表1　出生前診断（遺伝関連10学会による遺伝学的検査に関するガイドラインより）

（1）妊娠前半期に行われる出生前検査及び診断には，羊水，絨毛，その他の胎児試料などを用いた細胞遺伝学的，遺伝生化学的，分子遺伝学的，細胞・病理学的方法，及び超音波検査などを用いた物理学的方法などがある．

（2）出生前検査及び診断として遺伝学的検査及び診断を行うにあたっては，倫理的及び社会的問題を包含していることに留意しなければならず，とくに以下の点に注意して実施しなければならない．

（a）胎児が罹患児である可能性（リスク），検査法の診断限界，母体・胎児に対する危険性，副作用などについて検査前によく説明し，十分な遺伝カウンセリングを行うこと．

（b）検査の実施は，十分な基礎的研修を行い，安全かつ確実な検査技術を習得した産婦人科医により，またはその指導のもとに行われること．

（3）絨毛採取，羊水穿刺など，侵襲的な出生前検査・診断は下記のような場合の妊娠について，夫婦からの希望があり，検査の意義について十分な理解が得られた場合に行う．

（a）夫婦のいずれかが，染色体異常の保因者である場合

（b）染色体異常症に罹患した児を妊娠，分娩した既往を有する場合

（c）高齢妊娠の場合

（d）妊婦が新生児期もしくは小児期に発症する重篤なX連鎖遺伝病のヘテロ接合体の場合

（e）夫婦のいずれもが，新生児期もしくは小児期に発症する重篤な常染色体劣性遺伝病のヘテロ接合体の場合

（f）夫婦のいずれかが，新生児期もしくは小児期に発症する重篤な常染色体優性遺伝病のヘテロ接合体の場合

（g）その他，胎児が重篤な疾患に罹患する可能性のある場合

る。明確に出生前診断について聞きたいと来院される場合もあるが，時には出生前診断を受けようかどうしようかと迷いを持って来院することもある。また，自分は受けることに消極的だが，家族が受けるように勧める場合もある。そのようなときにはクライエントの不安に共感しつつ，迷いに寄りそい，自分の意見がまとまるまでカウンセリングを継続することもある。

続いて，当該の遺伝性疾患について，遺伝的なリスク，対応等の情報提供を改めて行いながら，夫婦の理解の差はないかどうかを確認する。提供する際には医学的な情報のみではなく，心理社会的な支援の状況などを加えながら伝えることが大切である。

出生前診断を受けると決めた夫婦には，特に次子が罹患だった場合にどうするかについて，十分に話し合うことが重要である。ある程度の時間をかけて話し合うことが望ましいため，妊娠前に遺伝カウンセリングを受けるのがより良いと思われる。妊娠した状況では時間の制約があり，冷静な判断は難しいことが多いと推測されるからである。夫婦の考える時間を十分に得るとともに，出生前診断に必要な遺伝学的な検査も技術的に可能かどうかを確認する必要がある。そのため，診断の希望がある夫婦は当該施設への紹介を早めに受けるとよいだろう。

一方で遺伝カウンセリングを受け，出生前診断を受けないと決める夫婦もいる。受けないと決めた夫婦の選択も尊重されるべきである。どんな子でも受け入れると決める夫婦もいれば，亡くなった児と同じ病気の子であることを望んで診断を受けないと決める夫婦もいる。次子が罹患児で出産する場合には，分娩体制について，産婦人科と連携をとり，調

表2　出生前診断の遺伝カウンセリングの流れ

遺伝カウンセリング外来受診	遺伝カウンセリング内容	心理的側面のフォロー
1回目 妊娠前	来訪の目的，発端者の情報の聴取，疾患の説明，遺伝カウンセリングプロセスの流れの説明，発端者の診断確定，（発端者の遺伝子検査，家族の多型解析，保因者診断），出生前遺伝子検査についての説明，遺伝子検査結果が陽性の場合について，意思を確認 出生前検査の受検希望があれば，発端者の遺伝子検査，家族の多型解析実施	クライエントの心理的状況，夫婦の価値観の把握，検査後のフォローの希望，児が亡くなって間もないようであれば危機介入
2回目	多型解析等結果開示，夫婦の意思再確認	結果が陽性だった場合についての考えを聞く
（必要に応じて倫理委員会に審査申請）		
3回目 妊娠判明時	出生前診断の意思確認，同意書に署名 検査結果が陽性だった場合についての意思確認と妊娠を諦める場合の医療機関の検討	
4回目 結果開示	検査結果開示，妊娠継続の場合のフォローアップ体制，出産体制の整備	結果が陽性：妊娠を諦める場合には，処置後フォローの連絡を入れ，面接．妊娠継続の場合には，産前，産後もフォロー 結果が陰性：1カ月後，フォローアップの連絡

整の役割を担う必要があるかもしれない。

妊娠が判明した場合には胎児の細胞を得る方法について再度説明をする。絨毛穿刺と羊水穿刺のどちらの方法がよいか，クライエントが利点やリスクとを十分に理解し，決めるプロセスが肝要である。結果は，約2～3週間で出ることが多い。

結果が陽性の場合には，重い選択がつきつけられることになる。結果を受けて，妊娠を中断する場合には心理的なフォローを受けられるように体制を整えることが大切である。心理的な葛藤は中絶のすぐ後で大きく，たいていの女性は時間を経て弱まるが，何人かの女性は苦悩の状態が続くとされる[3)]。したがって，中絶した悲しみからの回復には十分な時間がかけられるべきである。

児が罹患とわかって妊娠継続を選ぶ夫婦は出産までの長い間，お腹の児を受け入れていくためにさまざまな葛藤に直面するだろう。このような不安に対しても情緒面のサポートを行うとともに，出産後に社会的な資源を利用できるように手配を行う必要が出てくる。出生前診断を受けて，それぞれの夫婦が決定した選択に応じて，支援を考えることが遺伝カウンセリングに携わるスタッフに求められている。

おわりに

近年の周産期医療の技術の進歩により，胎児異常を指摘される妊婦が増えていると聞く。異常を指摘された妊婦が短い期間のうちに，自己選択をしなければならないが，それは容易なことではないと想像できる。新しい技術は人々を安心させるものとは限らず，知らなくてもよいことを知ってしまう現実もある。出生前診断に伴うクライエントや家族を支えるためにも，それぞれの状況に即した遺伝カウンセリングの意義は大きいと感じている。

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日本遺伝カウンセリング学会誌
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【原著】

脊髄性筋萎縮症における*SMN*遺伝子のコピー数解析と遺伝カウンセリングへの応用

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Copy number analysis of the *SMN* gene and genetic counseling using the results in spinal muscular atrophy

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【要旨】

脊髄性筋萎縮症（SMA：spinal muscular atrophy）は脊髄前角細胞の変性による筋萎縮と進行性筋力低下を特徴とする常染色体劣性遺伝性疾患である。SMAの原因遺伝子は*survival motor neuron 1*（*SMN1*）遺伝子であり，ホモ接合性欠失により発症する。同じように*SMN1*遺伝子欠失（0コピー）を示す症例でも，発症年齢や臨床的重症度に差が生じる。本研究では，臨床的重症度の差がなぜ生じるかを解明するために，SMA I～Ⅳ型患者33例において*SMN1*遺伝子と*SMN2*遺伝子のコピー数を調べた。その結果，臨床的に軽症なほど*SMN1*遺伝子の欠失範囲が小さくなり，*SMN2*遺伝子のコピー数が増加した。*SMN1*遺伝子欠失を示した症例では，*SMN1*遺伝子の欠失範囲と*SMN2*遺伝子のコピー数が重症度の差を生む要因であった。*SMN*遺伝子のコピー数情報は症状の重さや進行の予測などに利用できる可能性を示した。

キーワード：脊髄性筋萎縮症 spinal muscular atrophy（SMA），*SMN*遺伝子 *survival motor neuron*（*SMN*）gene，コピー数解析 copy number analysis，遺伝カウンセリング genetic counseling，発症年齢と臨床的重症度 onset and clinical severity

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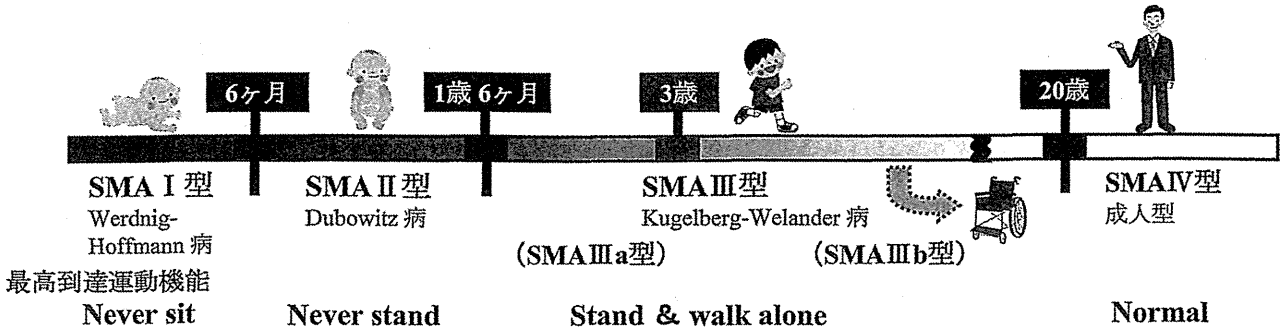
緒言

脊髄性筋萎縮症（SMA; OMIM 253300）は脊髄前角細胞の変性による筋萎縮と進行性筋力低下を特徴とする疾患である[1)]。SMAの頻度はおよそ出生児6,000～10,000人に1人であり，諸外国の保因者頻度は40～60人に1人である[2) 3)]。日本では2006年の全国調査によりSMAは約1,000人の患者数が推定される。小児期発症はⅠ型，Ⅱ型，Ⅲ型に，成人発症はⅣ型に分類される下位運動ニューロン病である[3 4)]。Ⅰ型（Werdnig-Hoffmann病）は6ヶ月までに発症し，生涯座位保持不可能である。2歳以降の生存のためには人工呼吸管理を必要とする。Ⅱ型（Dubowitz病）は1歳6ヶ月までに発症し，生涯起立・歩行の獲得が不可能である。Ⅲ型（Kugelberg-Welander病）は1歳6ヶ月以降に

図1　SMAの型と臨床症状

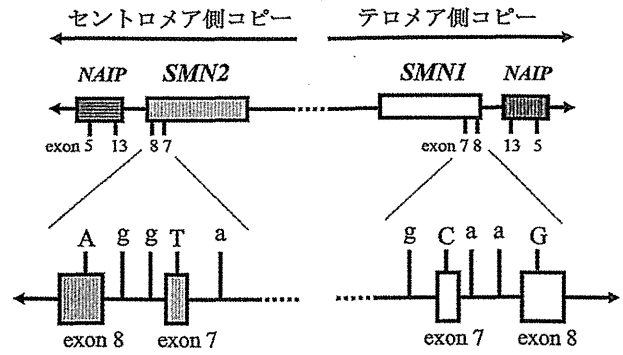


発症し，自立歩行が可能だが症状が進むにつれ不可能となる[3)-5)]。Ⅳ型は弧発性が多く，20歳以降に発症する[4)]（図1）。

小児期発症のSMAの原因遺伝子は*survival motor neuron 1*（*SMN1*）遺伝子であり，ホモ接合性欠失により発症する[6)]。*SMN1*遺伝子は第5染色体長腕5q13に存在し，同領域に向反性に重複した配列の*SMN2*遺伝子も存在する（図2）。テロメア側に位置する*SMN1*遺伝子とセントロメア側に位置する*SMN2*遺伝子の間には5つの塩基の違いがあり[6)]，翻訳領域にはexon 7に存在する1塩基の違い（*SMN1*遺伝子はc.840C，*SMN2*遺伝子はc.840T）しか存在しない[6) 7)]。1塩基の違いがexon 7でのスプライシングパターンに変化をもたらし，*SMN1*遺伝子は全長の*SMN1*転写産物を産生し，*SMN2*遺伝子はexon 7領域を欠いた（Δ7）*SMN2*転写産物を約85%，全長の*SMN2*転写産物を約15%産生する[4) 8)]。exon 7領域を欠いたタンパク質SMN Δ7は機能を持たず，すぐに分解される[9)]。全長の転写産物から翻訳された機能的なタンパク質SMNは脊髄神経細胞の核に存在し，RNAの代謝に関与している[10)]。また，*SMN*（*SMN1*，*SMN2*）遺伝子の下流には細胞のアポトーシスを抑制する蛋白質をコードする*neuronal apoptosis inhibitory protein*（*NAIP*）遺伝子が存在し，SMAの重症度に関連があると考えられている[6) 11)]。

SMAの遺伝学的検査としてはPCR-RFLP法を用いて*SMN1*遺伝子欠失を調べる方法が一般的である[6) 12)]。近年はReal time PCR法やMultiplex Ligation-dependent Probe Amplification（MLPA）法を用いて遺伝子のコピー数解析が行われるようになり，*SMN*遺伝子のコピー数が疾患の重症度と関連があるという報告がされている[13)-15)]。当センターではこれまでに*SMN1*遺伝子欠失を示すSMAにおいて，発症年齢や臨床的重症度に差を認める症例を確認している。本研究では，この臨床的スペクトラムの成因解明のために*SMN1*遺伝子欠失を示す症例のコピー数を調べ，コントロールと比較した。また，*SMN*遺伝子と*NAIP*遺伝子のコピー数の解析結果をどのように遺伝カウンセリングに活用できるかについて検討した。

図2　*SMN*遺伝子の構造



対象・方法

(1) 対象

SMA Ⅰ～Ⅳ型症例33例〔Ⅰ型　15例（0～6ヶ月），Ⅱ型　7例（7～18ヶ月），Ⅲ型　9例（19ヶ月～20歳未満），Ⅳ型　2例（20歳以上）〕，コントロール70例（20歳以上）。

20歳以上の対象者についてはインフォームドコンセントにて同意を取得し，20歳未満の対象者についてはインフォームドアセントを得て，同意能力を欠く場合には代諾者から同意を得た。また，本研究は東京女子医科大学倫理委員会の承認を受けて実施した。

(2) *SMN1*遺伝子exon 7，*NAIP*遺伝子exon 5欠失解析

ゲノムDNAは全血からQIAamp DNA Blood Mini Kit（QIAGEN）を用いて抽出し，最終濃度が100 ng/μlになるように調製した。*SMN1*遺伝子exon 7の欠失解析には，Lefebvreらの PCR-RFLP法を用いて検査を行った[6) 12)]。*NAIP*遺伝子exon 5の欠失解析には，Royらの PCR法を用いた[11)]。本方法はSMAの遺伝学的検査として，当センターで実施している。

(3) MLPA法を用いた*SMN*遺伝子，*NAIP*遺伝子コピー数解析

*SMN*遺伝子のコピー数解析にはSALSA MLPA KIT P021-A1 SMA（MRC-Holland）を用いた。キットには*SMN1*遺伝子と*SMN2*遺伝子exon 7に特異的なプローブ，

SMN1 遺伝子と *SMN2* 遺伝子 exon 8 に特異的なプローブ，*SMN1* 遺伝子と *SMN2* 遺伝子 exon 1, 4, 6, 8 に特異的なプローブ，*SMN* 遺伝子近傍の *NAIP* 遺伝子に特異的なプローブ，他の染色体に特異的なプローブ，リファレンスとなるプローブが含まれている。MLPA 反応後の DNA フラグメントは GeneMapper software v4.1 (Applied Biosystems) を用いて ABI 3130 Genetic Analyzer (Applied Biosystems) で解析を行った。

結果

(1) *SMN1* 遺伝子 exon 7，*NAIP* 遺伝子 exon 5 欠失解析

SMA 症例 33 例において *SMN* 遺伝子 exon 7 の欠失解析を行い，全症例において *SMN1* 遺伝子 exon 7 の欠失を確認した。SMA Ⅰ型では 8 例に *NAIP* 遺伝子 exon 5 の欠失を確認した。

(2) MLPA 法を用いた *SMN* 遺伝子コピー数解析

1) コントロールにおける *SMN1* 遺伝子コピー数解析

MLPA 法を用いてコントロールの *SMN* 遺伝子 (exon 1, 4, 6, 7, 8) のコピー数を解析した (図 3)。*SMN1* 遺伝子，*SMN2* 遺伝子をそれぞれ 2 コピーずつもつ症例〔*SMN1* : *SMN2* (コピー数比) = 2 : 2〕が最も多く，36 例 (51.4%) であった。次に *SMN1* 遺伝子を 2 コピー，*SMN2* 遺伝子を 1 コピーもつ症例 (*SMN1* : *SMN2* = 2 : 1) が 27 例 (38.6%) であった。その 2 つのタイプが 90% を占めた。*SMN1* 遺伝子を 1 コピー (*SMN1* : *SMN2* = 1 : 1) もしくは 3 コピーもつ症例 (*SMN1* : *SMN2* = 3 : 0 or 1) や，*SMN2* 遺伝子を 0 コピーもつ症例 (*SMN1* : *SMN2* = 2 or 3 : 0) を確認した。

2) SMA における *SMN* 遺伝子，*NAIP* 遺伝子コピー数解析

SMA 症例 33 症例について，MLPA 法により *SMN* 遺伝子，*NAIP* 遺伝子のコピー数を解析した (表 1)。

i SMA Ⅰ型

全症例 (15 例) で *SMN1* 遺伝子 exon 7, exon 8 の欠失 (0 コピー) を確認した。13.3% (2/15) は *SMN2* 遺伝子 exon 7 が 3 コピーであった。53.3% (8/15) は *NAIP* 遺伝子 exon 5 が欠失していた。

図 3　コントロール 70 例における *SMN* 遺伝子コピー数解析

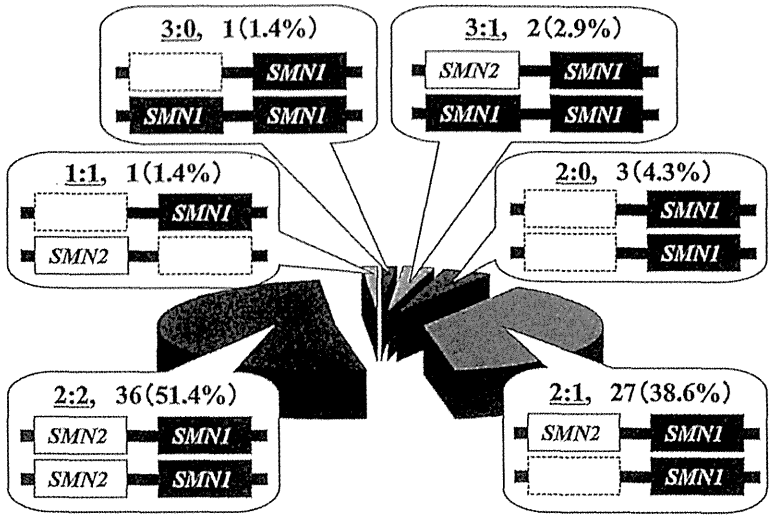


表 1　SMA 症例における *SMN* 遺伝子，*NAIP* 遺伝子コピー数解析

型 (合計人数)，該当人数 (%)	コピー数	*SMN2*		*SMN1*		*NAIP*	
		exon7	exon8	exon7	exon8	exon5	exon13
Ⅰ (n=15)	0	0	0	15 (100)	15 (100)	8 (53.3)	0
	1	0	0	0	0	7 (46.7)	15 (100)
	2	13 (86.7)	12 (80)	0	0	0	0
	3	2 (13.3)	3 (20)	0	0	0	0
Ⅱ (n=7)	0	0	0	7 (100)	5 (71.4)	1 (14.3)	0
	1	0	1 (14.3)	0	2 (28.6)	5 (71.4)	6 (85.7)
	2	4 (57.1)	1 (14.3)	0	0	1 (14.3)	1 (14.3)
	3	3 (42.9)	5 (71.4)	0	0	0	0
Ⅲ (n=9)	0	0	0	9 (100)	5 (55.5)	0	0
	1	0	0	0	0	3 (33.3)	2 (22.2)
	2	0	4 (44.4)	0	4 (44.4)	6 (66.6)	7 (77.7)
	3	9 (100)	2 (22.2)	0	0	0	0
	4	0	3 (33.3)	0	0	0	0
Ⅳ (n=2)	0	0	0	2 (100)	2 (100)	0	0
	1	0	0	0	0	0	0
	2	0	0	0	0	1 (50)	2 (100)
	3	2 (100)	0	0	0	1 (50)	0
	4	0	2 (100)	0	0	0	0

SMA Ⅰ～Ⅳ型患者 33 例 (Ⅰ型　15 例，Ⅱ型　7 例，Ⅲ型　9 例，Ⅳ型　2 例) についてコピー数の解析を行った。

ii SMA Ⅱ型

全症例（7例）で*SMN1*遺伝子exon 7の欠失が確認され，28.6%（2/7）は*SMN1*遺伝子exon 8が1コピーであった。42.9%（3/7）は*SMN2*遺伝子exon 7が3コピーであった。14.3%（1/7）だけが*NAIP*遺伝子exon 5の欠失を示した。

iii SMA Ⅲ型

全症例（9例）で*SMN1*遺伝子exon 7の欠失が確認され，44.4%（4/9）は*SMN1*遺伝子exon 8が2コピーであった。全症例（9例）で*SMN2*遺伝子exon 7が3コピーを示した。どの症例にも*NAIP*遺伝子exon 5の欠失は認められなかった。

iv SMA Ⅳ型

全症例（2例）で*SMN1*遺伝子exon 7，exon 8の欠失が確認された。全症例で*SMN2*遺伝子exon 7が3コピーを示した。どの症例にも*NAIP*遺伝子exon 5，exon 13の欠失は認められなかった。

考察

(1) コントロールにおける*SMN*遺伝子コピー数の多様性

コントロールにおける*SMN1*遺伝子コピー数解析の結果より（図3），*SMN1*遺伝子，*SMN2*遺伝子をそれぞれ2コピーもつ症例（*SMN1*：*SMN2*＝2：2）が最も多く（51.4%）観察されたが，*SMN1*遺伝子は1から3コピー，*SMN2*遺伝子は0から2コピーの範囲でバラツキが見られた。コントロールであっても*SMN*遺伝子のコピー数には多様性があり，染色体5q13領域は組換え等の変化が起こりやすい領域であると考えられた。アジアでは台湾や中国でもコントロールにおける*SMN1*遺伝子コピー数解析が大規模に行われている[16)-18)]。本研究と同様に*SMN1*遺伝子，*SMN2*遺伝子をそれぞれ2コピーもつ症例（*SMN1*：*SMN2*＝2：2）が最も多く（台湾56%，中国57.7%）観察され，*SMN1*遺伝子を2コピー，*SMN2*遺伝子を1コピーもつ症例（*SMN1*：*SMN2*＝2：1）が次に多く（台湾28%，中国26.2%）観察され，本研究の結果と同様の割合を示していた。また，*SMN1*遺伝子が1コピーの症例が70例中1例であった（図3）。日本のSMA保因者頻度はこの割合に近い数値であろう。諸外国の保因者頻度は40～60人に1人との報告がある[2) 3)]。アジアでは台湾で107,611人における大規模解析が行われており48人に1人[16)]，中国では1,712人について解析が行われ42人に1人と報告されている[18)]。今回の結果からは，日本人における保因者頻度は諸外国に比べると頻度が低いように思われるが，今回の解析は小規模であり，より正確な日本人の保因者頻度を算出するためには，大規模な解析が必要であると考えられた。

(2) 遺伝子の欠失範囲と重症度

PCR-RFLP法[6) 12)]を用いて*SMN*遺伝子exon 7欠失解析を行い，欠失が認められたSMA症例33例においてMLPA法によるコピー数解析を行った。全症例（33例）で*SMN1*遺伝子exon 7は欠失（0コピー）を示し，PCR-RFLP法による解析と同様の結果を得た。*NAIP*遺伝子exon 5の欠失はSMA Ⅰ型の53.3%（8/15）で認められ，PCR法，MLPA法ともに同様の結果を得た。PCR-RFLP法を用いて，東京女子医科大学附属遺伝子医療センターにおいて遺伝学的検査を実施したSMA症例322例では，*SMN*遺伝子の欠失はⅠ型で98%，Ⅱ型で95%，Ⅲa型で52%，Ⅲb型で42%，Ⅳ型で15%であった[19)]。SMA Ⅰ型では*NAIP*遺伝子exon 5を欠失する割合が41%確認されたが，その他の型では10%にも満たなかった。SMA Ⅰ型における*NAIP*遺伝子exon 5の欠失は本研究結果と近い割合（53.3%）であった（表1）。さらにMLPA法では片アレル欠失の有無の判断が可能であり，Ⅲa型の場合では片アレルだけ欠失の範囲が大きい場合（図4アレルA-2とA-4の組み合わせでもつタイプ）と両アレルとも欠失範囲が小さい場合（図4アレルA-2とA-2の組み合わせでもつタイプ）を比較すると欠失範囲の大きい前者の方が歩行不可能になる時期が早かった。特に10代で発症しているⅢb型は*SMN1*遺伝子exon 7のみの欠失の割合が多く（図4A-1），Ⅲa型との欠失範囲の大きさの差は顕著であった。

*SMN*遺伝子解析だけでなく*NAIP*遺伝子の解析をすることで*SMN*遺伝子近傍の欠失範囲を確認することができた。特にⅠ型では*SMN1*遺伝子だけでなく隣接する*NAIP*遺伝子も欠失している症例が多く見られ，欠失範囲が大きいことを示していた（図4A-4）。Ⅲ型，Ⅳ型では*SMN1*遺伝子の欠失の割合が少ないことは*SMN1*遺伝子以外の別の因子が原因であることも示唆された。

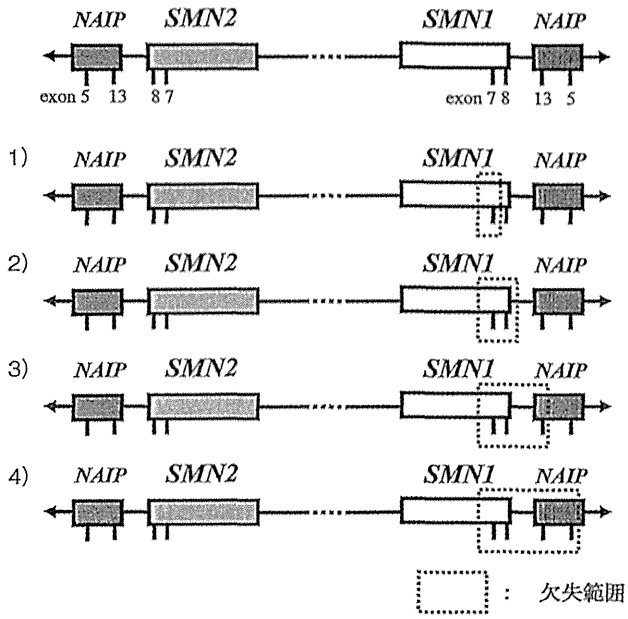
(3) *SMN2*遺伝子のコピー数と重症度

MLPA法解析により，*SMN2*遺伝子のコピー数の変化を確認した（表1，図5）。型別にコピー数の平均値を算出したところ，主に症状が軽くなるに従い，*SMN2*遺伝子のコピー数が増加する傾向にあり（図5），既報告と同様な傾向を示していた[13)-15)]。コピー数の増加は図4Bに示すように遺伝子変換が起こったためと考えられた。

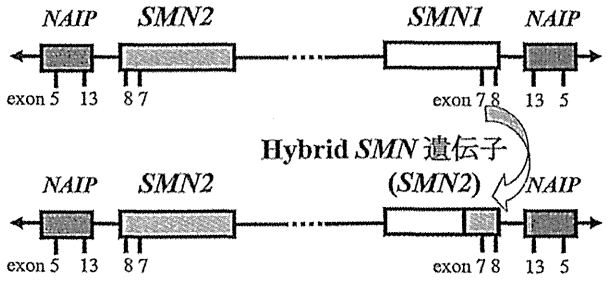
SMAは主に*SMN1*遺伝子を失うことで発症するが，*SMN1*遺伝子が完全に欠失するか，もしくは上述したような*SMN1*遺伝子と*SMN2*遺伝子間での遺伝子変換によるものと考えられる。*SMN1*遺伝子を完全に欠失した症例よりも遺伝子変換により*SMN1*遺伝子が*SMN2*遺伝子に変換した症例のほうが，症状が軽症化する傾向にあった。*SMN2*遺伝子は少ないながらも機能的なSMNタンパク質を産生する。*SMN2*遺伝子のコピー数が増加することで機能的なSMNタンパク質量が増え，症状の軽症化に繋がることが考えられた[20)]。さらには*SMN2*遺伝子からより多くの機能的なSMNタンパク質を産生させることが本症例の軽症化を目

図4　染色体5q13領域における変化

A. 欠失範囲の差



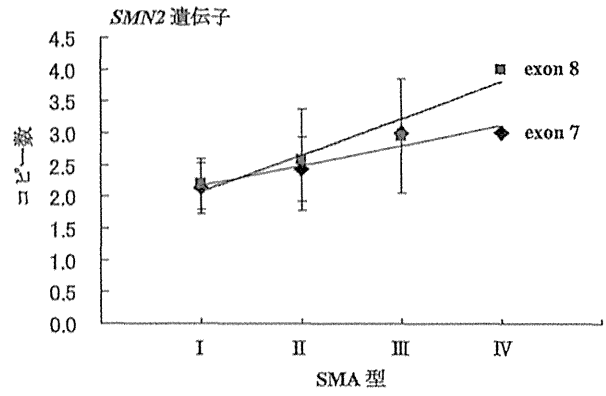
B. 遺伝子変換



A. 1) は *SMN1* 遺伝子 exon 7 のみの欠失，2) は *SMN1* 遺伝子 exon 7, 8 の欠失，3) は *SMN1* 遺伝子 exon 7 から *NAIP* 遺伝子 exon 13 付近までの欠失，4) は *SMN1* 遺伝子 exon 7 から *NAIP* 遺伝子 exon 5 付近までの欠失（もしくは全欠失）を示している。

B. *SMN2* 遺伝子の増加が確認される症例はこのような（*SMN1* から *SMN2* への）遺伝子変換が起こっている可能性が示唆された。

図5　SMA の型と *SMN2* 遺伝子コピー数の関係



型ごとにコピー数の平均値を算出した。I 型から IV 型にかけて *SMN2* 遺伝子コピー数の増加を示した。

指す治療に利用できると考えられた。

(4) 遺伝カウンセリング・診療への応用

SMA 症例の遺伝学的検査において，MLPA 法を用いることで *SMN* 遺伝子，*NAIP* 遺伝子のコピー数や欠失領域を知ることが可能になった。さらには，*SMN* 遺伝子，*NAIP* 遺伝のコピー数情報が重症度や進行の早さなどに関連していることが示唆された。

特に発症年齢に差がある III 型の場合にコピー数や欠失領域を診療に利用できると考えている。例えば *SMN2* 遺伝子（exon 7）のコピー数が同じ 3 症例を比較すると，欠失範囲が小さい（*SMN1* 遺伝子のみの欠失）2 症例は 50 歳を過ぎても歩行可能であったのに対し，欠失範囲が大きい（*SMN1* 遺伝子，*NAIP* 遺伝子ともに欠失）1 症例では 12 歳で歩行不可能になった。後者のような場合には，成長や発達過程を考慮しながら運動機能障害の進展の予測，関節拘縮予防などの理学療法の早期介入などの情報提供が有用であると考えられた。また，遺伝学的検査の解析結果によって，情報提供をする時期や内容を個別に検討し，患者・家族が SMA における運動機能障害の各段階を理解し，受容し，進展過程を少しでも抑制するためのサポートに応用可能であることが示唆された。

また，将来 *SMN2* 遺伝子をターゲットとした薬剤が利用可能になった場合，*SMN2* 遺伝子のコピー数情報がその薬剤の効果予測（例：*SMN2* 遺伝子のコピー数が多いと効果が高い可能性があるなど）に利用できるかもしれない。

結論

SMN 遺伝子欠失を示した SMA 症例では，遺伝子欠失範囲と *SMN2* 遺伝子のコピー数が発症の早さや症状の差を生む要因であった。また，コントロールにおいて *SMN* 遺伝子のコピー数は多様であり，5q13 領域は変化が起こりやすいことを示していた。*SMN* 遺伝子のコピー数情報を活用することで，症状の重さや進行の予測，保因者診断，治療の方向性など様々な情報を提供していくことが可能になった。

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Original article

Intragenic mutations in *SMN1* may contribute more significantly to clinical severity than *SMN2* copy numbers in some spinal muscular atrophy (SMA) patients

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Abstract

Background: Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder caused by deletion or intragenic mutation of *SMN1*. SMA is classified into several subtypes based on clinical severity. It has been reported that the copy number of *SMN2*, a highly homologous gene to *SMN1*, is associated with clinical severity among SMA patients with homozygous deletion of *SMN1*. The purpose of this study was to clarify the genotype-phenotype relationship among the patients without homozygous deletion of *SMN1*. *Methods:* We performed molecular genetic analyses of *SMN1* and *SMN2* in 112 Japanese patients diagnosed as having SMA based on the clinical findings. For the patients retaining *SMN1*, the PCR or RT-PCR products of *SMN1* were sequenced to identify the mutation. *Results:* Out of the 112 patients, 106 patients were homozygous for deletion of *SMN1*, and six patients were compound heterozygous for deletion of one *SMN1* allele and intragenic mutation in the retained *SMN1* allele. Four intragenic mutations were identified in the six patients: p.Ala2Val, p.Trp92Ser, p.Thr274TyrfsX32 and p.Tyr277Cys. To the best of our knowledge, all mutations except p.Trp92Ser were novel mutations which had never been previously reported. According to our observation, clinical severity of the six patients was determined by the type and location of the mutation rather than *SMN2* copy number. *Conclusion: SMN2* copy number is not always associated with clinical severity of SMA patients, especially SMA patients retaining one *SMN1* allele.

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Keywords: Spinal muscular atrophy; *SMN1*; *SMN2*; Copy number; Intragenic mutation

1. Introduction

Spinal muscular atrophy (SMA) is a common neuromuscular disease characterized by degeneration of lower motor neurons, leading to the axial and limb weakness associated with muscle atrophy. The incidence of the disease has been estimated at 1 in 10,000 newborns, with an expected carrier frequency of 1 in 50 [1]. Based on molecular epidemiological analysis using *SMN1* copy number, the worldwide carrier frequency of SMA is 1 in 40–70, suggesting a disease incidence of 1 in 6000–20,000 [2].

SMA is classified into four subtypes depending on the age of disease onset and the achievement of motor milestones [3]: namely, type 1 (severe form; onset age of 0–6 months old, unable to sit unaided), type 2 (intermediate form; onset age of <18 months old, unable to stand or walk unaided), type 3 (mild form; onset age of >18 months old, able to stand or walk unaided), and type 4 (milder form; onset age of >21 years old, able to stand or walk unaided).

All SMA subtypes have been mapped to chromosomal region 5q11.2–13.3 [4–7] and the survival motor neuron gene (*SMN*) and neuronal apoptosis-inhibitory protein gene (*NAIP*) were cloned as SMA-causing gene candidates [8,9]. The *SMN* gene exists as two highly homologous copies, *SMN1* (the telomeric copy) and *SMN2* (the centromeric copy) [8]. It is now established that SMA is caused by deletions or intragenic mutations of *SMN1*. *SMN1* is homozygously deleted in more than 90% of SMA patients [8,10], and deleteriously mutated in the remaining patients [8,11]. On the other hand, *NAIP*-deletion has been found only in 50% of type 1 patients, and much less frequently in type 2 and 3 patients. The presence or absence of *NAIP* may be associated with the clinical severity of SMA [9,10].

Increased *SMN2* copy number is related to improved survival outcomes and maintenance of motor function [12–16]. Both *SMN* genes, *SMN1* and *SMN2*, differ by only five nucleotides [8]. Of the five nucleotide differences between the two *SMN* genes, only one is present in the coding region at position +6 of exon 7 in *SMN1* (c.840C) and *SMN2* (c.840T). Although this mutation is translationally silent, the C-to-T transition alters the splicing pattern in *SMN2* exon 7 [17]. *SMN1* exclusively produces full-length (FL) *SMN1* transcripts, while *SMN2* produces ~90% of exon7-lacking (△7) *SMN2* transcripts and ~10% of FL-*SMN2* transcripts [18]. It is expected that high *SMN2* copy number may produce a large amount of FL-*SMN2* to compensate for the loss of *SMN1* to some degree.

However, most phenotype-genotype correlation studies have been conducted only in SMA patients with a complete loss of *SMN1*. The relationship between *SMN2* copy number and clinical severity are yet to be clarified in SMA patients retaining one *SMN1* allele. In this study, to understand the modifying factors in determining the clinical phenotype of SMA patients retaining one *SMN1* allele, we conducted a mutation analysis and investigated the contribution of *SMN2* copy number to the clinical severity in such patients.

2. Patients and methods

2.1. Patients

All 112 Japanese patients (51 males and 61 females) fulfilled the diagnostic criteria defined by the International SMA Consortium [19]. Here, patients with onset before 20 years old was classified into type 3, and those with onset after 21 years old was classified into type 4 [3]. Informed consent was obtained from these patients and/or their parents. This study project including genetic analysis was approved by the Ethical Committee of the Kobe University Graduate School of Medicine, Japan.

In this study, six patients (Patients 1–6) retaining one allele of *SMN1* exon 7, were found to carry intragenic mutations in *SMN1*. Patients 1 (female) and 2 (male) were type 1 patients reported previously to have one *SMN1* allele [20]. Patient 3 was a 19-day-old male with SMA type 1, referred to us because of respiratory insufficiency and swallowing difficulties. Patient 4 was a 7-year-old female with type 2 SMA. She was first diagnosed as having SMA type 2 close to type 3 because she could sit unaided and stand while holding onto something (such as a wall or table) for support. However, she rapidly lost such abilities at 2 years old. Finally, she was bound to artificial ventilator because of respiratory insufficiency at 3 years old. Patient 5 was a 13-year-old male with type 3 SMA, who had pain and heaviness in legs during exercise since the age of 11 years. He later developed symptoms including waddling gait, muscle weakness and atrophy in quadriceps, and attenuated patellar tendon reflex. Patient 6 was a 19-year-old female with type 3 SMA, who had noticed muscle weakness during swimming exercise at the age of 13 years. She gradually lost her running ability and could no

longer run as fast as the other classmates in her high school days.

2.2. SMN and NAIP deletion test

Genomic DNA was extracted from 3 ml of whole blood using a DNA extraction kit, SepaGene (Sanko Junyaku, Tokyo, Japan). For the *SMN* and *NAIP* deletion test, PCR and enzyme digestion reactions were performed according to the method of van der Steege et al. [21]. Exon 5 of the *NAIP* gene was detected using the PCR method of Roy et al. [9]. Here we adopted "exon 5" as a widely accepted exon number, although this exon has been denoted as "exon 4" by Chen et al. [22].

2.3. Copy number analysis of the SMN genes using real time PCR method

We determined the copy numbers of the *SMN* genes based on the real-time PCR method of Tran et al. [23]. Cystic fibrosis trans-membrane regulator gene (*CFTR* gene) was used as a reference gene for the relative quantification of copy numbers.

2.4. Messenger RNA analysis

For the assignment of the mutation to *SMN1* or *SMN2*, mRNA analysis was performed. Total RNA was extracted from leukocytes using the acid guanidiumthiocyanate-phenol–chloroform method. *SMN1* and *SMN2* mRNA species were amplified by reverse transcriptase (RT)-PCR method [16,24]. A new primer, ex1-F (5′-TGC GCA CCC GCG GGT TTG CT-3′), was designed for this study. The mRNA species encompassing exons 1–8 were amplified using primers ex1-F and 541C1120 [8], and the mRNA species encompassing exons 1–7 were amplified using primers ex1-F and 541C770 [8].

2.5. Nucleotide sequencing

The amplified PCR or RT-PCR products of *SMN* exons were purified and sequenced directly or after subcloning. The sequencing reaction was performed using a dye terminator cycle-sequencing kit (Life Technologies Corporation, Carlsbad, CA). The reaction product was electrophoresed on an ABI PRISM® 310 Genetic Analyzer (Life Technologies Corporation, Carlsbad, CA).

2.6. Computational algorithms

We predicted the mutation effects on the protein function using three computational algorithms: Sorting Intolerant from Tolerant amino acid substitutions (SIFT) [25], Polymorphism Phenotyping-2 (PolyPhen-2) [26], and Grantham score difference (Align-GVGD) [27].

2.7. Statistics

The correlation of copy number of *SMN2* with the clinical subtypes was compared by chi-square test and *t*-test. *P*-value of less than 0.05 was considered to indicate a significant difference. The software used for statistical analysis was Statistical Program for Social Science (SPSS) Version 16 (IBM Corporation, Paulo Alto, US).

3. Results

3.1. SMN1 and NAIP deletion test

SMN1 exon 7-deletion (herein after referred to as *SMN1*-deletion) was found in almost all SMA patients, regardless of clinical subtypes: 106 out of 112 (95%) patients with SMA in this study had *SMN1*-deletion and 6 patients (5%) had subtle mutations in *SMN1*. Out of 106 *SMN1*-deleted patients, 48 (45%) were type 1, 35 (33%) were type 2, 19 (18%) were type 3, and 4 (4%) were type 4 (Table 1).

In our study, 96 of 106 (91%) *SMN1*-deleted patients had deletion of *SMN1* exon 8. However, the other 10 patients (9.0%) retained *SMN1* exon 8. We confirmed that these patients had at least one copy of the hybrid gene with *SMN2* exon 7 and *SMN1* exon 8 using direct sequencing analysis of the PCR fragment amplified with the common primers for *SMN1* and *SMN2*.

NAIP exon 5-deletion (herein after referred to as *NAIP*-deletion) was always accompanied by *SMN1*-deletion (Table 1). In addition, *NAIP*-deletion was much more frequent in SMA type 1 than SMA non-type 1. *NAIP*-deletion was found in 29 out of 48 (60%) patients with *SMN1*-deleted SMA type 1, while it was found in only 8 out of 58 (14%) patients with *SMN1*-deleted SMA types 2, 3 and 4.

3.2. SMN2 copy number and clinical severity in patients with SMN1-deletion

We determined the *SMN2* copy numbers of all the patients enrolled in this study using the real-time PCR method. For the analysis of *SMN2* copy number and clinical severity, the"*SMN2* exon 7-*SMN1* exon 8 hybrid" gene is regarded as *SMN2*.

A significant relationship between *SMN2* copy number and clinical severity was observed in this study (Table 2). 38 out of 48 (79%) patients with *SMN1*-deleted SMA type 1 showed one copy or two copies of *SMN2*, 34 out of 35 (97%) patients with *SMN1*-deleted SMA type 2 showed three copies of *SMN2*, 18 out of 19 (95%) patients with *SMN1*-deleted SMA type 3 showed three or four copies of *SMN2*, and 3 out of 4 (75%) patients with *SMN1*-deleted SMA type 4 showed four copies of *SMN2*.

Table 1
SMN1 and *NAIP* deletion test ($n = 112$).

SMN1		*NAIP*	Type 1	Type 2	Type 3	Type 4	Total
Exon 7	Exon 8	Exon 5					
Del	Del	Del	29	6	1	1	37
Del	Del	Non-del	17	24	15	3	59
Del	Non-del	Non-del	2	5	3	0	10
Non-del	Non-del	Non-del	3	1	2	0	6
Total			51	36	21	4	112

Table 2
Clinical severity and *SMN2* copy number in patients with homozygous *SMN1*-deletion ($n = 106$).

	1 copy	2 copies	3 copies	4 copies	Mean	(SD)
Type 1	1	37	10	0	2.18	(0.44)
Type 2	0	1	34	0	2.97	(0.17)
Type 3	0	1	13	5	3.18	(0.51)
Type 4	0	0	1	3	3.80	(0.40)
Total	1	39	58	8		

Table 3
Clinical severity and *SMN2* copy number in patients retaining one *SMN1* allele ($n = 6$).

	Sex	Onset	Type	*SMN2* copy number	Nucleotide change (exon)	Amino acid change	Domain	References
Patient 1	F	5m	1	3	c.275 G > C (exon 3)	p.Trp92Ser	Tudor	[20]
Patient 2	M	6m	1	3	c.275 G > C (exon 3)	p.Trp92Ser	Tudor	[20]
Patient 3	M	0m	1	2	c.819_820 insT (exon 6)	p.Thr274Tyr fsX32	C-terminal	This study
Patient 4	F	12m	2	1	c.830 A > G (exon 6)	p.Tyr277Cys	C-terminal	This study
Patient 5	M	11y	3	1	c.5 C > T (exon 1)	p.Ala2Val	N-terminal	This study
Patient 6	F	13y	3	1	c.5 C > T (exon 1)	p.Ala2Val	N-terminal	This study

3.3. *SMN2* copy number and clinical severity in patients retaining one *SMN1* allele

In this study, we identified four different intragenic mutations in *SMN1* of six patients without *SMN1*-deletion (Patients 1–6) (Table 3). All of them were compound heterozygous for deletion of one *SMN1* allele and an intragenic point mutation of the other *SMN1* allele. The intragenic mutations included three missense mutations and one frame-shift mutation: c. 5C > T (p.Ala2Val) in exon 1, c. 275G > C (p.Trp92Ser) in exon 3, c.819_820insT (p.Thr274TyrfsX32) in exon 6, and c.830 A > G (p.Tyr277Cys) in exon 6. Three of the mutations, p.Ala2Val, p.Thr274TyrfsX32 and p.Tyr277Cys, are novel ones which have never been previously reported.

We predicted the effect of the missense mutations on the protein function using three computational algorithms: SIFT [25], PolyPhen-2 [26], and Align-GVGD [27]. All three types of missense mutation were predicted to damage the protein function.

Interestingly, the observed phenotype of patients carrying an intragenic mutation deviated from the expected correlations with the *SMN2* copy number (Table 3 and Fig. 1): type 3 patients with p.Ala2Val (Patients 5 and 6) carried only a single copy of *SMN2*, while type 1 patients with p.Trp92Ser (Patients 1 and 2) carried as many as 3 copies of *SMN2*. These findings suggested that intragenic mutations in *SMN1* influence the clinical phenotype more significantly than *SMN2* copy numbers in some patients.

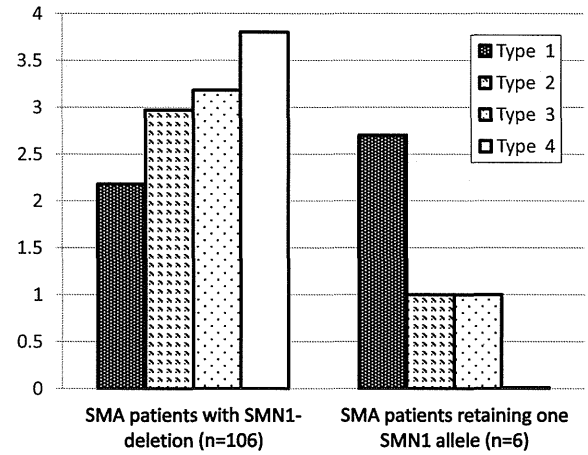

Fig. 1. Mean *SMN2* copy numbers in SMA patients. Patients with *SMN1*-deletion ($n = 106$) carried zero copies of *SMN1*. Patients retaining one *SMN1* allele ($n = 6$) which harbored intragenic mutations: p.Ala2Val, p.Trp92Ser, p.Thr274TyrfsX32 and p.Tyr277Cys.

4. Discussion

The identification of intragenic mutations, especially missense mutations, may help us to further elucidate the function of SMN and the pathogenic mechanism of SMA. In this study, we identified four different intragenic *SMN1* mutations in six SMA patients without *SMN1*-deletion. These intragenic mutations were p.Ala2Val, p.Trp92Ser, p.Thr274TyrfsX32, and p.Tyr277Cys.

The p.Ala2Val mutation, which is located in the N-terminal domain, has never been reported until now. Our two patients with p.Ala2Val were unrelated. However, another mutation in the same location, p.Ala2Gly, has previously been reported in three SMA patients; these patients were also unrelated individuals, but had the possibility of sharing an ancestral origin [28]. All patients with p.Ala2Gly carried only one *SMN2* copy, and two of them showed mild phenotype (type 3). The mutation effect of p.Ala2Val, as well as p.Ala2Gly, may be much less deleterious than other missense mutations identified in this study. However, *SMN2* may not be dispensable in these patients. The mild SMA mutation, p.Ala2Gly, by itself cannot rescue $Smn^{-/-}$ mice, suggesting that homomer of the mutant SMN is not functional [29]. According to the Workman et al. [30], the heteromer of mutant SMN and FL-SMN from a single copy of *SMN2* must have some function.

We previously reported the p.Trp92Ser mutation in two unrelated patients [20]. This mutation is located in the Tudor domain to which other proteins bind. [31]. Many of them are involved in small nuclear ribonucleoprotein (snRNP) biogenesis. SMN Tudor domain preferentially binds symmetric dimethylated arginine (sDMA) of Sm proteins which constitute Sm core of snRNP [32]. We have already reported that the binding ability of the mutated SMN with p.Trp92Ser to SmB and fibrillarin was reduced to half of normal levels [20]. Most recently, Tripsianes et al. [33] examined the relationship between mutated Tudor domain and the binding capacity to sDMA *in vitro*. According to them, p.Trp92Ser mutant was unfolded, as judged by fingerprint NMR spectra analysis, and did not bind sDMA [33].

The p.Thr274TyrfsX32 mutation is a frameshift mutation arising from a single nucleotide insertion in exon 6 and results in a truncated SMN protein lacking the C-terminal domain of *SMN*. A new isoform of SMN, axonal SMN (a-SMN), is expected to be produced in the patient, because a-SMN is a truncated, alternatively spliced isoform of *SMN1*, originating from the retention of intron 3 [37,38]. Although the role of a-SMN in the pathogenesis of SMA has not been clarified yet, the disease severity of the patient with this mutation suggests that a-SMN functions were not enough to fully compensate for the deleterious mutation.

The p.Tyr277Cys mutation is located in the C-terminal domain of SMN known as the YG box, which is essential for oligomerization or self-association of SMN [31]. Oligomerization defect destroys the function of SMN and correlates with clinical severity of SMA [34]. Many other mutations in the same domain have been frequently reported [35,36], although the p.Tyr277Cys mutation has not been reported up to now.

An interesting question arises as to which factor contributes more significantly to clinical phenotype in SMA, *SMN1* intragenic mutation or *SMN2* copy number. According to our analysis of the patients with homozygous *SMN1*-deletion (Table 2 and Fig. 1), increased *SMN2* copy number was associated with milder phenotype, which was compatible with previous reports [12–16]. However, the phenotype of patients without *SMN1*-deletion was not related to their *SMN2* copy number (Table 3 and Fig. 1). In our study, p.Ala2Val was found in two type 3 patients with one *SMN2* copy, p.Trp92Ser in two type 1 patients with three *SMN2* copies, p.Thr274TyrfsX32 in one type 1 patient with two *SMN2* copies, and p.Tyr277Cys in one type 2 patients with one *SMN2* copy. According to our findings, *SMN1* intragenic mutations appear to contribute much more significantly to clinical severity than *SMN2* copy numbers in some patients.

Since our patients carry various intragenic *SMN1*mutations, the next question is whether *SMN2* copy number effect is present or absent among the patients with the same *SMN1* mutation. Using the data of the SMA patients with missense mutations described in a review paper of Sun et al. [36], we analyzed the relationship between *SMN2* copy number and clinical severity in eleven patients with p.Tyr272Cys in *SMN1*. We observed that higher *SMN2* copy number was correlated with reduced disease severity: patients with three *SMN2* copies showed milder phenotype than the patients with one *SMN2* copy number. Thus, we speculate that *SMN2* copy number effect is present when the *SMN1* mutation is the same in the patients.

In conclusion, *SMN2* copy number is not always associated with clinical severity of SMA patients, especially SMA patients without *SMN1*-deletion. In these patients, clinical severity in SMA caused by *SMN1* mutations may be determined by the type and location of the intragenic mutation. Intragenic mutations in *SMN1* may contribute more significantly to clinical severity than *SMN2* copy numbers in some spinal muscular atrophy patients.

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Original article

Trinucleotide insertion in the *SMN2* promoter may not be related to the clinical phenotype of SMA

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Abstract

Background: More than 90% of spinal muscular atrophy (SMA) patients show homozygous deletion of *SMN1* (survival motor neuron 1). They retain *SMN2*, a highly homologous gene to *SMN1*, which may partially compensate for deletion of *SMN1*. Although the promoter sequences of these two genes are almost identical, a GCC insertion polymorphism has been identified at c.-320_-321 in the *SMN1* promoter. We have also found this insertion polymorphism in an *SMN2* promoter in an SMA patient (Patient A) who has SMA type 2/3.

Purpose: The aims of this study were to determine the frequency of the GCC insertion polymorphism in SMA patients, and to evaluate its effect on *SMN* transcription efficiency.

Patients and methods: Fifty-one SMA patients, including Patient A, were involved in this study. *SMN2* transcript levels in white blood cells were measured by real-time polymerase chain reaction. Screening of the GCC insertion polymorphism was performed using denaturing high-pressure liquid chromatography. The transcription efficiency of the promoter with the insertion mutation was evaluated using a reporter-gene assay.

Results: All SMA patients in this study were homozygous for *SMN1* deletion. Patient A retained two copies of *SMN2*, and showed only a small amount of *SMN2* transcript in white blood cells. We detected a GCC insertion polymorphism at c.-320_-321 only in Patient A, and not in 50 other SMA patients. The polymorphism had a slight but significant negative effect on transcription efficiency.

Discussion and conclusion: Patient A was judged to be an exceptional case of SMA, because the GCC insertion polymorphism rarely exists in *SMN1*-deleted SMA patients. The GCC insertion polymorphism did not enhance the transcriptional efficiency of

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SMN2. Thus, this GCC insertion polymorphism in the *SMN2* promoter may not be associated with the milder phenotype of the patient. Patient A suggests that there are other unknown factors modifying the clinical phenotype of SMA.

Keywords: Spinal muscular atrophy; *SMN1*; *SMN2*; Promoter; Polymorphism

1. Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder characterized by proximal muscular atrophy of the limbs and trunk, resulting from degeneration of motor neurons in the anterior horn of the spinal cord. The incidence of the disease has been estimated at 1 in 6000–10,000 newborns, with an expected carrier frequency of 1 in 40–50 [1].

SMA is classified into three clinical subtypes depending on the age of disease onset and the achievement of motor milestones [2]: type 1 (severe form, Werdnig-Hoffmann disease; age of onset 0–6 months, unable to sit unaided), type 2 (Dubowitz disease, intermediate form; age of onset <18 months, unable to stand or walk unaided), and type 3 (mild form; Kugelberg–Welander disease; age of onset >18 months, able to stand or walk unaided). Additionally, two other forms of the disease, with the most severe having prenatal onset and the mildest type manifesting after 20 years of age, have been reported as SMA type 0 (prenatal form) and SMA type 4 (adult form) [3].

Using linkage analysis, all clinical subtypes of SMA have been mapped to chromosome 5q11.2–13.3. The survival motor neuron (*SMN*) gene has been identified as a candidate for SMA [4]. *SMN* is in fact two highly homologous genes, *SMN1* (the telomeric copy) and *SMN2* (the centromeric copy) [4]. *SMN1* and *SMN2* encode the same protein; however, *SMN1* is now considered to be responsible for the development of SMA, because its homozygous deletion has been found in >90% of SMA patients, and subtle but deleterious intragenic *SMN1* mutations have been identified in non-deletion patients [4,5]. It has been accepted that *SMN2* may be a modifier gene of SMA. Owing to a single nucleotide difference between *SMN1* and *SMN2*, exon 7 of *SMN2* is alternatively spliced (more precisely, skipped) resulting in the production of an *SMN* transcript lacking exon 7 (Δ7-*SMN* transcript) and an unstable Δ7-SMN protein [6]. The single nucleotide change in *SMN2* exon 7, which is a C-to-T transition located at codon 280, increases Δ7-*SMN* transcript levels and, correspondingly, decreases full-length *SMN* (FL-*SMN*) transcript levels [7]. Even so, *SMN2* is also able to generate a small amount of full-length transcript, and thus it can partially compensate the loss of *SMN1* [8].

Generally, the clinical severity of SMA patients is inversely correlated with *SMN2* copy number. A high copy number of *SMN2* is associated with a milder phenotype, and a low copy number with a more severe phenotype. SMA type 1 patients typically have two copies of *SMN2*, SMA type 2 patients have three copies, and SMA type 3 patients typically have three or more copies [9]. More than four *SMN2* copies are associated with a milder phenotype of SMA type 3 [10]. However, the clinical severity cannot always be determined by the *SMN2* copy number alone.

The expression level of *SMN2* may also be correlated with the clinical severity of the disease and, therefore, analysis of the *SMN2* promoter is important. Echaniz-Laguna et al. and Boda et al. reported that the promoter sequences of *SMN1* and *SMN2* are identical, providing strong evidence for similar transcriptional regulation of these genes [11,12]. However, Monani et al. found more than 10 nucleotide differences between the promoter regions of these two genes [13,14]. One of them, a GCC insertion polymorphism, was specifically identified at c.-320_-321 in the *SMN1* promoter, leading to GCC duplication at c.-324–c.-318. Polymorphisms in the promoter region may have some effect on transcriptional activity.

We found the GCC insertion polymorphism in an *SMN2* promoter in a Japanese boy diagnosed as having SMA type 2/3 (Patient A). The location of the GCC insertion in the *SMN2* promoter in Patient A was corresponding to that of the GCC in the *SMN1* promoter reported by Monani et al. [14]. It is notable that the clinical phenotype of the patient was much milder than expected based on his *SMN2* copy number. In this study, we determined the frequency of the GCC insertion polymorphism in controls and SMA patients. We also evaluated the effect of the GCC insertion polymorphism on *SMN2* transcriptional activity.

2. Patients and methods

2.1. Patients

All 50 Japanese patients in this study fulfilled the diagnostic criteria defined by the 59th ENMC International Workshop [2]; 26 patients (aged 1–34 years) were type 1, 16 type 2, and eight type 3. The molecular genetic analysis was approved by the Ethical Committee of the Kobe University Graduate School of Medicine, Japan. Informed consent was obtained from the patients or their parents. Fifty healthy Japanese adults (aged 21–

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70 years) volunteered to participate in the study as control subjects.

Patient A was a 2-year-old Japanese boy who was clinically suspected as having a neuromuscular disorder with decreased muscle tonus. He was born as the third child to non-consanguineous and healthy parents. The pregnancy and delivery were non-eventful. Early developmental milestones were slightly delayed: head control was obtained at age 6 months, sitting without support at age 8 months, crawling at age 9 months, and standing and walking with support (ex. handrails) at age 18 months. However, he could never walk without support. He uttered his first word at 18 months, and a simple two-word sentence at 22 months. On admission, his weight and height were 85.5 cm (−0.7 SD) and 11.5 kg (−0.9 SD). His mental status was alert. Apparent facial anomaly was absent, but high-arched palate was present. Lung and heart auscultation revealed no abnormal findings. Abdominal examination was normal. Tongue fasciculation was absent. Muscle tonus was decreased: scarf sign, heel-to-ear sign, and loose-shoulder sign were observed. Muscle strength was also decreased especially in the proximal region of the legs. Deep tendon reflexes were absent or extremely diminished. Laboratory examination revealed no muscular damage (AST 28 IU/L, ALT 10 IU/L, CK 119 IU/L, ALD 7 IU/L, lactate 13 mg/dL, pyruvate 0.8 mg/dL). Muscle biopsy findings were compatible with those of SMA. Based on the muscle biopsy findings, together with the clinical phenotype, he was diagnosed as having SMA type 2/3.

2.2. *SMN1* deletion test and *SMN2* gene dosage analysis

Genomic DNA was extracted from peripheral white blood cells. The *SMN1* exon 7 deletion test was performed by the PCR-restriction fragment length polymorphism method of van der Steege et al. [15]. *SMN2* copy numbers were determined with a LightCycler 1.5 instrument (Roche Diagnostics GmbH, Mannheim, Germany) using FastStart DNA Master SYBR Green I (Roche Diagnostics) according to the method of Tran et al. [16].

2.3. RNA extraction, cDNA synthesis, and quantitative real-time PCR

Total RNA was isolated from peripheral white blood cells. cDNA was synthesized from total RNA with Transcriptor Reverse Transcriptase (Roche Diagnostics) according to the manufacturer's instructions.

Quantitative reverse-transcription-PCR was performed with a LightCycler 1.5 instrument (Roche Diagnostics) using FastStart DNA Master SYBR Green I (Roche Diagnostics). To evaluate transcript levels of the *SMN* genes, we amplified cDNA fragments of exons 1–2b, exons 7 and 8, and exons 5, 6 and 8. The cDNA fragment including exons 1–2b represented total *SMN* transcript, because the sequence of exons 1–2b is commonly included in all transcript isoforms. The cDNA fragment containing exons 7 and 8 represented the FL-*SMN* transcript, because it contained sequence beyond exon 7. The cDNA fragment including *SMN* exons 5, 6 and 8 represented the Δ7-*SMN* transcript, because it did not carry the sequence of exon 7. We used glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an endogenous reference gene, and the levels of *SMN* were normalized relative to those of *GAPDH*. The primers for the total-*SMN*, FL-*SMN*, Δ7-*SMN*, and *GAPDH* transcripts have been described previously [17,18]. Quantitation of the PCR products was performed with the second derivative maximum method of the LightCycler software.

2.4. Denaturing high-pressure liquid chromatography (DHPLC) detection of GCC insertion polymorphism in the SMN promoter

To screen for the GCC insertion polymorphism in SMA patients and controls, DHPLC analysis of PCR products was performed. PCR of the fragment including the polymorphism site was carried out with the primer set: 5′-tgcaatgagccgagatggtg-3′ and 5′-cctccccccttggaaaagtaa-3′. The PCR products were then directly loaded into the autosampler of an automated DHPLC system, the WAVE Nucleic Acid Fragment Analysis System, equipped with a DNASep cartridge (Transgenomic, Omaha, NE, USA). The samples were run under partially denaturing conditions at 54.6 °C (oven temperature). The buffer gradient conditions were the same as previously reported [19].

2.5. Sequencing

Direct and/or subcloned sequencing analyses of PCR-amplified products were performed. Sequencing reactions were performed using a dye terminator cycle-sequencing kit (Applied Biosystems/Life Technologies Corporation, Carlsbad, CA, USA), according to the supplier's instructions. The reaction products were automatically electrophoresed on an ABI PRISM 310 Sequencer (Applied Biosystems) and then analyzed using the Sequencing Software Module provided with the ABI PRISM 310 Sequencer.

2.6. Preparation of expression vectors

The PCR-amplified fragment containing GCCGCC polymorphism or GCC polymorphism was inserted into a firefly luciferase reporter plasmid, pGL2BTK (pGL2-Basic with a minimal herpes virus 1 thymidine kinase promoter). The GCCGCC and GCC fragment-containing plasmids were designated as 'pGCCGCC'

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and 'pGCC', respectively. The construct maps of pGL2BTK, pGCCGCC, and pGCC are shown in Fig. 1.

2.7. Transcription assay

The responses of the test plasmids (pGL2BTK, pGCCGCC, pGCC) to dibutyryl cAMP (dbcAMP; 0.5 mM), forskolin (20 μM), and a combination of dibutyryl cAMP and forskolin were determined in a human neuroblastoma cell line, BE(2)-C cells. The neuroblastoma cell lines have been used as useful experimental models of neuronal differentiation because the morphological, biochemical and electrophysiological properties of neuroblastoma cell lines are similar to those of neurons [20].

Neuroblastoma cells [2×10^5 cells in Minimum Essential Medium (MEM)] were cotransfected with a test plasmid (1.6 μg) and the phRL plasmid (a sea pansy luciferase reporter plasmid; Promega Corporation, Madison, WI, USA) (0.5 ng) using Lipofectamine 2000 (Invitrogen/Life Technologies Corporation). Twenty-four hours after transfection, dibutyryl cAMP, forskolin, or a combination of dibutyryl cAMP and forskolin was added to the MEM. The cells were harvested after culture for an additional 24 h.

Transcriptional activity of the test plasmids was measured using the dual-luciferase reporter assay system, in which sea pansy-luciferase activity was used as a control for the transfection efficiency of the test plasmids. Each transcriptional activity measurement was repeated three times and the data are expressed as the mean ± SD.

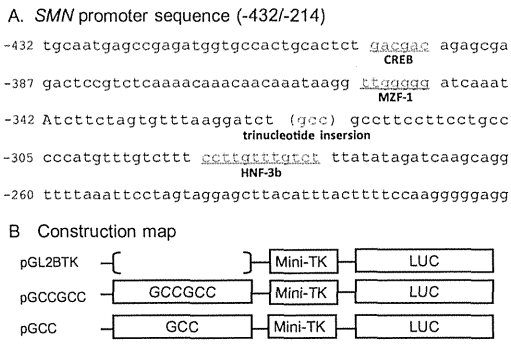

Fig. 1. *SMN* promoter sequence (A) and construction map (B). The *SMN* promoter sequence from c.-432 to c.-214 is shown in the upper part of the figure (A). The numbering of nucleotide in the promoter sequence is based on Monani et al. [14]. Trinucleotide insertion at c.-320_-321 is parenthesized. Putative transcription factor binding sites are underlined. Plasmid construction map is shown in the lower part of the figure (B). All constructs have a firefly-luciferase reporter gene, which is designated as LUC in the map. The pGL2BTK plasmid is a basic plasmid served as control. The GCCGCC and GCC fragment-containing plasmids were designated as 'pGCCGCC' and 'pGCC', respectively.

2.8. Statistics

Statistical analysis of the transcriptional activity data was performed using Microsoft Excel 2003 software and Statistical Package for the Social Sciences (SPSS Inc., Chicago, I, USA). The Student's *t*-test was conducted to evaluate differences between the plasmids. A probability of less than 0.05 was considered statistically significant.

3. Results

3.1. SMN1 deletion test and SMN2 gene dosage analysis

We performed an *SMN1* deletion test on Patient A, who was suspected as having SMA type 2/3. The patient carried zero copies of *SMN1* and two copies of *SMN2*. Based on molecular analysis, he was diagnosed as having SMA.

A nucleotide substitution in *SMN2* exon 7, c.859G>C, has been reported as a positive modifier of the SMA phenotype [21,22]. To check whether the mutation is present in Patient A, we performed a sequencing analysis of the exon 7. However, we did not find any substitutions including c.859G>C.

3.2. SMN2 transcript levels

Our aim of this study was to compare the *SMN2* transcript levels of Patient A to those of other SMA type 2 patients, because we hypothesized that *SMN2* transcript expression was the key determinant of the SMA phenotype. It would have been preferable to compare Patient A with SMA type 2 patients carrying two copies of *SMN2*. However, we did not have cDNA samples from SMA type 2 patients with zero copies of *SMN1* and two copies of *SMN2*. In this study, we determined the baseline transcript levels of total *SMN*, FL-*SMN*, and Δ7-*SMN* in the white blood cells of Patient A, five disease controls (DCs 1–5; they were all SMA type 2 patients with zero copies of *SMN1* and three copies of *SMN2*) and three healthy controls. All of the disease controls were able to sit without support, but could not stand or walk even with any support.

Total *SMN* transcript levels of Patient A, DC1, DC2, DC3, DC4, and DC5 were 38%, 76%, 66%, 181%, 232%, and 166% of the mean value of the healthy controls, respectively. This finding suggested that *SMN2* transcription in Patient A was significantly reduced compared with that of the disease controls.

The FL-*SMN* transcript levels of Patient A, DC1, DC2, DC3, DC4, and DC5 were 53%, 58%, 64%, 44%, 68%, and 95% of the mean value of the healthy controls, respectively. The Δ7-*SMN* transcript levels of Patient A, DC1, DC2, DC3, DC4, and DC5 were 167%, 206%, 130%, 130%, 97%, and 145% of the mean value of the healthy controls, respectively. These findings suggested

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