0–10 mM of VPA were 0.63–1.05 in OK11, 0.73–1.08 in AM21 and 1.3–1.86 in CO31 (arbitrary units relative to beta-actin; Fig. 3b). The change from the baseline only reached significance for the OK11 line at 1 mM VPA. Compared with the baseline levels of SMN protein (0 mM), we observed an approximately 1.5-fold increase in SMN protein expression in SMA cell lines treated with 1–10 mM.

The baseline SMN protein levels in the SMA type II cells, AM21, were slightly higher than those in the SMA type I cells, OK11. However, during VPA treatment, the SMN protein levels were almost identical. *SMN*2 copy number did not contribute to the increase in SMN protein levels in both of our SMA cell lines.

3.3. VPA modulates splicing factor expression

Brichta et al. reported that VPA increased the levels of the splicing factors SF2/ASF, Htra2-β1 and SRp20 [33]. However, they did not describe the effect of VPA on other splicing factors, such as hnRNPA1. Thus, we studied whether VPA treatment changes SF2/ASF or hnRNPA1 protein levels relative to the baseline (mock status) levels in cultured fibroblasts.

Transcript levels of the splicing factors, SF2/ASF or hnRNPA1, upon treatment with 0–10 mM of VPA were fluctuated in our fibroblast cell lines, showing no tendency to increase or decrease with VPA amount (data not shown). However, western blotting revealed an increase in SF2/ASF protein expression in all three cell lines with 1–10 mM VPA (Fig. 4a). The maximum changes in SF2/ASF protein levels were 0.59–0.91 in OK11, 0.65–1.25 in AM21 and 0.96–1.56 in CO31 (arbitrary units relative to beta-actin). Compared with the baseline (mock status), we observed a 1.5–2-fold increase in SF2/ASF protein expression in all cell lines treated with 1 and/or 10 mM VPA (Fig. 4a). On the other hand, treatment with 1–10 mM VPA led to a significant decrease in hnRNPA1 protein expression in all three cell lines (Fig. 4b). The changes in hnRNPA1 protein levels were 0.56–0.29 in OK11, 0.77–0.29 in AM21 and 0.85–0.51 in CO31. Compared with the baseline (mock status), we observed an approximately 2-fold decrease in hnRNPA1 protein expression in all cell lines treated with 10 mM VPA. It should be noted that the ratio of SF2/ASF to hnRNPA1 also increased with increase in the VPA concentration (Fig. 4c).

4. Discussion

4.1. VPA induction of FL-SMN transcript and SMN protein

We showed in this study that VPA treatment increased total-*SMN* transcripts, FL-*SMN* transcripts and SMN protein levels in SMA fibroblast cell lines. VPA is an HDAC inhibitor [44,45]. Treatment with HDAC inhibitors increases acetylated histone levels and relaxes chromatin structure, resulting in the activation of many genes including *SMN2* [33–39,46]. Our SMA cell lines demonstrated an approximately 2-fold increase in FL-*SMN* transcripts and a 1.5-fold increase in SMN protein levels after 16 h incubation with 10 mM VPA. These results are consistent with previous studies of the effect of VPA on *SMN* expression [33,34].

Brichta et al. reported that a 16 h incubation with VPA at concentrations less than 0.5 mM increased FL-*SMN* transcripts in SMA fibroblast cell lines [33]. These authors also reported that 0.5 nM–0.5 mM VPA increased the production of FL-*SMN2* transcripts and SMN protein levels 2 to 4-fold. They also predicted that a considerable increase in SMN protein could be obtained at even lower VPA concentrations than those used in epilepsy treatment, because the serum VPA level required for epilepsy therapy is 0.48–0.7 mM [47].

In contrast, Sumner et al. reported that a 24 h incubation with 1 mM VPA did not increase FL-*SMN* transcripts in SMA fibroblast cell lines [34]. This agrees with our data showing that a 16 h incubation with 1 mM VPA did not increase FL-*SMN* expression in our SMA fibroblast cell lines. The effective concentration of VPA may depend on the responsiveness of *SMN* to VPA in the cell line.

With regards to *SMN2* copy number, our study show that, before VPA treatment, the OK11 fibroblast cell lines with zero *SMN1* and two *SMN2* copies showed significantly lower baseline levels of FL-*SMN* transcripts and SMN protein than those of the AM21 fibroblast cell lines with zero *SMN1* and three *SMN2* copies (Figs. 2b and 3). However, during VPA treatment, both fibroblast cell lines produced similar levels of FL-*SMN* transcript and SMN protein. Contrary to our expectations, our SMA fibroblast cell lines responded similarly to the VPA treatment, regardless of their different *SMN2* copy numbers. This suggests that the *SMN2* copies are not equivalent in response to VPA treatment.

4.2. Modulation of splicing factor expression by VPA

Brichta et al. and Sumner et al. reported that, in SMA fibroblasts, the FL/Δ7 ratio increased 1.5–2-fold after VPA treatment [33,34]. Both of these reports concluded that the increase in VPA-induced FL-*SMN* could be explained not only by *SMN2* transcriptional activation but also *SMN2* exon 7 splicing correction. The upregulation of the FL/Δ7 ratio suggested modulated production of splicing factors in the cells.

VPA activates not only *SMN2*, but also many other genes, including those encoding splicing factors. Brichta et al. showed that VPA induced overexpression of the splicing factor, Htra2-β1, in the SMA cells, and these authors speculated that overexpressed Htra2-β1 plays

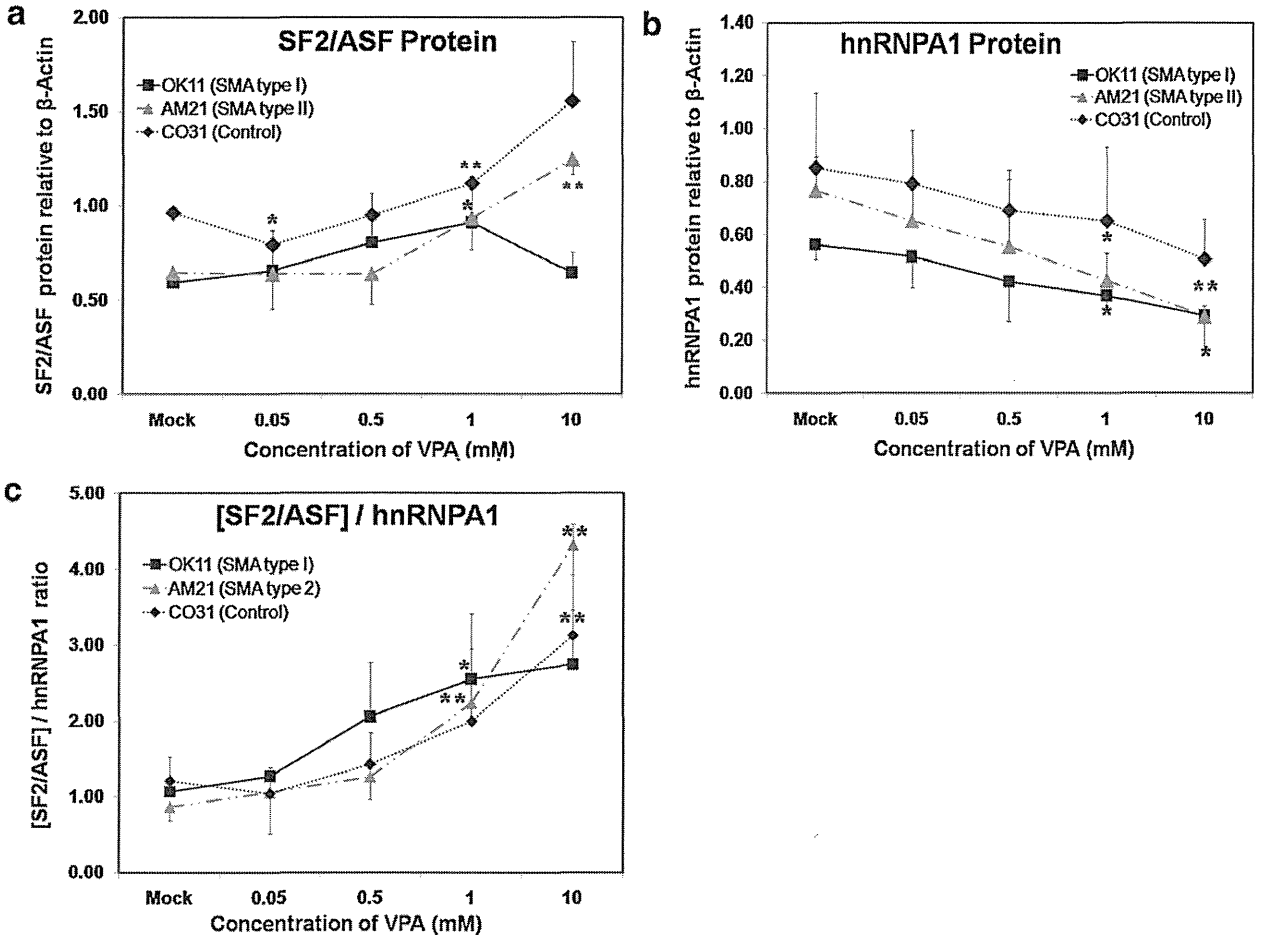

Fig. 4. Quantitative analysis of SF2/ASF protein. (a) Upregulation of SF2/ASF protein levels determined by western blotting in the cell lines, incubated for 16 h with different concentrations of VPA. The data are expressed as mean ± SD in arbitrary units relative to beta-actin. Densitometry revealed increasing SF2/ASF protein levels with increasing concentration of VPA. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* the baseline (mock). (b) Downregulation of hnRNPA1 protein levels determined by western blotting in the cell lines, incubated for 16 h with different concentrations of VPA. The data are expressed as mean ± SD in arbitrary units relative to beta-actin. Densitometry indicated decreasing hnRNPA1 protein levels with increasing VPA concentration. Asterisks indicate a significant decrease compared with the mock status in each cell line. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* the baseline (mock). (c) The calculated [SF2/ASF]/hnRNPA1 ratios showed modulation of splicing factor expression induced by VPA. The FL/Δ7 ratio was not affected by the increase in the [SF2/ASF] / hnRNPA1 ratio, suggesting that *SMN2* gene splicing was not affected by modulation of SF2/ASF and hnRNPA1 levels. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* the baseline (mock).

an important role in correcting the splicing pattern [33]. This notion was supported by previous studies showing that Htra2-β1 overexpression can promote the inclusion of *SMN2* exon 7 [39,48,49].

We examined two crucial splicing factors: SF2/ASF, related to *SMN1* exon 7 inclusion, and hnRNPA1, related to *SMN2* exon 7 exclusion. In our study, VPA increased SF2/ASF and decreased hnRNPA1 at the protein level (Fig. 4a and b). It should be noted here that VPA does not always activate gene expression, but sometimes inactivates gene expression indirectly. For example, HDAC inhibitors such as trichostatin A and VPA suppress the expression of steroidogenic gene *CY11A1* and reduce CY11A1 levels in adrenocortical cell lines [50].

According to Kashima et al., *SMN2* exon 7 inclusion was enhanced by treatment with small interfering RNAs against hnRNPA1 [19]. Thus, it can be expected that decreased hnRNPA1 expression prevents the skipping of *SMN2* exon 7, correcting the splicing of *SMN2* exon 7. In contrast, our experiment shows that VPA did not change the FL/Δ7 ratio in any cell line, indicating that the decrease in hnRNPA1 caused by VPA did not contribute to a splicing correction of *SMN2* exon 7. This may be explained by at least three possibilities. The first explanation is that the decrease in hnRNPA1 was insufficient for splicing correction. The second is that additional effects of VPA may weaken the influence of decreased hnRNPA1 expression in correcting the splicing. As an example of the latter case, if VPA could

increase or retain the amount of hnRNPA2 which is related and potentially redundant with hnRNPA1 [51], it may attenuate the influence of decreased hnRNPA1 expression in splicing correction. The third is the possibility that in our cell lines, VPA could not increase Htra2-β1. If VPA could increase the amount of Htra2-β1, the ratio of FL/Δ7 would have been corrected. Further analysis of the effects of VPA on splicing factors, including Htra2-β1, is required.

4.3. Clinical application of VPA in SMA treatment

Brichta et al. showed the effect of VPA treatment in 20 SMA patients [40]. There was an increase in *SMN* transcript and SMN protein levels in blood cells in 7 patients (1 of 5 in type I; 4 of 11 in type II and 2 of 4 in type III). According to these authors, there are two types of SMA patients: responders and nonresponders to the VPA treatment, mantaining serum VPA levels of 0.48–0.70 mM [40]. Responders showed a 1.6–3.4-fold elevation of FL-*SMN* transcripts while nonresponders showed only minor changes in FL-*SMN* transcript levels. For nonresponders, a higher dose of VPA may be required to obtain a sufficient increase in FL-*SMN* transcripts or SMN protein. However, Brichta et al. did not demonstrate an effect of VPA on the improvement of clinical symptoms including muscle strength.

Weihl et al. presented evidence for improvement in muscle strength in seven adult patients with SMA type III/IV patients after VPA treatment [52]. These authors collected quantitative data of muscle strength determined by hand-held dynamometry [53]. They also reported the patient's subjective benefits from VPA treatment, such as feeling stronger, ease of breathing, ease of rising from chairs, ability to dress themselves, ability to comb hair and pick grapes and walking endurance in a marching band.

Tsai et al. reported that age may play a role in the improvement of muscle strength in six SMA patients (two SMA type II and four SMA type III) with VPA treatment [41]. The therapeutic effects of VPA in SMA patients were inversely related to age. These authors assessed the muscle strength of SMA patients using the Medical Research Council score (from 0 to 5) [41]. Two SMA children showed some improvement in muscle power, and two SMA adolescents showed a slight increase. In contrast, two SMA adult patients showed no response to VPA.

Swoboda et al. [54] reported results similar to those of Tsai et al. and found that the Modified Hammersmith Functional Motor Scale showed some improvement in gross motor function in 27 SMA type II children upon VPA treatment. However, no children $\geqslant$5 years of age showed a six-point improvement after 1 year. In 8 of 16 children (50%) under 5 years of age, the improvement was significantly more pronounced.

In light of these data, the question arises as to whether VPA can have a negative effect on the motor neuron function. Rak et al. found that VPA can increase *Smn* expression and lead to reduced growth cone size and decreased excitability in axon terminals of the motor neurons of SMA mice ($Smn^{-/-}$; *SMN2*) [55]. These authors reported that the potential positive effects of VPA are counteracted by its negative effects of on neuronal excitability and axon growth. Similarly, Van der Berg et al. reported that VPA reduced neuronal excitation through sodium channels [56]. Recently, Swoboda et al. have demonstrated no benefit from six months treatment with VPA and L-carnitine in a young non-ambulatory cohort of subjects with SMA [57]. However, their patients may have included responders and non responders to VPA. We should rather emphasize the importance of estimating prior to the treatment whether the SMA patients are responders or non responders to VPA. If we can identify the responders for VPA treatment before clinical trial, feasible outcome would be gained.

We can say that it is worthwhile treating SMA patients with VPA, especially who are VPA-responders, because it is a safe, widely-used and long-term drug used in epileptic children. Based on the studies using cells derived from SMA patients, including ours, the responsiveness to VPA of *SMN2* expression may vary from patient to patient. Some patients require a small dose of VPA to activate *SMN2* expression and improve clinical symptoms, but others require a much larger dose. Thus, dose-related side effects should always be considered. In fact, children need a higher dose of VPA per kilogram than adults to obtain an equivalent serum concentration [58]. Regular monitoring of serum VPA levels is necessary to avoid any harmful side effects.

5. Conclusion

We demonstrated here that VPA increases FL-*SMN2* transcript and SMN protein levels, suggesting that VPA is a candidate drug for SMA. Because splicing correction of *SMN2* exon 7 was not observed, the increase in FL-*SMN2* transcript and SMN protein levels may be explained mainly by the activation of *SMN2* transcription.

In addition, we also demonstrated that VPA modulates the expression of the splicing factors SF2/ASF and hnRNPA1 in our cell lines: VPA increased SF2/ASF protein levels and decreased hnRNPA1 protein levels. Based on our data, *SMN2* exon 7 splicing may be regulated not only by SF2/ASF and hnRNPA1 but also by other factors as well. However, the number of SMA cell lines in our study was very limited: we could not neglect the possibility that these findings were specific to our SMA cell lines. Thus, it is necessary to study

the VPA-effects on expression of the splicing factors using a larger number of cell lines.

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＝原著論文＝

Duchenne 型筋ジストロフィー児への病気の説明に関する調査

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要旨

【目的】Duchenne 型筋ジストロフィー（DMD）児への病気説明には，さまざまな困難があり，専門医がどのように説明を行っているかを検討する．

【方法】日本小児神経学会認定小児神経専門医 1,022 名に DMD 児の仮想事例を用いた質問紙調査を行った．

【結果】311 名から回答を得た．患児への説明をカテゴリー化した結果，症状説明など医学的な視点だけでなく，生きがいを伝えるなど人間的な支えの視点もみられた．説明時には両親の理解や受容が重要であり，病名を伝える際には心理職などの必要性や一定年齢以上なら病名を伝える考えに同意した医師が多かった．

【結論】患児への病気説明時に重要な要因および医師の考えの現状が明らかとなった．

見出し語　Duchenne 型筋ジストロフィー，告知，病気説明，質問紙調査

はじめに

一般に，小児難病患者への告知は患児の精神発達の段階に応じて，適切な説明を行っていくことが望ましいとされる．Duchenne 型筋ジストロフィー（DMD）のような緩やかに進行していく疾患では，成長に合わせた説明が必要であるとされ[1]，小児がんなどで行われている告知や説明[2]とは異なっている．

これまで，DMD ではステロイドの運動機能維持効果が報告され，標準的治療法とされているものの[3]，根本的な治療法は確立していなかった．しかし，治療に関する研究の進展[4)5)]により，遺伝子治療，新規薬物治療，幹細胞治療なども治験段階に入りつつある．それゆえ，今後は治療機会の確保のために確定診断を行い，若年のうちに告知や病気に関する説明が行われる機会は増加すると予測される．病名の告知や説明についての考えや実践には，文化的背景による差違がある可能性もあるが[2]，DMD における告知や病気の説明については，海外のガイドラインでも十分に触れられていない[6]．患児，家族と医師のコミュニケーションには困難が伴う場合もあり[7]，病気についての説明がいかになされているかを検討することは意義があると考え，本研究を計画した．

第 53 回日本小児神経学会総会推薦論文

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I　対象・方法

1. 対象

調査開始時点で郵送によりアンケートが送付可能であった，日本小児神経学会認定小児神経専門医 1,022 名とした．

2. 方法

郵送によるアンケート調査とした．調査票は 2010 年 8 月から 2011 年 2 月までの期間に配布，回収された．

3. 調査内容

配布したアンケートは仮想事例を提示し，自由記述で対応を問うもの，病気の説明をするに当たって，どのような要因が重要であるか，医師の告知についての考え等を問う設問から構成された．仮想事例は，以下の通りである．

「患児は現在小学 5 年生 11 歳で，1 歳半時に DMD と診断を受けた．小学 4 年時より，立ち上がりや歩行の困難から車椅子を使用．現在は年 2 回程度で数年通院している．明らかな精神遅滞はない．母親の話によると，学校で『生きること』について考える授業をしたので，それをきっかけに，患児は漠然と将来のことも考えており，自身の病気をもっと知りたいと希望している．筋肉の病気であり，日常生活で注意が必要なことは説明されているが，将来や予後については，話しておらず，家でもそういった話はしてこなかった．母親自身どのように説明したら良いのか分からず，医師に相談を持ちかけた」．このような場合に患児にどのように対応するかについての自由記述を求めた．患児への説明の際に重要と考える要因では，0（全く重要でない），1（あまり重要でない），2（ある程度重要），3（非常に重要）の 4 段階で評定を求めた（図 2）．また，病名を伝えることについての医師の考えを問う設問では，0（まったくそう思わない），1（あまりそう思わない），2（ある程度そう思う），3（非常にそう思う）までの 4 段階で評定を求めた（図 3）．

なお，本研究の実施に当たっては，国立病院機構刀根山病院

表1 患児に説明する内容についてのカテゴリ

カテゴリ	カテゴリの枠組み	n
症状説明	筋ジストロフィーの疾患特性，筋肉の病気であることなどの現状の説明	160
病気の経過・予後	現在の症状が今後どのように経過していくのかということの見通し，場合によっては生命予後までを伝える	114
医療的対応	現在，今後出てくる症状に対してできる医療的対応を伝える	59
将来の治療の可能性・希望（治療の可能性）	将来治療法が開発される可能性もあり希望があること	38
生きがい・生の楽しみ（生きがい）	できないこともあるが，その中で生きがいや人生の楽しみを見つけていくこと	32
患者会の紹介，同病の方の例（患者会等）	患者会や同病の方の例を紹介する	32
協働・周囲のサポートがあること（協働・サポート）	医師が患児と協働して支えていくこと，周囲のサポートがあるということ	30
病気について伝えないこと（伝えないこと）	病名，病気については伝えないこと	21
人生・いのちについての医師の自己開示（医師の自己開示）	医師自身の人生観，生命観についての自己開示	17
筋力が低下してもできること（できること）	今後筋力は低下していくが，その中でもできることはあると伝えること	12
個人差があり不確定	病気の進行には個人差があり，今後のことははっきりとはわからないと伝えること	7
自分の病気を知ること	自分自身の病気を知ることは意味があると伝えること	5
肯定的見方・積極性	自身の病気について肯定的に捉えられるように伝え，患児の積極性を引き出すようにする	5

患児への対応として得られた自由記述から，説明の内容に関する記述を分類し，13カテゴリを作成した．表中のnは，そのカテゴリに対応する内容を記述した医師の数を示す．

臨床研究審査委員会の審査を経た．

4. 分析

自由記述により得られたデータは，意味の単位ごとに区切り，内容の類似したものをカテゴリ化して分類した．分類されたカテゴリに，その内容を示すものとしてカテゴリ名をつけた．また，分析の妥当性を確保するため，作成されたカテゴリについて，筋ジストロフィーの心理支援に関する研究に2年以上携わった経験のある2名が独立に評定を行った．統計的解析にはSPSS 17.0®を用いた．有意性検定においては，$p \leqq 0.05$ を有意差ありとし，post hoc testではBonferroniの修正を行った．

Ⅱ 結　　果

宛先不明で返送された13通を除き，回答が得られたのは311名であった（回収率30.8%）．本研究では筋ジストロフィーの診療経験がないと回答した27名のデータを除いた284名を分析の対象とした．なお，質問項目の一部のみ無回答であった場合は，その回答のみ除外し，項目ごとに有効回答を集計した．対象者の年齢は35～86歳（平均年齢50.8歳）で，小児神経疾患に関する臨床経験年数は4～55年（平均20.7年），筋ジストロフィー診療に関する臨床経験年数は1～55年（平均13.7年）であった．

1. 患児への対応についての自由記述回答の分析

自由記述の設問に回答した251名を対象として分析を行った．自由記述の回答をその意味内容ごとに分類した結果，説明の内容と説明の仕方に分かれ，説明の内容については13カテゴリ（表1），説明の仕方については8カテゴリに分類した（表2）．カテゴリの分類評定では，評定者2名による評定は95%一致しており，カテゴリ分類の妥当性が確認された．説明の内容は多岐にわたっていたが，多くみられたものは「症状説明」「病気の経過・予後」「医療的対応」「将来の治療の可能性・希望」などであった．多くの医師は現在の患児が持っている症状に関する説明を行い，それらが今後どのように経過していくのかということ，それらについての医療的対応を伝えていた．さらに，現時点では治療法はないが今後研究の進展により治療法が開発されてくるという希望を伝えていることが多かった．また，生きがいを見つけることや医師が協働していくといった人間的支えに関する内容もみられた．説明の仕方についての回答では「本人の希望を確認して答える」「説明の仕方を工夫する」「患児の特性に合わせて説明する」といった回答が多く見られた．

これらのカテゴリで，n＝10以上であったカテゴリを用いてコレスポンデンス分析を行い，それぞれの反応の類似度を2次

表2　患児への説明の仕方についてのカテゴリ

カテゴリ	カテゴリの枠組み	n
本人の希望を確認して答える（本人の希望）	説明する際には患児の希望や疑問点を確認して，それらに対して応えていく	49
説明の仕方を工夫する（説明の工夫）	説明する際には，資料を用意する，別に時間を取る，数回に分けて繰り返し説明をして伝えていくなどの工夫をする	31
患児の特性に合わせて説明（患児の特性）	患児の知識や理解力，患児の価値観などの特性を把握した上で説明する	30
家族の意向を確認する（家族の意向）	家族が患児の知りたいという欲求をどう受け止めているか，伝えることに関しての意向を確認する	21
前向きに伝える	今後前向きに生活を送れるよう，伝えるときの言葉を選ぶ	19
サポート体制を構築して説明（サポート体制）	医療者側の連携や，親，学校と患児を支えていけるような体制を作った上で説明をする	13
説明する人を選ぶ	医師からあるいは家族から説明するのかを選択する	7
専門医を頼む	筋ジストロフィーの専門病院に紹介する	1

患児への対応として得られた自由記述から，説明の仕方に関する記述を分類し，8カテゴリを作成した．表中のnは，そのカテゴリに対応する内容を記述した医師の数を示す．

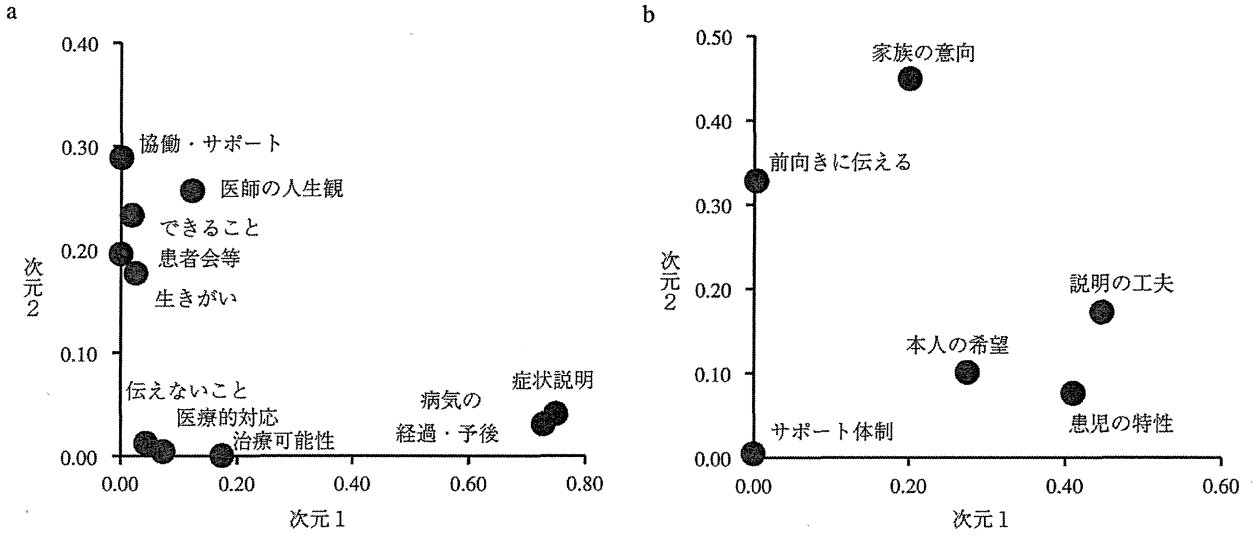

図1　患児に説明する内容，患児への説明の仕方についてのカテゴリの分布

a：患児に説明する内容，b：患児への説明の仕方

表1に示した患児への説明の内容についてのカテゴリ，および表2に示した患児への説明の仕方についてのカテゴリで，n=10以上であったカテゴリを用いてコレスポンデンス分析を行い，カテゴリ間の距離を2次元軸上に表現した．図中の次元1，次元2の軸はカテゴリの寄与率を示す．図中の点はカテゴリの座標位置を示し，カテゴリ間の距離が近いほどカテゴリの類似度が高いことを示す．

元軸上に表現した（図1-a，b）．コレスポンデンス分析は，カテゴリデータを類似度から，カテゴリを座標軸上にプロットし，カテゴリ間の関係の近さを視覚的に表現する分析法である．2次元軸上に表現されたカテゴリの座標位置の距離が近いほど，それぞれのカテゴリが類似したものであることを示す．図中の次元は，それぞれの軸におけるカテゴリの寄与率を示す．

説明の内容については，症状や予後について説明するということ，医療的対応や治療の可能性を伝える，病名や予後について伝えないなど，医療的立場から支えることなどが横軸方向に布置された．また，医師が患児と協働して支えていく姿勢や医師の自己開示など，患児の人生を支えていくという立場から伝える人間的支えのカテゴリが縦軸方向に布置された．

説明の仕方については説明の工夫や患児の特性に合わせる，本人の希望を確認するなど説明に関する配慮に関することが布置された．また，サポート体制の構築，前向きに伝える，家族の意向についてはそれぞれが分かれて布置された．

2. 説明をする際に重要と考える要因

これらの設問では4段階の評定を求めたが，「全く重要でない」と回答した医師がいなかったため，3段階での評定の分布を示した（図2）．「非常に重要」と回答した医師が多かった項目は，「両親の病気の理解」（78%），「医師と家族との信頼関係」（73%），「両親の疾患受容」（72%），「医師と患児の

信頼関係」(71%),「患児の心理状態」(71%) などであった.「病院と学校との連携」(26%),「病院としての支援体制」(31%),「家庭の生活状況」(32%),「学校や地域のサポート」(34%) などでは,「非常に重要」としていた医師は他の項目と比較して少なかった.

3. 病名を伝えることについての医師の考え

病名を患児に伝えることについての医師の考えを問う設問では,臨床心理士やケースワーカーなどの専門職による援助が必要であること,一定年齢以上であれば伝えることについて,およそ半数の医師が「非常にそう思う」と回答していた(図3).また,客観的な理解にとどめるべき,希望をなくすのではないかという内容については「まったくそう思わない」,「あまりそう思わない」と回答した割合と「ある程度そう思う」,「非常にそう思う」と回答した割合がそれぞれ半数程度であった.

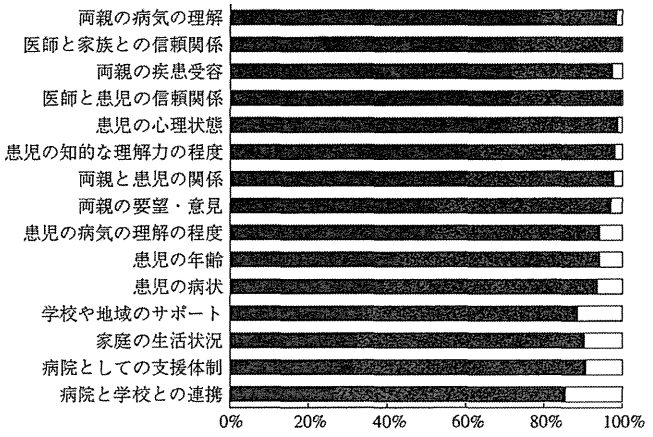

図2　患児への説明の際に重要と考える要因

患児への説明の際に重要と考える要因として,両親の理解や疾患受容,医師との信頼関係などを重要とした割合が高かった.

■:非常に重要,■:ある程度重要,□:あまり重要でない

これらの考えについて,年齢や臨床経験年数によって差異があるかを検討するために,年齢および臨床経験年数をそれぞれ3群に分け,それらを独立変数とした分散分析を行った.その結果,客観的理解にとどめること,一定年齢以上の場合には原則的に説明するということについて,年齢,臨床経験年数によって群間に差異がみられた(表3).客観的な理解にとどめるという考えについては,56歳以上,小児神経疾患の臨床経験が26年以上の医師はその考えにより強く同意していた($p<0.001$, $p<0.001$).一定年齢以上であれば伝えるという考えについては,小児神経疾患の臨床経験が26年以上,筋ジストロフィーの臨床経験年数が20年以上の医師は,より強く同意していた($p=0.050$, $p=0.028$).また,患児が何歳以上なら伝えるかという設問については,全体の平均は12.7歳であったが,56歳以上,小児神経の臨床経験が26年以上,

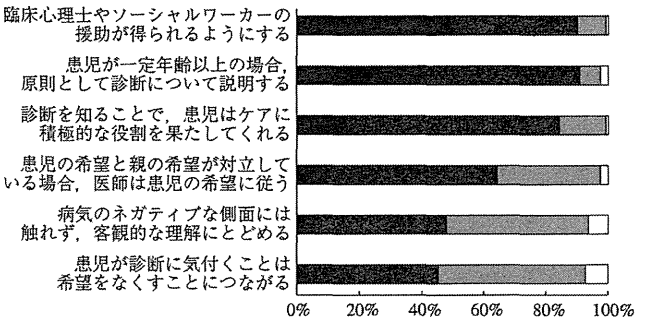

図3　患児に病名を伝えることについての医師の考え

患児に病名を伝えることについては,臨床心理士やケースワーカーなどの専門職による援助が必要,一定年齢以上であれば伝えることについてそう思うと回答した医師が多かった.

■:非常にそう思う,　■:ある程度そう思う,

▨:あまりそう思わない,□:全くそう思う

表3　年齢,臨床経験年数による病名を伝えることへの考え方の違いについての比較

	医師年齢(歳)			小児神経疾患の臨床経験(年)			筋ジストロフィーの臨床経験(年)		
	a. 45歳以下	b. 46～55歳	c. 56歳以上	a. 19年以下	b. 20～25年	c. 26年以上	a. 9年以下	b. 10～19年	c. 20年以上
病気のネガティブな側面には触れず,客観的な理解にとどめる(評定平均値)	1.28 (n=79)	1.38 (n=114)	1.82 (n=71)	1.32 (n=105)	1.43 (n=86)	1.73 (n=73)	1.44 (n=82)	1.42 (n=84)	1.56 (n=82)
	$p<0.001$ a, b<c			$p<0.001$ a, b<c			$p=0.400$		
患児が一定年齢以上の場合,原則として診断について説明する(評定平均値)	2.23 (n=78)	2.27 (n=119)	2.38 (n=73)	2.20 (n=106)	2.31 (n=91)	2.45 (n=74)	2.24 (n=83)	2.19 (n=85)	2.45 (n=85)
	$p=0.357$			$p=0.050$ a<c			$p=0.028$ b<c		
何歳以上なら伝えるか(歳)	13.5 (n=60)	12.5 (n=74)	11.5 (n=44)	13.5 (n=76)	12.3 (n=55)	11.5 (n=47)	13.2 (n=55)	13.4 (n=59)	11.4 (n=55)
	$p=0.003$ c<a			$p=0.001$ c<a			$p<0.001$ c<a, b		

告知の考え方について年齢,小児神経疾患および筋ジストロフィーの臨床経験年数を3群に分け,それらを独立変数とした分散分析を行った.表中の p 値は分散分析における有意確率を示す.Post hoc test においては,Bonferroni の修正を行い5%水準で有意差のあったものを示した.

筋ジストロフィーの臨床経験が20年以上の医師は，平均11.4～11.5歳と，より若い年齢で伝えるとしていた（$p=0.003$，$p=0.001$，$p<0.001$）．

Ⅲ 考　　察

本研究は，DMD児に対して医師がいかに病気の説明を行っているのか，その際に考慮すべき点などについて，どのように医師が考えているか調査を行い検討した．

患児に対する対応の自由記述をカテゴリ化した内容から，医師は医学的な病気の説明だけでなく，医療的な支えを行っていくこと，さらには人間的な支えも行っていくという実践がうかがえた．また，医師が患児に対して説明を行うにあたり，それぞれの医師がさまざまな内容や方法を考慮しているということが明らかとなった．さらに，医療的な説明に限らず，医師自身の考えの自己開示や生きがいを見つけていくことなども伝えられていた．これはDMDには根本的治療法がないことや進行性の疾患であるために，医師は医療的支えだけでなく人間的支えも必要であると考え，そのような実践をしているものと考えられた．DMDは病気の進行により，自己を確立していく時期である思春期に喪失体験を重ねる疾患であるため，精神的支えが重要となる．我々が先行研究として行った筋ジストロフィー病棟に勤務する医師を対象とした研究[8]では，医師が患児に対して病気のことを伝えていく際に果たす役割として，医学的な専門家という役割，患児をサポートして支えていく役割などを見出した．今回の調査においても同様の傾向が見られ，病気のことを伝える告知の場面では，医学的な視点だけでなく人間的な支えという視点も意識して関わっている医師が少なからずいると考えられた．単に病名や病態を伝えるのみで，病気に対する適切な対処法や希望，支えがあると伝えない場合は，患児や両親に絶望感や不安感を与えてしまう危険性があるという指摘[9]があるように，病気に対して過剰に絶望することなく，生活を続けていける支えがあるということ，病気を抱えながら生きていく道があると伝えることは患児や家族にとって必要であろう．

医師が子どもに説明する際に考慮する要因として重要なものは両親に関することが多く見られ，両親の病気の理解や信頼関係，疾患受容などであった．患児に関連するものとしては，信頼関係，心理状態，理解力などがあげられていた．特に，信頼関係については，患児と両親の両者で重要と捉えられている割合が高かった．医師がこのような告知を行う際には，医師との間に信頼関係が形成されていくことにより，単に医療提供者とケアを受ける側ということ以上の関係が形作られていくことを表していると考えられ，そのような関係性が患児，家族に大きく影響している[10]．難病患児に病名，病態を伝えることは，伝えられ，知る側である患児やその家族だけでなく，伝える側である医師にとっても困難な体験となることがある[11]．そのために，医師が患児や家族に対してうまく説明できないと感じ，不十分な説明で終わってしまうことが生じる可能性が指摘されている[12]．しかし，医師と病気について話し合うことによって，子どもや家族が希望を持てるようになる[13]という場合もあり，医師が患児や家族とどのように関わりを持つのかは重要である．

医師が患児に病名を伝える際には，臨床心理士やソーシャルワーカーなどの他の専門家の支援が有用であると認識されていた．診断を伝えることは希望をなくすことになる，ネガティブな側面には触れないという考えについては意見が分かれており，診断を伝えるということが医師の側にとっても難しい問題となっていることがうかがえた．また，年齢や経験年数による考え方の差異も見られた．概して，年齢が高く臨床経験の長い医師は，病名の告知や説明はより早い時期に行うが，ネガティブな側面には触れないような説明を行うとしていた．病気の説明を行う上で，ネガティブな面には触れざるを得ないが，より早期に病気を患児に伝えていくためにネガティブな側面を強調しすぎない説明を行っていると考えられた．このような傾向は筋ジストロフィーの臨床経験だけでなく，年齢や小児神経疾患の臨床経験でも同様の傾向があったため，筋ジストロフィーの臨床に特有のものではなく，経験の蓄積によるものであると考えられる．その判断は臨床経験の豊富さから行われていると考えられ，臨床経験を積み重ねていくことによって，その判断基準が変化していくことが示唆された．

患児や家族が情緒的な苦痛を体験する場面は，医師にとっても同様に苦痛な場面であるが[12]，医療的，人間的支えという視点から患児や家族を支えているということが明らかとなった．結果からは，各々の医師は，画一的ではなく，患児や家族に配慮した説明を行っていることがうかがわれた．いかに患児や家族に説明していくのかといった問いには唯一の解決はないが，この結果は医師がより適切な説明を行うための情報に資すると考えられる．

本研究の限界として，仮想事例を用いた調査であったことがあげられる．実際場面ではより複雑な因子や条件が関与する中で，患児，家族，医師それぞれの意思決定を行っていると考えられるが，本研究では限られた情報から構成された仮想事例を用いた一時点での調査であった．そのため，臨床場面での患児，家族と医師の間のコミュニケーションについては，十分に捉えられていなかった．本研究は，病気の説明についてのプロセスから，その一場面について調査を行ったものであるが，告知という場面は医師の一方的な態度によって行われるものではなく，患者との相互作用的な関わりの中で行われる．患者との対話を通じて，医師は患者が実際に望んでいる情報の量を把握しやすくなり，知りたくない情報を知らないでいる権利を尊重することにもつながる．そのような視点から考えると，医師側の因子と患者側の因子の両者を踏まえた研究が必要であろう．今後これらの視点を取り入れた研究を行っていくことが望まれる．

本研究に協力いただいた小児神経専門医の先生方に深謝いたします．本研究は，厚生労働省精神・神経疾患研究開発費20委-11「筋ジストロフィーの集学的治療と均てん化に関する研究」班の助成により，日本小児神経学会共同研究支援委員会の支援（共同研究10-3）を受けて実施された．本研究の実施にあたり，貴重なご意見をいただきました国立病院機構刀根山病院の藤村晴俊先生に深謝いたします．分析にご協力いただいた大阪大学大学院人間科学研究科岩田優子氏，結城奈穂氏に感謝いたします．

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Survey for Assessing How Duchenne Muscular Dystrophy is Explained to Children with the Disorder

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Objective: There are many difficulties in disclosing Duchenne muscular dystrophy (DMD) to children with the disorder. The purpose of this study was to assess the explanation of DMD given to affected children by child neurologist.

Methods: The questionnaire was mailed to board-certified child neurologists of the Japanese Society of Child Neurology. The questionnaire consisted of questions on how physicians explained the condition to children with DMD (their patterns of explanation) and their attitude towards the children while explaining the disease.

Results: We received 311 replies. The contents of physicians' explanations were categorized and correspondence analysis revealed "medical support" (explanation about the symptoms, prognosis, medical responses) and "humanistic support" (telling purpose in life, patient group introduction). Parents' understanding of the disease, acceptance, and trust relationships were considered important factors for disease explanation by the physicians. Physicians agreed with the need of clinical psychologist and other psychological professionals when they tell their diagnosis, and agreed with telling the diagnosis to a DMD child reached a certain age.

Conclusions: It was revealed that physicians' explanation were largely categorized into two groups, and the important factors for disease explanation and physicians' attitudes towards disclosure of the diagnosis. This information will help in explaining the disease to children with DMD.

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Allele-specific PCR for a cost-effective & time-efficient diagnostic screening of spinal muscular atrophy

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***Background & objectives*: Genetic diagnosis of spinal muscular atrophy (SMA) is complicated by the presence of *SMN2* gene as majority of SMA patients show absence or deletion of *SMN1* gene. PCR may amplify both the genes non selectively in presence of high amount of DNA. We evaluated whether allele-specific PCR for diagnostic screening of SMA is reliable in the presence of high amount of genomic DNA, which is commonly used when performing diagnostic screening using restriction enzymes.**

***Methods*: A total of 126 blood DNA samples were tested in amounts ranging 80-200 ng, referred for the genetic diagnosis of SMA using both conventional PCR-RFLP and allele-specific PCR.**

***Results*: The results from both methods showed agreement. Further, allele-specific PCR was found to be a time-efficient and cost-effective method.**

***Interpretation & conclusions*: Our study demonstrated the accuracy of our allele-specific PCR and the results were comparable compatible with that of PCR-RFLP, indicating its practical application in SMA diagnostic screening.**

Key words Allele-specific PCR - PCR-RFLP - SMA diagnostic screening - *SMN1* - spinal muscular atrophy

Conventional PCR-RFLP for genetic diagnosis of spinal muscular atrophy (SMA)[1] has been considered time consuming and expensive. It requires restriction enzyme (RE) digestion which uses a consi derably high amount of PCR product. If the amount of the PCR product is higher than necessary, this may lead to partial RE digestion resulting in the appearance of undigested PCR product on gel electrophoresis (*i.e.* false-negative results).

The responsible genes for SMA are *Survival Motor Neuron* (*SMN*) genes. The *SMN* genes consist of two highly identical genes; *SMN1* (telomeric *SMN*) and *SMN2* (centromeric *SMN*) which share over 99.8 per cent sequence homology over a 30 kb segment. *SMN1* and *SMN2* can be distinguished by only five nucleotides differences located in intron 6, exon 7, intron 7 and exon 8[2]. Ninety five per cent of SMA patients showed

an absence of *SMN1* gene due to either deletion or conversion, thus demonstrating that *SMN1*, not *SMN2*, is the SMA-causing gene[3]. Thus, genetic diagnosis of SMA (*i.e.* detection of *SMN1* deletion) was complicated by the presence of *SMN2* because PCR may amplify the genes unselectively, especially in the presence of high amount of genomic DNA, while all patients carry *SMN2* gene. Allele-specific PCR for the genetic diagnosis of SMA has been described elsewhere from as early as 1999[4-9]. However, to the best of our knowledge studies involving high amount of DNA, the amount of which is routinely used for genetic diagnosis, provided conflicting results regarding chances of *SMN2* mis-amplification. In addition, no study has been done to evaluate the cost-effectiveness and time-efficiency of this method over conventional PCR-RFLP. Using relatively larger sample size, we studied the reliability of allele-specific PCR by comparing the test results against conventional PCR-RFLP using high amount of genomic DNA.

Material & Methods

This study was carried out from 2003 to 2008 in the Department of Paediatric, School of Medical Sciences, Universiti Sains Malaysia, Kelantan. A total of 126 patients were randomly selected from the patients sent for SMA genetic diagnosis (Table I). Whole blood (3-5 ml) was collected from patients. Informed consent was taken prior to blood taking. The study protocol was approved by the Research Ethics Committee (Human) of the Universiti Sains Malaysia. Sample size was calculated using single proportion formula. Genomic DNA was extracted using commercially available kit (GeneAll Biotechnology Co. Ltd., Korea).

PCR-RFLP (Method A): All samples were analyzed twice, each using two different methods, method A and method B. Method A refers to the PCR-RFLP as previously described[1]. This method consisted of two steps, PCR amplification and enzyme digestion which used *DraI* restriction enzyme for exon 7 *SMN*.

Allele-specific PCR (Method B): Method B refers to allele-specific PCR using primer pairs described previously[4], telSMNex7forw 5'-TTTATTTTCCTTACAGGGTTT*C*-3' and telSMNint7rev 5'-GTGAAAGTATGTTTCTTCCAC*gT*A-3'. Italic uppercase characters indicate the position of nucleotide difference between *SMN1* and *SMN2*, while lowercase character indicates the position of a deliberate mismatch. The primers specifically amplify exon 7 of *SMN1*, not *SMN2*. This method consisted of only one PCR amplification step. Positive or negative interpretation was determined visually on agarose gel electrophoresis by the absence or presence of the *SMN1* exon 7, respectively. To monitor the efficiency of PCR amplification, a housekeeping gene (β-globin) was used as a reference gene, with the primers 5-ACCTCACCCTGTGGAGCCAC-3 and 5-CTCACCACCAACTTCATCCAAG-3. One

Table I. Clinical features of the patients

Clinical features			Number of patients	Total
Clinically SMA	Sex	Male	25	
		Female	38	63
	Age on diagnosis (Month)	0-6	11	
		6-18	13	
		>18	39	63
	Type of SMA	I	31	
		II	23	
		III	9	63
	Tongue Fasciculation	+ve	35	
		-ve	20	55*
	EMG	+ve	20	
		-ve	1	21*
Clinically Not SMA	Sex	Male	30	
		Female	33	63
	Age on diagnosis (Month)	0-6	20	
		6-18	19	
		>18	22	61*

EMG; electromyography, *Data not available for some patients

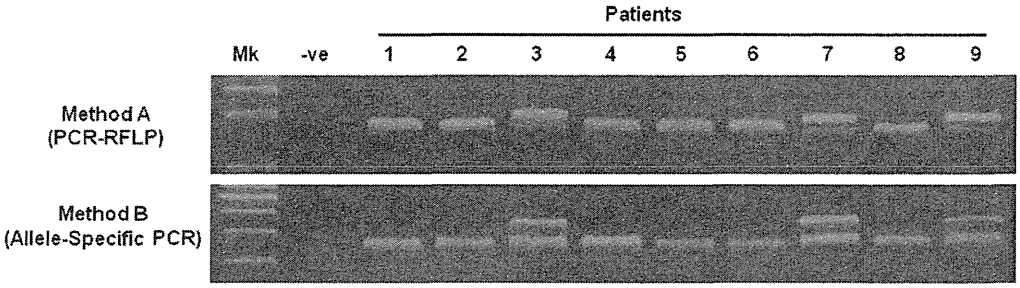

Fig. *SMN1* (exon 7) deletion analysis using method (A) and (B). In method (A), deletion or non-deletion is indicated by the absence or presence of the first band (188 bp). The second band (164 bp) shows the presence of *SMN2* (exon 7). In method (B), deletion or non-deletion is indicated by the absence or presence of the first band (307 bp). The second band (240 bp) indicated the presence of reference gene (β-globin). Molecular markers were electrophoresed in the "Mk" lane and a control PCR product in the "–ve" lane. Patients 1, 2, 4, 5, 6, 8 show deletion of *SMN1*. Patients 3, 7, 9 show non-deletion of *SMN1*.

reaction of 20 µl of PCR mixture contained 80 - 200 ng of genomic DNA, 0.4 µl of 10 mM dNTPs, 1.2 µl of 25 mM $MgCl_2$, 4.0 µl of 5x PCR buffer, 0.75U *Taq* DNA polymerase (Promega Corporation, Madison, USA) and 1.5 µl of each 10 pmol of an allele specific primer pairs and 0.5 µl of each 5 pmol of an internal control primer pairs (Sigma-Proligo, The Woodlands, TX, USA). The PCR cycles included an initial denaturation at 94°C for 7 min, followed by 33 cycles of 94°C for 1 min, 59.7°C for 1 min, 72°C 1 min before a final extension at 72°C for 7 min. The PCR product was then directly visualized under the UV light, using 2 per cent agarose gel. PCR product of *SMN1* was visualized at the corresponding size of 307 bp, while that of β-globin was at the size of 240 bp.

Cost and time evaluation: The cost and time for both methods were compared to evaluate the cost-effectiveness and time-efficiency of allele-specific PCR over PCR-RFLP.

Results & Discussion

Of the 126 samples tested, 54 (43%) were found to have *SMN1* exon 7 deletions. The findings (Fig.) from both methods were in complete agreement, suggesting that both methods performed with the same reliability. Evaluation of time-efficiency for both methods showed that method B was five times more rapid than method A. Evaluation on the cost-effectiveness showed that method B was more cost-effective (68%) compared to method A (Table II).

Table II. Comparison of time-efficiency and cost-effectiveness between PCR-RFLP and allele-specific PCR in SMA genetic diagnosis

Items	PCR-RFLP (A) (min)		Allele-specific PCR (B) (min)	
A. Time-efficiency comparison				
1. PCR mixture preparation	30		30	
2. PCR thermocycling	150		150	
3. Gel electrophoresis I	40		40	
4. PCR-RFLP preparation	10		-	
5. Incubation	960		-	
6. Gel electrophoresis II	40		-	
Total	1230		220	
B. Cost-effectiveness comparison	PCR-RFLP		Allele-specific PCR	
	(INR)	(US$)	(INR)	(US$)
1. PCR reagents	949	16.9	949	16.9
2. Restriction enzyme	2336	41.6	-	-
3. Gel electrophoresis	190	3.4	95	1.7
4. Consumables	219	3.9	95	1.7
Total	3694	65.8	1139	20.3

*based on US$ 1 = INR 56.15

Table III. Summary of previous studis on diagnosis of SMA using allele specific PCR

Author	Primers	Technique	Outcome
Feldkötter *et al*[4]	Forward (c.840C>T) Reverse (c.888+214A>G)	Quantitative real-time PCR of *SMN1* using LightCycler instrument (Roche Diagnostics)	*SMN1*-specific amplification from 11.25ng of genomic DNA.
Xu *et al*[8]	Specific reverse primer (c.840C>T)	PCR	Lower analytical sensitivity/specificity, especially with lower (≤ 0.1 pg) and higher (≥ 1 pg) DNA amount.
Moutou *et al*[5]	Specific reverse primer (c.840C>T)	PCR with fluorescent technology	*SMN1*-specific amplification from up to 1ng of genomic DNA.
Zeng *et al*[9]	Specific reverse primer (c.840C>T)	PCR	*SMN1*-specific amplification from 50ng of genomic DNA.
Ravard-Goulvestre *et al*[6]	Specific reverse primer (c.840C>T)	PCR with fluorescent technology	*SMN1*-specific amplification from up to 700ng of genomic DNA.
Simsek *et al*[7]	Specific forward primer (c.840C>T)	PCR	Successfully amplified *SMN1* by using 100ng of DNA amount and the results were comparable with the PCR-RFLP.

We have been using the primers described by Feldkötter *et al*[4] for gene-specific copy number analysis of *SMN1* and *SMN2* as a diagnostic procedure in our laboratory and found that our results were consistent[10,11]. However, the experiments were done with relatively low amount of DNA, by which the chance for *SMN2* mis-amplification was very small.

In this study, 54 samples showed deletion in *SMN1* gene exon 7. Among the remaining 72 patients without *SMN1* deletion, nine were categorized as clinically SMA (Table I). Patients without apparent deletion of *SMN1* may not be excluded from the SMA diagnosis since possibility remains that they might carry point mutation which can only be identified through DNA sequencing.

Our analyses showed that allele-specific PCR was a time-efficient and cost-effective method. It may also reduce the risk for experimental errors since it involves fewer steps.

The main differences of the method described here compared to other similar methods is the design of the primers, the usage of conventional PCR method and the usage of a relatively higher amount of genomic DNA than the amount which is routinely used for SMA genetic diagnosis. Using primer pairs described by Feldkotter *et al*[4]. We could specifically amplify *SMN1* in the existence of relatively high DNA amount (80 - 200ng), without mis-amplifying *SMN2*, thus enabling the use of the method for routine genetic testing of SMA. However, the use of higher amount of DNA in this study has not provided evidence if the test is still reliable in the presence of much lower DNA amount as described elsewhere[5,8]. This could be a major hurdle for applications such as preimplantation genetic diagnosis (PGD).

The primers were selected because these fulfilled the criteria for highly-efficient allele-specific amplification for utilizing two nucleotides differences between *SMN1* and *SMN2* in exon 7 (c.840C>T) and intron 7 (c.888+214A>G) and a deliberate primer mismatch simultaneously in one PCR reaction. *SMN1* carries the C and A in its exon 7 and intron 7, respectively. The forward primer (telSMNex7forw) incorporated the C at its first nucleotide at the 3' end, while the reverse (telSMNint7rev) combined an incorporation of A at its 2nd nucleotide before the 3' end and a deliberate mismatch at its 3rd nucleotide before the 3'end (G instead of A). Newton *et al*[12] showed that a deliberate mismatch near to the primer's 3'end increased its amplification specificity. Therefore, the primer pair described by Feldkotter *et al*[4] contained three characteristics, a specific forward, a specific reverse and a deliberate mismatch.

Similarly, specific primers may be designed to detect the presence or absence of *SMN1* exon 8. However, we concentrated only on exon 7 of the *SMN1* gene in this study because it is the only region with the clinical significance for the diagnostic screening of SMA[13]. The summary of the previous studies were shown in Table III.

In conclusion, our study demonstrated the reliability of allele-specific PCR for diagnostic screening of SMA. The accuracy of this method was comparable with that of PCR-RFLP, and it was cost-

effective. Thus, it can be applied to routine diagnostic screening of SMA.

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ORIGINAL ARTICLES

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Spinal Muscular Atrophy Patient Detection and Carrier Screening Using Dried Blood Spots on Filter Paper

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Aim: Spinal muscular atrophy (SMA) is a common autosomal recessive neuromuscular disorder. It is caused by mutations in the *SMN1*, and its clinical severity is modified by copy number variations of the *SMN2*. According to previous studies, deletion of *SMN1* exon 7 is the most frequently observed in patients with SMA. Therefore, molecular analyses exploiting this genetic lesion could be beneficial in the diagnosis of SMA. Unfortunately, in many geographical regions, physicians do not have the latest molecular screening technologies at their immediate disposal. Thus, to overcome this issue, we developed an SMA-diagnosing system using dried blood spots (DBS) placed on filter paper to facilitate remote diagnosis. *Methods:* In this study, we validate the applicability of DBS on Flinders Technology Associates (FTA) filter paper for detecting *SMN1* exon 7 deletions and copy number variations of *SMN1* and *SMN2*. To detect exon 7 deletions in *SMN1*, polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis was conducted by using DNA extracted from the DBS on FTA filter paper that had been stored at room temperature for a period of up to 4 years. To determine the copy numbers of *SMN1* and *SMN2*, we carried out SYBR green-based real-time PCR by using the same blood specimens. *Results:* The results obtained from the DBS on FTA filter paper were in complete concordance with those analyses using fresh blood specimens. This indicates that DBS on filter papers is a reliable method for SMA patient detection and carrier screenings. *Conclusion:* The SMA-diagnosing system, combined with the mailing of DBS on filter paper, will be beneficial for patients suffering from neuromuscular disorders in areas with limited or no access to diagnostic facilities with molecular capabilities.

Introduction

SPINAL MUSCULAR ATROPHY (SMA) is an autosomal recessive disease characterized by degeneration of motor neurons in the spinal cord, resulting in atrophy of skeletal muscles. SMA is clinically divided into four subtypes based on age at onset and the type of motor function achieved: SMA type I (Werdnig-Hoffman disease, severe type), SMA type II (intermediate type), SMA type III (Kugelberg-Welander disease, mild type), and SMA type IV (adult onset type or very mild type) (Lunn and Wang, 2008).

The gene responsible for SMA is the survival motor neuron (*SMN*) gene. This gene has two nearly identical copies, *SMN1* and *SMN2* (Lefebvre *et al.*, 1995). It has been shown that SMA is caused by a homozygous disruption of *SMN1*, and further, the clinical severity of the disease is modified by copy number variations of *SMN2* (Feldkötter *et al.*, 2002). To a limited degree, the presence of *SMN2* copies can compensate for the deletion of *SMN1*, and it has been shown that in the absence of *SMN1*, increased copy numbers of *SMN2* can improve the clinical severity of the disease (Harada *et al.*, 2002). Additionally, the presence of the neuronal apoptosis inhibitory protein (*NAIP*) gene is also associated with the clinical severity (Glotov *et al.*, 2001).

Most cases of SMA present with the complete absence of *SMN1* (Parsons *et al.*, 1998). Patients with an SMA-like presentation should be tested for the presence of a homozygous deletion of *SMN1* by using methods that are 98.4%–99% sensitive and 98.3%–100% specific (Beck *et al.*, 2001; Mailman *et al.*, 2002). Therefore, the detection of *SMN1* deletions is highly beneficial in diagnosing SMA. Additionally, copy number analysis of *SMN1* may also be valuable for carrier testing, because all carriers have only 1 copy of *SMN1*.

When discussing the topic of molecular testing, it is essential to note that the means to conduct molecular analysis is not universally available in all geographical regions of the world.

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With this in mind, if a simple method of collection, storage, and shipment of samples could be established, then all patients can gain access to the benefits of molecular analysis regardless of geographical or industrial limitations. This is true not only for SMA but also for other inherited disorders as well (Aggarwal *et al.*, 1992). In this study, we proposed a novel SMA-diagnosing system using dried blood spot (DBS) on filter paper, which effectively facilitates the simplified collection, storage, and shipment of patient samples to centralized testing facilities.

Patients and Methods

Patients

ID-1 (woman, SMA type 1). She was referred to Kobe University Hospital for the evaluation of muscle weakness at 1 month old. She did not obtain head control or sit by 3 years.

ID-2 (woman, SMA type II). She was referred to Kobe University Hospital for the evaluation of muscle weakness at 6 months old. She obtained head control at 4 months old, but she could not sit without aid. She underwent tracheostomy at 2 years of age.

ID-3 (woman, SMA type III). She was referred to Kobe University Hospital for the evaluation of muscle weakness affecting dominantly lower limbs at 12 years old. Her gait disturbance had been noticed since she was 3 years old.

ID-4 (woman, SMA type I). She was referred to Kobe University Hospital for the evaluation of muscle weakness at 1 year 5 months old. She did not obtain head control or sit without aid.

ID-5 (man, SMA type III). He was referred to Kobe University Hospital for the evaluation of muscle weakness affecting dominantly lower limbs at 5 years of age.

Before the molecular analysis, informed consent was obtained from the parents of the patients. This study was approved by the Ethical Committee in Kobe University.

Extraction of DNA from Flinders Technology Associate elute microcard™

For the collection of blood samples from SMA suspicious patients, we adopted the use of FTA® Elute Cards (Whatman® Inc, Schleicher & Schuell, Clifton, NJ, art no. WB 120410). Storage periods of the Flinders Technology Associate (FTA) Elute Cards varied from 1 to 4 years. All FTA Elute Cards were stored at room temperature. To extract DNA from the DBS, 5 circles were punched out from the center of a blood spot by using a
F1 ▶ Φ3 mm hole puncher, as shown in Figure 1. The punched circles were put into a sterile 1.5 mL microfuge tube and washed by adding 500 µL of sterile water and 3 cycles of pulse vortexing. Excess water used for washing was removed from the tube by using a sterile pipette. After this step, the circles obtained were further centrifuged for 5 s, and excess water was removed again by pipetting. After washing, these punched circles were completely immersed in 100 µL of TE buffer, used as an eluant followed by heating at 95°C for 30 min. At the end of the incubation period, the tubes were pulse vortexed ~60 times. The eluant was stored at −20°C until being used as a DNA source for further examination (Whatman, Inc., Clifton, NJ).

Deletion test

Polymerase chain reaction (PCR) was performed according to the methods described by van der Steege *et al.* (1995). For each reaction, 300–500 ng of DNA was used. The oligonucleotide primers for exon 7 in the *SMN1* and *SMN2* were R111 (Lefebvre *et al.*, 1995) and ×7-Dra (van der Steege *et al.*, 1995), and those applied for use on exon 8 of both genes were 541C950 (Lefebvre *et al.*, 1995) and 541C1120 (Lefebvre *et al.*, 1995). To discriminate between *SMN1* and *SMN2* products, PCR amplicons were completely digested with *Dra*I (Takara Biomedical, Tokyo, Japan) for exon 7 and *Dde*I (Takara Biomedicals) for exon 8. The restriction enzymes *Dra*I and *Dde*I only cleave the amplified fragments from *SMN2* exon 7 and 8, respectively. PCR amplification of the *NAIP*-specific sequence, exon 5, was performed according to the method reported by Roy *et al.* (1995).

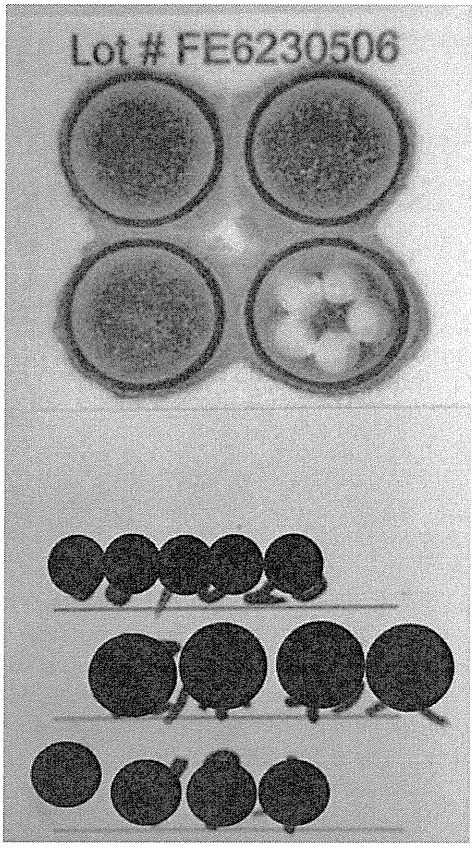

FIG. 1. Image of FTA Elute microcard™ after being punched. On filter paper, five holes in the center of one well can be observed. To avoid cross contamination between samples, a micropunch was carried out thrice on a clean piece of filter paper followed by wiping of the holepuncher with 95% ethanol. This was done between all FTA samples. FTA, Flinders Technology Associates.

Sequencing analysis

To identify and confirm the presence of hybrid genes, direct sequencing analysis was performed by using a BigDye Terminator V3.0 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and a genetic analyzer (ABI Prism 310; Applied Biosystems), with DNA Sequencing Analysis Software (Applied Biosystems). PCR amplicons were used as templates along with the forward primer of intron 6 and reverse primer of exon 8 for sequencing reactions.

Copy number analysis

For determination of *SMN1* and *SMN2* copy numbers, we used a quantification method utilizing real-time PCR and a LightCycler instrument (Roche Diagnostics, Mannheim, Germany). *SMN1* exon 7 was amplified with a primer set of telSMNex7forw (5′-TTT ATT TTC CTT ACA GGG TTT C-3′) (Feldkötter *et al.*, 2002) and telSMNint7rev (5′-GTG AAA GTA TGT TTC TTC CAC GTA-3′) (Feldkötter *et al.*, 2002). *SMN2* exon 7 was amplified with a primer set of cenSMNex7forw (5′-TTT ATT TTC CTT ACA GGG TTT TA-3′) (Feldkötter *et al.*, 2002) and cenSMNint7rev (5′-GTG AAA GTA TGT TTC TTC CAC GCA-3′) (Feldkötter *et al.*, 2002). The *CFTR* was used as a reference gene for the relative quantification of *SMN1* and *SMN2*. *CFTR* was amplified with a primer set of CF621F (5′-AGT CAC CAA AGC AGT ACA GC-3′) (McAndrew *et al.*, 1997) and CF621R (5′-GGG CCT GTG CAA GGA ATG TTA-3′) (McAndrew *et al.*, 1997). The details of the experimental procedure we applied have been described elsewhere (Tran *et al.*, 2008).

Statistical analysis

To evaluate the performance of the FTA Elute Microcard method described in this study, sensitivity and specificity were calculated by using standard statistical analyses.

Results

DNA concentration or recovery of DNA from the DBS on FTA elute microcard

To assess the performance of FTA Elute microcards (Whatman Inc, Schleicher & Schuell, art no. WB 120410), we selected cards based on their storage time (Table 1). The storage time of the individual cards varied from 1 to 4 years.

TABLE 1. DNA CONCENTRATION FROM DRIED BLOOD SPOT ON FLINDERS TECHNOLOGY ASSOCIATES ELUTE MICROCARD™

Age of dried blood on FTA elute Microcard™	*Personal ID*	*DNA concentration (mg/L TE Buffer)*	*OD 260: OD280*
1 year	16	255.9	1.68
	17	151.4	1.75
	9	200.1	1.62
	19	244.8	1.61
2 year	3	66.5	1.76
	8	73.6	1.86
	5	215.3	1.67
	6	321.3	1.61
	7	239.9	1.67
	4	270.5	1.57
	12	180.2	1.64
	13	223.6	1.66
3 year	1	143.8	1.56
	14	314.8	1.61
	10	180.4	1.61
	11	200.7	1.67
	18	226.4	1.75
	20	268.0	1.77
4 year	2	187.6	1.81
	15	123.2	1.62

Sample was prepared from five punched circles that had been (Φ3 mm) incubated in 100 μL of TE buffer.

Concentration of DNA extracted from the cards ranged between 66.5 and 321.3 mg/L (mean 204.4 mg/L). DNA samples had OD 260: OD 280 values of 1.56 and 1.86, respectively. The concentration and purity of the isolated DNA was sufficient for subsequent PCR analysis.

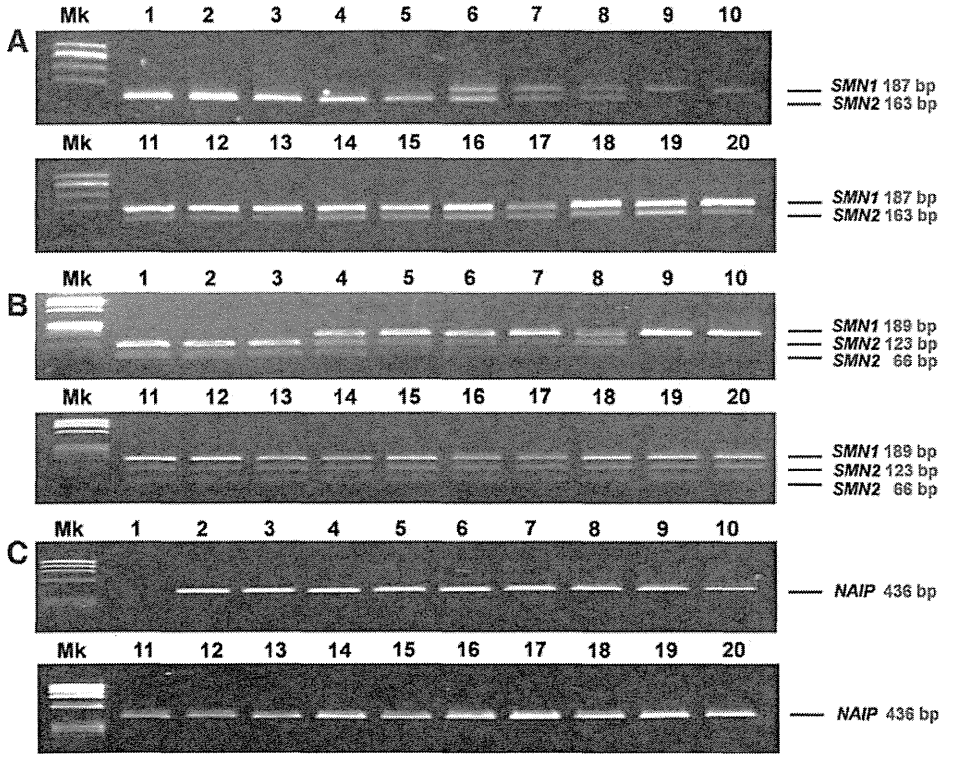

FIG. 2. **(A)** *SMN1* exon 7 deletion test. About 5/20 samples showed a homozygous deletion of *SMN1* exon 7. About 2/20 samples showed a homozygous deletion of *SMN2* exon 7. **(B)** *SMN1* exon 8 deletion test. About 3/20 samples showed a homozygous deletion of *SMN1* exon 8. 2/20 samples showed a hybrid *SMN* gene. Another 2/20 samples showed a homozygous deletion of *SMN2* exon 8. **(C)** *NAIP* exon 5 deletion test. About 1/12 samples showed a homozygous deletion *NAIP* exon 5. Lane numbers in this figure are identical to personal ID of the samples in Tables 1 and 2.

TABLE 2. SURVIVAL MOTOR NEURON-DELETION TEST AND *SMN1* AND *SMN2* COPY NUMBER ANALYSIS USING DRIED BLOOD ON FLINDERS TECHNOLOGY ASSOCIATES ELUTE MICROCARD

			Deletion test					*Copy number* SMN1				*Copy number* SMN2			
			SMN *Exon 7*		SMN *Exon 8*			*Freshly prepared sample*		*Card DNA*		*Freshly prepared sample*		*Card DNA*	
	Personal ID	*Clinical status*	SMN1	SMN2	SMN1	SMN2	*NAIP Exon 5*	*CNR*	*Copy number*	*CNR*	*Copy number*	*CNR*	*Copy number*	*CNR*	*Copy number*
Patient	1	SMA type I	Del	Nondel	Del	Nondel	Del	–	0	–	0	1.96	2	1.91	2
	2	SMA type II	Del	Nondel	Del	Nondel	Nondel	–	0	–	0	3.00	3	2.96	3
	3	SMA type III	Del	Nondel	Del	Nondel	Nondel	–	0	–	0	3.32	3	3.25	3
	4	SMA type I	Del	Nondel	Nondel	Nondel	Nondel	–	0	–	0	2.67	3	2.87	3
	5	SMA type III	Del	Nondel	Nondel	Nondel	Nondel	–	0	–	0	3.65	4	3.72	4
Carrier	6	Father of Patient no 5	Nondel	Nondel	Nondel	Nondel	Nondel	0.95	1	0.93	1	1.75	2	2.38	2
	7	Mother of Patient no 5	Nondel	Nondel	Nondel	Nondel	Nondel	1.05	1	0.66	1	2.65	3	2.58	3
	8	Mother of Patient no 3	Nondel	Nondel	Nondel	Nondel	Nondel	0.95	1	1.00	1	2.97	3	3.13	3
Control	9	Healthy	Nondel	Del	Nondel	Del	Nondel	1.99	2	1.91	2	–	0	–	0
	10	Healthy	Nondel	Del	Nondel	Del	Nondel	1.96	2	1.91	2	–	0	–	0
	11	Healthy	Nondel	Nondel	Nondel	Nondel	Nondel	1.84	2	1.55	2	0.93	1	1.07	1
	12	Healthy	Nondel	Nondel	Nondel	Nondel	Nondel	2.30	2	2.30	2	1.00	1	1.14	1
	13	Healthy	Nondel	Nondel	Nondel	Nondel	Nondel	2.20	2	2.32	2	1.15	1	1.20	1
	14	Healthy	Nondel	Nondel	Nondel	Nondel	Nondel	1.87	2	2.23	2	1.80	2	2.20	2
	15	Healthy	Nondel	Nondel	Nondel	Nondel	Nondel	1.88	2	1.90	2	1.97	2	1.74	2
	16	Healthy	Nondel	Nondel	Nondel	Nondel	Nondel	1.97	2	2.04	2	2.05	2	2.16	2
	17	Healthy	Nondel	Nondel	Nondel	Nondel	Nondel	1.95	2	1.90	2	2.00	2	2.19	2
	18	Healthy	Nondel	Nondel	Nondel	Nondel	Nondel	2.28	2	2.22	2	2.22	2	1.52	2
	19	Healthy	Nondel	Nondel	Nondel	Nondel	Nondel	2.02	2	1.96	2	2.84	3	3.19	3
	20	Healthy	Nondel	Nondel	Nondel	Nondel	Nondel	2.96	3	2.71	3	1.91	2	2.26	2

These data obtained from analyses using DNA extracted from DBS on the FTA Elute microcards were compatible with those obtained from analyses using DNA prepared from EDTA-blood. (The data of *SMN*-deletion test using DNA prepared from EDTA-blood not shown).

[a]The deletion test using card blood was completely compatible with the freshly prepared sample.

DBS, dried blood spot; FTA, Flinders Technology Associates; CNR, calibrator-normalized ratio; del, deletion; nondel, nondeletion.

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