the GeneAmp PCR system 9700 (PE Applied Biosystems) under the following conditions: initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 96 °C for 30 s, annealing at 60–65 °C for 30 s, extension at 72 °C for 2 min, and post-extension at 72 °C for 7 min. The PCR products served as templates for direct sequencing by the fluorescent dye-terminator cycle sequencing method [1]. Then, we examined the risk (C) allele of the new SNP (rs2275294). The genotypes with the risk allele are CC homozygous and CT heterozygous, and one without the risk allele is TT homozygous.

2.3. Retrospective observation

We conducted a retrospective review and evaluated both the outcomes and clinical manifestations of these patients. Survival time, which was an endpoint in this study, was defined as the number of months from symptom onset until death from any cause or of tracheostomy for institution of permanent mechanical ventilation, and incidence of non-invasive ventilation. The patient characteristics entered for the univariate analyses were the age at onset, sex, site (bulbar/spinal) of symptom onset, time from onset to diagnosis and riluzole use. In regard to riluzole use, we defined riluzole use as continuous treatment with 100 mg drug daily. The longest retrospective observation period of time for ALS patients was 72 months.

2.4. Statistical analysis

For general statistical analyses, we used the SPSS v.11.0.1 program. Chi-square test for independent testing was applied to a two-by-two contingency table with sex, site (bulbar/spinal) of symptom onset, and history/no history of riluzole use. Student's *t*-test was applied to the mean age at onset and the mean survival time. Mann–Whitney's *U*-test was applied to the mean time from onset to diagnosis. The effects of prognostic factors on the survival were assessed using the Kaplan–Meier life-table method for all 176 patients according to the rs2275294 C allele carrier status. The log-rank test was used to assess the equality of the outcome functions. Multivariable analysis was performed with the Cox proportional hazard model. The following variables were included in the model: the risk allele (C allele; with vs. without), sex (male vs. female), the age at onset (included as continuous variable), the site of onset (bulbar vs. spinal), riluzole (use vs. no), and the time from onset to diagnosis (included as continuous variable). All tests were two-tailed and significance was set at $P<0.05$.

3. Results

The risk allele (C allele; CC and CT genotypes) of the new SNP (rs2275294) in the ZNF512B gene was detected in 128 (72.7%) of the 176 patients with ALS. The mean age at onset of ALS was 62.7 (±11.1) years in patients without the risk allele and 63.8 (±10.7) years in those with the risk allele, the difference not being significant (*t*-test, $P=0.56$, Table 1). The site (bulbar/spinal) of onset was 19/29 in patients without the risk allele and 43/85 in those with the risk allele, the difference not being significant (chi-square test, $P=0.46$, Table 1). The mean time from onset to diagnosis was also not significantly different between the two groups (Mann–Whitney's *U*-test, $P=0.32$, Table 1). Furthermore, no influence of the use of riluzole was found in this study (chi-square test, $P=0.98$, Table 1).

We could confirm the endpoints of 72 patients among the analyzed 176 patients without censure. The mean survival time was 36.6 (±19.7) months in patients without the risk allele and 24.3 (±13.8) months in those with the risk allele, so the mean survival time with the risk allele was shorter than the one without the risk allele, the difference being significant (*t*-test, $P<0.01$, Fig. 1). Moreover in regard to the prognosis after onset, the Kaplan–Meier survival curves for patients with versus without the risk allele according to the rs2275294 C allele carrier status revealed a significantly lower survival probability in those with the risk allele (log-rank test, $P<0.01$, Fig. 2).

In the multivariate analysis accounting for all investigated variables, significant effects for the risk allele ($P=0.043$), the age of onset ($P=0.002$), the site of onset ($P=0.049$) and the time from onset to diagnosis ($P<0.001$) were found. The genotype with the risk allele (C allele; CC and CT) was an independent prognostic factor (hazard ratio, 1.807; 95% confidence interval, 1.018–3.209). The values for sex and riluzole were not significant (Table 2).

4. Discussion

The results suggest that ALS patients with the risk (C) allele of the new SNP (rs2275294) might have shorter survival compared with ALS patients without the risk allele. In the survival time and the Kaplan–Meier analysis, the patients with the risk allele had a markedly shorter survival by 72 months. The original study of Iida et al. [1] included 1305 ALS patients while there were only 176 ALS patients in our study. This might have influenced the results. Moreover in the study of Iida et al. [1] the risk (C) allele frequency is approximately 50%, while it is almost 75% in our study. The greatest reason for this difference of the two studies is the restriction of definite ALS patients in our study, excluding probable ALS patients. Thus we considered that the sample size of our study was smaller than the original study, but this had reliability as well as originality.

Previous studies have reported a number of prognostic factors in ALS patients; the most consistent are the age at onset, site of symptom onset and longer time from the first symptom to diagnosis [10–13]. In addition, some investigators have reported male gender as a favorable prognostic factor, although the incidence between men and women is about the same in familial disease [13,14] and recently Byrne et al. reported that patients with ALS and the *C9orf72* repeat expansion seem to present reduced survival [15]. Although in our study the *C9orf72* repeat expansion was not analyzed, the frequency of the *C9orf72* repeat expansion among Japanese patients is

Table 1
Baseline characteristics of ALS patients.

Characteristic	Without the risk allele (N=48)	With the risk allele (N=128)	*P* value
Sex (male/female)	30/18	67/61	0.23 (Chi-square test)
The mean age at onset (SD) (year)	62.7 (±11.1)	63.8 (±10.7)	0.56 (Student's *t*-test)
The site of onset (bulbar/spinal)	19/29	43/85	0.46 (Chi-square test)
Riluzole (±)	25/23	67/61	0.98 (Chi-square test)
The mean time from onset to diagnosis (SD) (month)	16.8 (±14.2)	13.1 (±9.3)	0.32 (Mann–Whitney's *U*-test)

Bulbar/spinal = bulbar/spinal-onset ALS, SD = standard deviation.

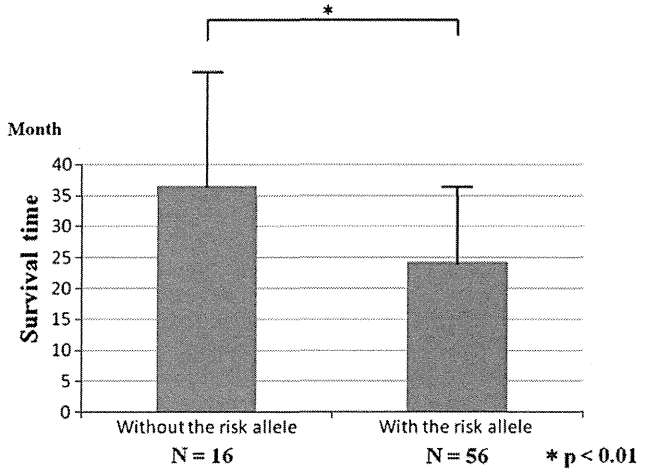

Fig. 1. Survival time for ALS patients: We could confirm the endpoints of 72 patients among the 176 analyzed patients. The mean survival time was 36.6 (±19.7) months in patients (N=16) without the risk allele and 24.3 (±13.8) months in those (N=56) with the risk allele (C allele; CC and CT genotypes). The mean survival time with the risk allele was shorter than the one without the risk allele, the difference being significant (*t*-test, $P<0.01$).

much lower than in Western populations [16]. Thus we considered that the *C9orf72* repeat expansion had not influenced our study and knowledge of the pattern of disease progression and survival time is of utmost importance for the clinical care of ALS patients and appropriately directed research on ALS. Evaluation of early prognostic variables and a proper understanding of "at first exam" factors related to survival may influence the selection of patient cohorts for clinical trials and identification of practical indicators that clinicians might find useful in the management of ALS patients. In this retrospective study of patients diagnosed as having ALS, we found that the ZNF512B gene was a prognostic factor influencing survival, independent of the sex, age at onset, time from onset to diagnosis, site (bulbar/spinal) of symptom onset and riluzole use [17]. This study revealed that the survival probability of patients with the risk allele (rs2275294, C allele) in the ZNF512B gene was significantly lower than that in those without the risk (C) allele.

The attempts to establish the genetic basis of survival for sALS by identifying susceptibility genes have had little success [18,19]. To date results from candidate gene studies have identified several

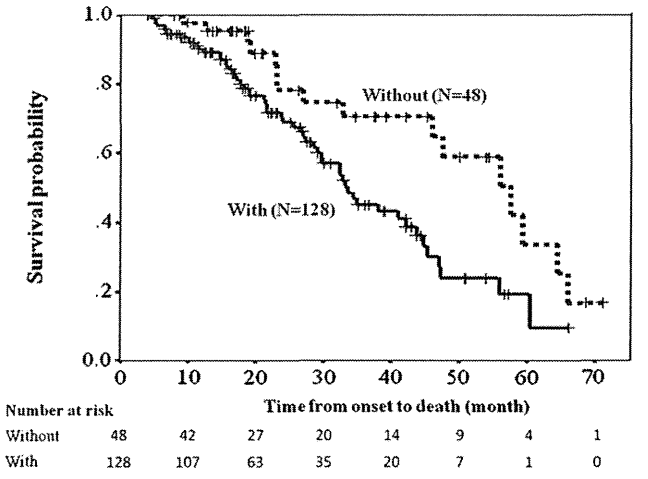

Fig. 2. Kaplan–Meier survival curves for ALS patients: Kaplan–Meier survival curves for ALS patients (N=48) without (broken line) versus those (N=128) with (solid line) the risk allele (C allele; CC and CT genotypes) for all 176 patients according to the rs2275294 C allele carrier status. Vertical bars indicate censured data. A significant difference was found between the two survival curves (log-rank test, $P<0.01$).

Table 2
Cox regression analysis.

Characteristic	Hazard ratio	95% CI	*P* value
The risk allele (C allele; CC/CT)	1.807	(1.018–3.209)	0.043[a]
Sex (male)	0.673	(0.403–1.125)	0.131
The age at onset	1.004	(1.016–1.072)	0.002[a]
The site of onset (bulbar)	1.716	(1.001–2.942)	0.049[a]
Riluzole (no)	1.333	(0.805–2.209)	0.264
The time from onset to diagnosis	0.960	(0.939–0.982)	<0.001[a]

CI is confidence interval.
[a] Significant covariates.

susceptibility genes [20], although the mechanism by which risk is conferred is not known [13]. But recently Chiò et al. reported that the SNP (rs12608932) located in the intron of the UNC13A gene associated with susceptibility to ALS significantly influenced survival in Italian ALS patients [21]; the mechanism of the effect of the UNC13A gene on ALS survival is still unclear. On the other hand we could indicate the mechanism of the ZNF512B gene on ALS survival as follows.

To date, studies on the ZNF512B gene and ALS have been based mainly on in vitro analysis results of studies conducted by Iida et al. [1]. However, our study indicated that the new SNP influenced the phenotype of ALS patients. Proteomics analysis has suggested that the protein encoded by the ZNF512B gene is a transcription factor that promotes the expression of a downstream gene in the signal transduction pathway of TGF-β [5], which is essential for the protection and survival of neurons [6–8], and several studies have reported elevated serum and plasma levels of TGF-β in ALS patients [22,23]. However, ZNF512B gene expression is reduced in ALS patients with the risk (C) allele of the new SNP, and their serum and plasma levels of TGF-β decrease, resulting in the weakening of their neuronal protection signals. Thus the survival probability of ALS patients with the risk (C) allele decreases (Fig. 3). On the basis of this hypothesis, development of therapies based on the neuroprotective ability of TGF-β has been expected. Day et al. conducted a study in which they administered intraperitoneal injections of TGF-β to a mouse model of ALS that carried a disease-related SOD1 mutation [24]; they demonstrated that the motor performance of the mouse model of ALS improved. Thus, their study revealed that systemic treatment with TGF-β may protect motor neurons from toxic protein insults in the short term, although the long-term effects of TGF-β on ALS have yet to be determined. The disruption of TGF-β signaling is an important molecular event in the pathogenesis of motor neuron diseases [25] and Nakamura et al. reported that activation of the TGF-β

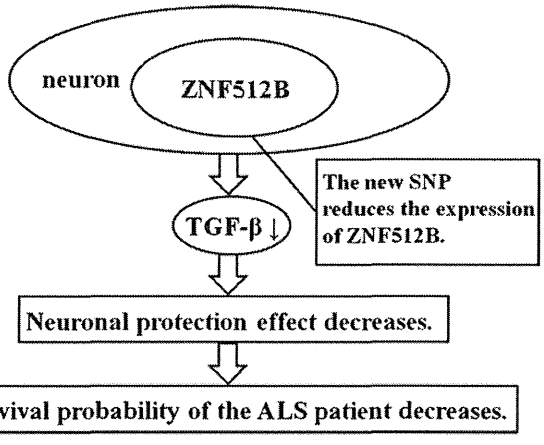

Fig. 3. The mechanism by which the ZNF512B gene might serve as a prognostic factor in ALS patients: In patients with the new SNP (rs2275294), the ZNF512B gene expression is reduced, which results in the weakening of the neuronal protection signals by TGF-β and, therefore, reduction in the survival probability of the ALS patients with the risk allele (C allele; CC and CT genotypes).

signaling system is protective against aggregate formation of cytoplasmically mislocalized TDP-43 and may be a potential therapeutic approach to delay the progression of ALS [26]. Our study might also indicate the beneficial effect of TGF-β for the protection and survival of neurons in actual ALS patients. Taking these reports together with our data, it may be reasonable to suggest that the ZNF512B gene may serve as a new clinical prognostic factor in ALS patients. Thus, it is very important to examine the ZNF512B gene in ALS patients for prognosis prediction and appropriate treatment selection.

In conclusion, an association between the SNP rs2275294 within the ZNF512B gene and survival in ALS has been confirmed. Our retrospective study revealed that the genotype of the ZNF512B gene is a very useful new prognostic factor in patients with ALS. This study is the first, as per our knowledge, to indicate that the association between the new susceptibility gene for ALS and its pathway could be identified and the findings may open the path to new insights concerning therapy for this devastating disease.

Conflict of interest

The authors have no conflict of interests to declare.

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References

[1] Iida A, Takahashi A, Kubo M, Saito S, Hosono N, Ohnishi Y, et al. A functional variant in ZNF512B is associated with susceptibility to amyotrophic lateral sclerosis in Japanese. Hum Mol Genet 2011;20:3684–92.
[2] Simpson CL, Al-Chalabi A. Amyotrophic lateral sclerosis as a complex genetic disease. Biochim Biophys Acta 2006;1762:973–85.
[3] Schymick JC, Talbot K, Traynor BJ. Genetics of sporadic, amyotrophic lateral sclerosis. Hum Mol Genet 2007;16:233–42.
[4] Iida A, Takahashi A, Deng M, Zhang Y, Wang J, Atsuta N, et al. Replication analysis of SNPs on 9p21.2 and 19p13.3 with amyotrophic lateral sclerosis in East Asians. Neurobiol Aging 2011;32:757e13–4.
[5] Colland F, Jacq X, Trouplin V, Mougin C, Groizeleau C, Hamburger A, et al. Functional proteomics mapping of a human signaling pathway. Genome Res 2004;14:1324–32.
[6] Henrich-Noack P, Prehn JH, Krieglstein J. Neuroprotective effects of TGF-beta 1. J Neural Transm Suppl 1994;43:33–45.
[7] Iwasaki Y, Shiojima T, Tagaya N, Kobayashi T, Kinoshita M. Effect of transforming growth factor beta 1 on spinal motor neurons after axotomy. J Neurol Sci 1997;147:9–12.
[8] Krieglstein K, Strelau J, Schober A, Sullivan A, Unsicker K. TGF-beta and the regulation of neuron survival and death. J Physiol Paris 2002;96:25–30.
[9] Brooks BR, Miller RG, Swash M, Munsat TL. El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. Amyotroph Lateral Scler Other Motor Neuron Disord 2000;1:293–9.
[10] Atsuta N, Watanabe H, Ito M, Tanaka F, Tamakoshi A, Nakano I, et al, Research Committee on the Neurodegenerative Diseases of Japan. Age at onset influences on wide-ranged clinical features of sporadic amyotrophic lateral sclerosis. J Neurol Sci 2009;276:163–9.
[11] Czaplinski A, Yen AA, Appel SH. Amyotrophic lateral sclerosis: early predictors of prolonged survival. J Neurol 2006;253:1428–36.
[12] Talbot K. Motor neuron disease: the bare essentials. Pract Neurol 2009;9:303–9.
[13] del Aguila MA, Longstreth Jr WT, McGuire V, Koepsell TD, van Belle G. Prognosis in amyotrophic lateral sclerosis: a population-based study. Neurology 2003;60:813–9.
[14] Kiernan MC, Vucic S, Cheah BC, Turner MR, Eisen A, Hardiman O, et al. Amyotrophic lateral sclerosis. Lancet 2011;377:942–55.
[15] Byrne S, Elamin M, Bede P, Shatunov A, Walsh C, Corr B, et al. Cognitive and clinical characteristics of patients with amyotrophic lateral sclerosis carrying a C9orf72 repeat expansion: a population-based cohort study. Lancet Neurol 2012;11:232–40.
[16] Ogaki K, Li Y, Atsuta N, Tomiyama H, Funayama M, Watanabe H, et al, Japanese Consortium for Amyotrophic Lateral Sclerosis research (JaCALS). Analysis of C9orf72 repeat expansion in 563 Japanese patients with amyotrophic lateral sclerosis. Neurobiol Aging 2012;33:e11-6 [2527].
[17] Lacomblez L, Bensimon G, Leigh PN, Guillet P, Meininger V. Dose-ranging study of riluzole in amyotrophic lateral sclerosis. Amyotrophic Lateral Sclerosis/Riluzole Study Group II. Lancet 1996;347:1425–31.
[18] Traynor BJ, Nalls M, Lai SL, Gibbs RJ, Schymick JC, Arepalli S, et al. Kinesin-associated protein 3 (KIFAP3) has no effect on survival in a population-based cohort of ALS patients. Proc Natl Acad Sci U S A 2010;107:12335–8.
[19] Orsetti V, Pegoraro E, Cima V, D'Ascenzo C, Palmieri A, Querin G, et al. Genetic variation in KIFAP3 is associated with an upper motor neuron-predominant phenotype in amyotrophic lateral sclerosis. Neurodegener Dis 2011;8:491–5.
[20] Beleza-Meireles A, Al-Chalabi A. Genetic studies of amyotrophic lateral sclerosis: controversies and perspectives. Amyotroph Lateral Scler 2009;10:1–14.
[21] Chiò A, Mora G, Restagno G, Brunetti M, Ossola I, Barberis M, et al. UNC13A influences survival in Italian amyotrophic lateral sclerosis patients: a population-based study. Neurobiol Aging 2013;34:e1–5 [357].
[22] Houi K, Kobayashi T, Kato S, Mochio S, Inoue K. Increased plasma TGF-beta1 in patients with amyotrophic lateral sclerosis. Acta Neurol Scand 2002;106:299–301.
[23] Ilzecka J, Stelmasiak Z, Dobosz B. Transforming growth factor-beta 1 (tgf-beta 1) in patients with amyotrophic lateral sclerosis. Cytokine 2002;20:239–43.
[24] Day WA, Koishi K, Nukuda H, McLennan IS. Transforming growth factor-beta 2 causes an acute improvement in the motor performance of transgenic ALS mice. Neurobiol Dis 2005;19:323–30.
[25] Katsuno M, Adachi H, Banno H, Suzuki K, Tanaka F, Sobue G. Transforming growth factor-β signaling in motor neuron diseases. Curr Mol Med 2011;11:48–56.
[26] Nakamura M, Kaneko S, Ito H, Jiang S, Fujita K, Wate R, et al. Activation of transforming growth factor-β/Smad signaling reduces aggregate formation of mislocalized TAR DNA-binding protein-43. Neurodegener Dis 2012;10.

A homozygous mutation of *C12orf65* causes spastic paraplegia with optic atrophy and neuropathy (SPG55)

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ABSTRACT

Background Autosomal recessive hereditary spastic paraplegias (AR-HSP) constitute a heterogeneous group of neurodegenerative diseases involving pyramidal tracts dysfunction. The genes responsible for many types of AR-HSPs remain unknown. We attempted to identify the gene responsible for AR-HSP with optic atrophy and neuropathy.

Methods The present study involved two patients in a consanguineous Japanese family. Neurologic examination and DNA analysis were performed for both patients, and a skin biopsy for one. We performed genome-wide linkage analysis involving single nucleotide polymorphism arrays, copy-number variation analysis, and exome sequencing. To clarify the mitochondrial functional alteration resulting from the identified mutation, we performed immunoblot analysis, mitochondrial protein synthesis assaying, blue native polyacrylamide gel electrophoresis (BN-PAGE) analysis, and respiratory enzyme activity assaying of cultured fibroblasts of the patient and a control.

Results We identified a homozygous nonsense mutation (c.394C>T, p.R132X) in *C12orf65* in the two patients in this family. This *C12orf65* mutation was not found in 74 Japanese AR-HSP index patients without any mutations in previously known HSP genes. This mutation resulted in marked reduction of mitochondrial protein synthesis, followed by functional and structural defects in respiratory complexes I and IV.

Conclusions This novel nonsense mutation in *C12orf65* could cause AR-HSP with optic atrophy and neuropathy, resulting in a premature stop codon. The truncated *C12orf65* protein must lead to a defect in mitochondrial protein synthesis and a reduction in the respiratory complex enzyme activity. Thus, dysfunction of mitochondrial translation could be one of the pathogenic mechanisms underlying HSPs.

INTRODUCTION

Hereditary spastic paraplegias (HSPs) comprise a large and heterogeneous group of genetic disorders mainly affecting the pyramidal tracts of the legs. The cardinal pathological findings in HSPs are the result of a dying back degeneration of the corticospinal tracts in the spinal cord. The longest fibres, innervating the lower extremities are mostly affected. HSPs are divided into two subtypes that comprise pure and complex forms. The pure form of HSP is characterised by progressive bilateral leg spasticity, weakness, exaggerated tendon reflexes and positive pathological reflexes, whereas the complex form of HSP shows the following additional symptoms: peripheral neuropathy, cerebellar atrophy, thin corpus callosum, optic atrophy, retinal degeneration, mental impairment, convulsions and extrapyramidal signs.[1]

HSPs can be inherited in an autosomal-dominant (AD), autosomal-recessive (AR) or X-linked recessive (XR) manner. To date, at least 52 spastic paraplegia gene (SPG) loci have been assigned, and approximately 30 genes have been identified. The pure form is usually transmitted as an AD trait, whereas the complex form is transmitted as an AR or XR one. The most common AD-HSP is SPG4 with the *spastin* gene mutation, accounting for 40–45% of AD-HSP.[2] Meanwhile, the most frequent AR-HSP might be SPG11 with the *spatacsin* gene mutation, showing a complex phenotype including dementia and thin corpus callosum.[3] The genes most responsible for AR-HSPs, however, remain unknown.

Several pathogenic mechanisms underlying HSPs have been suggested. HSPs might result from disruption of the axonal transport of molecules, organelles and other cargos, which mainly affects the distal parts of motor neurones.[4] Axonal transport might be impaired by mutations of the *SPAST* gene[5] and kinesin heavy chain *KIF5A*.[6] An animal model of spastin deletion shows progressive axonal degeneration restricted to the central nervous system leading to a late and mild motor defect. The degenerative process is characterised by focal axonal swelling associated with abnormal accumulation of organelles and cytoskeletal components.[7] The intracellular transport of molecules and organelles to and from nerve terminals also depends on the mitochondrial function.

An abnormal mitochondrial function also leads to several HSPs: SPG7 with the *paraplegin* gene mutation and SPG13 with the heat-shock protein 60 (*HSPD1*) one. For instance, paraplegin is a part of the metalo-protease AAA (ATPases associated with diverse cellular activities) complex with AFG3L2,[8] an ATP-dependent proteolytic complex located at the mitochondrial inner membrane, which controls protein quality and regulates ribosome assembly.[9] A homozygous mutation of *AFG3L2* also leads to spastic ataxia-neuropathy syndrome (SPAX-5 in OMIM).[10] Paraplegin-deficient mice are affected by distal axonopathy of spinal and peripheral axons, characterised by axonal swelling and degeneration caused by

massive accumulation of organelles and neurofilaments, similar to those observed in the animal model of spastin deletion.[11]

Here, we report a novel homozygous nonsense mutation in the chromosome 12 open reading frame 65 (*C12orf65*) gene in patients with AR-HSP with optic atrophy and neuropathy found on linkage analysis involving single nucleotide polymorphism (SNP) and exome sequencing. Furthermore, we revealed that this mutation led to a mitochondrial translation dysfunction in a patient (proband). This is the first report that a *C12orf65* mutation causes HSP.

PATIENTS AND METHODS

Patients

The present study involved two patients from a family with spastic paraplegia, optic atrophy and peripheral neuropathy described elsewhere.[12] The family pedigree is shown in figure 1A. The parents were first cousins. Two affected (IV-3 and 6) and three unaffected members (III-1, 2 and IV-4) of the family underwent neurological examinations and nerve conduction studies, except for III-2 and IV-4.[12] Presently, the unaffected members are all deceased.

Linkage analysis

Genomic DNA was extracted from blood samples from the two affected individuals (IV-3 and 6) with written informed consent (figure 1A), and then multipoint parametric linkage analysis involving a SNP high-throughput linkage analysis system (SNP HiTLink) was performed.[13] With this system, SNP chip data for the Mapping 100 k/500 k array set and Genome-Wide Human SNP array 6.0 (Affymetrix, Santa Clara, California, USA) can be directly imported and passed to a multipoint parametric linkage analysis programme Allegro.[14] Parametric LOD scores were calculated using Allegro V.2 with the parameter setting of an AR model with 100% penetrance.

Copy-number variation detection

We performed array-based comparative genomic hybridisation (aCGH) analysis for copy-number alteration detection in the candidate gene areas. We developed custom aCGH arrays against the candidate areas in the four chromosomes using a Human Genome CGH Microarray Kit 244K (Agilent Technologies, Santa Clara, California, USA) according to the manufacturer's protocol.[15 16] We made one CGH probe every 160 bp on average against the candidate gene areas.

Exome sequencing

We collected a blood sample from one affected individual (IV-6) and performed massively parallel sequencing. Genomic DNA was extracted from leukocytes from the case and then sheared. An adaptor-ligated library was prepared and clustered on the cBOT system (Illumina, San Diego, California, USA). Exon capture was performed with a SureSelect Human All Exon kit (Agilent). Paired-end sequencing was carried out on an Illumina Hiseq 2000 that generated 91-bp reads. For sequence alignment, variant calling and annotation, the sequences were aligned with the human genome reference sequence (hg19 build) using a Burrows-Wheeler Aligner. Substitution calling was carried out with a Genome analysis tool kit (GATK). SNP calls were made with a GATK Unified Genotyper, and indel calls were made with a GATK IndelGenotyper V2. SNP calling was performed with reference to dbSNP131 and dbSNP134. All variants were annotated with reference to consensus coding sequences (CCDS) (NCBI release 20090902) and RefSeq (UCSC dumped 20101004).

Sanger sequencing

The coding exons and flanking intronic sequences of *C12orf65* were amplified using the genomic DNA of the patients, and using an Marshall Scientific (MS) Research Thermal Cycler. The primer sequences were as follows: Ex2-F: aac atg gca gac agt gca ag, Ex2-R: ggc tga tcc cat tca cac tt, Ex3-F: ttc tga ggt cct gtc cat ttt t, and Ex3-R: gcc cag ccg agt ttt att ct. Sanger sequencing was performed according to an established standard protocol on an Applied Biosystems (ABI) 3730 capillary sequencer (Applied Biosystems, Carlsbad, California, USA). We sought additional *C12orf65* mutations by Sanger sequencing of the coding regions of two exons and their flanking sequences of *C12orf65* in 74 index Japanese AR-HSP patients without known HSP gene (SPG1/2/3A/4/5/6/7/8/10/11/13/17/20/21/31/33) substitutions established by the Japan Spastic Paraplegia Research Consortium, and one case in a family with Charcot–Marie–Tooth disease (CMT) and optic atrophy with AR transmission. Genomic DNA samples from 200 Japanese subjects without apparent neurologic disorders were also analysed as controls.

Immunoblotting

Mitochondria were isolated from primary fibloblasts from the patient and two controls. Mitochondrial protein (10 μg per lane) was fractionated by 12% NuPAGE Gel (Life Technologies) and transferred to polyvinylidene fluoride (PVDF) membranes. The filters were preincubated for 1 h with Block One (Nacalai), followed by incubation for 1 h with Can Get Signal solution 1 (Toyobo) containing C12orf65 antibodies (Abcam) or voltage dependent anion channel (VDAC) antibodies (MitoSciences). The filters were washed four times with phosphate-buffered saline (PBS) containing 0.1% Tween 20, incubated for 1 h with Can Get Signal solution 2 (Toyobo) containing horseradish peroxidase-conjugated antirabbit or mouse immunoglobuline G (igG) (GE Healthcare), and then washed with PBS containing 0.1% Tween 20. Protein bands were visualised using enhanced chemiluminescence (ECL) prime western blotting reagents (GE Healthcare).

Mitochondrial protein synthesis assaying[17]

The patient's (IV-6) skin fibroblasts were obtained by skin biopsy with informed consent. Fibroblasts derived from two non-mitochondrial disease cases were used as controls. (^{35}S) Methionine, cysteine incorporation into mitochondrially encoded proteins was analysed essentially as described previously,[18 19] using the patient's and control fibroblasts in culture. Semiconfluent cells in 6-well plates were labelled with (^{35}S) EXPRESS protein labelling mix (Perkin Elmer) for 30 min, in the presence of emetine (0.1 mg/ml) in methionine-free DMEM supplemented with dialysed 10% fetal bovine serum (FBS). The cells were lysed in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 1% sodium dodecyl sulfate (SDS). Total cellular proteins (10 μg/lane) were fractionated by 15–20% gradient SDS-PAGE. The gel was stained with Quick-coomassie brilliant blue (CBB) PLUS (Wako) and dried. The gel was exposed to an imaging plate, and the labelled polypeptides were located with a bioimaging analyser (BAS3000, Fuji Photo Film).

Blue native polyacrylamide gel electrophoresis (BN-PAGE) and western blotting for immunodetection[20]

Mitochondrial proteins were isolated from cultured fibroblasts from patient IV-6.[21] A mixture of mitochondrial proteins from 10 non-mitochondrial disease cases was used as a control. For the detection of individual complexes, the mitochondrial fraction (20 μg protein) was solubilised with 0.5% (w/v)

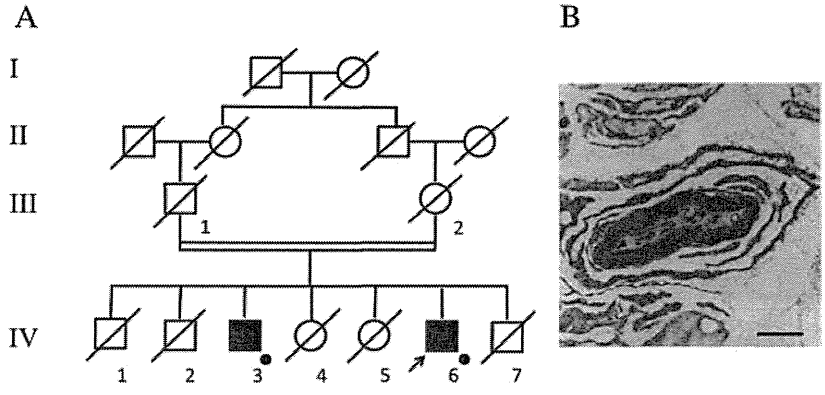

Figure 1 Family pedigree and sural nerve biopsy findings. (A) Family pedigree. The proband (IV-6) and his older brother (IV-3) show the same clinical phenotype. Other siblings are all deceased. The parents (III-1 and 2), who were first cousins, were neurologically asymptomatic before their death. Filled squares, affected males; open squares, unaffected males; open circles, unaffected females; slashes, deceased; arrow, proband; dots, examined subjects. (B) Sural nerve biopsy findings. An electron microscopic image of a sural nerve from the proband revealed the formation of an onion bulb-like structure. Bar, 2 μm.

n-dodecyl-β-D-maltoside, and for the detection of supercomplexes, the mitochondrial fraction (30 μg protein) was solubilised with 1% (w/v) digitonin, respectively. Electrophoresis was performed on 3–12% gradient polyacrylamide gels (Invitrogen).[21 22] Following BN-PAGE, the gels were blotted onto polyvinylidene fluoride membranes using an iBlot transfer system (Invitrogen). Subunit-specific primary antibodies were used to immunodetect protein complexes. The cocktail of primary antibodies comprised NDUFA9 (complex I, Invitrogen) (2.0 μg/ml), SDHA (complex II, Invitrogen) (0.02 μg/ml), UQCRC2 (complex III, Abcam) (0.2 μg/ml), MTCO1 (complex IV, Invitrogen) (0.2 μg/ml), and ATP5B (complex V, Invitrogen) (2.0 μg/ml). After removing the cocktail of primary antibodies, alkaline phosphatase-conjugated secondary antibodies were added, and then chemiluminescent detection was performed with a bioimaging system (LAS4000 mini, GE Healthcare).

Enzymatic activity of respiratory chain complexes[20]

The enzymatic activity of individual mitochondrial respiratory complexes was determined using the mitochondrial fractions (1 μg protein) isolated from cultured fibroblasts from patient IV-6 and 10 controls according to Trounce *et al*[23] with modifications. The activities of complexes I, II, III and IV were measured using a multiwell plate reader spectrophotometric system (SPECTROstar Nano, BMG Labtech). Citrate synthase (CS) activity was used for normalisation. All measurements were performed in triplicate and averaged.

RESULTS

Clinical features

The proband (IV-6) was a 32-year-old man who was admitted to our hospital for evaluation of slowly progressive weakness of the lower extremities and decreased visual acuity. He noticed the reduced visual acuity at age 7 years. At about age 10 years, his leg weakness and drop feet led to a steppage gait. He could not execute fine finger movement at age 16 years. On ophthalmologic examination, he exhibited 20/100 vision in the right eye and 20/200 in the left one. Fundoscopic examination demonstrated bilateral optic atrophy with central scotoma. On neurologic examination, bilateral leg spasticity, and anterior tibial muscle weakness and atrophy were noted. Mild distal arm weakness without atrophy was observed. Tendon reflexes,

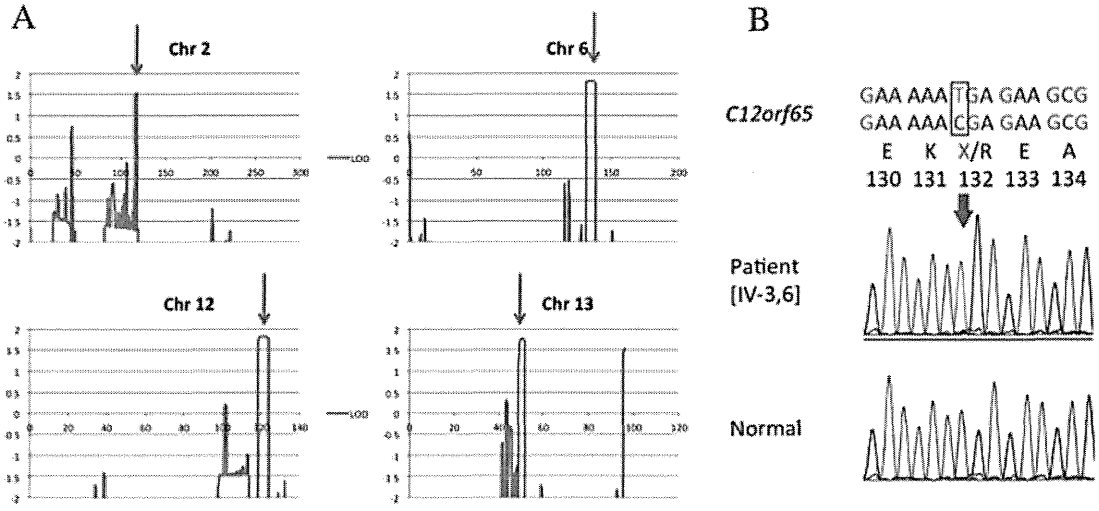

Figure 2 Linkage analysis and mutation of *C12orf65* in the proband. (A) Linkage analysis. Linkage analysis involving single nucleotide polymorphisms revealed the highest lod scores (about 1.8) in parts of chromosomes 2, 6, 12 and 13 (arrows). These four areas were thought to be candidate areas in which the causative gene was located. (B) Mutation of *C12orf65* in the proband. Sanger sequencing confirmed the homozygous nonsense mutation (c.394C>T, p.R132X9) of the C12orf65 gene identified in the proband (IV-6). The affected brother (IV-3) showed the same mutation.

except for normal ankle jerks, were exaggerated in all extremities. Bilateral planter responses were flexion. Superficial and vibratory sensations were diminished in the distal legs with a glove and stocking distribution and preserved position sense. The results of blood and cerebrospinal fluid examinations were within normal ranges. The urine organic acid pattern was normal. EEG, ECG and brain CT showed no remarkable findings. A nerve conduction study disclosed mild decreases of motor and sensory nerve conduction velocities in the upper extremities, but they were not evoked in the lower extremities. High-amplitude NMUs, and a reduced number of NMUs, were observed in the extremities on needle electromyography (EMG). Motor-evoked potential examination revealed prolongation of the central motor conduction times in the corticospinal tracts. Microscopic examination of a muscle biopsy specimen showed grouped atrophy in the tibialis anterior muscle. A sural nerve biopsy revealed a decreased number of nerve fibres of large diameter, the formation of an onion bulb-like structure, and endoneural fibrosis (figure 1B).

The second case (IV-3), a brother of the proband, exhibited essentially the same clinical phenotype. He noticed the visual difficulty at age 7 years, the onset of slowly progressive muscle atrophy in the lower extremities at age 10 years, and pes equinovarus deformities at age 12 years. Neurologic examination at age 42 years showed bilateral optic atrophy with central scotomas and diminished visual acuity (10/200 on right and 16/200 on left). In the lower extremities, marked muscular atrophy, spasticity and pes equinovarus were noted. Deep tendon reflexes were exaggerated in all extremities with left patellar clonus and extensor planter reflexes bilaterally. Superficial sensation and vibration were diminished in a glove and stocking distribution. Position sense was normal. Needle EMG showed neurogenic patterns. A nerve-conduction study disclosed slight decreases in motor nerve conduction velocities and normal sensory nerve conduction velocities in the upper extremities, but they were not evoked in the lower extremities.

Identification of candidate chromosome areas

We found linkages, that were not statistically significant, to chromosomes 2 (rs116837089-rs118552355), 6 (rs131535042-rs138306536), 12 (rs119856515-rs125250432) and 13 (rs67990237-rs70707721), with maximum cumulative logarithm of the odds (LOD) scores of 1.8 (figure 2A). Array CGH analysis revealed no pathological copy-number alterations in the four candidate chromosome areas. These four areas contained neither previously identified HSP loci nor CMT loci.

Exome sequencing allowed identification of the candidate gene substitutions

The presence of consanguinity, and the fact that the parents appeared asymptomatic, suggested that the patients had homozygous disease-causing mutations, and therefore, a homozygous autosomal-recessive model was applied. Exome sequencing covered 98.65% of the target region, and the average sequence depth on target was 41.47.

We identified three homozygous, non-synonymous single nucleotide variants (c.394C>T (p.R132X) in *C12orf65*, c.136G>A (p.E46K) in *COQ5*, and c.6599T>G (p.I2200S) in *KNTC1*) in the chromosome 12 candidate area with reference to dbSNP131. No such variations were detected in the chromosome 2, 6 and 13 areas. We could subsequently exclude the two candidate gene mutations in *COQ5* and *KNTC1* as benign polymorphisms, because the two variants had been registered as SNPs to the dbSNP134 and 1000 genomes (rs139585780: c.136G>A in *COQ5*, A allele frequency=0.0112 and rs140880563: c.6599T>G in *KNTC1*, G allele frequency=0.0056 in Japanese). The remaining candidate gene, *C12orf65*, was confirmed to have a homozygous nonsense mutation (c.394 C>T, p.R132X) on Sanger sequencing in the two patients, IV-3 and 6 (figure 2B). This nonsense mutation was not found in 200 Japanese control genomic DNAs.

Mitochondrial respiratory function is impaired by the mutation in *C12orf65*

Very recently, two homozygous *C12orf65* 1 bp deletion mutations were identified in patients with Leigh syndrome, optic atrophy and ophthalmoplegia.[24] The C12orf65 protein shows high sequence similarity to mitochondrial class I peptide release factors (RFs), and it has been reported that mutations in *C12orf65* cause a mitochondrial translation defect. Analysis of the assembly of mitochondrial phosphorylation complexes showed decreases of complexes I, III, IV and V. This disease entity is called combined oxidative phosphorylation deficiency 7 (COXPD7) (online Mendelian inheritance in man (OMIM) #613559). To determine how this nonsense mutation affects the mitochondrial function, we first performed immunoblot analysis and mitochondrial protein synthesis assaying of patient fibroblasts. Immunoblot analysis showed that a smaller C12orf65 protein was generated in the patient's fibroblasts than the wild-type protein in controls (figure 3A). Marked reductions of the synthesised polypeptides were observed in patient IV-6 compared with those in controls (16% for COX I and COX II, figure 3B), implying the aberrant statuses of respiratory complexes. Actually, complexes I and IV were severely impaired in their enzymatic functions (29% and 13%, respectively, figure 4A), and holoenzyme structures (the average value for two experiments, 17% and 23%, respectively, figure 4B) in patient IV-6, probably due to the mitochondrial translation defect, as shown in figure 3B. Furthermore, the amounts of respiratory supercomplexes that consist of complexes I, III and IV were also significantly reduced in patient IV-6 (about 30%, figure 4B). Thus, the disrupted protein integrity (function and structure) of complexes I and IV, which is closely associated with the aberrant mitochondrial bioenergetics in patient IV-6, would constitute the molecular pathogenicity in a patient carrying a mutation in *C12orf65*.

DISCUSSION

In the present study, we identified a novel *C12orf65* nonsense mutation (c.394C>T, p.R132X) in patients with spastic paraplegia with optic atrophy and neuropathy with AR inheritance (SPG55). The possibility remains that there is another undetected mutation in the four candidate chromosome areas because the exome sequencing did not cover all exons in the areas. However, our patients carried the nonsense mutation of *C12orf65* that had been identified as the causative gene for the neurological disorder COXPD7. In COXPD7, it had been reported that deletion mutations of *C12orf65* lead to a decrease in mitochondrial translation and combined oxidative phosphorylation (OXPHOS) deficiencies. In the present study, we clearly demonstrated that a nonsense mutation of *C12orf65* led to a mitochondrial protein synthesis defect and respiratory complex enzyme activity reduction, similarly. Thus, the spastic paraplegia phenotype in our patients could be a novel and distinct clinical entity based on the *C12orf65* mutation.

The C12orf65 protein is considered to belong to the mitochondrial class I peptide RFs.[24] This protein is a soluble matrix, one that is not coprecipitated with mitochondrial ribosomes,[25]

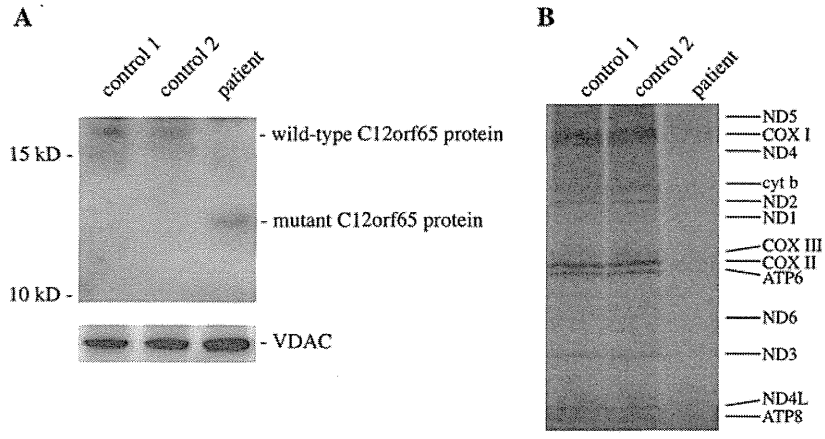

Figure 3 Immunoblot analysis of the patient's fibroblasts and mitochondrial protein synthesis assay. (A) Immunoblot analysis of the patient's fibroblasts. Immunoblot analysis of the C12orf65 protein in two controls and the patient's fibroblasts. Mitochondrial protein extracts (10 μg) were fractionated, transferred to PVDF filters, and then probed against C12orf65. VDAC was used as a loading control. Immunoblot analysis showed that a smaller C12orf65 protein (about 13 kDa) was generated in the patient's fibroblasts than the wild-type proteins (about 17 kDa) in controls. (B) Mitochondrial protein synthesis assay. Fibroblasts from a patient (IV-6) and two controls were labelled with (^{35}S) EXPRESS protein labelling mixing, and incorporated into mitochondrial encoded proteins. The fibroblasts were cultured with containing medium in the presence of emetine, as a cytosolic translation inhibitor. Total cell proteins (10 μg) were fractionated by 15–20% gradient SDS-PAGE and visualised by autoradiography. The autographic image shows the marked reduction of mitochondrial DNA translation in the patient fibroblasts compared with that in control fibroblasts. The synthesised polypeptides of combined oxidative I and II in the patient fibroblasts were reduced to 16% in density compared with those in the control fibroblasts.

and that has no peptidyl-tRNA hydrolase activity.[24] Therefore, although the actual function of C12orf65 is still not clear, it might play a role in recycling abortive peptidyl-tRNAs that have been prematurely released from ribosomes during polypeptide elongation. The nuclear C12orf65 gene comprises three exons, and its protein-coding region comprises 501 bp derived from its exons 2 and 3. The C12orf65 protein consists of 166 amino acids that include a RF-1 domain (amino acid numbers 53–146) and a glycine-glycine-glutamine (GGQ) motif (amino acid numbers 71–73). Our patients had a homozygous nonsense mutation (c.394 C>T) in exon 3, that is predicted to result in a stop codon at 132. Previously reported COXPD7 patients had two homozygous deletion mutations (c.210delA and c.248delT), and both mutations resulted in a premature stop codon at 84 in exon 2.[24]

The RT-PCR product derived from the *C12orf65* mRNA was not significantly reduced in COXPD7 patients[24] or our patients compared with that in normal controls (data not shown). We suggest that truncated C12orf65 proteins were generated in these COXPD7 and our patients (figure 3A). COXPD7 and our patients share a few clinical symptoms, that is, optic atrophy and peripheral neuropathy. We consider that our patients could be included in COXPD7 with a different clinical phenotype. However, the cardinal clinical feature in COXPD7 patients is Leigh syndrome, while that in our patients is spastic paraplegia. The former shows a more severe phenotype than the latter. We found that our patient has a relatively larger C12orf65 protein (131 amino acids) than that in COXPD7 cases (83 amino acids). Moreover, COXPD7 patient's fibroblasts have been reported to exhibit severe decreases in complexes I, IV and V. Meanwhile, our patient's fibroblasts showed decreases in complexes I and IV, and a milder decrease in complex V. In several reports, patients with mtDNA translation defects were found to be associated with an unaffected amount of complex III or a milder decrease than of other complexes.[26–29] In the case of COXPD7 patient's fibroblasts, there was a milder decrease in complex III, while our patient's fibroblasts had an unaffected amount of complex III. Therefore, our patients might have a preserved C12orf65 protein function compared with that in COXPD7.

A study involving cultured neurones showed a complex I deficiency could increase mitochondrial reactive oxygen species (ROS) production, and increase neuronal death attenuated by ROS scavengers.[30] Accumulated intracellular oxidative damage to neurones could be a causative mechanism for neurodegeneration caused by mitochondrial respiratory deficiencies.

To date, several HSP genes related to mitochondria have been identified: *paraplegin* and *HSPD1*. The SPG31 protein, receptor expression-enhancing protein 1 (REEP1), coordinates ER shaping and microtubule dynamics,[31] although another report has proposed that REEP1 is a mitochondrial protein.[32] SPG7 is an AR-complicated HSP resulting from *paraplegin* gene mutations. The clinical features of SPG7 are varied with associated symptoms: cerebellar signs, optic atrophy, distal amyotrophy