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Ⅳ．研究成果の刊行物・別刷

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神経症候群（第2版）

—その他の神経疾患を含めて—

II

III　変性疾患

運動ニューロン疾患　脊髄性筋萎縮症

5番染色体性劣性遺伝性脊髄性筋萎縮症

脊髄性筋萎縮症
[SMA I型（infantile acute SMA, Werdnig-Hoffmann病），SMA II型（infantile chronic SMA, Dubowitz病）]

西尾久英

運動ニューロン疾患
脊髄性筋萎縮症
5番染色体性劣性遺伝性脊髄性筋萎縮症

脊髄性筋萎縮症[SMA I型(infantile acute SMA, Werdnig-Hoffmann病), SMA II型(infantile chronic SMA, Dubowitz病)]

Spinal muscular atrophy type I and type II

西尾久英

Key words：脊髄性筋萎縮症I型，脊髄性筋萎縮症II型，*SMN1*遺伝子，*SMN2*遺伝子，*NAIP*遺伝子

1. 概念・定義

脊髄性筋萎縮症(spinal muscular atrophy: SMA)は，脊髄前角細胞の脱落に伴い，体幹・四肢近位部優位の筋緊張低下・萎縮を生じる遺伝性運動ニューロン病の一つである．本稿では，5番染色体関連のSMA(5q-SMA)を中心に記述し，特に断らないかぎり，SMAとは5q-SMAを指す．

SMAは，発症時期と運動発達の程度により，3つの病型に分類されてきた[1]．I型(infantile acute SMA, Werdnig-Hoffmann病)は生後6カ月までに発症する重症型で，自力での座位保持が困難である．II型(infantile chronic SMA, Dubowitz病)は生後18カ月までに発症する中等症型で，自力で座位保持は可能だが，自力で立って歩行できない．III型(juvenile SMA, Kugelberg-Welander病)は18カ月以降に発症する軽症型で，自力で立って歩行できる．自力歩行は，幼児期に限られる例もあれば，青年期以降も可能な例もある．なお，最近では，胎児期に発症したと考えられる最重症型を0型，20歳以降に発症した軽症例をIV型とすることが多くなった[2]．0型，III型，IV型は，本誌別稿で記述される．

SMAの責任遺伝子は*SMN1*遺伝子である．*SMN1*遺伝子変異に基づくSMAは常染色体劣性遺伝疾患であり，90％以上のSMA患者は*SMN1*遺伝子欠失のホモ接合体であることが報告されている[3]．

2. 疫　　学

*SMN1*遺伝子欠失を有する保因者の頻度は，人種差はなく，40-70人に1人である．このことから，患者の発生頻度(すべての病型を含む)は，世界のどの地域においても，出生10,000あたり0.5-1人であると推定される[4]．

3. 症　　状

乳幼児期に発症するSMA I型・SMA II型患者は，フロッピー・インファント特有の姿位(カエル型姿勢)を取り，体幹・四肢近位部優位かつ左右対称性の筋トーヌス低下，筋力低下，腱反射減弱～消失を示す．

SMA I型は生後6カ月までに発症する重症型である．頸定が得られないことも多く，座位保持能力の獲得に至らない．I型患者では，嚥下障害と呼吸器合併症が問題になる．呼吸不全のため，2歳までに死亡の転帰をとる例も多かったが，近年，人工呼吸管理が普及し，長期生存例も増えた．

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表1　SMA患者の遺伝子解析(神戸大学 1996-2011年)

1. SMA患者95例の病型と遺伝子欠失

SMN1 エクソン7	*SMN1* エクソン8	*NAIP* エクソン5	I型	II型	III型	IV型	
欠失	欠失	欠失	26	6	0	1	33
欠失	欠失	残存	16	18	11	2	47
欠失	残存	残存	2	6	2	0	10
残存	残存	残存	2	0	3	0	5
			46	30	16	3	95

2. *SMN1* 欠失患者90例の病型と *SMN2* 遺伝子コピー数

SMN2 遺伝子コピー数	I型	II型	III型	IV型	
1	1	0	0	0	1
2	34	2	0	0	36
3	9	28	9	1	47
4	0	0	4	2	6
	44	30	13	3	90

3. *SMN1* 欠失患者90例の病型と *NAIP* 遺伝子の有無

I型 (44例)		II, III, IV型 (46例)	
NAIP(+) 18例 (41%)	*NAIP*(−) 26例 (59%)	*NAIP*(+) 39例 (85%)	*NAIP*(−) 7例 (15%)

SMA II型は生後18カ月までに発症する中等症型である．自力での座位保持が可能なところまで運動能力を獲得するが，自力で立って歩行する能力は獲得できない．II型患者では，嚥下障害や排痰障害のほかに，脊柱変形(側彎など)が問題になる．

4. 病因(遺伝子レベル)

1) *SMN1* 遺伝子

1995年に，5番染色体(5q13領域)からクローニングされたsurvival motor neuron 1(*SMN1*)遺伝子がSMAの責任遺伝子候補であると報告された[3]．その後，*SMN1* 遺伝子の完全な欠失(*SMN1* 遺伝子欠失のホモ接合体)がSMA患者の90%以上に認められることや，*SMN1* 遺伝子内の微小変異によって発症したSMA患者も存在することより，*SMN1* 遺伝子こそがSMAの責任遺伝子であると考えられるようになった．表1-1に，著者らの症例の病型と *SMN1* 遺伝子エクソン7，エクソン8の欠失の頻度を示す．

2) *SMN2* 遺伝子

SMAの遺伝子座である5q13領域には大規模な重複が生じていて，テロメア側とセントロメア側に2つの相同遺伝子が並ぶ．テロメア側の *SMN1* 遺伝子に対応するセントロメア側の相同遺伝子が *SMN2* 遺伝子である．SMA患者には *SMN2* 遺伝子が必ず残存している(*SMN* 1遺伝子と *SMN2* 遺伝子が同時に欠失すれば胎生致死になると考えられている)．

SMN1 遺伝子と *SMN2* 遺伝子はほとんど同一の塩基配列を有する相同遺伝子である．遺伝子全体では合計5個の塩基しか違っておらず，コーディング領域の違いといえばエクソン7の6番目の塩基の違いのみであるが，これはアミノ酸置換を引き起こさない．しかし，このエクソン7の塩基の違いがスプライシングに大きな影

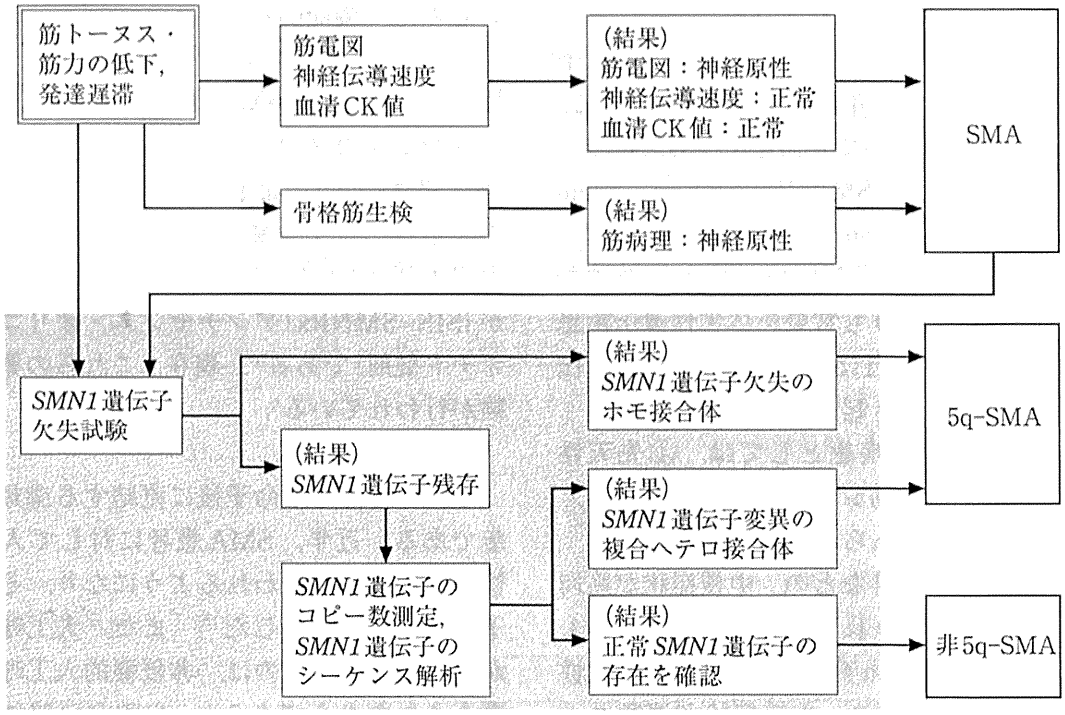

図1 SMA診断のアルゴリズム

響を与えている[5]. この*SMN2*遺伝子から転写されるmRNAの大部分はエクソン7が組み込まれておらず, ここから翻訳されて産生されるタンパク質は機能的なものではない.

しかし, *SMN2*遺伝子から転写されるmRNAの一部にはエクソン7が組み込まれていて, 少量ではあるが機能的なSMNタンパク質が産生される. これが, *SMN1*遺伝子の欠失をある程度補完する. 興味深いことに, SMA軽症型(II型, III型, IV型)では, SMA重症型(I型)患者よりも*SMN2*遺伝子コピー数が多い傾向がある[6]. **表1-2**に, 著者らの症例の病型と*SMN2*遺伝子コピー数を示す. これらの所見から, *SMN2*遺伝子コピー数はSMAの症状の修飾因子であると考えられる.

3) *NAIP*遺伝子

1995年に, *SMN1*遺伝子の近傍にneuronal apoptosis inhibitory protein(*NAIP*)遺伝子も, SMAの責任遺伝子候補として報告された[7]. *NAIP*遺伝子エクソン5の欠失はSMA1型の50%強, それ以外の型についてはずっと低い頻度で認められる. **表1-3**に, 著者らの症例の病型と*NAIP*遺伝子の欠失率の相関を示す. これらの所見から, *NAIP*遺伝子もSMAの重症度と関連する因子であると考えられる.

5. 病態(タンパク質レベル)

*SMN1*遺伝子から産生されるSMNタンパク質は, 神経細胞にも, 非神経細胞にも発現している. SMNタンパク質は他のタンパク質と複合体を形成し, snRNPsの生合成, 細胞骨格の制御, 運動ニューロンの軸索輸送, 神経筋接合部の成熟などに関与していることが次第に明らかになってきた[4].

*NAIP*遺伝子から産生されるNAIPタンパク質は, バキュロウイルスのアポトーシス抑制タンパク質と相同性があることから, 神経細胞死との関連が考えられている[7].

6. 診断と鑑別診断

図1にSMAの診断のアルゴリズムを示す. *SMN1*遺伝子欠失試験に関しては様々な方法が開発されてきたが, 最近はMLPA(multiplex ligation-dependent probe amplification)法を用いて, *SMN1*遺伝子・*NAIP*遺伝子欠失試験, *SMN1*遺伝子・*SMN2*遺伝子のコピー数の判定

が行われることが多くなった[8].

*SMN1*遺伝子が正常であっても，他の遺伝子の異常に基づくSMA(非5q-SMA)が存在する．このようなSMAについても知見が次第に蓄積されつつある．例えば，SMARD1(spinal muscular atrophy with respiratory distress type 1)は，横隔膜麻痺による顕著な呼吸障害が特徴的なSMAである．SMARDも常染色体劣性遺伝疾患であり，11番染色体(11q13)の*IGHMBP2*遺伝子の変異によって引き起こされる[9].

ほかに鑑別すべき疾患としては，①先天性ミオパチー，先天性筋ジストロフィーなどの，中核症状が骨格筋にあらわれる遺伝性疾患群，②Prader-Willi症候群などの，中核症状が筋肉以外の臓器にあらわれる遺伝性疾患群，③Charcot-Marie-Tooth病(遺伝性運動性感覚性ニューロパチー)などの，中核症状が運動ニューロンと末梢神経にあらわれる遺伝性疾患群が挙げられる．

7. 治　　療

1) 薬物治療

SMAの根本的な治療法はまだ確立していないものの，機能的なSMNタンパクの産生増加を目指して，①*SMN2*遺伝子プロモーターを活性化する戦略(治療戦略1)と，②*SMN2*遺伝子エクソン7のスプライシングを修正する戦略(治療戦略2)が考案された．治療戦略1に基づく薬剤がバルプロ酸(ヒストン脱アセチル化酵素阻害剤)である[10,11]．治療戦略2に基づく薬剤がISIS-SMNRx(アンチセンス・オリゴヌクレオチド製剤)である[12]．現在，これらの薬剤の治験が行われている[4].

2) 医学的管理

呼吸障害は，生命予後に直結する深刻な合併症である．近年，SMA患者に対して人工呼吸管理が積極的に行われるようになり，その生命予後は著しく改善した[13]．また，人工呼吸管理が急速に普及したのは，非侵襲的人工呼吸法が導入されたからである[14]．夜間非侵襲的人工呼吸療法などを含む呼吸管理の向上に伴い，栄養管理では，経鼻胃管栄養のほかに胃瘻増設術も選択肢に入るようになったし，整形外科的管理では，装具療法のほかに側彎矯正手術も選択肢に入るようになった[15].

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Chapter 9

Coagulation and Fibrinolysis Abnormalities in Patients with Muscular Dystrophy

Toshio Saito

Additional information is available at the end of the chapter

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1. Introduction

The cause of the Duchenne muscular dystrophy (DMD) is deficiency of the dystrophin protein leading to dysfunction of many organs. Originally it was thought that the natural history of this disease limits the lifespan of the patients to 20 year. However, positive therapeutic interventions for heart failure, respiratory failure, nutritional management, spinal surgery and the rehabilitation raised the lifespan of patients with DMD in Japan above 30 years of age. (Ishikawa Y, et al. 2011) (Matsumura T, et al. 2011) (Saito T, et al. 2011). Consequently, nowadays complications accompanying the higher survival age of DMD patients should also be considered. This chapter describes a coagulation and fibrinolysis abnormality of muscular dystrophy, and its involvement in the microcirculation disorder accompanying this disease.

2. The old tale of DMD as a microcirculation disorder

Historically before the discovery of dystrophin, a hypothesis was proposed that blood circulation insufficiency due to intravascular obstruction causes muscle necrosis in DMD. This hypothesis was based on muscle histopathology findings similar to necrosis caused by circulation insufficiency. There were some reports that tried to model the pathologic condition of DMD with impaired circulation. However, these trials to reproduce the DMD pathology were unsuccessful. (Bradley WG, et al. 1975) (Gudrun B, et al. 1975) (Leinonen H, et al. 1979)

Meanwhile Miike T, et al. described vascular obstruction and vascular endothelial hyperplasia, namely the blister-like swelling of vascular endothelial cells in the muscle histopathology of non-symptomatic children with DMD, and put forward a hypothesis of the blood flow abnormality that affects the progress of DMD (Miike T, et al. 1987). After the discovery of

dystrophin, the mainstream theory for the pathogenesis of DMD became the muscle destruction due to the membranous fragility related to dystrophin defects. Since then the vascular disorders in DMD have been regarded not important.

3. Thrombosis and embolization as complication of DMD

There are some reports related to thrombosis or embolization of patients with DMD.

In 1982, Matsuishi T, et al. reported a basilar artery occlusion in a case of DMD, in which the pathogenesis of infarction was uncertain. (Matsuishi T, et al. 1982) In 1989, Gaffney JF, et al reported left ventricular thrombus and systemic emboli complicating the cardiomyopathy of DMD. (Gaffney JF, et al. 1989) Authors showed anteroseptal mural thrombus and right atrial thrombus as autopsy findings. Riggs T also reported three terminal DMD cases of cardiomyopathy and pulmonary emboli. (Riggs T. 1990) Author showed thrombus formation of ventricle and pulmonary embolus with a ventilation perfusion scan.

The epidemiologic aspects of DMD-related thromboembolism were addressed by Biller J, et al., who reported the frequency of cerebral infarction in patients with inherited neuromuscular diseases including DMD, Becker muscular dystrophy (BMD), myotonic dystrophy, and Freidrich ataxia. According to their data cerebral infarction was seen in 1. 5% of the cases with these diseases and concluded that cerebral infarction is uncommon in neuromuscular diseases.

4. Pioneer studies of coagulation and fibrinolysis abnormalities of DMD in Japan

Among annual reports of clinical research group for muscular dystrophy in Japan, some reports described cases of cerebral infarction and pulmonary embolism in patients with DMD. Ishihara T, et al. reported series of 15 autopsied cases of DMD/BMD with hemorrhagic pulmonary infarction in 1990. He pointed out that this disorder is an important cause of death in DMD. Matsuka Y, et al. reported a DMD case of cerebral infarction and thrombus formation in the left ventricle in 1991, and described elevated levels of thrombin-antithrombin complex (TAT) and platelet factor 4 (PF4) among many DMD cases with CTR>50% in 1993. Hanajima, et al. reported the occurrence of cerebral infarction of DMD in muscular dystrophy wards in Japan to be 5 / 269 DMD patients aged from 16 to 20 years old. Authors concluded that cerebral infarction is not a rare complication of DMD. (Hanajima, et al. 1996)

Based on these findings the clinical research team for the genetic counseling and the clinical research of the pathology and treatment in muscular dystrophy patients (from 1996 to 1998), that was directed by Ishihara T, proposed a research and intervention project to investigate the blood coagulation disorder complicating the muscular dystrophy. In next section, results of the research in this team are described.

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5. Abnormal coagulation and fibrinolysis in DMD

Saito Y, et al. reported hypercoagulable state in patients with DMD. (Saito Y, et al. 1997) By the blood coagulation test of patients with DMD and other neuromuscular diseases at rest condition, the authors showed that abnormal findings appear in many coagulation and fibrinolysis parameters such as thrombotest, TAT, and plasmin –α2 Plasmin inhibitor complex (PIC) in DMD. Namely, level of thrombotest, which reflects coagulation activity including effect of PIVKA (used for monitoring warfarin treatment), was low compared to normal range in 78% of DMD, TAT level was elevated in 61% of DMD, and PIC level elevated in 40. 3% of DMD. Abnormality of the coagulation and fibrinolysis was found in most patients with DMD. The frequency of abnormality was high compared with other neuromuscular diseases.

In this report, the ratio of abnormal value of D-dimer and fibrin and fibrinogen degradation products (FDP) was low in DMD, authors described that coagulation cascade is more enhanced than fibrinolysis cascade in patients with DMD. The coagulation and fibrinolysis abnormality was not associated with age, respiratory function, cardiac activity, and activities of daily living. Authors concluded that muscular dystrophy itself is a risk factor for thrombosis.

Based on examination of relation with the muscle destruction Saito T, et al. reported that coagulation and fibrinolysis abnormality is strongly present in younger patients with DMD, BMD, and Fukuyama congenital muscular dystrophy (FCMD). (Saito T, et al. 2001) They showed significant correlation between serum levels of FDP and MM isozyme of creatine kinase (CK-MM), irrespective of type of dystrophy. Figure 1 shows correlation between FDP and CK-MM of patients with DMD, whereas Figure 2 shows correlation between FDP and D-dimer. Levels of FDP were higher at ambulatory young boy with high CK DMD. Authors speculated that enhanced coagulation and fibrinolysis in DMD, BMD, and FCMD is induced by some components that leak from destructed muscle. It is inferred that the disturbances of the coagulation and fibrinolysis result from the muscle destruction. Increase of both plasma levels of D-dimer and serum levels of FDP is an indirect proof of thrombus having been present in vivo. It means that microcirculation disorder is possibly present in DMD, BMD, and FCMD potentially.

In this study advanced DMD patients with low CK showed no abnormal elevation of FDP and D-dimer. However, even DMD patients in advanced stage, whose CK levels were within normal range, showed coagulation abnormalities, if serum CK increased as a consequence of muscle destruction induced by various causative factors. Saito T, et al. reported activated coagulation cascade in a case of advanced DMD that showed transient elevation of serum CK due to convulsion. (Saito T, et al. 2003) These phenomena are possible sources of pulmonary emboli accompanying DMD. Nakayama T, et al. established that CK elevation preceded the development of pulmonary embolism in patients with DMD (Nakayama T, et al. 2000).

6. Abnormal coagulation and fibrinolysis in cases of dystrophinopathy with heart failure

There is evidence for association between cardiac dysfunction and coagulation disorder. Saito T, et al. reported that levels of TAT and prothrombin fragment (F1+2) in DMD patients

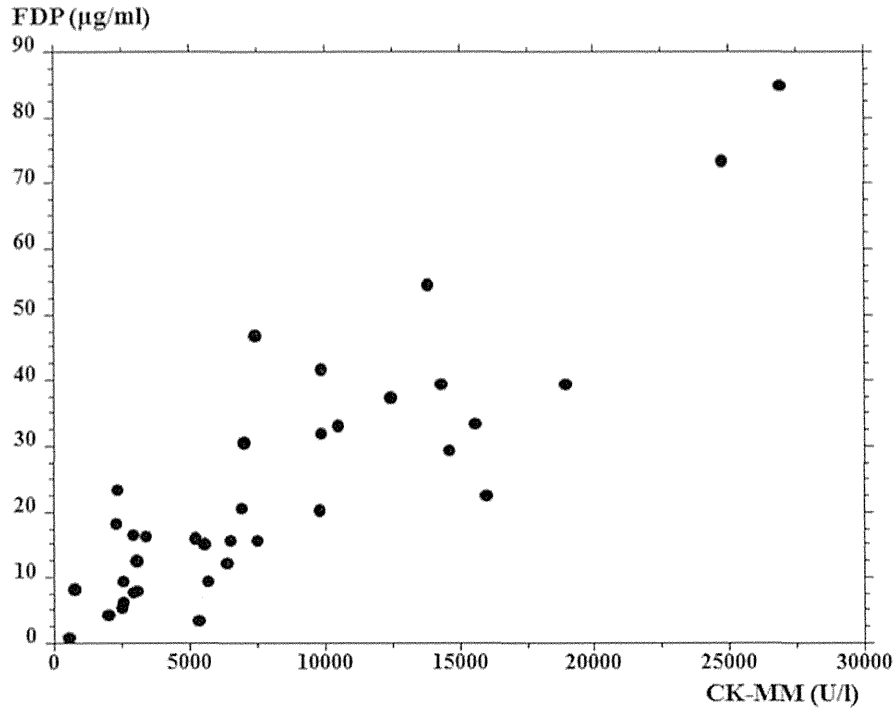

Figure 1. Correlation of serum FDP and CK-MM in patients with DMD, Serum CK-MM level is significantly correlated with FDP. n=36, (modified figure of literature, Saito T, et al. 2001)

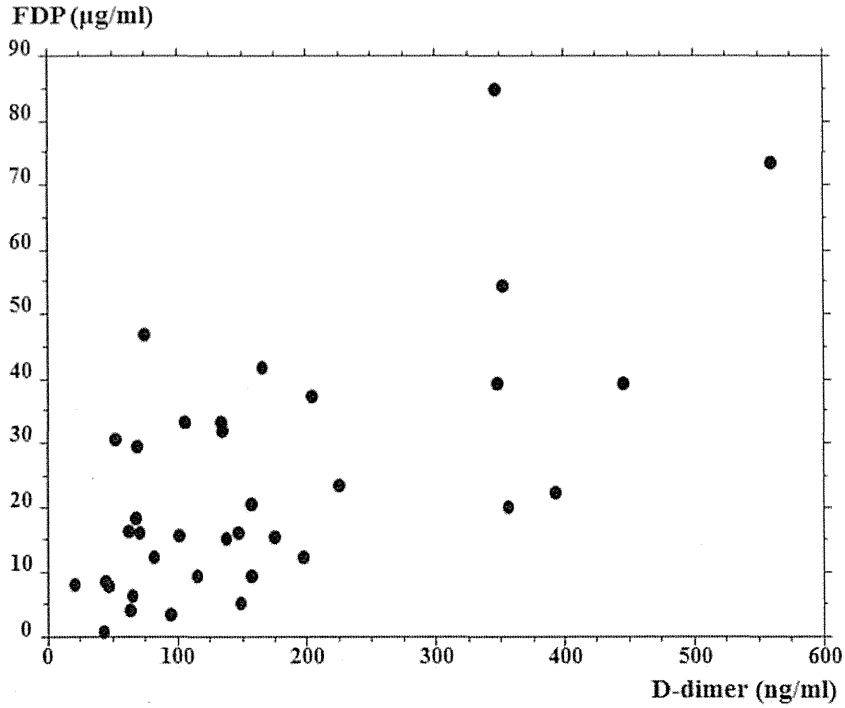

Figure 2. Correlation of serum FDP and plasma D-dimer in patients with DMD, Although correlation between FDP and D-dimer was not significant, both FDP and D-dimer elevated in DMD patients. n=36 (modified figure of literature, Saito T, et al. 2001)

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with the markedly depressed cardiac function were significantly elevated compared to DMD patients with preserved cardiac function. Authors concluded that activated coagulation is associated with cardiac dysfunction in patients with DMD. (Saito T, et al. 2005) Porreca E, et al. also reported similar findings in patients with dystrophinopathy including BMD. (Porreca E, et al. 1999) These abnormalities probably induce cerebral infarction through a mechanism similar to the one observed in idiopathic cardiomyopathy. Ikeniwa C, et al. reported two cases of DMD with dilated cardiomyopathy and cerebral infarction. (Ikeniwa C, et al. 2006)

7. Studies of other factors affecting coagulation and fibrinolysis status

In addition to the cases described above, the clinical research group for muscular dystrophy in Japan reported that infectious diseases activate coagulation cascade by increasing the level of fibrinogen resulting in elevation of D-dimer. However, this acute-phase reaction induced by infection is observed generally in normal subjects too.

An interventional study was also proposed in the form of a clinical trial to administer warfarin for DMD/BMD patients with high risk of thrombosis. Within its framework information regarding the coagulation status of 190 DMD/BMD patients in muscular dystrophy wards in Japan was collected abnormal rate of TAT was 36. 0%, and that of F1+2 was 51. 2% in DMD patients, which demonstrated that enhanced blood coagulation was dominant in DMD patients. However, the number of patients recruited in this clinical trial was too small, so the trial was not started. Instead of clinical trial, they proposed substitute treatment, namely improving congestion in venous return of bedridden patients with DMD, and prevention of dehydration.

8. Platelet abnormalities in DMD

Forst J, et al. reported a significant deficiency of platelet adhesion and ristocetin induced aggregation as well as a marked reduction of expression of glycoprotein IV, although normal plasmatic coagulation and a slight but not significant increase of bleeding time was observed in DMD patients (Forst J, et al. 1998). Authors speculated that the platelet function deficiency occurs because of a decompensation of platelet adhesion as well as aggregation capacity in major spinal surgery, although the deficiency of platelet function in DMD patients does not affect ordinary life or minor surgery.

Further, Matsumura T, et al. reported a case of DMD complicated by thrombotic thrombocytopenic purpura (TTP). In their report, TTP was confirmed by decreased activity of von Willebrand factor cleaving protease and activity plasma exchange was successful for the patient (Matsumura T, et al. 2003).

9. Pathogenetic aspects of the coagulation abnormalities in Duchenne muscular dystrophy

From the point of view that coagulation disorders induce microcirculation abnormalities, Saito T, et al. speculated that hypoxic and ischemic condition might exist in DMD. They reported that elevated levels of VEGF are observed in dystrophinopathy patients, and supposed that these are induced by relative hypoxic and ischemic condition. (Saito T, et al. 2009) However, these conditions were marked in advanced DMD patients rather than young boy with DMD.

On the other hand, it has been considered that circulation abnormality may participate in disease progression of DMD, which has not been evaluated for a long time since dominance of membrane theory. (Lombard JH. 2011) Functional muscle ischemia has been reported in patients with DMD. (Sander M, et al. 2000) Defect of nNOS due to dystrophin absence induce functional muscle ischemia related muscle exercise, which can induce microcirculation insufficiency of muscle tissue. Asai A, et al. reported effectiveness of Phosphodiesterase-5 Inhibitor to mouse model of muscular dystrophy by improving microcirculation of muscle tissue. (Asai A, et al. 2007)

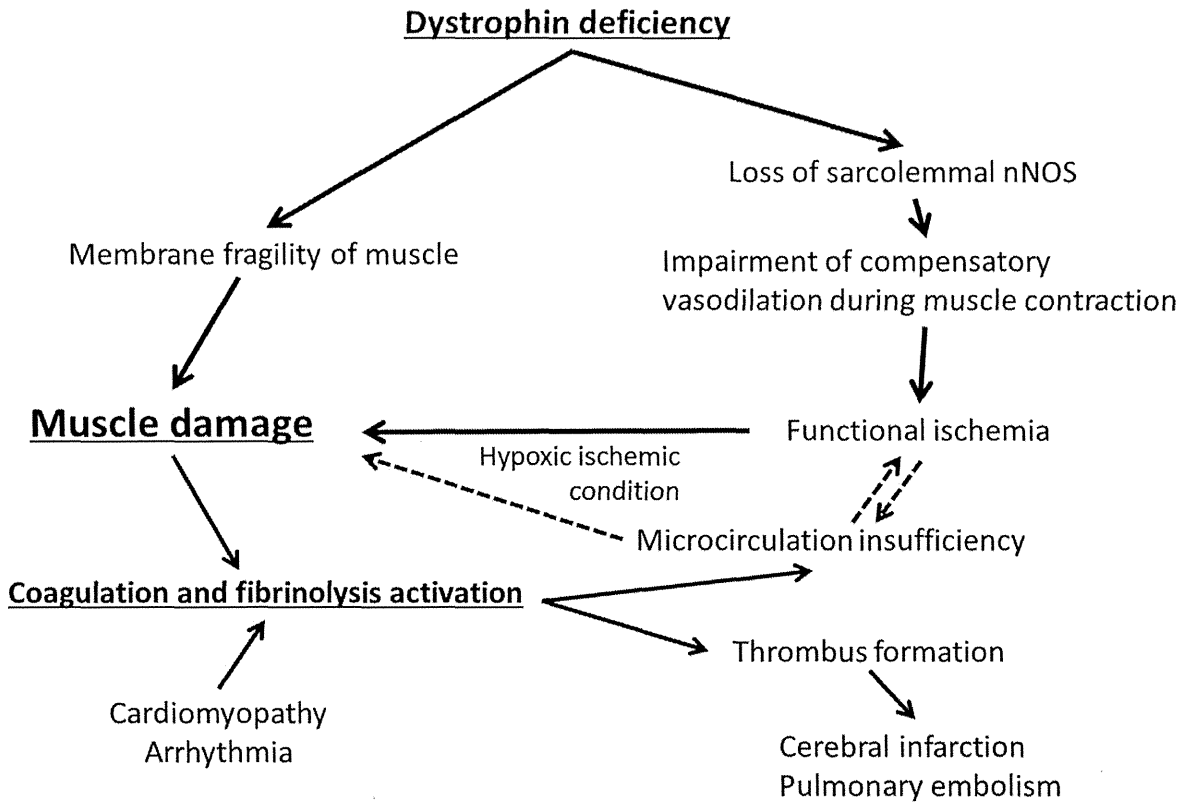

Figure 3. Muscle destruction process and the relation to coagulation and fibrinolysis adnormalities in DMD patients

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In Figure 3, I summarize the muscle destruction process and the relation to coagulation and fibrinolysis adnormalities in DMD patients. The origin of DMD is dystrophin deficiency. Dystrophin deficiency induces functional muscle ischemia as well as membrane fragility of muscle, leading to muscle destruction. Muscle destruction activates coagulation and fibrinolysis cascade (, which may be similar to rhabdomyolysis). Activated cascade induces microcirculation insufficiency affecting functional muscle ischemia derive from dystrophin deficiency. On the other hand, cardiomyopathy and arrhythmia cause thrombus formation with mechanism similar to idiopathic dilated cardiomyopathy, which can cause cerebral infarction and pulmonary embolism. Moreover, transient muscle damage even in advanced DMD patients activates coagulation cascade leading to cerebral infarction and pulmonary embolism.

Therefore, improving microcirculation insufficiency, and coagulation and fibrinolysis abnormalities may lead to improving disease progression and prevention of complications in DMD patients. Now, the level of peripheral circulating CD34 positive cells, namely endothelial circulating progenitor cell related with vascular homeostasis, functional maintenance and angiogenesis, is evaluated whether it can be the biomarker reflecting microcirculation abnormality and disease progression of DMD (Saito T, et al. 2013).

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ORIGINAL ARTICLE

A new method for *SMN1* and hybrid *SMN* gene analysis in spinal muscular atrophy using long-range PCR followed by sequencing

Yuji Kubo[1,2,3], Hisahide Nishio[4,5] and Kayoko Saito[1,2]

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder characterized by progressive loss of motor neurons in the spinal cord. Approximately 95% of SMA patients have a homozygous deletion of the survival motor neuron 1 (*SMN1*) gene, whereas 5% harbor compound heterozygous mutations such as an *SMN1* deletion allele and an intragenic mutation in the other *SMN1* allele. It is difficult to detect intragenic mutations in *SMN1* because of the high degree of homology shared between *SMN1* and *SMN2*. Current methods analyze a restricted region from exon 2a to exon 7 in *SMN1*. We propose a new, efficient long-range polymerase chain reaction (PCR) method for detecting intragenic mutations in *SMN1* (exon 1–8) and hybrid *SMN* genes. We analyzed 20 unrelated SMA patients using *SMN* copy number analysis, and the new long-range PCR method followed by sequencing. We thus confirmed a novel mutation in *SMN1* exon 1 (c.5C>T) in three patients with SMA type III who also had an *SMN1* deletion allele. Moreover, we confirmed three hybrid *SMN* gene types in eight patients. We report a novel *SMN1* mutation responsible for a relatively mild SMA phenotype and three hybrid *SMN* gene types in patients with SMA type III.
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INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder characterized by degeneration of anterior horn cells in the spinal cord, leading to progressive proximal muscle weakness and atrophy.[1] Disease incidence has been estimated at 1 in 6000–10 000 live births, with a carrier frequency of 1 in 40–60.[2,3] SMA is a lower motor neuron disease and is clinically classified into four phenotypes: childhood-onset types I–III and adult-onset type IV.[4] SMA type I (also known as Werdnig–Hoffmann disease; OMIM 253300) is the most severe form, with onset before the age of 6 months. Unable to sit without support, patients must be ventilated to survive after the age of 2 years. SMA type II (OMIM 253550) is the intermediate form, with onset before the age of 18 months; patients with this form of SMA never gain the ability to stand and walk. SMA type III (also known as Kugelberg–Welander disease; OMIM 253400) is a mild form, with onset after the age of 18 months; patients are able to walk early in the disease course, but lose this ability as the disease progresses.[5] Adult-onset SMA is referred to as SMA type IV (OMIM 271150) and manifests after the age of 20.[4]

SMA is caused by deletion of the survival motor neuron (*SMN*) gene located on chromosome 5 (5q13). *SMN* is present in two homologous copies, a telomeric *SMN1* and a centromeric *SMN2*; the difference between these two genes is only five base pairs.[6] Both *SMN* genes encode the SMN protein, which has a role in pre-messenger RNA (mRNA) splicing in the anterior horn cells in the spinal cord.[7] Although transcription of *SMN1* produces full-length mRNA, transcription of *SMN2* yields only 15% full-length mRNA, whereas 85% of the mRNA is incomplete (lacking exon 7).[4]

SMN1 is the SMA-determining gene; ~95% of patients have homozygous disruptions of *SMN1* owing to deletion or conversion of *SMN1 to SMN2*.[8,9] Homozygous deletions of *SMN1* exon 7 are the result of a gene conversion of *SMN1 to SMN2*, yielding a hybrid *SMN* gene.[10,11] Approximately 5% of patients are compound heterozygotes with a deletion and an intragenic mutation in one *SMN1* allele.[12] *SMN2* copy numbers also vary among patients and are associated with disease severity.[13–15]

If no *SMN1* deletion is detected in a patient with suspected SMA, *SMN1* copy number analysis and intragenic mutation screening should be performed.[16] Real-time polymerase chain reaction (PCR) and multiplex ligation-dependent probe amplification are used to analyze *SMN1* copy number. Intragenic mutation screening of *SMN1* should be performed to determine whether *SMN1* or *SMN2* carries any intragenic mutations, because the sequences are largely homologous. Current methods include reverse-transcription PCR of mRNA

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or long-range PCR of genomic DNA, both of which have limitations.[6,14,17,18] It can be difficult to construct SMN1 complementary DNA because of the low expression level of SMN1 mRNA in peripheral blood leukocytes. Moreover, the current method does not detect intronic mutations. Although strategies have been developed to overcome some of the problems associated with this method, it remains limited to a restricted region (13.2 kb) from exon 2a to exon 7 in *SMN1* (20 kb). Therefore, the current method cannot be used to analyze upstream regions such as the 5′-untranslated region and exon 1 or regions associated with the hybrid *SMN* gene, such as exon 7, intron 7 and exon 8.

We have developed a more efficient and broadly applicable method using long-range PCR for specific amplification of *SMN1*. This new method was evaluated using controls and a sample from a previously reported patient with SMA type I, who is a confirmed compound heterozygote for *SMN1*, with one deleted *SMN1* allele and an intragenic mutation (c.275G>C, p.W92S) in the other allele.[19] We identified a novel missense mutation in *SMN1* exon 1 (c.5C>T), leading to an alanine-to-valine substitution at amino acid 2 (p.A2V) in three Japanese patients with SMA type III. We also identified three hybrid *SMN* gene types in eight Japanese patients with homozygous deletions of *SMN1* exon 7.

MATERIALS AND METHODS

Ethics statement

This study was approved by the Ethics Committee of Tokyo Women's Medical University and was performed with the written informed consent of all patients.

Patients

We analyzed 10 controls and 20 unrelated patients with SMA type I ($n=1$), type III ($n=18$) and type IV ($n=1$). All patients met the diagnostic criteria for proximal SMA established by the International Consortium for SMA.[5] Some patients did not clearly fit a single category; for those patients, we assigned SMA type by giving priority to each patient's highest function over age of onset. Our new method was evaluated in Patient 9 with SMA type I. Patient 9, as reported previously,[19] was known to be compound heterozygous for *SMN1*, with one deleted *SMN1* allele and the other allele containing an intragenic mutation (c.275G>C, p.W92S). The remaining 19 patients (patients 1–8 and 10–20) were analyzed to demonstrate and characterize the presence of homozygous or heterozygous deletions in *SMN1* exon 7, intragenic mutations and hybrid *SMN* genes. Family members 1–1 and 1–2 were analyzed as part of our evaluation of Patient 10.

DNA extraction and *SMN1* deletion test

Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and adjusted to a final concentration of 100 ng μl^{-1}. The *SMN1* exon 7 deletion was detected by PCR-restriction fragment length polymorphism.[6,20]

SMN copy number analysis using the multiplex ligation-dependent probe amplification method

We used the SALSA multiplex ligation-dependent probe amplification KIT P021-A1 SMA (MRC-Holland, Amsterdam, Netherlands) to determine *SMN* copy numbers. This kit contains a mixture of probes specific to exon 7 of the *SMN1* (NM_000344) and *SMN2* genes (NM_017411); exon 8 of the *SMN1* and *SMN2* genes; exons 1, 4, 6 and 8 of the *SMN1* and *SMN2* genes; and probes for genes located near *SMN* (for example, the *NAIP* and *H4F5* (*SERF1*) genes); other chromosomes; and reference probes. After multiplex ligation-dependent probe amplification, DNA fragments were analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with GeneMapper software v4.1 (Applied Biosystems).

Specific amplification of *SMN1* by long-range PCR

Conventional long-range PCR was performed using a specific *SMN1* exon 7 reverse primer to amplify a 13.2-kb region that includes exons 2a–7 of *SMN1*. Our new long-range PCR (nLR-PCR) method for specific amplification of *SMN1* was performed using forward primer hybridization—654 bp from the transcription initiation site and a specific *SMN1* exon 8 reverse primer to amplify a 28.2-kb region that includes exons 1–8 of *SMN1* (Figure 1). The reaction was performed with KOD FX Neo polymerase (TOYOBO, Osaka, Japan) by step-down cycle PCR in a 50 μl reaction volume, with 25 μl of 2× PCR Buffer, 0.4 mM of each dNTP, 0.15 μM of each primer (SMN_FL_(ex1-654)_F and SMN_FL_ex8_R), 1 U of polymerase and 100 ng of genomic DNA (Supplementary Table 1). nLR-PCR was performed as follows: initial denaturation at 94 °C for 2 min, followed by 5 cycles of denaturation at 98 °C for 10 s, annealing and extension at 71.2 °C for 15 min, followed by 5 cycles of denaturation at 98 °C for 10 s, annealing and extension at 69.2 °C for 15 min, followed by 5 cycles of denaturation at 98 °C for 10 s, annealing and extension at 67.2 °C for 15 min, and 20 cycles of denaturation at 98 °C for 10 s, annealing and extension at 65.2 °C for 15 min and a final extension at 65.2 °C for 7 min. Expected 28.2-kb products were confirmed by 0.7% agarose gel electrophoresis. Amplified nLR-PCR products were excised, extracted with the QIAEX II Gel

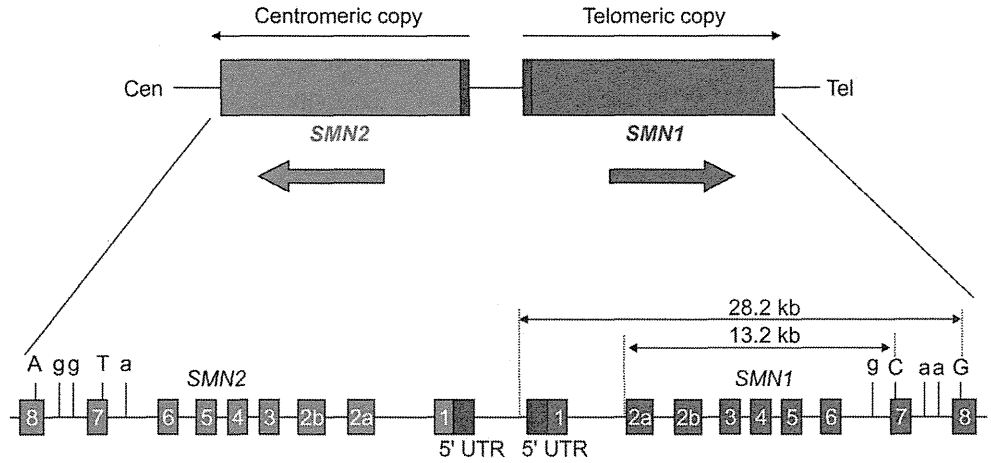

Figure 1 Strategy for specific amplification of *SMN1* by long-range PCR. *SMN1* and *SMN2* lie, respectively, on the telomeric and centromeric halves of an inverted duplication in chromosome region 5q13. Long-range PCR (13.2 kb) of the region including exons 2a–7 of *SMN1* was reported by Clermont *et al.*[17] The new long-range PCR (28.2 kb) encompasses the region including exons 1(-654)–8 of *SMN1*. We specifically amplified *SMN1* using the 1-base difference in exon 8. A full color version of this figure is available at the *Journal of Human Genetics* online.

Extraction Kit (Qiagen) and eluted in 20 µl of elution buffer. The nLR-PCR products were quantified using the ImageJ (NIH) software.

Intragenic mutations and hybrid *SMN* gene analysis by sequencing

We used 1 µl of the purified nLR-PCR product as a template to amplify each *SMN1* exon by nested PCR. Supplementary Table 1 lists the sequencing PCR primers and their annealing temperatures. Amplification of exon 1 was performed with KOD FX polymerase (TOYOBO) by two-step cycle PCR in a 25 µl reaction volume, with 12.5 µl of 2× PCR Buffer, 0.4 mM of each dNTP, 0.4 µM of each primer, 0.5 U of polymerase and 1 µl of template (Supplementary Table 1). PCR was performed under the following conditions: initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 98 °C for 10 s and annealing and extension at 68 °C for 45 s. Other targets were amplified using the Ex Taq polymerase (TAKARA) by three-step cycle PCR in a 25 µl reaction volume with 2.5 µl of 10× Ex Taq Buffer, 0.2 mM of each dNTP, 0.4 µM of each primer, 1.25 U of polymerase and 1 µl of template (Supplementary Table 1). PCR was performed under the following conditions: initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 45 s, followed by a final extension at 72 °C for 5 min. Each *SMN1* exon product was purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced on an ABI 3130 Genetic Analyzer (Applied Biosystems) using the BigDye Terminator v3.1 Cycle Sequencing Kit. Mutations reported here have been submitted to a Leiden Open Variation Database (http://www.LOVD.nl/SMN1).

Family analysis

Family members 1–1 and 1–2 were the mother and younger brother of Patient 10, respectively. Copy number and sequencing analyses were performed for all family members of Patient 10.

In silico analysis

The Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/) and Align-GVGD (http://agvgd.iarc.fr/) classification tools were used to determine the amino-acid changes that were most likely to be responsible for the loss of protein function.[21,22] The dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), 1000 Genome Project databases (http://www.1000genomes.org) and Human Genetic Variation Database (http://www.genome.med.kyoto-u.ac.jp/SnpDB/index.html) were used to determine whether the identified variants are polymorphisms.

RESULTS

SMN1 deletion test and *SMN* copy number analysis

The *SMN1* deletion test and *SMN1* copy number analysis in 20 patients with SMA type I, SMA type III or SMA type IV revealed the absence of *SMN1* exon 7 in all of these patients (Supplementary Table 2). The overall distribution of deletion types was as follows: eight patients with homozygous deletion of *SMN1* exons 7 and 8 (patients 1–8); four patients with heterozygous deletion of *SMN1* exons 7 and 8 (patients 9–12); and eight patients with homozygous absence of *SMN1* exon 7 but not exon 8 (patients 13–20). Regarding the *NAIP* and *H4F5* (*SERF1*) genes located near *SMN*, the overall distribution of deletion types was as follows: one patient with homozygous deletion of *NAIP* exon 5 (patient 1); eight patients with heterozygous deletion of *NAIP* exon 5 (patients 2, 6, 8, 10–12, 15 and 16); three patients with heterozygous deletion of *H4F5* (*SERF1* exon 1) (patients 10–12).

Specific *SMN1* analysis by long-range PCR

Eight control subjects (controls 1–8) had two *SMN1* copies and eight patients (patients 1–8) had *SMN1* deletions. Products, 28.2-kb in size, were confirmed for all controls, whereas the bands were faint in the patients (Figure 2a). Band intensity for the controls was four times higher than that for the patients (patients 1 and 2 or patients 3–8 versus controls 6–8; $P<0.05$; Figure 2b). Controls 1 and 2 had the *SMN2* deletion and, therefore, their samples produced the highest-intensity bands (controls 1 and 2 versus controls 6–8; $P<0.05$). *SMN1* intron 6, exon 7 and intron 7 were amplified from the nLR-PCR products by nested PCR using SMN-ex7-F and R primers and sequenced to verify *SMN1* specificity (Figure 2c).

Direct sequencing for patient 9, who had a known intragenic mutation (c.275G>C), revealed an abnormal heteroduplex signal (blue: Cytosine, black: Guanine) in exon 3 of *SMN1* and *SMN2* (Figure 3). Only *SMN1* regions were isolated by nLR-PCR; *SMN1* exon 3 was amplified by nested PCR from nLR-PCR products; sequencing revealed increased cytosine and decreased guanine signal intensity (Figure 3). These findings suggested that the cytosine was derived from *SMN1* and that the mutation was present in *SMN1* exon 3. *SMN1* intron 6, exon 7 and intron 7 were also sequenced from nLR-PCR products to verify *SMN1* specificity (data not shown).

Novel intragenic mutations and family analysis

We screened all exons of *SMN* for novel intragenic mutations by direct sequencing of genomic DNA. Patient 10, with SMA type III, produced an abnormal heteroduplex signal (blue: Cytosine, red: Thymine) in exon 1 of *SMN1* and *SMN2* (Figure 4a), indicating an intragenic mutation in exon 1 of *SMN1* or *SMN2*. To determine which gene carried the mutation, *SMN1* nLR-PCR products were sequenced. A single signal (red: Thymine) was detected in *SMN1* exon 1, indicating that the mutation was present in *SMN1* exon 1 (Figure 4a). This C-to-T mutation at position 5 (c.5C>T) causes an alanine-to-valine substitution at amino acid 2 (p.A2V). This mutation was also identified in patients 11 and 12 (Table 1).

Copy number and sequencing analyses were performed for relatives (family members 1–1 and 1–2) of patient 10 (II–1; Figure 4b). The mother (family member 1–1; I–2) carried one *SMN1* copy and two *SMN2* copies; the brother (family member 1–2; II–2) carried two *SMN1* copies and two *SMN2* copies. The intragenic mutation in patient 10 (II–1) was absent in both of the family members tested (I–2 and II–2).

In silico analysis

The c.5C>T mutation was not observed in 100 normal Japanese control samples. This mutation has not been documented in dbSNP, the 1000 Genome Project database or the Human Genetic Variation Database. Functional significance was evaluated by referring to Polyphen-2, SIFT and Align-GVGD. The mutation was assumed to lead to a hazardous change in protein function because all three programs returned evaluations of 'DAMAGING (PolyPhen-2 score: 0.939, SIFT score: 0.01)' and 'Class C65.' Thus, in SMA type III patients 10–12, the disease was attributed to a compound heterozygous mutation, including one *SMN1* allele deletion and a c.5C>T mutation in the other *SMN1* allele.

Hybrid *SMN* gene analysis by long-range PCR and sequencing

Patients 13–20, carrying a homozygous absence of *SMN1* exon 7 but not exon 8, were assessed for the presence of the hybrid *SMN* gene by nLR-PCR amplification of a region that includes exons 1–8 of *SMN1* and by sequencing of intron 6, exon 7 and intron 7 (Table 2). We identified three hybrid *SMN* gene types (Table 2 and Figure 5). The sequences of hybrid *SMN* intron 6, exon 7, intron 7 and exon 8 were as follows: patient 13, aTagG; patients 14 and 16–20, aTggG; and patient 15, gTaaG.