るし、斉藤（1972）は『病院・医院』という著書において『要綱』を基に原価計算を解説しているだけである。さらに森（1969）も、『要綱』の方法をベースとしつつ部門別原価計算をさらに診療科別原価計算に展開すべきことを論じているだけである。

本格的な理論的研究をしている研究者は限られており、病院原価計算について体系的に研究した山元昌之氏（山元，1944；1957a,b；1958a,b；1967他）と神馬新七郎・神馬駿逸両氏（神馬，1955a,b；神馬・神馬，1964；神馬，1964；1965；1975a；1975b）、診療報酬設定との関係で病院原価計算について研究した染谷恭次郎氏（染谷，1956；1961；宮沢・染谷・勝沼，1961）くらいに限定される。山元氏は英米における研究を紹介しつつ、主にイギリスにおける研究成果を基に日本病院協会の『要綱』を検討し、賛成及び改善提案を行っている。一方、両神馬氏は英米の研究を踏まえたうえで、主にアメリカの研究成果を基に日本における病院原価計算を研究した。この他に、旧病院管理研修所の研究員である石原信吾氏も理論的な考察を行い、経営管理目的の違いにより実施すべき原価計算の種類が違うことを指摘し、その理解不足と各種類の原価計算の利用方法に関する知識不足が、原価計算が期待されながら結局普及していかない理由であると指摘している（石原，1956a；1956b；1957a；1957b）。

3.2 病院実務家らによる実践研究

本節では、実務者らによる病院管理研究の発表の中心となってきた雑誌『病院』注(4)に掲載された病院実務家による原価計算実践報告を基に、『要綱』発表以降の実態を明らかにする。

まず武蔵野日赤病院では、『要綱』発表後、何度か『要綱』にしたがって原価計算を実施した（神崎，1963）。武蔵野日赤病院事務部長の森（1966）は、病院の設立・拡張当初から病院が企業体であるという考え方が徹底しないために、原価計算作業に相当の時間と労力を費やすが、その計算結果の正確性は低いと述べている。また森（1966）は、『要綱』による原価計算は全部原価計算であり、われわれの欲する財務的情報を全部提供してくれるわけではない、とその限界も指摘していた。

また『要綱』発表以降、消毒・洗濯・仕上処理サービス（橋本，1956a）、完全寝具サービス（橋本，1956b）、理学的治療・温泉治療サービス（大内，1961）、をそれぞれ対象分野とした給付別原価計算が実施報告されている。国立姫路病院家政係である橋本氏は、消毒・洗濯・仕上げの各過程において、処理対象物の重量を等価係数とした等級別原価計算を特殊原価調査的な実際原価計算として実施している。また完全寝具サービスにかかる原価の計算も特殊原価調査的な実際原価計算として実施し、採算性の検討をしている。一方、国立別府病院理学療法科医長である大内氏は、温泉治療・理学的治療の治療別原価計算を特殊原価調査として実施している。

またこの時期においては、多くの病院では原価計算を基本的には診療報酬改定のための資料として理解していたように思われる。たとえば大内（1961）は、温泉治療・理学的治療の原価計算を特殊原価調査として実施し、その結果を報告することにより診療報酬改定の参考に供したいと述べていた。また武蔵野日赤病院長の神崎（1963）は、原価計算をするのは、病院経営の危機を一般に納得させ診療報酬の引き上げを求めるには、抽象的に危機を訴えるのではだめで、数字を示す以外にないからであると主張していた。また石原（1957a）も、従来から原価計算の用途は診療単価決定資料としてしか理解

されないことが多かったため、診療単価改訂の要望が高まってくるにつれて病院で熱心に原価計算が行われるが、そうした気運が過ぎると関心は大抵いつのまにか薄れてしまっていたと述べていた。

このように『要綱』公表後、病院における原価計算実践は盛んになったが、部門別原価計算であれ、給付別原価計算であれ、特殊原価調査として実施されたものであり、経常的な原価計算として実施されたものではなかった。またその多くは診療報酬改定を目的としたものであり、経営管理目的としては十分に利用されていなかった。だからこそ、経常的な原価計算は不要で、特殊原価調査だけが行われていたのだと考えることもできる。

3.3　厚生省及び病院団体の調査

『要綱』公表後まず1960年には、厚生省が病院経営実態調査のなかで、協力病院に部門別原価計算調査を実施してもらっている。また1966年には全国公私病院連盟が病院経営実態調査の一環として、1967年には中央社会保険医療協議会が、部門別原価計算調査を実施している。その後も1973年に日本病院協会と全国自治体病院協議会が協力して、部門別原価計算調査を実施した。さらに1975年には、全国公私病院連盟が全国自治体病院協議会、日本病院会の協力の下に、部門別原価計算調査を実施し、その後3年ごとに継続していた（病院・医院経営実務研究会, 1990）。

このような調査は数年に一度であったが、調査協力病院を中心に部門別原価計算への関心を高める効果を持ったと考えられる。しかしそれとともに、厚生省『試案』で当初目標とされた診療行為別原価計算は忘れ去られる傾向を生んだ。またこれらの調査は、診療報酬適正化を主要目的としているにも関わらず、診療報酬点数（収益）比による原価配賦が多く見られ、目的適合性を欠いたものとなっていた。加えて、これら調査での収益比配賦の多用は、各病院での原価計算実務に影響を与えたと考えられる。

4.　関心が薄れた停滞期：1970年代末まで

病院原価計算に関するさかんな理論的及び実践的研究は1960年代まで続いたが、1970年代になるとほとんど見られなくなってしまう。1970年代には雑誌『病院』上、病院団体などによる部門別原価計算調査報告以外には、個々の病院の実務家らによる実践報告はまったく見られなくなった。この背景にはいくつかの理由がある。1つの理由は、1960年代以降，国民皆保険の達成と順調な経済成長を背景として医療需要が着実に伸びつづけ，1970年代には病院経営が安定してきたことがある。また採算が悪化した場合には，原価側に問題があるのではなく診療報酬が低いためであるとして医療界は診療報酬の引き上げを要求し、日本経済の高成長を背景に比較的容易に引き上げを実現してきたからである。

しかし病院界における原価計算の展開自体にも，あまり関心が持たれなくなっていった理由がある。その1つの理由は，病院団体などによる調査が定着し，部門別原価計算が特殊原価調査としてではあるが、一定の普及を見たことである。また当時の原価計算の第一義的な目的は診療報酬改定目的であり、経営管理目的はあまり意識されていなかったことから，病院団体による調査の定着で、個々の病院が原価計算を実施する意義が薄くなったと認識されたことも理由の1つである。

5. 実務界における関心の再燃期：1990年代半ばまで

1980年代に入り財政悪化から診療報酬が徐々に制限されるようになり、病院経営の危機が迫ってくるにつれて、一方では診療報酬改定を目的として、また一方で現実には診療報酬の大幅な改善は期待できないことからいよいよ経営管理を目的として、病院実務界において再び原価計算が注目されるようになってきた。また病院実務界における原価計算に対する関心の再燃の背景には、1980年代を通じた病院へのコンピュータの導入の本格化と、とりわけ1990年代に入ってからの統合的な病院情報システムの発達という、原価計算の経済的な実施可能性を高める病院情報処理環境の飛躍的な改善がある。

まず、1980年代に入ると、診療報酬改定を目的とした従来型の病院原価計算に関する実践研究が再び見られるようになった。南大和病院院長である池田（1982）は、診療報酬点数の低さに対する外科系臨床医の不満が大きいことから手術室の原価計算を試み、外科手術報酬が妥当であるか検討し、既存の手術点数が実際の手術室原価にはるかに及ばないことが明らかになったと指摘している。また二木（1988）は、診療報酬改定を目的としてリハビリテーション部門の原価計算調査を実施した。

一方で、1980年代に入ると、原価計算の目的が依然として第一義的には診療報酬改定であることの裏返しであるが、経営管理を目的として原価計算を実施・活用すべきとの主張が見られるようになる。虎ノ門病院事務部長である黒田（1983）は、結果が医療費の是正に利用されることが少ない現状では原価計算に対する期待も薄いが、原価計算の本来の目的に立ち返り、良い医療をよりよい条件のもとにより活発化させる施策をたてるための具体的資料として考えてみれば、大いに活用すべきであると主張している。また実際に、部門別原価計算を経営管理目的として利用する病院も現れるようになった。

たとえば花巻病院では、部門別原価計算の経常的な実施により、①財務会計より詳細に経営内容が把握できる、またこれにより将来予測もより的確にできる、②中間管理職へのデータ公開により彼らの参加意識が高まり、経営改善のために中間管理職が努力する、③経営改善の協力要請のための説得力のある資料が得られる、④財務会計との連携により入力ミスが容易に発見できる、⑤保険点数の改定による影響を容易に知ることができる、⑥見積もり・発注が能率的になる、⑦財務会計との対比により請求漏れがチェックできる、⑧原価管理への関心が高まる、⑨採算部門と不採算部門とが明らかになる、⑩不採算の原因が漸次明らかになる、といった経営上のメリットを得られたと報告されている（佐藤，1984；1986；1987；後藤，1985）注(5)。

また1980年代末以降になると、病院情報システム全体との関係のなかで部門別原価計算が論じられるようになってきた。牧田総合病院電算室部長の堀内（1989：1994）は、部門別原価計算システムと業務別のシステムの両者を同時に整備し、両者の連携を図ることの重要性を主張した。経理業務のコンピュータ化の遅れとともに、こうした部門別原価計算と各業務システムとの関連性の欠如がデータ収集の困難さを生み、それが部門別原価計算が一般化しない理由の1つであると述べている。

さらに1990年代に入ってくると、部門別の固定費段階的差引計算により各部門及び部門長の責任の所在と業績を明らかにした部門別原価

計算を導入し、これにより医長の給与を決定する病院も見られるようになったという（川渕, 1994）。

しかし、少なくとも中核的な病院は今後は部門別の原価計算を行わなければいけないとの指摘（二木, 1992）が見られるように、1990年代半ばまでにおいては、関心は高まっていたものの、中核的な病院ですら、多くの場合、実施できていなかったようである。六角（1996）も「病院の経営安定化のために管理会計としての部門別原価計算を導入する病院も現れている（9頁）」と述べており、部門別原価計算がまだ1990年代半ばにおいてはごく一部の病院のみでの実践であり、十分に普及していなかったことを示唆している。ただしアンケート調査によると、77%の病院管理職員が、①損益戦略の明確化、②各部門の貢献度の把握、③日々の業務指針、④業務評価の明確化、のために部門別原価を把握する必要性を感じており（六角, 1996）、実践はまだであったものの、経営管理を目的とした経常的な部門別原価計算への関心が非常に高まっていたことは確かである。

6. 部門別原価計算の実践期：1990年代後半以降

1990年代後半になると、単に関心が高まるだけでなく、実際に部門別原価計算を実践する病院も増えていった。2002年末に実施したアンケート調査によれば、すでに実施していた病院の大半は1990年代後半以降に実施し始めた（Arai, 2006）。その結果、特殊原価調査による随時的な実施も多く含まれているものの、病床数の多い大規模病院を中心に、2000年初頭には6割強の病院が診療科別原価計算を実施するようになった。また経常的な部門別原価計算に限定しても、大規模かつ経営管理に積極的な病院群においては、2002年末には4割弱の病院が実施するようになった[注6]。

しかし、まだ2000年代前半においては、特殊原価調査によるものを含めても3割5分、経常的な原価計算については6割強以上の病院が、部門別原価計算を実施しておらず、十分な普及はなされていなかった。この背景には、原価計算の必要性の認識段階（厳しくない病院経営環境、経営トップの原価計算に対する認識不足）、実施段階（経営管理体制の欠陥、管理会計能力の高い経営管理職員の不在、医療職員の経営管理意識の低さ）、定期的実施への展開段階（原価計算の情報システム化の困難性）、の3段階における多様な要因があった（荒井, 2001）。

すでに原価計算を実施している病院においては、インタビュー調査によれば、①職員の原価管理（経営管理）意識の向上、②部門別採算把握・損益分岐分析、③部門原価構造の把握・分析による業務改善、④部門別予算設定の基礎資料、⑤業績評価（各部門の努力評価）、⑥各部門の器材設備の購入評価、⑦長期的な経営方針・経営計画の策定、といった目的で実施している。こうした目的のために、原価計算情報は、院長、理事長、事務部長などの少数の幹部のみにより活用されている（Arai, 2006）。また基本的に組織上の部門を原価部門としており、診療報酬の対象となるサービスを直接的に提供している各診療科・各病棟及び各中央診療部門を利益センターとし，診療報酬対象サービスを直接には提供していない各補助部門を原価センターとしているのが一般的である。

原価配賦方法に関しては、労務費及び薬剤費を中心に、費目から部門への直課割合は5割程度の病院が多く、間接費の配賦基準ベースとしては、フロア面積・食数（85%）や診

療報酬・収入（77%）、職員数（69%）、患者数（62%）が多く用いられており、サービス単位件数（31%）や、調剤件数など各種件数（23%）、時間（8%）は十分活用されていない（Arai, 2006）。こうした状況はインタビュー調査においても同様であった。また部門間の原価配賦に関しては、補助部門・中央診療部門・診療科及び病棟の順に段階的に配賦していく、直接配賦法と階梯式配賦法の折衷的な方法である段階的配賦法が多くの病院で用いられている。その際の配賦基準ベースとしては、一般に収益や件数・患者数・職員数・ベッド数・床面積が用いられており、問題の多い収益比による配賦が著しく多用されている。また件数比についても部門内の各種サービス間の原価の違いを反映させない単純な件数比が用いられている。さらに、部門原価を一括して1つの配賦基準ベースで配賦していて，変動費・固定費別の複数基準で配賦している病院は見られない。そもそも原価態様を考慮した複数基準の配賦法はその存在すら認知されていないようである。

以上のような各病院レベルでの原価計算実践に加えて、政府関連の病院原価計算研究も特に2000年代に入ると盛んになってきた。これらの研究は、基本的には一国の医療提供システムを経営するための原価計算制度に関するものであるが（荒井，2007）、研究協力病院群はもちろんのこと、他の病院も含めて、各病院での原価計算実践を促進していると考えられる。

7. 病院原価計算対象の多様化期：2000年代後半以降

日本病院界全般としては、ようやく部門別原価計算がある程度普及しはじめた状況であるが、2000年代後半以降、診断群分類（DPC）別包括払い制の拡大や大幅かつ継続的な診療報酬抑制を背景として、一部の先駆的な病院においては、診断群分類別原価計算も実施されるようになってきている。ただし、材料費だけでなく全原価要素を含めた、全DPC種類を対象とした、経常的な診断群分類別原価計算はあまり見られず、多くは一部のDPC種類を対象とした特殊原価調査による随時的な全部原価計算であったり、材料費のみによる全DPC種類を対象とした経常的な原価計算であったりする。また前年度実績値や過去の調査データを用いた見積もり計算といった性格の計算も見られ、必ずしも当年度の実績値ベースの実際原価計算ではない。しかしそうしたなかにあっても、電子カルテ記録データが医事会計システムと原価計算システムに流れ、その際に物流システムから仕入れ単価が付加され、毎日自動的に患者別に原価が積み上げられ、そこから全DPCを対象とした経常的な全部原価計算ができるシステムを導入しているところもある注(7)。

またこうした診断群分類別原価計算の登場とともに、手術領域や検査領域を中心に、部門内各種サービス別原価計算も試みられるようになりつつある。ただし、一部の各種サービスのみを対象とした特殊原価調査によるものや材料費のみによる原価計算も多く、すべての部門内各種サービスを対象に全部原価ベースで経常的に算出できる原価計算システムを導入しているところは少ない。さらに一部の先駆的な病院では、医師別原価計算の試みも見られるようになってきた。

こうした原価計算対象の多様化に加えて、まだ一部の病院においてではあるものの、原価計算の洗練化も見られつつある。収益比配賦の多用に変えて一部ではあるがウエイト付けされた件数比を用いる病院や、組織上の部門区分を基

礎としつつも活動の観点からの原価部門の細分化をする病院など、伝統的な収益比配賦法からABC（活動基準原価計算）への洗練化を試みている病院も見られる。また正確性の向上だけではなく、原価情報の目的適合性の向上や原価計算システムの職員行動への影響機能の考慮化などの面からも、原価計算の洗練化が徐々に見られつつある。

8. おわりに

以上に明らかにしてきたように、製造業などと比べると、病院界においてはまだまだ原価計算の実践は不十分である。しかし終戦後、各時代の要請を背景として、提唱期、発達期、停滞期、再燃期を経て、実践期、多様化期へと、徐々にではあるものの病院原価計算の実践は広がってきた。とりわけ2000年代以降の現在においては、部門別原価計算を中心としていよいよ病院原価計算が本格化している。日本病院界も遅ればせながら、しっかりとした原価情報に基づく経営管理を要求する病院経営環境の変化に対応しつつあるといえる。もっとも、日本病院界の経営環境は現在さらに一歩変化（診断群分類別包括払い制の拡大）しており、診断群分類別定額払い制導入後のアメリカ病院界における迅速な対応（荒井, 2007）のように、日本病院界も迅速に診断群分類別原価計算などの導入を図っていく必要がある。より多くの病院が各時代の経営環境に適切に対応し、病院経営を安定させ、継続的に医療サービスを提供しつづけることが期待されている。

注(1)　現在の国立保健医療科学院の前身であり、また戦後から病院管理の教育・研究の中心的な機関としての役割を担ってきた病院管理研修所の後身である。

注(2)　たとえば検査部門の検査数、看護部門の1患者1入院日、手術部門の手術数などが例として挙げられる。

注(3)　山元氏はすでに戦前に病院会計に関する理論的研究書を出しており、英米の病院原価計算研究を踏まえた提言を行っている（山元, 1944）。

注(4)　雑誌『病院』は、昭和24年から現在まで続いている病院管理の領域では非常に歴史のある代表的な雑誌であり、また幅広く読まれている雑誌であるため、雑誌『病院』を中心に考察した。

注(5)　花巻病院のより詳細な原価計算実務に関しては、部門別原価計算全般については病院長の佐藤氏らによる文献（佐藤, 1984;1986;1987他;高橋, 1985）を、また臨床検査部門及び医事部門における部門別原価計算実施に伴う結果については、それぞれ後藤（1985）及び三好（1985）を参照されたい。

注(6)　2000年代後半に入り、その後も部門別原価計算の実施は着実に広がってきている。筆者らの医療経済研究機構部門別収支研究班による88病院に対する調査（2007年11月実施：回収率96.6%）では60%の病院が原価計算を実施しているが、そのうち58%の病院が2003年度以降に開始したと回答している。

注(7)　2000年代後半以降の先駆的病院における現状についての詳細は、近刊予定の荒井（2009）第6章を参照されたい。

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J Neurol
DOI 10.1007/s00415-008-0941-4

ORIGINAL COMMUNICATION

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Frequency of nocturnal sudden death in patients with multiple system atrophy

Received: 8 October 2007
Received in revised form: 6 March 2008
Accepted: 14 March 2008
Published online: 28 July 2008

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Abstract Sudden death has been reported in patients with multiple system atrophy (MSA), although the frequency of this event has not been well delineated. We investigated the frequency and potential causes of sudden death in patients with MSA. During the 5-year observation period, 10 of 45 patients with probable MSA died. The causes of death included sudden death of unknown etiology (seven patients), aspiration pneumonia (one patient), asphyxia after vomiting (one patient), and lung cancer (one patient). The mean survival time of patients with sudden death was 63.0 ± 24.7 months (range, 39–116 months). Among seven patients who experienced sudden death, six were found to have died during sleep. Among these patients, two had been treated with tracheostomy and three with continuous positive airway pressure (CPAP) or noninvasive positive pressure ventilation (NPPV) during sleep, suggesting that these treatments do not always prevent sudden death in patients with MSA. Nocturnal sudden death should be recognized as the most common mechanism of death in patients with MSA.

Key words multiple system atrophy · sudden death · tracheostomy · continuous positive airway pressure · noninvasive positive pressure ventilation

Introduction

Sudden death has been reported in patients with multiple system atrophy (MSA) [7–9, 13], although the frequency of this event has not been well delineated. The mechanism underlying sudden death remains unclear; it may be explained by laryngeal obstruction due to vocal cord abductor paralysis (VCAP) [3]. Tracheostomy, which bypasses the obstruction of vocal cords, is usually considered as the optimal treatment for VCAP; continuous positive airway pressure (CPAP) or noninvasive positive pressure ventilation (NPPV), which prevents upper airway obstruction, has been shown to increase the survival time in patients with MSA [2]. In this study, we investigated the frequency of sudden death in patients with MSA.

Patients and methods

Ethical approval for this study was obtained from the Ethical Committee of Niigata University School of Medicine. In accordance with the consensus statement criteria [1], we enrolled consecutive Japanese patients with probable MSA who were admitted to our hospital between May 2001 and April 2004, and followed through June 2006. We retrospectively evaluated the frequency of sudden death. A hyperintense putaminal rim and a "hot cross bun" sign on MRI [5] were used to support the diagnosis. Patients were excluded from the study

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if they showed evidence for hereditary spinocerebellar ataxias by a molecular genetic test [10]. The onset of symptoms was determined as the time at which the initial symptoms of movement impairment were first noted by the individual and close family members. Screening for dysphagia was performed by videofluorography, and percutaneous endoscopic gastrostomy (PEG) was used for tube feeding when dysphagia or aspiration was observed. Standard nocturnal polysomnography (PSG) and laryngoscopy were performed as described previously [10]. Patients who had VCAP or nocturnal oxygen desaturation that fulfilled CT90 (cumulative percentage of time spent at saturations below 90 %) greater than 10 % were treated with CPAP or NPPV. Ventilator settings were adjusted to prevent apnea, hypopnea, snoring, stridor, and desaturation. Patients who had recurrent aspiration pneumonia and VCAP were treated with conventional surgical tracheostomy. The data represent mean ± SD.

Results

We enrolled 47 patients with probable MSA; however, two patients (4.3 %) were lost during the follow-up period. On the basis of their clinical presentation, 45 patients were subdivided into those with prominent parkinsonism (MSA-P, n = 11) and those with prominent cerebellar ataxia (MSA-C, n = 34). The mean age at onset was 58.8 ± 5.3 years (range, 41–73 years). Among them, ten patients died during the study period and the mean survival time from disease onset to the time of death was 63.0 ± 24.7 months (range, 39–116 months). The causes of death included sudden death of unknown etiology (seven patients), aspiration pneumonia (one patient), asphyxia after vomiting (one patient), and lung cancer (one patient) (Table 1). One patient who preferred not to be treated with PEG tube feeding died as a result of aspiration pneumonia.

We analyzed the clinical features of the seven patients with MSA who experienced sudden death. Studies before their death revealed that four patients had VCAP. Two patients exhibited bilateral partial abduction restriction of the vocal cords, and two patients exhibited bilateral complete abduction restriction of the vocal cords. Daytime stridor during wakefulness was observed in two patients. Hypercapnea ($PaCO_2$ > 45 mmHg) was present in 3 of 7 patients. None of the patients had pneumonia, hypoxemia, arrhythmia, cardiac failure, or choking prior to 24 hours of the day of sudden death. Six patients had nocturnal death. The mode of the sudden death was unclear due to lack of any witnesses at the time of death. Among seven patients, two had been treated with tracheostomy and three with CPAP or NPPV during sleep. Although patient 3 (Table 1) had neither VCAP nor recurrent aspiration pneumonia, he underwent tracheostomy because he had frequent episodes of transient respiratory arrest during sleep. Three of these seven patients died within five years after the onset of the disease. The two patients treated with tracheostomy died 3 and 4 months, respectively, after the tracheostomy, and the three patients treated with CPAP or NPPV died 2, 4, and 21 months, respectively, after the treatment. We

Table 1 Summary of clinical features of MSA patients who died

Patient	Cause of death	Sex	Age at onset	Type of MSA	OH	UI	Dysphagia	VCAP	Daytime stridor	PaCO2 (mmHg)	Time of death	Treatment	PEG	Survival time (months)	AHI (/hour)	Type of apnea	CT90 (%)
1	sudden death	F	55	P	(−)	(+)	(+)	NE	(−)	46.5	6:30 PM	none	(−)	73	4.1	0	0
2	sudden death	M	48	C	(+)	(+)	(+)	NE	(+)	67.2	4:00–6:00 AM	none	(−)	74	NE	NE	NE
3	sudden death	M	51	C	(+)	(+)	(−)	(−)	(−)	48	4:00–6:30 AM	tracheostomy	(−)	88	11.6	O > M	2.4
4	sudden death	F	65	C	(−)	(+)	(+)	(+) BP	(−)	39.8	2:00–3:00 AM	tracheostomy	(+)	50	poor study	poor study	poor study
5	sudden death	F	40	C	(+)	(−)	(−)	(+) BP	(−)	40.8	6:45 AM	CPAP (14)	(−)	116	0.7	O	0.6
6	sudden death	F	53	C	(−)	(+)	(+)	(+) BC	(−)	48	4:00–5:00 AM	NPPV (14/4)	(−)	45	18.3	O	30.4
7	sudden death	F	62	P	(+)	(+)	(−)	(+) BC	(+)	34.9	4:30 AM	NPPV (6/4)	(−)	39	poor study	poor study	O
8	aspiration pneumonia	M	67	C	(−)	(+)	(+)	(−)	(−)	36.4		none	(−)	43	2	O	1.1
9	lung cancer	M	49	C	(+)	(+)	(+)	(−)	(−)	42.5		CPAP, auto mode (4–20)	(−)	57	17.6	O	13.9
10	asphyxia after vomiting	F	72	C	(+)	(+)	(+)	(−)	(−)	39.9		NPPV (8/4)	(+)	45	50.7	O > C	23.6
			56.7 ± 10.0	CP = 8:2										63.0 ± 24.7			

C multiple system atrophy with clinical predominance of cerebellar symptoms (MSA-C); P multiple system atrophy with clinical predominance of parkinsonism (MSA-P); *OH* orthostatic hypotension; *UI* urinary incontinence; *VCAP* vocal cord abductor paralysis; *NE* not examined; *BP* bilateral partial abduction restriction of vocal cords; *BC* bilateral complete abduction restriction of vocal cords; *CPAP* continuous positive airway pressure; *NPPV* noninvasive positive pressure ventilation; *PEG* percutaneous endoscopic gastrostomy; *AHI* apnea-hypopnea index; *O* obstructive type; *M* mixed type; *C* central type; *CT90* cumulative percentage of time spent at saturations below 90 %. Survival time indicates the time from disease onset to death. Number in parentheses indicates the pressures of CPAP or NPPV used

confirmed that at least two of these three patients were wearing a nasal mask at the time of death.

Discussion

We conducted an analysis of mortality among patients with MSA. The incidence of death associated with aspiration pneumonia was lower than previously reported [11], suggesting that careful screening and treatment for dysphagia might reduce mortality by preventing deaths associated with aspiration pneumonia. We found that sudden death of unknown etiology was the most common cause of death in our cohort. We also found that the time interval between the onset of the disease and sudden death was diverse, and that sudden death can occur in the early phase of the disease. Little information about the mode of death was obtained, because six of the seven patients died during sleep without apparent warning symptoms such as arrhythmia and pneumonia. However, because we encountered one patient suffered a respiratory arrest without cardiac arrest, we speculate that cardiac arrest during sleep might have occurred subsequent to respiratory arrest, which might have been caused by central dysfunction of the respiratory regulation system [9] and impaired chemosensitivity to hypoxemia [12].

We noted two patients who developed sudden death despite tracheostomy. These findings suggest that tracheostomy does not always prevent sudden death as previously reported [6, 11] and that sudden death is caused by a mechanism other than upper airway obstruction such as VCAP and obstructive sleep apnea syndrome. A recent study has reported that tracheostomy might aggravate central sleep apnea, thereby increasing the risk of nocturnal sudden death in MSA [4]. In the present study, the interval between tracheostomy and death was diverse (3, 4, 5, and 13 months). PSG would be an appropriate study to elucidate whether tracheostomy aggravates central sleep apnea and causes nocturnal sudden death. We also noted three patients who developed nocturnal sudden death despite CPAP or NPPV treatment. This indicates that, as with tracheostomy, CPAP or NPPV also does not always prevent sudden death, although it is important to maintain a high compliance rate and adequate ventilator settings during the treatment.

A comparison of the clinical features of the patients who experienced sudden death with those of patients who died but not suddenly might help us to elucidate the effect of therapeutic interventions on survival. Unfortunately, because of lack of sufficient numbers of patients treated with CPAP, NPPV, or tracheostomy who died but did not die suddenly, the present study did not provide information about this. Further studies should be performed to identify the role of these treatments in sudden death in patients with MSA.

Acknowledgments This study was supported in part by grants for Surveys and Research on Specific Diseases and Ataxias and Neurodegenerative Diseases from the Ministry of Health, Labor, and Welfare of Japan.

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ARTICLES

Published Ahead of Print on June 25, 2008 as 10.1212/01.wnl.0000311277.71046.a0

Total deletion and a missense mutation of *ITPR1* in Japanese SCA15 families

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ABSTRACT

Background: Spinocerebellar ataxia type 15 (SCA15) is a progressive neurodegenerative disorder characterized by pure cerebellar ataxia, very slow progression, and distinct cerebellar atrophy. The locus for SCA15 was first mapped to 3p24.2-3pter in an Australian family. We have subsequently mapped two Japanese families presenting with ataxia and postural tremor of the head, arm, or trunk to the SCA15 locus. Recently, partial deletions involving both the type 1 inositol 1,4,5-triphosphate receptor (*ITPR1*) and sulfatase modifying factor 1 (*SUMF1*) genes have been identified in Australian and British families with SCA15.

Methods: We conducted fine haplotype analysis on the region including *ITPR1*. To identify the deletion, we conducted gene dosage analysis and array-based comparative genomic hybridization (aCGH) analysis. Gene expression analysis was performed using quantitative real-time reverse transcription PCR. Mutational analyses of *ITPR1* and *SUMF1* were also performed.

Results: We have identified a 414-kb deletion including the entire *ITPR1* and exon 1 of *SUMF1* in patients in family A. The expression levels of *ITPR1* and *SUMF1* mRNAs of the patient were half those of the normal control. Furthermore, in family B, we have identified a C-to-T substitution at position 8581 of *ITPR1*, resulting in the amino acid substitution of leucine for proline at codon 1059, which is highly conserved among species.

Conclusions: Our results strongly confirm that *ITPR1* is the causative gene for SCA15 and suggest that we need to investigate the point mutation in *ITPR1* in the patients with autosomal dominant cerebellar ataxia and tremor. ***Neurology*® 2008;71:1-1**

GLOSSARY

aCGH = array-based comparative genomic hybridization; **LCR** = low-copy repeat; **NAHR** = nonallelic homologous recombination; **NHEJ** = nonhomologous end joining; **SCA15** = spinocerebellar ataxia type 15.

Spinocerebellar ataxia type 15 (SCA15) was first reported on the basis of a single large Australian family with pure cerebellar ataxia, very slow progression, and marked cerebellar atrophy, particularly in the vermis.[1,2] The disease locus was mapped to chromosome 3p24.2-3pter.[1,2] We have subsequently identified two Japanese families with autosomal dominant cerebellar ataxia characterized by postural and action tremor. The disease locus for these Japanese SCA families was mapped to 3p25.3-26.1 with the highest cumulative multipoint lod score of 3.30 at D3S3728.[3] Interestingly, the critical region for our families partly overlapped with the locus for the Australian SCA15 family. The overlapping region was 7.9 cM between D3S1620 and D3S1304. Clinical features, including a very slow progression, tremor of the head, arm, or trunk and distinct cerebellar atrophy without brainstem atrophy, are shared among these fam-

e-Pub ahead of print at www.neurology.org.

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Supported in part by KAKENHI (Grant-in-Aid for Scientific Research) on Priority Areas "Advanced Brain Science Project," "Applied Genomics," the 21st Century COE Program "Center for Integrated Brain Medical Science," and a Grant-in-Aid for Scientific Research (A) and (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a grant for the Research for the Future Program from the Japan Society for the Promotion of Science, a grant for "the Research Committee for Ataxic Diseases" of the Research on Measures for Intractable Diseases from the Ministry of Health, Labor and Welfare, Japan, Grant for Promotion of Niigata University Research Projects, and Grant-in-Aid for Young Scientists (B).

Disclosure: The authors report no disclosures.

ilies, raising the possibility that the causative gene for the Australian SCA15 family is allelic to that for the two Japanese SCA15 families.[1] However, some of the clinical features of the Japanese families are distinct from those of the Australian family as regards to the extracerebellar signs. Postural tremor of the head, arm, or trunk is more predominant in the Japanese patients than in the Australian patients.[3] Subsequently, additional families have been reported to link to the SCA15 locus.[4]

Recently, partial deletions of type 1 inositol 1,4,5-triphosphate receptor (*ITPR1*) and sulfatase modifying factor 1 (*SUMF1*) genes have been identified in the Australian SCA15 family and two British families with pure cerebellar ataxia, suggesting that *ITPR1* is the causative gene for SCA15.[5] Furthermore, a partial deletion of *ITPR1* has been identified in the Japanese SCA16 family.[6] However, it is unclear whether *ITPR1* is solely responsible for SCA15, because the deletions also involve *SUMF1*. Here, we report that the two families mapped to the SCA15 locus have the mutations in *ITPR1*, including a missense mutation, which strongly confirms that *ITPR1* is the causative gene for SCA15.

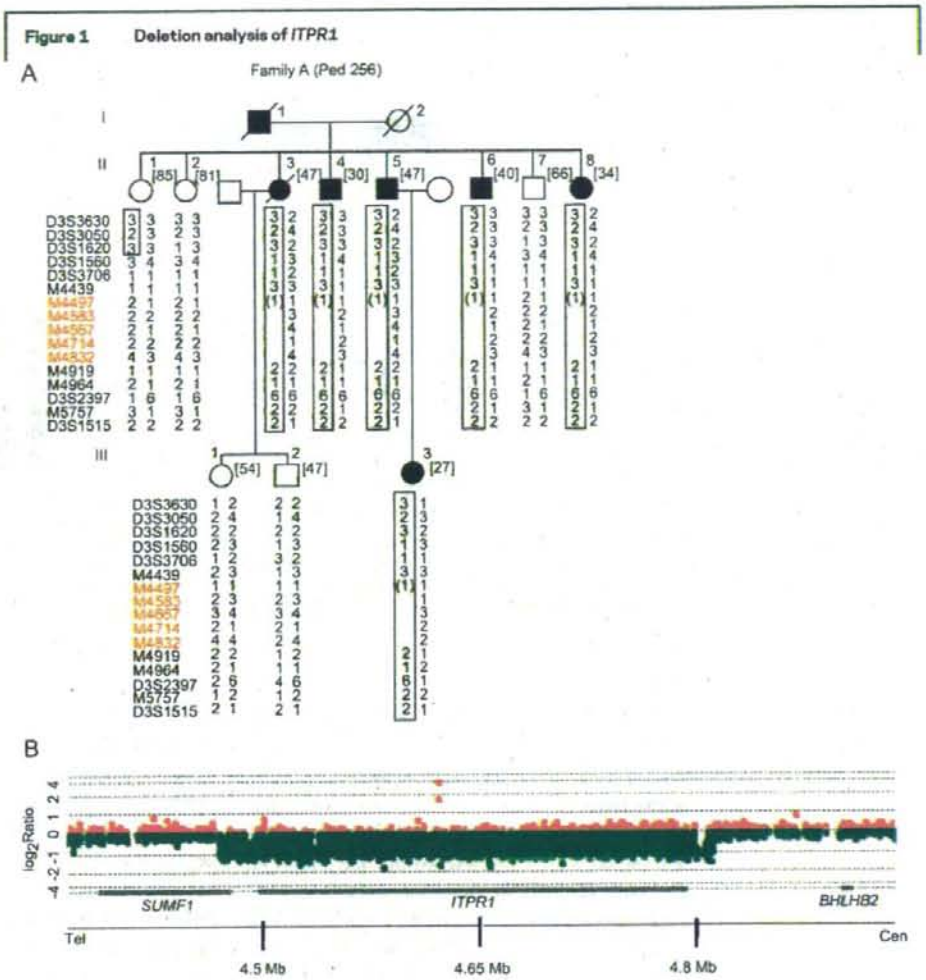
METHODS SCA families. The family members of the two Japanese families mapped to the SCA15 locus were analyzed in the present study. In addition, 54 autosomal dominant SCA families in which abnormal expansions of CAG repeats in the responsible genes for SCA1, SCA2, Machado-Joseph disease/SCA3, SCA6, SCA8, SCA17, and dentatorubral-pallidoluysian atrophy have been excluded are also enrolled for gene copy number analysis of *ITPR1*. High-molecular-weight genomic DNA was extracted from peripheral leukocytes after obtaining informed consent from the patients. The present study was approved by the Institutional Review Board of Niigata University.

Genetic analysis. For fine haplotype mapping, we established nine new microsatellite markers, namely, M4439, M4497, M4583, M4667, M4714, M4832, M4919, M4964, and M5757 in the region between D3S3706 and D3S1515 including *ITPR1*. Gene dosage was analyzed using a quantitative real-time PCR technique with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). We also employed the custom high-definition array-based comparative genomic hybridization (aCGH) microarrays (Agilent Technologies Inc., Palo Alto, CA).[7] A region of approximately 3.8 Mb from *CNTN4* (Gene ID: 152330) to D3S1303 was included in the microarray at an average interval of 200 bp for the probes. For gene expression analysis, RNA was extracted from cultured skin fibroblasts from an affected individual (II-4) and a control. The amplified products of all 58 exons of *ITPR1* were subjected to a cycle sequence reaction using BigDye Terminator version 3.0 (Applied Biosystems).[2] Detailed methods are available in table e-1 and e-Methods on the *Neurology*® Web site at www.neurology.org.

RESULTS Identification of deletion involving entire *ITPR1* in family A (Ped 256). To investigate whether a common haplotype is shared among the affected individuals in the two Japanese ataxia families linked to the SCA15 locus,[3] we conducted fine haplotype analyses based on the linkage analysis. We did not find any common founder haplotypes between these Japanese ataxia families. However, all of the affected individuals in family A (Ped 256) lost heterozygosity from M4497 to M4832 (figure 1A). Furthermore, alleles from M4583 to M4832 were not transmitted from the affected individual II-5 to his affected daughter III-3 (figure 1A). These findings strongly suggested that the affected individuals in this family (Ped 256) had a heterozygous deletion in the region containing two genes: *ITPR1* and *SUMF1* (figure e-1A). We performed quantitative real-time PCR analyses for *ITPR1* and *SUMF1*. The dosages of exons 2, 26, and 58 of *ITPR1* and exon 1 of *SUMF1* in the affected individuals were one-half those of unaffected individuals (figure e-1B). These findings indicated that the affected individuals had the deletion of the entire *ITPR1* and exon 1 of *SUMF1*.

Identification of breakpoint sequence in *ITPR1*. To confirm the deletion of *ITPR1*, we performed aCGH analysis using oligonucleotide probes spanning the region from *CNTN4* to D3S1303 on chromosome 3p from three affected individuals (II-5, II-6, and II-8) and two unaffected individuals (II-7 and III-2) in family A. The $\log_2$ R ratios for the affected individuals for the probes from the nucleotide position 4,476,024 to 4,887,327 were decreased to ~−1, whereas those for the probes from the nucleotide position 4,142,899 to 4,471,831 and from the nucleotide position 4,887,691 to 5,242,899 were ~0, confirming the extent of the deletion (figure 1B). In contrast, we detected no deletion in this region in an affected individual in family B (Ped 2216). Furthermore, aCGH analysis was applied to 54 autosomal dominant SCA families; however, no deletion of *ITPR1* or *SUMF1* was detected in these families.

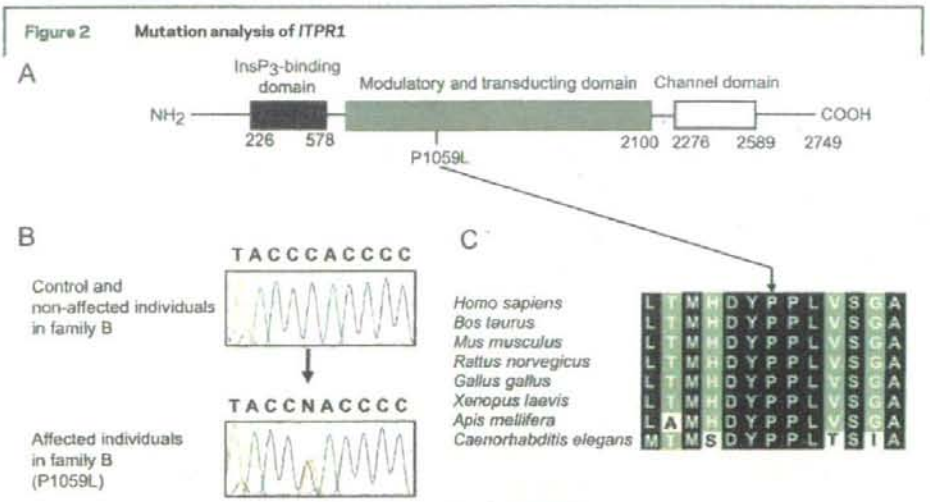
To determine the breakpoint, we developed the primers for each end and performed PCR, obtaining a ~2,300 bp fragment from the affected individuals, but not from the unaffected individuals (figure e-2A). The sequence analysis of this junction fragment revealed that no significant homology was observed when the junction sequence was aligned with the reference genomic sequence at the proximal and distal breakpoints and the sequences at proximal and distal breakpoints showed a two nucleotide overlap (figures e-2B and e-2C). RepeatMasker (http://www.repeatmasker.org/) revealed that



(A) Haplotype analysis of family A (Ped 256) employing microsatellite markers spanning the SCA15 locus at 3p26.1. Squares indicate males. Circles indicate females. Affected individuals are indicated by solid symbols, and unaffected individuals by open symbols. Deceased individuals are indicated by diagonal lines. Age at death or current age are indicated (n in brackets) in unaffected individuals. Age at onset is indicated (n in brackets) in affected individuals. Haplotypes linked to the disease are boxed. It cannot be determined whether the M4497 locus is included in the deletion, since all the affected individuals had the identical allele "1" at M4497; parentheses. (B) Deletion of entire *ITPR1* identified by high-density oligonucleotide array-based CGH analysis. A part of the results of array-based CGH analysis of the affected individual II-5 are plotted as a function of chromosomal position (Mb). Regions of loss (green) and gain (red) are color-coded.

the distal breakpoint was embedded within an AT dinucleotide repeat. The proximal breakpoint was embedded immediately before the *AluSx* element. The deletion in this family is thus 414,018 bp in size, including the entire *ITPR1* and exon 1 of *SUMF1*.

Chromosomal deletion is mainly mediated by two mechanisms: nonallelic homologous recombination (NAHR) or nonhomologous end joining (NHEJ).[8] In the case of NAHR, the chromosomal reengagement occurs between large, highly homologous low-copy repeat (LCR) structures, AT-rich palindromes, and pericentromeric repeats.[8] To determine whether the breakpoints are embedded within LCR structures, we compared the 1-Mb genomic sequence surrounding *SUMF1* and *ITPR1* against itself using PipMaker.[9] The sequence analyses of the junction in our case and those in previously reported cases showed that distal breakpoints were scattered within a ~65 kb region and proximal breakpoints were scattered within a ~223 kbp region[5,6](figure e-3). These breakpoints are frequently embedded within or beside repetitive sequences; however, none of the breakpoints were embedded within LCR structures, AT-rich palindromes, and pericentromeric repeats (figure e-3).[5,6] In addition, the sequences at breakpoints showed a 2–5-nucleotide overlap, which is frequently observed in chromosomal rearrangements



(A) Functional domains of *ITPR1* and location of missense mutation (P1509L). (B) Direct sequencing revealed a heterozygous C-to-T substitution at position 8581 of *ITPR1*, resulting in the amino acid substitution of leucine for proline at codon 1059. (C) Amino acid sequences at the mutation site in *ITPR1* and the orthologs of *ITPR1* aligned by ClustalW. Conserved amino acid residues are shaded using GeneDoc. Each shade represents a degree of conservation (black, 100%; dark gray, 80%).

mediated by NHEJ (figure e-2C).[5,6,10,11] Taken together, the results indicate that *ITPR1* deletion is mediated by NHEJ.

Consequences of *ITPR1* and *SUMF1* deletion at mRNA level. The quantitative real-time PCR analysis using mRNA from cultured skin fibroblasts from an affected individual (II-4) revealed that the mRNA expression levels of *ITPR1* exons 5–6, *ITPR1* exons 25–26, *ITPR1* exons 44–45, and *SUMF1* exons 6–7 of the affected individual were one-half those of the normal control, indicating that *ITPR1* and *SUMF1* from the deleted allele were not expressed (figure e-4).

Identification of missense mutation in *ITPR1* in family B (Ped 2216). We performed nucleotide sequence analyses of the entire exons and splice junctions in *ITPR1* and *SUMF1* of the affected individuals in family B and identified one missense mutation, C8581→T (resulting in substitution of leucine for proline: P1059L) in exon 25 of *ITPR1* in all the affected individuals in the heterozygous state, whereas unaffected individuals did not have this substitution (figure 2, A and B). This nucleotide change was not observed in 234 normal chromosomes in Japanese controls. We found no nucleotide substitutions in *SUMF1*. P1059L was located in the modulatory and transducing domain in *ITPR1* (figure 2A). Amino acid sequence alignment of *ITPR1* using ClustalW[12] revealed that the proline residue at codon 1059 is highly conserved among species (figure 2C).

DISCUSSION In this study, we found the total deletion of *ITPR1* and the decrease in *ITPR1* mRNA expression level by half in family A. Partial deletions of *ITPR1* and *SUMF1* have been reported in one Australian SCA15 family and two British families with pure cerebellar ataxia.[5] Deletions involving *ITPR1* were not observed in the other 54 families with undetermined autosomal dominant ataxia, suggesting that deletions of *ITPR1* are not frequent in Japanese autosomal dominant SCA families. All the deletions identified in the SCA15 families, including our cases, also included *SUMF1*. *SUMF1* is a causative gene for multiple sulfatase deficiency, which is an autosomal recessive disorder characterized by mental retardation, seizure, and leukodystrophy.[13] Patients with a heterozygous mutation in *SUMF1* are clinically healthy, suggesting that the partial deficiency of *SUMF1* does not likely cause neurodegenerative disorders. In addition, in family B, we first identified the missense mutation P1059L in *ITPR1*. Although we did not investigate the function of mutant *ITPR1* with P1059L, we consider that this P1059L mutation causes cerebellar ataxia, because this mutation was not present in 234 chromosomes in Japanese controls and showed complete cosegregation with the disease. Moreover, the mutated proline residue at codon 1059 is highly conserved among species. Recently, a partial deletion of *ITPR1* has been identified in a Japanese SCA16 family.[6] Taken together, these results strongly confirm that *ITPR1* is the responsible gene for cerebellar ataxia in humans.

How do the deletions or the missense mutation of *ITPR1* cause cerebellar ataxia in patients with SCA15? ITPR1 is a major inositol 1,4,5-

triphosphate receptor, which mediates Ca^{2+} release from the endoplasmic reticulum in various neurons, including CA1, basal ganglia, and the thalamic neurons, particularly Purkinje neurons.[14,15] Intracellular Ca^{2+} homeostasis is important for maintaining the function of neurons particularly Purkinje neurons.[16] Indeed, mice homozygous for null *ITPR1* develop ataxia and epilepsy without apparent morphologic abnormalities.[17,18] On the other hand, mice heterozygous for null *ITPR1* develop mild motor discoordinaton without apparent morphologic abnormalities in the cerebellum.[19] Thus, the haploinsufficiency of *ITPR1* may result in dysfunctions confined to Purkinje neurons, and the complete loss of *ITPR1* results in dysfunctions in both cortical and Purkinje neurons. The finding indicates that Purkinje neurons are particularly vulnerable to the gene dosage of *ITPR1*. Indeed, none of the individuals with SCA15 with *ITPR1* mutation had epilepsy or abnormal electroencephalograms, and the clinical phenotype was limited to cerebellar ataxia with tremor even in the elderly.[1-3,5] The neuropathologic findings of individuals with *ITPR1* deletion or missense mutations will confirm this speculation.

Dysregulation of intracellular Ca^{2+} homeostasis by haploinsufficiency or missense mutation of *ITPR1* results in dysfunction of Purkinje neurons, and ultimately might result in degeneration of Purkinje neurons in humans. The study of the molecular mechanism underlying Purkinje cell degeneration caused by *ITPR1* will provide new insights into the mechanism of ataxias and eventually the development of new therapeutic approaches for preventing the degeneration of Purkinje neurons.

Electronic database information. NCBI accession numbers: *Homo sapiens* ITPR1, AAB04947.2; *Bos taurus* ITPR1, NP_777266.1; *Mus musculus* ITPR1, NP_034715.2; *Rattus norvegicus* ITPR1, NP_001007236.1; *Gallus gallus* ITPR1, XP_414438.2; *Xenopus laevis* ITPR1, NP_001084015.1; *Apis mellifera* ITPR1, XP_392236.3; *Caenorhabditis elegans* ITPR1, NP_001023174.1.

Received September 21, 2007. Accepted in final form January 31, 2008.

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We thank the clinical, genetic, pathology, and technical staff of the collaborating centers for making information and DNA/tissue samples available for this study. We also thank the families of patients whose generosity made this research possible.

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TDP-43 Mutation in Familial Amyotrophic Lateral Sclerosis

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder. Accumulating evidence has shown that 43kDa TAR-DNA–binding protein (TDP-43) is the disease protein in ALS and frontotemporal lobar degeneration. We previously reported a familial ALS with Bumina bodies and TDP-43-positive skein-like inclusions in the lower motor neurons; these findings are indistinguishable from those of sporadic ALS. In three affected individuals in two generations of one family, we found a single base-pair change from A to G at position 1028 in TDP-43, which resulted in a Gln-to-Arg substitution at position 343. Our findings provide a new insight into the molecular pathogenesis of ALS.

Ann Neurol 2008;63:538–542

Amyotrophic lateral sclerosis (ALS) is a fatal and incurable neurodegenerative disorder. One of the neuropathological hallmarks of ALS is the presence of ubiquitinated neuronal cytoplasmic inclusions (NCIs) in lower motor neurons.[1,2] Recently, the 43kDa TAR-DNA–binding protein (TDP-43) has been identified as the major component of NCIs in sporadic ALS (SALS) and superoxide dismutase 1 (SOD1)–negative familial ALS (FALS), as well as sporadic and familial frontotemporal lobar dementia (FTLD).[3–7] Furthermore, the abnormal-molecular-weight fragments of TDP-43 were

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Received Oct 9, 2007, and in revised form Feb 23, 2008. Accepted for publication Feb 27, 2008.

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Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/ana.21392

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detected in affected nervous tissues.[3–5,8] These results suggest that TDP-43 is a causative protein in these disorders. However, the significance of TDP-43 in the pathogenesis of these neurodegenerative disorders remains to be elucidated.

We have reported the case of a SOD1-negative FALS family, in which one autopsied case showed degeneration limited to the lower and upper motor neuron systems, with Bunina bodies and ubiquitinated skeinlike inclusions in the remaining lower motor neurons; the clinical and pathological findings are indistinguishable from those in SALS.[9] Subsequently, we demonstrated that the ubiquitinated inclusions were immunoreactive for TDP-43.[6] Here, we report a missense mutation in *TDP-43* in the case's family. Furthermore, we found a widespread distribution of TDP-43–immunoreactive neuronal and glial cytoplasmic inclusions, as well as abnormal-molecular-weight fragments of TDP-43 in the spinal cord of an affected individual with this mutation.

Subjects and Methods

Mutational Analysis of TDP-43

Sixteen ALS families in which mutation of the *SOD1* gene had been excluded were enrolled. High-molecular-weight genomic DNA was extracted after obtaining patients' informed consent. RNA was extracted from the spinal cord of an affected individual (Fig 1A; Subject II-2) and a control subject.[9] We also analyzed genomic DNA from 92 clinically diagnosed SALS patients, 20 autopsy-confirmed SALS cases, and 4 autopsy-confirmed cases with related disorders: frontotemporal lobar degeneration with ubiquitin-positive inclusion (FTLD-U) in 1 case; FTLD with motor neuron disease in 2 cases; and primary lateral sclerosis in 1 case. We amplified all the exons of *TDP-43* (NM_007375) with the use of a series of primers, followed by sequence reaction. This study was approved by the Institutional Review Board of Niigata University.

TDP-43 Immunohistochemistry

In an autopsied case (see Fig 1A; Subject II-2), the distribution of TDP-43–immunoreactive NCIs and glial cytoplasmic inclusions (GCIs) was studied.[9] We prepared 4μm-thick, paraffin-embedded sections. These sections were immunostained by the avidin-biotin-peroxidase complex method with the use of a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and a rabbit polyclonal antibody against TDP-43 (10782-1-AP; 1:4,000; ProteinTech Group, Chicago, IL).

Plasmid Constructs

Full-length, wild-type, human TDP-43 complementary DNA (cDNA) was isolated from the human whole-brain

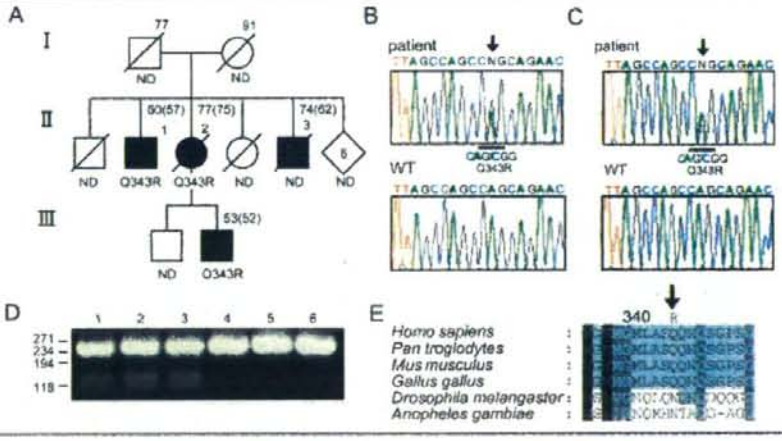

Fig 1. Detection of TDP43 *mutation in familial amyotrophic lateral sclerosis (FALS). (A) Partial family pedigree. Q343R, A1028G change in genomic DNA in heterozygous state. Age at death or current age and age at disease onset (n [m]) are indicated. (B) DNA sequence of genomic polymerase chain reaction (PCR) product.* Arrow *indicates A1028G in the patient. The resulting Q343R is shown at the bottom of the chromatogram of the patient. (C) DNA sequence of reverse transcribed polymerase chain reaction (RT-PCR) product from spinal cord of the autopsied case.* Arrow *indicates A1028G in the case. (D) Analysis of A1028G in genomic PCR products.* MspI *digestion of PCR products is shown; fragment sizes in base pairs are indicated on the left. Lanes 1 to 3 indicate patients; lanes 5 to 7 indicate normal control subject. (E) Sequence alignments of TDP-43 in different species. An amino-acid sequence of* Homo sapiens *NP_031401.1,* Pan troglodytes *XP_001135199.1,* Mus musculus *NP_663531.1,* Gallus gallus *XP_417612.1,* Drosophila melanogaster *NP_477400.1, and* Anopheles gambiae *XP_309663.2 were multiply aligned with the use of the ClustalW program, version 1.81.[11] The numbering on top of the alignments correlates with the human amino acid sequence. Amino acid 343, which is the site of Q343R, is indicated. ND = not determined.*

cDNA library (Clontech, Palo Alto, CA) and subcloned into the pcDNA DEST-40 (Invitrogen, Carlsbad, CA) or pEGFP-C3 vector (Clontech). TDP-43 mutant (Q343R) cDNA was generated with the use of a GeneTailor site-directed mutagenesis system (Invitrogen) and subcloned into the pcDNA DEST-40 or pEGFP-C3 vector to express green fluorescent protein-tagged TDP-43 in mammalian cells.

Fractionation of Frozen Spinal Cord Tissues and Immunoblotting

Proteins from the spinal cord of the autopsied case (see Fig1A; Subject II-2), five SALS patients, and three control subjects was extracted as described previously.[10] In brief, frozen tissues were homogenized in buffer A (10mmol/L Tris-HCl [pH 7.5], 1mmol/L EGTA, 1mmol/L dithiothreitol, 10% sucrose) and centrifuged. The resulting pellets were then extracted in buffer A containing 1% Triton X-100 (Sigma, St. Louis, MO) and centrifuged. These pellets were subsequently homogenized in buffer A containing 1% sarkosyl, incubated for 1 hour, and centrifuged. The sarkosyl-insoluble pellets were solubilized in 8mol/L urea buffer. After centrifugation, the supernatants were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by immunoblotting with the anti–TDP-43 rabbit polyclonal antibody (10782-1-AP; 1:1,000; ProteinTech Group) and anti-neurofilament L rabbit polyclonal antibody (NA 1214; 1:1,000; BIOMOL International L.P, Exeter United Kingdom).

Cell Culture and Confocal Microscopy

COS-7, human embryonic kidney 293, and C6 cells were grown and transfected with the use of lipofectamine 2000 (Invitrogen). All images were acquired with an inverted microscope (TE-300NT; Nikon, Tokyo, Japan) and a confocal microscope (CSU-10; Yokogawa, Tokyo, Japan) equipped with a 40× objective.

Results

Mutation Detection

We analyzed the sequence of *TDP-43* in the 16 ALS families in which mutation of the *SOD1* gene had been excluded. We found a single base-pair change at position 1028 from A to G (A1028G) in exon 6, which resulted in a Gln-to-Arg substitution at position 343 (Q343R) in *TDP-43* in three affected individuals in two generations in one FALS family (see Figs 1A, B).[9] The sequence analysis of the reversed transcribed polymerase chain reaction products of messenger RNA, which was extracted from the spinal cord of the autopsied case (see Fig 1A; II-2), showed that both normal and mutant alleles were transcribed (see Fig 1C). This substitution results in the generation of a novel *MspI* restriction site (see Fig 1D). By polymerase chain reaction restriction fragment length polymorphism analysis, we found that the A1028G substitution did not exist in any of 267 unrelated healthy individuals, 92 patients clinically diagnosed with SALS, 20 cases with autopsy-confirmed SALS, or 4 cases with autopsy-confirmed sporadic neurodegenerative disorders (FTLD-U [n = 1], FTLD with motor neuron disease [n = 2], primary lateral sclerosis [n = 1]). Furthermore, glutamine at position 343 is well conserved in several species (see Fig 1E).[11]

Clinical Features and Pathology

The age at onset of FALS is from 52 to 75 years (see Fig 1A).[9] All affected individuals predominantly experience development of dysarthria and dysphagia, which are features similar to those of bulbar-type SALS.[9] Furthermore, the neuropathological findings that include the presence of Bunina bodies and ubiquitin-positive NCIs in lower motor neurons are quite similar to those of SALS.[6] TDP-43–immunoreactive NCIs and GCIs were also observed in various regions of the subjects' central nervous systems (Table).

TDP-43 Expression Analysis

To characterize TDP-43 protein biochemically, we sequentially extracted proteins from the spinal cord of the autopsied case using buffers with increasing abilities to solubilize proteins and then analyzed the proteins by immunoblotting with an anti–C-terminal TDP-43 antibody. In addition to the band corresponding to full-length TDP-43, an approximately 25kDa band and an approximately 45kDa band were detected for all soluble and insoluble fractions (Fig 2A). Analysis of 1% sarkosyl-soluble fractions extracted from the spinal cord of the control subjects, SALS patients, and FALS patients with Q343R demonstrated that an approximately 25kDa band and an approximately 45kDa band were more distinctly observed in the FALS patient with Q343R than in the control subjects and SALS patients (see Fig 2B).

Because a TDP-43–positive inclusion is cytoplasmic and abolishes normal nuclear TDP-43 staining,[4,6] we investigated the consequences of the Q343R substitution at the cellular level. We transiently transfected wild-type or Q343R-myc– or GFP-tagged TDP-43 cDNA constructs into COS-7, human embryonic kidney 293, and C6 cells. Both the wild-type and Q343R TDP-43 proteins localized to the nucleus with a punctuated pattern, which partially colocalized with coilin, suggesting that the Q343R mutation has no adverse effect on subcellular localization of TDP-43 (see Fig 2C).[12]

Discussion

In this study, we show Q343R substitution in *TDP-43* in a family with ALS. Although we did not obtain DNA from unaffected individuals and we failed to show abnormal localization of TDP-43 with Q343R, we consider this Q343R substitution to be associated with the ALS phenotype in the family for the following reasons. First, Q343R was not present in 534 chromo-

Table. **Distribution of TDP-43–Immunoreactive Neuronal and Glial Cytoplasmic Inclusions**

Regions	NCIs	GCIs
Cerebral cortex		
Frontal	−	−
Motor	+	+
Parietal	+	−
Temporal	+	−
Insular cortex	+	+
Entorhinal	−	−
Subcortical areas		
Hippocampus	−	−
Amygdala	+	−
Basal nucleus of Meynert	NE	NE
Caudate and putamen	+	+
Globus pallidus	−	−
Internal capsule		−
Thalamus	+	+
Subthalamic nucleus	NE	NE
Brainstem		
Midbrain tectum	+	+
Reticular formation	+	+
Red nucleus	+	+
Substantia nigra	+	−
Cerebral peduncle		+
Locus ceruleus	NE	NE
Faciál nucleus (motor)	+	+
Pontine nuclei	−	−
Superior olivary nucleus	−	−
Hypoglossal nucleus	+	+
Dorsal vagal nucleus	+	−
Nucleus ambiguus	+	−
Inferior olivary nuclei	+	−
Pyramis		+
Spinal cord		
Anterior horn	+	+
Intermediate lateral nucleus	−	−
Clarke nucleus	−	−
Posterior horn	+	−
White matter		−

NCI = neuronal cytoplasmic inclusions; GCI = glial cytoplasmic inclusions; + = present; − = absent; NE = not examined.

somes in Japanese control subjects. Second, the glutamine at position 343 is well conserved in several species. Third, Q343R was present in three affected individuals, including one autopsy-confirmed case, in two generations.[9]

We observed widespread distribution of TDP-43–immunoreactive NCIs and GCIs in the autopsied case who had this substitution.[6] The autopsied case demonstrated neuronal loss and gliosis restricted to the lower and upper motor neuron systems; however, the case demonstrated the TDP-43–immunoreactive NCIs and GCIs in various regions of the central nervous system (see the Table), suggesting that the case had a multisystem neuroglial proteinopathy of TDP-43.[6,9] Similar findings in SALS have also been described.[6] Interestingly, none of the affected individuals in the family showed dementia.[9] Although a few TDP-43–immunoreactive NCIs were found in the parietal, temporal, and insular cortices (no such inclusions were observed in the hippocampal dentate granule cells), no neurodegenerative features were evident in the autopsied case, indicating that Q343R substitution is not sufficient to cause FTLD.[6,9]

In this study, we failed to show abnormal functional consequence of Q343R substitution in *TDP-43*. However, we found abnormal-molecular-weight fragments of TDP-43 in the spinal cord of the autopsied case; these fragments are observed in SALS and in SOD1-negative FALS.[3–5,7] This result suggests that the Q343R substitution accelerates the production of abnormal-molecular-weight fragments of TDP-43. Furthermore, the Q343R substitution is located in the C-terminal region of TDP-43; this region is essential for binding to heterogeneous nuclear ribonucleoproteins and exhibits RNA-splicing inhibitory activity.[13] The result suggests that the substitution alters the ability of TDP-43 to bind to heterogeneous nuclear ribonucleoproteins and disturb RNA splicing.

Although the frequency of the mutation of the coding sequence of TDP-43 is rare,[14] further study for the functional consequences of Q343R substitution in neurons and glia should clarify the molecular basis for the association between Q343R substitution and the ALS phenotype, and may provide new insights into the pathomechanism of ALS.

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas "Applied Genomics" from the Ministry of Education, Culture, Sports, Science and Technology of Japan (17019006, O.O.) and the Research Committee on Neurodegenerative Diseases, Ministry of Health, Labor and Welfare, Japan (200731015, HT).

We thank the family for their participation.

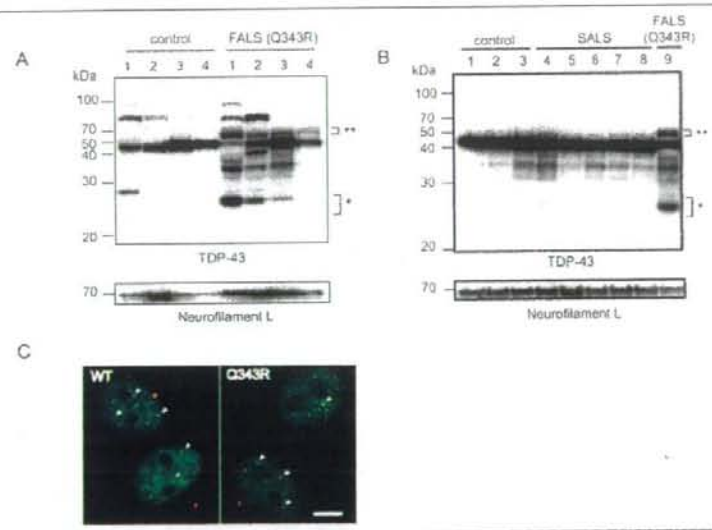

Fig 2. Biochemical analysis of 43kDa TAR-DNA–binding protein (TDP-43) in familial amyotrophic lateral sclerosis (FALS). (A) Immunoblots of extracts from spinal cords of control subjects and ALS patient with TDP-43 mutation probed with anti-TDP-43 (top) and anti-neurofilament L (bottom) antibodies. The 43kDa bands are observed for all the fractions. The approximately 25kDa (asterisks) *and approximately 45kDa bands* (double asterisks) *are detected for the patient fractions. Lane 1, low salt; lane 2, high salt with 1% Triton X-100; lane 3, 1% sarkosyl; lane 4, 8mmol/L urea. (B) One percent sarkosyl-soluble fractions from spinal cords were subjected to immunoblot analysis with anti-TDP-43 (top) and anti-neurofilament L (bottom) antibodies. Lanes 1 to 3, control subjects; lanes 4 to 8, sporadic ALS (SALS); lane 9, FALS with Q343R. (C) TDP-43 localization. COS-7 cells transfected with wild-type or Q343R GFP-tagged TDP-43 and TurboRed-tagged coilin are shown. The merged image shows TDP-43 (green) and coilin (red).* Arrowheads *indicate colocalization of TDP-43 and coilin. Scale bar = 10μm.*

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