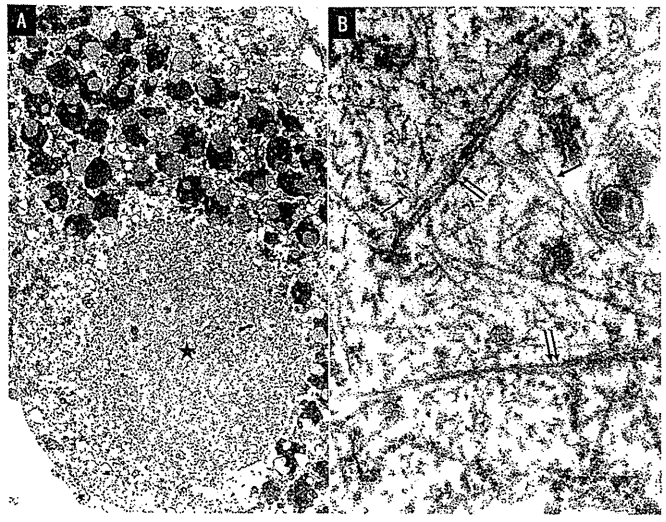

図 6
A）ALS 前角細胞円形封入体(★)の電子顕微鏡像．B）拡大像．神経細糸様の細い線維(→)と異常な太い線維(二重矢)とが混在している．A 2,000 倍，B 10,000 倍．

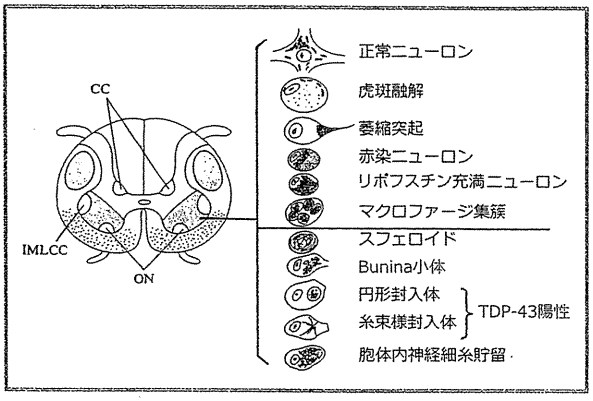

図 8　ALS 脊髄病変と LMN の細胞病理

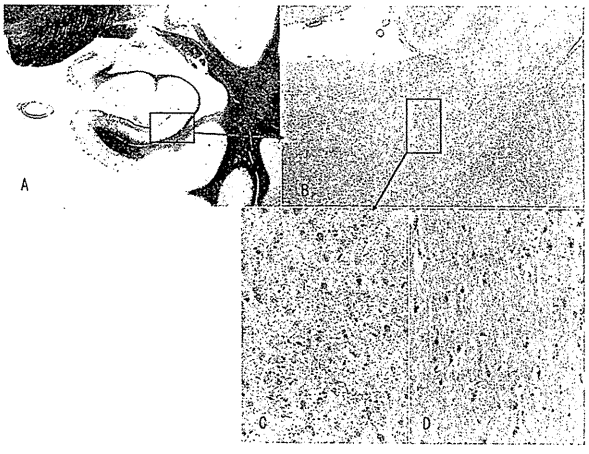

図 9　認知症を伴う ALS(ALSD)の海馬 CA1-支脚移行部変性
A）海馬足の anatomy．B）ALSD の CA1-支脚移行部．HE 染色，5 倍．C）B の拡大図．錐体細胞消失とアストロサイトーシスが著明．HE 染色，50 倍．D）対照．HE 染色，50 倍．

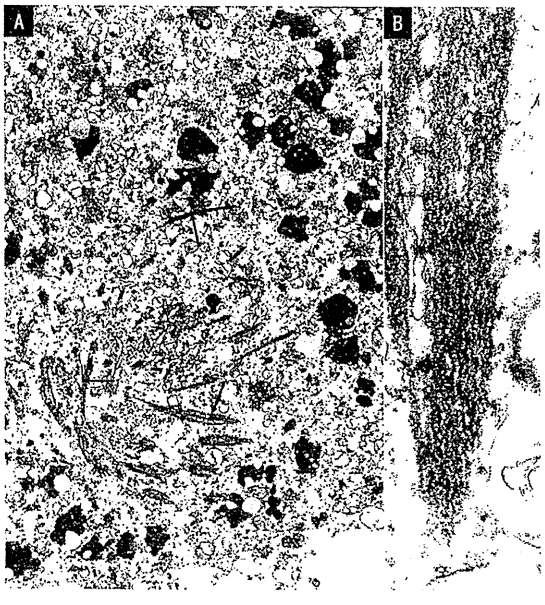

図 7　ALS 前角細胞の糸束様封入体の電子顕微鏡像
A）線維状構造(→)が集簇傾向を示している．B）拡大像．異常な太い線維の束であり，集簇傾向を呈している．A 2,000 倍，B 10,000 倍

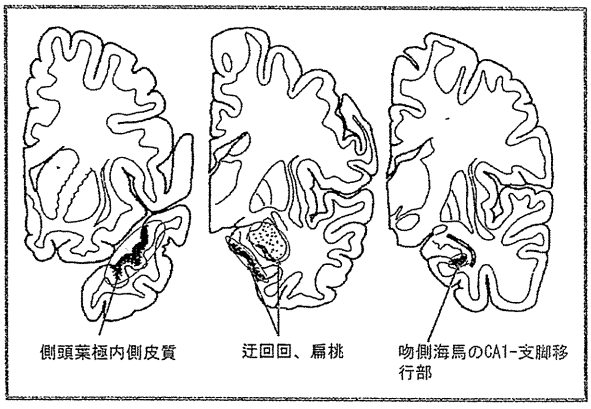

図 10　認知症を伴う ALS の側頭葉病変
左）側頭葉極内側，中）迂回回と扁桃核，右）海馬足の CA1-支脚移行部

ない症候のように思われる．今後，sALS の病変が初発部位(例えば手)から途中を飛び越えて遠隔部位(例えば足)に出現するか否かという目で観察していく必要がある．

sALS の中に特有の認知症(前頭側頭型認知症)を呈する症例(認知症を伴う ALS：ALSD)が知られていた．ALSD の最大の特徴は病識の欠如である．これは単に精神症状のみでなく，身体症状に対しても当てはまる．呼吸不全に陥って肩呼吸をしているにも拘わらず，呼吸苦を訴えず，歩ける場合は病棟を徘徊する．sALS と前頭側頭葉変性症の ubiquitin 化封入体の構成蛋白がいずれも TDP-43 であることが見出されて，両者は TDP-43 proteinopathy というカテゴリーの中に含まれるようになった．ただし，これはあくまで蓄積蛋白を切り口としてのまとめであり，TDP-43 がどの程度まで sALS の病態(運動ニューロン死)に関わっているかという点の解明は今後の課題である．

神経病理

sALS の症候と最も緊密に関係する病理は，UMN と LMN の進行性かつ選択的変性脱落であることから，ここでは sALS(Charcot 病)の，主として運動ニューロン病理に限定する．

1．肉眼所見

sALS では大脳の萎縮はなく，中心前回の萎縮も通常はみられず，大脳の前額断面でも中心溝の内側に位置する中心前回皮質にも萎縮や変色はみられない．ただし，中心前回の変性が高度の場合にはその萎縮と，前額断面での中心前回皮質の萎縮と変色が観察され，錐体路の変性も認められる．

有髄線維の束である根が白いのは髄鞘が白色であるからである．軸索が消失すると髄鞘も崩壊してその白さが失われる．ALS では運動ニューロンの消失により軸索が変性し，前根の髄鞘が崩壊して，白さが失われる．これは運動性有髄線維を含むいずれの根でも生じるはずであるが，経験的には頸髄前根で最も明瞭であり，腰髄では判定が難しい(図 1)．

2．脊髄のセミマクロ所見

図 2 は ALS と正常対照の頸髄の Klüver-Barrera 染色を併置したものである．この ALS 例では経過が短いため，前角の背腹方向への萎縮は目立たないが，錐体側索路・前索路(★)はともに明らかに淡明になっている．また，錐体路以外の前索・側索も対照よりは淡明化しており，正常の色調を保持しているのは後索のみである．ALS の多くの脊髄では前角が前後方向に萎縮して，その前縁が扁平になり，外側の角が先鋭になる．前角の萎縮は，主として前角 LMN の脱落につれてその樹状突起が消失することによると考えられる．

興味深いのは，後脊髄小脳路は正常でも錐体側索路より淡明に見えることである(図 2 矢印)．これはこの線維路がほぼ大径有髄線維から構成されていることに由来し，染色のトリックで淡く見えるのである(図 3)．この機序は内包後脚における皮質脊髄路の染色性に関しても当てはまる[11]．

3．組織所見

ALS の細胞病理像は，① 運動ニューロンが死に行く過程と，② 封入体の出現とに分けられる．

A．LMN 病変

1）LMN 消失への過程

前角の残存ニューロンは“正常 LMN”像のほかに，Nissl 小体中心崩壊，細胞体と樹状突起の萎縮，核の偏在，萎縮した細胞質の赤染，リポフスチンによる細胞体占拠，核萎縮と濃縮など，種々の細胞病理を示す(図 4)．

Golgi 装置抗体(MG-160 抗体)免疫染色では，残存ニューロンの多くが Golgi 装置の断片化を呈している[12]．ALS における運動ニューロン死はアポトーシスによるとの考えが提唱されたが，異論もある[13,14]．Yamazaki らの観察は，ALS の LMN では DNA の断片化は神経細胞変性の最末期に生じ，アポトーシスとは異なるメカニズムの細胞死が生じている可能性を示している[14]．一般に，ALS，Parkinson 病，Alzheimer 病などの神経変性疾患ではアポトーシスの組織学的所見と考えられているアポトーシス小体は認められない．

2）LMN の異常構造物

a）封入体

残存ニューロンには Bunina 小体(図 5)と TDP-43 陽性封入体が出現する．

Bunina小体は好酸性を示す微少な細胞質内封入体(図5A, B)で，ALSに特異的な重要な封入体である．その構成蛋白や由来は不明であるが，cystatin C，あるいはtransferrinに対する抗体で陽性に染まる．電子顕微鏡的には，限界膜を持たず，電子密度の高い顆粒が密に集簇した構造である(図5C)．周辺には壊れた膜構造が付着している．内部にしばしば空隙(封入体外の細胞質が陥入あるいはトラップされたものである可能性がある)を有し，神経細糸を含んでいることも希でない．

TDP-43陽性封入体には，円形をしたもの(円形封入体)と線条に見える封入体(糸束様封入体)とがある．円形封入体はHE染色では文字通り丸く見えるが，多くは内部が不規則に網目状に見えて周辺は空胞で囲まれている．その超微形態は異常な太い線維と神経細糸様の細い線維とが混合している構造である(図6)．糸束様封入体は，円形封入体内の異常に太い線維のみが束になった構造であり，限界膜はない．糸束様封入体が集簇(図7)して円形封入体に移行している像が時に認められる．糸束様封入体はしばしば二重膜を有する小胞で囲繞されており，ユビキチン化の後にリソゾーム系で処理されることが推測される[15]．

b）スフェロイド

スフェロイドはLMN軸索近位部に神経細糸が貯留して類球形に腫大した構造である．早期に死亡した症例で多くみられる．神経細糸は遅い軸索流に関連していることから，ALSの運動ニューロン死には軸索流の障害が関係しているとの説もある．

B．UMN

その病理像はLMNほど精緻には観察されていない．錐体路の淡明化(軸索脱落)がしばしば認められる．軸索脱落が軽微な場合は淡明化が捉えにくいので，鍍銀軸索染色で確認する必要がある．

中心前回では，Betz細胞の変性・萎縮とその消失跡へのマクロファージの集簇が認められる．

図8に脊髄におけるALS病理のまとめを示す．

C．ALSDの非運動ニューロン病変

大脳では基本的には前頭側頭葉変性を呈するが，その葉性萎縮の程度は様々である．組織学的には，皮質表層の多少曖昧な海綿状変化を呈し，脳の広範な領域のニューロンとグリアにTDP-43陽性封入体が出現する[16]．その他に，初期病変と思われる変化が側頭葉内側面に出現し，中でも側頭葉極内側面皮質の変性と海馬吻側のCA1-支脚移行部の限局性神経細胞脱落が特徴である[17](図9, 10)．黒質にもLewy小体の出現を伴わない明らかな変性がみられる．

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of laxity on outcome measures such as grip strength measured by myometry in the normal population (children from 4 years to adults). Our results suggest that children and adults with upper limb joint laxity have reduced power in grip when compared to values for normal. In children with neuromuscular disorders, the problems of joint laxity, when combined with weakness, can prevent or delay functional activities including propping, getting to sitting, crawling and pulling to stand. It can delay walking, cause tip-toeing, knee pain and fatigue. At the same time in some conditions such as DMD joint laxity can confer advantages. We suggest that joint laxity, which appears to confer both advantages and disadvantages to children with neuromuscular disorders, must be considered when looking at natural history studies as it might allow the further stratification of different populations of patients.

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P1.9

Glucocorticoid therapy in a non-ambulant six year old boy with Duchenne muscular dystrophy

U. Schara[a], A. Sprinz[b], S. Lutz[a]
[a] *University of Essen, Pediatric Neurology, Essen, Germany;* [b] *Outpatient Centre Haus Walstedde, Pediatric Neurology, Drensteinfurt, Germany*

Duchenne muscular dystrophy is a X-linked progressive muscular dystrophy with an incidence of about 1:3500 male newborns. Symptoms usually appear before the age of 6 but also as early as infancy. They include delay of motor, mental and speech skills. The boys usually show pseudohypertrophic calf muscles as well as worsening muscle weakness, frequent falls, positive phenomenon of Gowers, a dull gait and loss of ambulance around nine to ten years of life. There is no known cure so far, but a drug therapy with steroids to start at the age of five in usually ambulant patients is recommended to slow the decline of muscle strength and function. We report on a boy first seen in our outpatient clinic at the age of two years. In the context of a common cold blood samples were taken and showed a markedly increased result of creatine kinase level of about 36,000 U/l. Genetic analysis (sequencing) showed a deletion of two bases in exon 27 in the dystrophin gene leading to an out-of-frame-mutation confirming the diagnosis of Duchenne muscular dystrophy. Until this age the boy showed a motor delay in sitting stable (age 27 months), in crawling (age 30 months), in babble (age 12 months). Until the age of six he was not able to walk independently, only a few steps with assistance. Nevertheless we decided to treat the patient with daily Deflazacort (0.9 mg/kg daily). The next four to six weeks he started to walk independently and by that he improved his social skills. Despite missing values or standards in non ambulant patients with a very early manifestation of Duchenne muscular dystrophy a medical treatment with steroids should be considered. Although there is no evidence, we think the acquisition of ambulance might be a consequence of steroid therapy.

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P1.10

Comparative pharmacokinetics (PK) in primates and humans of AVI-4658, a phosphorodiamidate morpholino oligomer (PMO) for treating DMD patients

S.B. Shrewsbury[a], P. Sazani[b], F. Muntoni[c]
[a] *AVI BioPharma, Clinical Development, Bothell, United States;* [b] *AVI BioPharma, Preclinical development, Bothell, United States;* [c] *Institute of Child Health, London, United Kingdom*

AVI-4658 is a PMO that skips dystrophin exon 51, restores the reading frame and enables dystrophin expression in a substantial subset of Duchenne muscular dystrophy (DMD) patients. To characterize the plasma PK and understand the PK/Pharmacodynamic relationship, data has been collected in non-dystrophic non-human primates (NHP) and DMD patients. This paper studied the PK profile of AVI-4658 to assess whether the non-metabolized PMO PPK behavior is similar between animals and humans, and to better guide development assumptions for progressing additional PMOs into development. (1) Cynomolgus monkeys were dosed IV with 0, 5, 40 or 320 mg/kg (Maximum Feasible Dose) and 320 mg/kg subcutaneously weekly for 12 weeks, with blood and urine collected for 24 h post 1st and 11th dosing as part of a GLP IND-enabling toxicology study. (2) 19 DMD patients were dosed with 0.5, 1.0, 2.0, 4.0, 10.0 or 20.0 mg/kg weekly for 12 weeks, with blood and urine collected after 1st, 6th and 12th doses. The PK profile of AVI-4658, a neutral, non-metabolized 30-mer PMO, was consistent across species and time points with similar $T_{1/2}$ (NHP: 1.6–3.9 h; DMD: 1.6–3.6 h); dose-proportional C_{max} and AUC and steady clearance (NHP: 3.6–6.7 mL/min/kg; DMD: 3.9–10.2 mL/min/kg). The subcutaneous dosing in NHP at MFD provided at least 100% bioavailability. AVI-4658, the first PMO for DMD, demonstrates consistent PK across dose, species and disease states, with rapid urinary elimination (unmetabolized) from the plasma. If preclinical data suggests consistent behavior across different sequences and different length oligomers, this may support acceleration of the development of other PMOs for additional DMD genotypes.

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CARDIORESPIRATORY: POSTER PRESENTATIONS

P1.11

Comparison between courses of home mechanical ventilation patients with muscular dystrophy and mechanical ventilation inpatients

T. Saito[a], K. Tatara[b], S. Shinno[a], S. Sakoda[a], H. Fujimura[a]
[a] *National Hospital Organization Toneyama National Hospital, Neurology, Toyonaka, Japan;* [b] *National Hospital Organization Tokushima National Hospital, Pediatrics, Tokushima, Japan*

In Japan, there are 27 hospitals specializing in muscular dystrophy treatment, which have managed wards for inpatients with muscular dystrophy and other neuromuscular disorder. We have been conducted survey of inpatients of these wards and home-mechanical ventilation patients (HMV patients) with muscular dystrophy and neuromuscular disorder annually since 1999, to construct the muscular dystrophy database. To evaluate efficacy of mechanical ventilation therapy for HMV patients and ventilation-dependent inpatients (MV inpatients) with those wards. By using these databases, we analyzed the courses of HMV patients and those of MV inpatients of wards, those of both groups started mechanical ventilation after 1999. Examination points are mechanical ventilation periods, outcome and caregiver (for HMV patients). HMV patients group included 434 patients; 262 patients with Duchenne muscular dystrophy (DMD), 60 myotonic dystrophy (MD), 14 spinal muscular atrophy (SMA), and so on. MV inpatients group included 583 inpatients; 339 DMD, 103 MD, 16 SMA, and so on. The range of mechanical ventilation introduction age for HMV patients was $6.3 \sim 72.8$ years old (mean 37.6), and that of MV inpatients was $10.0 \sim 76.0$ years old (31.7). The number of NPPV introduction cases of HMV patients was 417, and that of MV inpatients was 418. Survival analysis showed that 75% life time of HMV patients was 1689 days, while that of inpatients surpassed the observation period. The number of death cases of HMV patients was 57, and that of MV inpatients was 65. The former included more sudden death cases than latter, and had some accidental cases. Caregivers for 80% of HMV patients were patients' families. The course of HMV patients was fairly good. However, burden of caregivers was

supposed to be severe. The muscular dystrophy wards may be requested to offer the circumstances for those who have difficulties in continuing HMV.

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P1.12

Duchenne muscular dystrophy and optoelectronic plethysmography: A longitudinal study of respiratory function

M.G. D'Angelo [a], M. Romei [b], A. Lo Mauro [c], E. Marchi [d], S. Gandossini [a], S. Bonato [a], D. Colombo [d], A.C. Turconi [a], A. Pedotti [c], N. Bresolin [e], A. Aliverti [c]

[a] *IRCCS E. Medea, NeuroRehabilitation Dept., Bosisio Parini-Lecco, Italy;* [b] *IRCCS E. Medea, BioEngineering Dept., Bosisio Parini-Lecco, Italy;* [c] *Politecnico Milano, BioEngineering Dept, Milano, Italy;* [d] *IRCCS INRCA, Casatenovo-Lecco, Italy;* [e] *IRCCS Fondazione Policlinico University of Milano, Neuroscience Dept, Milano, Italy*

Respiratory muscle weakness starts as early as the second decade of life in Duchenne Muscular Dystrophy (DMD) but signs and symptoms are subtle and not easy to detect. Identify early markers of respiratory insufficiency and rule out the role of pharmacological and surgical therapies (i.e. steroids and spinal fusion) through a longitudinal study in a large group of DMD patients, in which multidisciplinary clinical evaluations are combined with Optoelectronic Plethysmography (OEP). 114 DMD patients spanning the whole range of disease severity (age range of 3–30 years). Clinical protocol: Muscular and functional evaluation (MRC and MFM scale, North Star Ambulatory Assessment and 6 min walk test), degree of scoliosis – cardiac function (ECG, 24 h ECG and echocardiography) – respiratory function (spirometry, MIP, MEP, PCF, polysomnography). OEP: abdominal contribution to chest wall volume variations (ABD) during quiet breathing, slow vital capacity and cough. The DMD patients subdivided into 4 groups according to age, showed that ABD during quiet breathing is a strong indicator of diaphragm impairment, which occurs at different times in different patients. A subgroup of 40 adolescent DMD patients showed differences in ABD related to the time spent with low oxygen saturation during night, despite similar spirometric parameters. The influence of steroids, scoliosis and spinal fusion are under analysis. Additionally, the inadequate pre-inspiration and insufficient expiratory flow, particularly of the rib cage muscles, seemed to be the cause underlying the progressive inefficient cough typical of the natural course of the disease. Our results are able to identify early signs of respiratory impairment and initial alterations of some mechanisms such as cough, that could improve the correct timing of the interventions with non invasive ventilation or cough-assisting devices.

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P1.13

Respiratory pattern during water swallowing in patients with Duchenne muscular dystrophy and myotonic dystrophy type 1

S. Nozaki [a], S. Sugishita [b], Y. Umaki [b], T. Matsumura [c], K. Tatara [b]

[a] *Hyogo University of Health Sciences, Hyogo, Japan;* [b] *Tokushima National Hospital, Tokushima, Japan;* [c] *Toneyama National Hospital, Osaka, Japan*

To analyze the respiratory change during swallowing is important for the management of dysphagia in patients with muscular dystrophy. We evaluated the respiratory cycle at rest and after water swallowing, and apnea/hypopnea during water swallowing. We evaluated the respiratory patterns during water swallowing in 10 patients with Duchenne muscular dystrophy (DMD) (19.6 ± 3.2 years old, %FVC: 22.6 ± 18.1%), 10 patients with myotonic dystrophy type 1 (MD-1) (46.5 ± 11.6 years old, %FVC: 55.2 ± 16.6%), and 10 healthy volunteers as control subjects (43.9 ± 10.3 years old). The respiratory patterns were evaluated by the simultaneous recording of cervical swallowing sound during water swallowing. A thermistor was used for pneumography and a hypersensitive microphone was used for detecting cervical sound. The means of four continuous respiratory cycles at rest and after 3 ml water swallowing were used for analysis. The respiratory cycle, in which the amplitude was smaller than half of that at rest, was defined as apnea/hypopnea. In DMD patients, the respiratory cycle was 2.8 ± 0.6 s at rest, and 2.9 ± 0.7 s after swallowing. In MD-1 patients, that was 2.6 ± 0.5 s at rest, and 3.0 ± 0.6 s after swallowing. In control subjects, that was 4.2 ± 0.8 s at rest and 4.0 ± 0.9 s after swallowing. The apnea/hypopnea duration during water swallowing were 8.4 ± 4.1 s in DMD patients, 3.7 ± 1.6 s in MD-1 patients, and 3.2 ± 1.6 s in control subjects. The respiratory cycles in DMD and MD-1 patients were shorter than that in control subjects. The apnea/hypopnea duration in DMD patients was longer than those in MD-1 patients and control subjects. Prolonged apnea/hypopnea was observed during water swallowing in DMD patients.

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P1.14

Cardiac transplantation in Duchenne muscular dystrophy: A case report

L. Cripe, K. Kinnett, K. Uzark, P. Eghtesady, B. Wong, R. Spicer

Cincinnati Children's Hospital Medical Center, The Heart Institute, Cincinnati, United States

Despite significant advances in the management of Duchenne muscular dystrophy (DMD), dilated cardiomyopathy and heart failure remain causes of morbidity and mortality. Although cardiac transplantation is an accepted form of therapy for most patients with end stage dilated cardiomyopathy, the severely limited donor pool excludes patients with poor long-term survival and quality of life. We report the case of a 14-year-old patient with intermediate DMD and severe dilated cardiomyopathy who underwent successful cardiac transplantation. The patient was diagnosed at age three, when he presented with delayed motor milestones and weakness. Genetic testing demonstrated duplication of exon 2 in the dystrophin gene and <2% dystrophin in skeletal muscle. He was treated with deflazacort for six years, and remained ambulatory. Cardiac screening at age 10 years demonstrated dilated cardiomyopathy. He was treated with digoxin, enalapril, and carvedilol. Decompensated congestive heart failure ensued at age 14 and he was referred for cardiac transplantation. Although ambulatory, he had marked activity intolerance. Echocardiography demonstrated a left ventricular shortening fraction of 12%. He was treated with percutaneous intravenous home milrinone infusion with clinical improvement. Pulmonary function testing demonstrated mild respiratory muscle weakness which predicted he would not require ventilatory support for 5–10 years. Following discussions with medical, surgical, and bioethical personnel, he was listed. Two months later, he underwent uncomplicated cardiac transplantation. His cardiac immunosuppression includes Prograf, Imuran, and Prednisone, and he has had no rejection or serious infections. He remains ambulatory four years following cardiac transplantation. Our experience suggests that cardiac transplantation can be successful in selected patients with DMD who have a phenotype of severe cardiomyopathy with preserved pulmonary function and skeletal muscle integrity.

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症例報告

脳MRIで大脳基底核，視床枕，後頭・側頭葉皮質病変を呈し，抗グルタミン酸受容体抗体が陽性であった若年女性脳炎の1例

齊藤　利雄[1)*]　斎藤　朋子[1)]　高橋　幸利[2)]　穀内　洋介[3)]　藤村　晴俊[1)]

要旨：妊娠・出産後に複視・ふらつきで初発し，精神症状，運動障害などの症状を呈し，進行性の経過を辿った脳炎の25歳女性症例を経験した．本例は副腎皮質ステロイドが症状改善に有効であったが，急性増悪をきたし第197病日に死亡した．抗グルタミン酸受容体ε2抗体が陽性であった点，妊娠・出産後に発症している点，発症初期に大脳基底核主体の病変がみられ，進行期に視床，後頭・側頭葉皮質病変を呈した点などが特徴的であった．

（臨床神経 2011;51:192-196）

Key words：抗グルタミン酸受容体抗体，脳炎，Pulvinar sign

緒　言

脳炎の原因は多岐にわたるが，われわれは，感冒様症状，出産後に複視とふらつきで発症した脳炎の25歳女性症例を経験した．副腎皮質ステロイドが症状改善に有効であったが，急性増悪をきたし第197病日に死亡した．抗グルタミン酸受容体（GluR）ε2抗体は陽性で，脳炎の病態あるいは病勢を反映していることが示唆された．発症初期には大脳基底核主体の病変がみられ，進行期に視床枕，後頭・側頭葉皮質病変を呈した．

症　例

25歳女性

主訴：意識障害，痙攣

家族歴：母は統合失調症．父は大動脈瘤・脳梗塞で死亡．姉は精神疾患罹患．

既往歴：24歳時，左眼一過性視力低下を自覚したが，眼科では異常指摘されず．甲状腺疾患，膠原病の既往なし．

出産歴2回　海外渡航歴なし．

現病歴：2007年3月，総合病院で第3子出産．妊娠中著変なく経過したが，出産一週間前から咳嗽があった．出産3日後（第1病日）から，複視とふらつき，両側水平方向の突発的な眼球不随意運動が出現した．呼名反応は低下し，左下肢から左上肢，左顔面に拡大する痙攣が出現し転倒した．痙攣は15分程度で消失したが，37度程度に発熱した．翌日に約30分，第4，5病日にも10分程度の同様の発作が出現した．脳MRI T_2強調画像，FLAIR像で左尾状核頭と被殻に高信号域をみとめた（Fig. 1 A）．髄液細胞数は15/mm^{3}と軽度上昇していたが，脳波に異常をみとめなかった．

第13病日，大学病院に入院した．意識清明，MMSE25/30，髄膜刺激症状をみとめず．眼位は両側軽度内転位，両側外転障害をみとめ，右方両眼視時に複視を訴えた．徒手筋力テストは上下肢4$^+$～5/5で，深部腱反射正常，病的反射をみとめず．不随意運動なし．筋トーヌス，感覚系，協調系に異常をみとめず．立位保持は安定していたが，継ぎ足歩行は左へ偏位した．第24病日，右不全麻痺，自殺念慮，うつ傾向が出現し，フマル酸クエチアピンとアルプラゾラムが投与されたが，意思疎通可能，経口摂取可能であった．

ツベルクリン反応は陰性であったが，喀痰抗酸菌塗末でガフキー1号，胃液抗酸菌PCR陽性が判明し，第47病日からイソニアジド，リファンピシン，エタンブトール，ピラジナミドを投与．第53病日，結核治療のため当院へ転院した．

転院時，臥床状態で発語なく，項部硬直をみとめた．瞳孔正円同大，対光反射迅速であった．追視可能であったが衝動的であった．瞬目は保たれたが，閉眼すると随意的開眼は困難であった．提舌は不可能．右上肢は随意的に動かせなかったが，左上肢挙上可能で，離握手の指示にしたがえた．膝立不可能であったが，左下肢は膝屈曲肢位での保持は可能であった．深部腱反射は左右差なく正常～軽度減弱し，バビンスキー徴候は陰性であった．

抗結核薬は転院後も投与されたが，37～38度の高体温が持続し，運動機能障害は徐々に増悪した．第56病日は，呼びか

*Corresponding author: 国立病院機構刀根山病院神経内科〔〒560-8552　大阪府豊中市刀根山5—1—1〕

1)国立病院機構刀根山病院神経内科

2)国立病院機構静岡てんかん・神経医療センター小児科

3)大阪大学医学部附属病院神経内科

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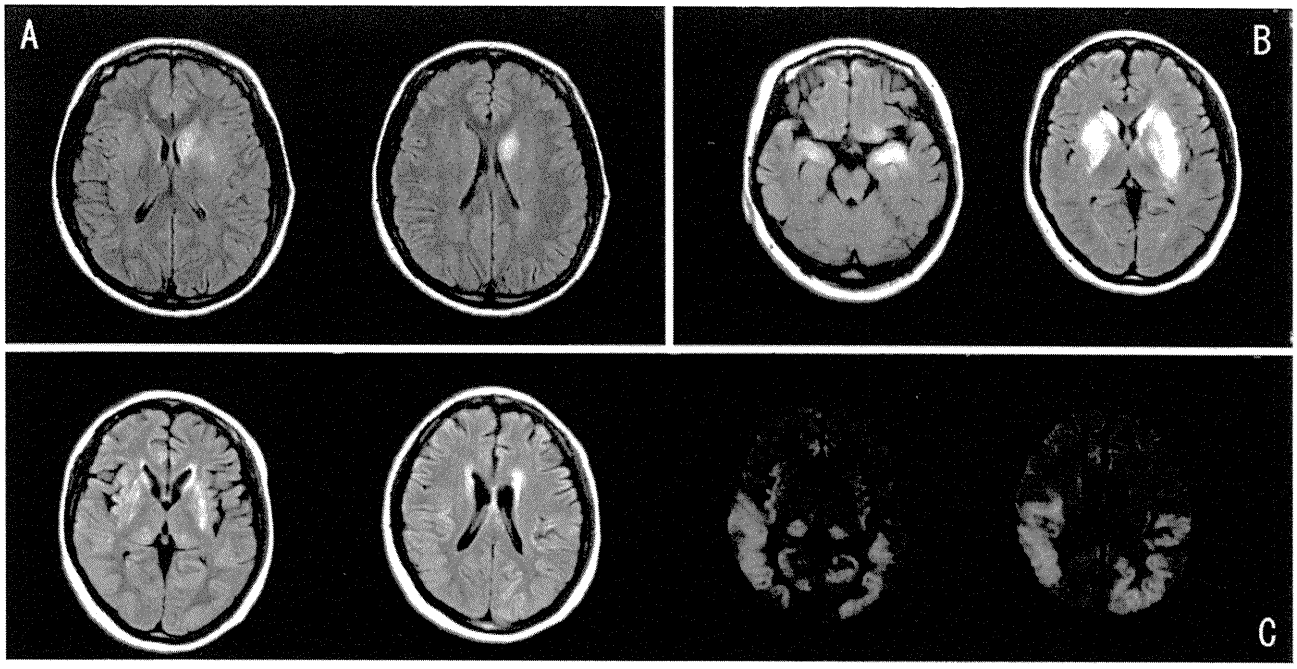

Fig. 1 Brain MR images.
(A) High intensity areas can be seen in the left caudate nucleus head and putamen in fluid-attenuated inversion recovery (FLAIR) images (axial; TR 7,000 ms, TE 112.5 ms).
(B) The high intensity areas are shown spread to the bilateral hippocampus and basal ganglia in FLAIR images (axial; TR 8,000 ms, TE 144 ms).
(C) The high intensity lesions are reduced in the bilateral putamen and globus pallidus in FLAIR images, while brain atrophic changes have progressed (axial; TR 8,000 ms, TE 144 ms). High intensity lesions were also observed in the bilateral pulvinar nucleus, occipital lobes, and temporal lobes in diffusion-weighted images (DWI). (1.5 T; TR 6,000 ms, TE 88.7 ms, b value = 1,000 secs/mm^3).

けに開眼していたが，第 61 病日には，随意的な開眼・開口が不可能となった．第 73 病日には，口頭の指示にはしたがおうとはしたが，左手の離握手も不可能となった．

第 74～76 病日にメチルプレドニゾロン 1,000mg/日のステロイドパルス療法（パルス療法）を施行した．第 78 病日には右方向への共同偏視が出現したが，第 80 病日には追視可能となり，第 83 病日には随意的開閉眼可能となった．第 78 病日の脳 MRI の FLAIR 像では，両側海馬と，両側尾状核頭，被殻，淡蒼球など基底核を中心に高信号域が拡大していた（Fig. 1 B）．

第 86～88 病日に 2 回目のパルス療法を施行した後，随意的開口可能となり，第 92 病日には右手離握手，左手指のわずかな随意運動が可能となった．

第 99～101 病日に 3 回目のパルス療法を施行した．第 102 病日，意味のある発話は不可能であったが，タ・カ行など小声で発音可能となり，開閉眼や左手指の動きで可否の意思伝達が可能となった．ゼリー嚥下可能となり，経口摂取を開始した．全身状態は徐々に改善し，簡単な受け答えが可能となった．

第 121～123 病日に 4 回目のパルス療法を施行した．経過中，後療法の副腎皮質ステロイド投与はおこなわなかったが，パルス療法の度に臨床症状は改善した．脳 MRI の FLAIR 像での異常信号域の範囲は徐々に縮小したが，側脳室拡大，脳溝拡大など脳萎縮は徐々に進行した．

第 138 病日，前兆なく意識障害，顔面・眼瞼・上肢などに間代性痙攣が出現した．フェノバルビタール，ジアゼパム投与で痙攣は抑制されたが，痙攣出現 2 日後の第 140 病日になっても，痛み刺激で開眼する程度の意識レベルで発語もなかった．項部硬直をみとめ，左上肢筋緊張は亢進し，四肢の運動をみとめなかった．第 140 病日の脳 MRI では，FLAIR 像の尾状核頭，被殻，淡蒼球などの高信号域は軽減～消失していたが，大脳は萎縮し，拡散強調像（DWI）では視床枕，後頭・側頭葉などに高信号域をみとめた（Fig. 1 C）．

第 141～143 病日，第 150～152 病日にパルス療法を，第 141～145 病日にアシクロビルを投与したが，徐々に除皮質肢位を呈するようになった．以後，意識レベルの回復なく経過した．

第 190 病日頃から下痢が出現，輸液，抗生剤投与などで対応したが，第 195 病日頃からいちじるしい腹部膨満が出現した．体温は 39 度台に上昇し，炎症反応増加，白血球著増をみとめ，敗血症をうたがい，免疫グロブリン，副腎皮質ステロイド，シベレスタットナトリウムなどを投与したが，第 197 病日に死亡した．剖検はえられなかった．

検査所見：当院転院時の検血，一般生化学に著変をみとめ

Table 1　Summary of antibody titers to virus.

	Serum	Cerebrospinal fluid
Measles (EIA)	IgM negative (day 54) IgG positive (day 54)	IgG negative (day 67)
Herpes simplex (EIA)	IgM negative (day 54) IgG positive (day 54)	IgG 0.32 (normal < 0.2)(day 67)
HHV-6 (EIA) HHV-6	IgM negative (day 68) IgG positive (day 68)	 PCR negative (day 85)
Herpes zoster (EIA)	IgM negative (day 54) IgG positive (day 54)	
Mumps (EIA)	IgM negative (day 65) IgG positive (day 65)	
Cytomegalovirus (EIA)	IgM negative (day 68) IgG positive (day 68)	
Rubella (EIA)	IgM negative (day 54) IgG positive (day 54)	
Japanese encephalitis (CF) Japanese encephalitis Japanese encephalitis (JaGAr)(HI)	× 4 (normal<4)(day 82) × 20 (normal<10)(day 86)	negative (day 85) virus RNA (RT-PCR) negative (day 85) negative (day 85)

Table 2　Summary of laboratory data of cerebrospinal fluid.

day	17	30	40	59	67	85	140	149	170
cell (/mm^3)	4	3	5	47	7	6	35	24	20
protein (mg/dl)	28	28	27	31	33	25	38	70	37
sugar (mg/dl)	46	46	49	60	69	75	57	52	53
IgG index	0.46		0.577	0.8168			0.5873	0.5857	0.6296
NSE (ng/ml)							179	24.3	11.4
MBP (pg/ml)								79.9	80.8

NSE, neuron-specific enolase; MBP, myelin basic protein

なかった. 第59病日施行の髄液検査での細菌・真菌・抗酸菌培養は陰性，抗酸菌PCR陰性，細胞診も陰性であった. 甲状腺機能は正常，抗甲状腺抗体は陰性であった. リウマチ因子（RF）は19（正常<10）と陽性であったが，各種自己抗体は陰性であった. CA19-9 81.5U/ml，CEA 2.9ng/ml，CYFRA 2.3ng/ml，pro-GRP 28.7pg/mlと，腫瘍マーカーの上昇はみとめなかった.

血清各種ウイルス抗体価は既感染を示す結果であった（Table 1）. 髄液中単純ヘルペスIgG抗体は0.32（<0.2）とごく軽度上昇していたが，麻疹IgG抗体，HHV6 PCRは陰性であった. 血清の日本脳炎ウイルス抗体はCF法で4倍（<4），ジャガー株ではHI法で20倍（<10）であったが，髄液の日本脳炎ウイルスRNA（RT-PCR）は陰性，ウイルス抗体（CF法）も陰性，ジャガー株抗体（HI法）も陰性であった.

血清可溶性IL-2レセプター抗体は，第39病日で881U/ml，第67病日で1,020U/mlと上昇していた.

第54病日の血液中乳酸，ピルビン酸は正常であった. 髄液中乳酸は16.6mg/dl，ピルビン酸は0.94mg/dlと軽度の上昇をみとめたが，ミトコンドリア遺伝子変異はみとめなかった.

第170病日の髄液T-tau蛋白は1,401pg/ml（>1,300），14-3-3蛋白は陽性（半定量法）であった.

髄液所見の経時変化をTable 2に示す. 全経過を通じ細胞数上昇は軽度であった. 第138病日の病状変化後は，蛋白，NSEの増加をみとめた.

抗GluRε2抗体は，第67・140病日髄液ともイムノブロット法で陰性であったが，第68病日血清で，IgM陰性，IgG（±），第140病日血清で，IgM陰性，IgG陽性であった. 67病日髄液のELISA法ではN末，C末に対する抗体が陽性であった.

脳波は，徐波を主体とし，てんかん性波型やPSDはみとめなかった. 胸部CT・腹・骨盤部CT/MRIで，腫瘍性病変をみとめなかった.

考　察

本症例は，妊娠・出産後に複視・ふらつきで発症し，記憶障害，情動変化などの辺縁系によると考えられる症状に止まらず，意識障害，運動障害など多彩な症状を呈し，副腎皮質ステ

ロイドへの反応性を示したものの，進行性の経過を辿った．MRI 上脳病変は皮質や基底核などが中心で，脱髄性疾患は否定的，ミトコンドリア異常症も否定的で，臨床経過からは脳炎の可能性がもっとも高いと考えられた．

急性脳炎・脳症は，ウイルス直接侵襲脳炎，傍感染性脳炎・脳症，傍腫瘍性脳炎・脳症，全身性膠原病合併脳炎・脳症，分類不能に分けられる[1]．本例では腫瘍は発見されず，甲状腺機能も正常，RF は陽性であったが，自己抗体は陰性であった．肺結核罹患が判明したが，中枢神経系結核は否定的で，ウイルス抗体価の有意な上昇をみとめなかった．抗 GluRε2 抗体が陽性であった点，妊娠・出産後に発症している点，急性増悪時に視床枕，後頭・側頭葉皮質病変を呈した点などが，特徴的であった．

妊娠に関連した脳症・脳炎の報告は散見されるが，発症にいたるメカニズムは明らかでない[2)~4)]．本例では，発症前に咳嗽があり感染がうたがわれる病歴もあった．発症の契機は感染か妊娠かは特定困難だが，血清抗 GluRε2 抗体は陽性で，脳炎の病態を反映することが推定される．

抗 GluRε2 抗体は，ラスムッセン脳炎など自己免疫が関与する疾患などで検出される病態マーカーであるが，疾患特異性はなく，傍腫瘍性脳炎，ウイルス感染に起因する辺縁系脳炎・急性脳炎などでも陽性となる．抗 GluRε2 抗体陽性例の初発神経症状として言動異常が多いが[1)]，本例でも自殺念慮やうつ傾向がみとめられた．抗 GluRε2 抗体の関連する病態機序として，感染などにともなう刺激で産生された GluRε2 分子と交差反応をおこす血液中の抗体が，血液脳関門の破綻などで中枢神経系にいたり，病態形成に関連する可能性が考えられているが[5)]，回復期・慢性期には血液脳関門の回復で髄液中濃度は低下，症状は回復するとされる．本例の抗 GluRε2 抗体は，第 68 病日髄液では ELISA 法で N 末，C 末に対する抗体が陽性，血清でもイムノブロット法で第 68 病日では（±），病状増悪時の第 140 病日では陽性であった．これは本症例の病状変化が，抗 GluRε2 抗体変化と並行することを示し，本症例の病態に抗 GluRε2 抗体が深く関連することを示すものと推定される．

本例の病状増悪期 MRI では，FLAIR 像の被殻，淡蒼球などの高信号域は縮小していたが，DWI で後頭・側頭葉を中心とした大脳皮質や両側視床枕に高信号域をみとめた．前者は，それまでの病状が安定してきていた経過を示し，後者は急性期変化を反映するものと推察するが，後者の視床枕病変は，変異型クロイツフェルト・ヤコブ病（vCJD）様の画像所見を呈した．一方，本例では第 170 病日の髄液 14-3-3 蛋白は陽性であった．髄液 14-3-3 蛋白の CJD の感度は決して高くなく[6)]，海外渡航歴など感染の機会はなかったと考えられるものの，臨床経過のみからの CJD の否定は慎重にならざるをえない．

視床枕に高信号域を呈する疾患は，いくつか報告されている[7)]．vCJD での MRI での両側対称性の視床枕高信号域は pulvinar sign と呼ばれ[8)9)]，大脳皮質や線条体の高信号よりも強いとされる[7)]．本例の異常信号の輝度はいずれの部位も同程度で，vCJD とはことなった機序が推定される．

本例は，「若年女性に好発する急性非ヘルペス性脳炎（AJFNHE）」の一群との異同にも留意する必要がある[10)11)]．AJFNHE にも抗 GluR 抗体が検出されるが，脳 MRI 画像上は大きな変化がないことが多く，本症例のような基底核を中心とした異常像の所見はみとめない．また，本例では，検索範囲内では婦人科系腫瘍もみとめず，本例は AJFNHE の範疇には入らぬと考えられる．本例では剖検はえられなかったが，同様の症例の病理学的検討による症例蓄積が望まれる．

謝辞：ミトコンドリア遺伝子変異を検索してくださった，国立精神・神経医療研究センター神経研究所　後藤雄一先生，T-tau 蛋白，14-3-3 蛋白を測定してくださった，長崎大学大学院感染分子解析学　佐藤克也先生，長崎大学医学部・歯学部附属病院へき地病院再生支援・教育機構　調　漸先生に深謝いたします．

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Abstract

Encephalitis associated with positive anti-GluR antibodies showing abnormal appearance in basal ganglia, pulvinar and gray matter on MRI—Case report

Toshio Saito, M.D.[1], Tomoko Saito, M.D.[1], Yukitoshi Takahashi, M.D.[2], Yosuke Kokunai, M.D.[3] and Harutoshi Fujimura, M.D.[1]

[1]Division of Neurology, National Hospital Organization Toneyama National Hospital

[2]National Epilepsy Center, Shizuoka Institute of Epilepsy and Neurological Disorders

[3]Department of Neurology, Osaka University Graduate School of Medicine

We treated a 25-year-old woman with encephalitis. Following delivery, the patient developed fever, consciousness disturbance, cognitive dysfunction, and progressive motor dysfunction. In addition, mycobacterium tuberculosis was found in the lung, though there was no evidence of such infection in the central nervous system. Cerebrospinal fluid analysis revealed a slight elevation of mononuclear cells with a normal protein level, indicating a possible viral infection. We could not find the origin of the infection, though the serum anti-glutamate ε2 receptor antibody was positive. Intravenous administration of methylprednisolone (1,000 mg/day for 3 days) was temporarily effective for improvement of the clinical signs and symptoms. However, she finally demonstrated rapid deterioration resulting in death. Diffusion-weighted brain magnetic resonance imaging demonstrated abnormal high intensity lesions in the bilateral pulvinar and gray matter, with an abnormal appearance mimicking pulvinar sign.

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Key words: anti-glutamate receptor antibody, encephalitis, Pulvinar sign

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Original article

Valproic acid increases *SMN2* expression and modulates SF2/ASF and hnRNPA1 expression in SMA fibroblast cell lines

Indra Sari Kusuma Harahap [a,b], Toshio Saito [c], Lai Poh San [d], Naoko Sasaki [a], Gunadi [a], Dian Kesuma Pramudya Nurputra [a], Surini Yusoff [a], Tomoto Yamamoto [a,e], Satoru Morikawa [a,e], Noriyuki Nishimura [a,e], Myeong Jin Lee [a], Yasuhiro Takeshima [e], Masafumi Matsuo [e], Hisahide Nishio [a,e,*]

[a] *Department of Community Medicine and Social Healthcare Science, Kobe University Graduate School of Medicine, Kobe, Japan*
[b] *Department of Neurology, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia*
[c] *Department of Neurology, Toneyama National Hospital, Toyonaka, Osaka, Japan*
[d] *Department of Pediatrics, Yong Loo Lin School of Medicine, National University of Singapore, Singapore*
[e] *Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Japan*

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Abstract

Spinal muscular atrophy (SMA) is a common autosomal recessive neuromuscular disorder that is caused by loss of the survival motor neuron gene, *SMN1*. SMA treatment strategies have focused on production of the SMN protein from the almost identical gene, *SMN2*. Valproic acid (VPA) is a histone deacetylase inhibitor that can increase SMN levels in some SMA cells or SMA patients through activation of *SMN2* transcription or splicing correction of *SMN2* exon 7. It remains to be clarified what concentration of VPA is required and by what mechanisms the SMN production from *SMN2* is elicited. We observed that in two fibroblast cell lines from Japanese SMA patients, more than 1 mM of VPA increased *SMN2* expression at both the transcript and protein levels. VPA increased not only full-length (FL) transcript level but also exon 7-excluding (Δ7) transcript level in the cell lines and did not change the ratio of FL/Δ7, suggesting that *SMN2* transcription was mainly activated. We also found that VPA modulated splicing factor expression: VPA increased the expression of splicing factor 2/alternative splicing factor (SF2/ASF) and decreased the expression of heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1). In conclusion, more than 1 mM of VPA activated *SMN2* transcription and modulated the expression of splicing factors in our SMA fibroblast cell lines.

1. Introduction

Spinal muscular atrophy (SMA) is a common autosomal recessive neuromuscular disorder characterized by progressive muscular atrophy of the limbs and trunk, resulting from degeneration of α-motor neurons in the spinal cord. The incidence of the disease is approximately 1 in 6,000 live births, and the carrier frequency is 1/40–1/50 [1]. SMA can be classified into three groups: SMA type I (Werdnig–Hoffman disease; severe form), SMA type II (intermediate form) and SMA type III (Kugelberg–Welander disease; mild form) [2]. This classification is based on the age of onset and the achievement of motor milestones. The gene responsible for SMA is the survival motor neuron (*SMN*), which is present as two highly homologous copies within the SMA gene region on chromosome 5q11.2–13.3: telomeric *SMN* (*SMN1*) and centromeric *SMN* (*SMN2*) [3–6].

* Corresponding author at: Department of Community Medicine and Social Healthcare Science, Division of Epidemiology, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan. Tel.: +81 78 382 5540; fax: +81 78 382 5559.
E-mail address: nishio@med.kobe-u.ac.jp (H. Nishio).

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SMN1 and *SMN2* are identical apart from several nucleotide differences. There is a single nucleotide change in the coding region: nucleotide +6 in exon 7 is C in *SMN1* and T in *SMN2*. However, *SMN1* and *SMN2* encode the same protein, because the nucleotide change is synonymous. *SMN1* is homozygously deleted or interrupted in more than 95% of SMA patients [7,8], and deleteriously mutated in the remaining patients [9–11]. Although it encodes the same protein, *SMN2* does not fully compensate for the loss or dysfunction of *SMN1*. In addition, *SMN2* is deleted in approximately 5% of normal individuals [6]. Based on these findings, *SMN1*, but not *SMN2*, has been recognized as the SMA-causing gene.

Interestingly, *SMN2* has never been reported as absent in SMA patients. In addition, the *SMN2* copy number correlates inversely with the disease severity: a higher *SMN2* copy number may ameliorate the clinical phenotype [1,12]. Accordingly, a study using SMA model mice reported that increased copies of *SMN2* could rescue embryonic lethality in mice, indicating modulation of phenotypic severity [13]. Thus, it is thought that *SMN2* may compensate for the loss of *SMN1* to some degree by modifying the severity of the disease through the production of a small amount of functional SMN protein. Increased expression of *SMN2* thus may provide a treatment strategy for SMA, for which there is currently no effective therapy.

SMN1 and *SMN2* show different splicing patterns. All *SMN1*-derived transcripts contain exon 7, i.e., the full-length *SMN* transcript (FL-*SMN* transcript), while the majority of *SMN2*-derived transcripts lack exon 7 (Δ7-*SMN* transcript), because the C–T change in *SMN2* at nucleotide position +6 in exon 7 induces exon skipping [14,15]. In *SMN1*, a heptamer sequence motif including the C at nucleotide position +6 in exon 7 forms a splicing factor 2/alternative splicing factor (SF2/ASF) binding site as an exonic splicing enhancer (ESE) leading to exon 7 inclusion [16–18]. Meanwhile, the corresponding T nucleotide in *SMN2* disrupts the ESE motif and forms a heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) binding site as an exonic splicing silencer (ESS), leading to exclusion of exon 7 [19].

Two SMA treatment strategies targeting *SMN2* have been proposed. The first strategy involves splicing correction which would prevent exon 7 skipping in *SMN2*, thus facilitating the inclusion of exon 7 in the *SMN2* mRNA. Several pharmacological compounds and synthetic nucleotides have been reported as suitable for the first strategy. These pharmacological compounds include aclarubicin (known as an anthracycline antibiotic) [20], sodium vanadate (a phosphatase inhibitor) [21], hydroxyurea (a cell cycle inhibitor) [22] and salbutamol (a β2-adrenoceptor agonist) [23–25]. The synthetic nucleotides are termed ESSENCE (exon-specific silencing enhancement by small chimeric effectors) [16], TOES (targeted oligonucleotide enhancers of splicing) [26] and antisense oligonucleotides masking exonic splicing suppressors [27–29]. Recently, a tetracycline-like compound, PTK-SMA I, has been identified as an alternative to synthetic nucleotides in stimulating splicing of exon 7 [30]. The second strategy is through the activation of *SMN2* transcription. Drugs known to activate *SMN2* transcription are interferons [31] and histone deacetylase (HDAC) inhibitors. HDAC inhibitors which have been reported to activate *SMN2* transcription include sodium butyrate [32], valproic acid (VPA) [33,34], phenylbutyrate [35], benzamide M344 [36], suberoylanilide hydroxamic acid (SAHA) [37], Trichostatin A (TSA) [38] and hydroxamic acid LBH589 [39]. Among these HDAC inhibitors, sodium butyrate, benzamide M344 and VPA are also able to indirectly correct the splicing abnormality, mainly through the upregulation of splicing factors.

VPA has been approved by the U.S. Food and Drug Administration and is already widely used for the treatment of epileptic patients. Some studies have shown that VPA increases the expression of FL-*SMN2* transcript [33,34]. According to Brichta et al., VPA may activate the *SMN2* promoter and correct abnormal splicing of *SMN2* exon 7 in SMA fibroblasts [33]. However, some patients did not respond to VPA treatment at the normal dose given to epileptic patients [40,41]. In this study, we determined the effect of VPA dose on *SMN2* expression in fibroblasts from two Japanese SMA patients. In addition, we also demonstrated changes in two trans-acting splicing factors, SF2/ASF and hnRNPA1, which regulate the splicing of *SMN1* and *SMN2* exon 7.

2. Materials and methods

2.1. Cell culture and VPA treatment

Fibroblast cell lines from a type I SMA patient (OK11, skin biopsy at 10 months old, passage 3–10), a type II SMA patient (AM21, skin biopsy at 25 years old, passage 3–10) were used in this study. Both SMA cell lines are deleted for *SMN1*. OK11 cells carry two copies of *SMN2*, and AM21 cells carried three copies of *SMN2*. In the control cells (CO31), two copies of *SMN1* and *SMN2* are present respectively. These fibroblast cell lines were maintained in Dulbecco's Modified Eagle's medium (Sigma–Aldrich, St.Louis, MO) containing 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum (Biological Industries, Haemek, Israel) in a 5% CO_2 atmosphere at 37 °C. VPA (Sigma–Aldrich, St. Louis, MO) was dissolved in aqua dest and was freshly prepared before each use [37]. For the dose-dependency experiment, the fibroblasts were incubated with VPA

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at final concentrations of 0 (mock), 0.05, 0.5, 1 and 10 mM for 16 h in a 5% CO_2 atmosphere at 37 °C. This optimal time was established in previous experiment [33].

This study was approved by the ethical committee of Kobe University, and inform consent was obtained from the patients and/or their parents.

2.2. Cell viability assay

To evaluate the cell viability and cytotoxicity of VPA, the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was performed. The cytotoxic effect of 10 mM VPA was studied using OK11 fibroblasts. One hundred microliters of the cell suspension (1×10^5 cells/ml) was placed in the wells of a 96-well culture dish (Iwaki, Chiba, Japan) and incubated in a 5% CO_2 atmosphere at 37 °C. When the cells reached 90% confluency, VPA was added to each well. The final concentration of VPA in the assay was 10 mM. The cell viabilities 2, 4, 8, 16 and 24 h after the addition of VPA were determined using a CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI) which measures the conversion of MTS to violet formazan by dehydrogenases in metabolically active, proliferating cells.

2.3. RNA extraction and cDNA synthesis

Total RNA was isolated from fibroblast cultures in 6-well plates using Sepasol RNA I reagent (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocols. After DNase treatment with DNaseI Amplification Grade (Invitrogen, Carlsbad, CA), total RNA was denatured for 10 min at 65 °C and chilled on ice. Reverse transcription was performed at 55 °C for 30 min in a total volume of 20 μl containing 1 μg of total RNA, 60 μM of random hexamer primers, 1 mM dNTPs, 50 mM Tris/HCl, 30 mM KCl, 8 mM $MgCl_2$ pH 8.5, 20 U of protector RNase inhibitor and 10 U of Transcriptor reverse transcriptase (Roche Diagnostics GmbH, Mannheim, Germany).

2.4. Development of a method for quantification of SMN and splicing factor gene transcript levels

To assess whether VPA is able to influence SMN transcript and protein expression, we treated three passages from each of the two *SMN1*-deleted fibroblast cell lines and the control cell line (OK11, AM21 and CO31, respectively) for 16 h with different concentrations of VPA ranging from 0.05 to 10 mM. After reverse transcription of RNA extracted from the fibroblasts, *SMN* and splicing factor gene transcript levels were determined by quantitative real-time PCR (qRT-PCR).

2.5. Quantitative real-time PCR

qRT-PCR was performed on a LightCycler 1.5 instrument (Roche Diagnostics GmbH, Mannheim, Germany) using FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany). To evaluate the total transcript levels of the SMN genes, we amplified cDNA fragments encompassing SMN exons 1, 2a and 2b. The FL-SMN and Δ7-SMN transcript levels were quantitated from the levels of the products encompassing SMN exons 7 and 8, and SMN exons 5, 6 and 8, respectively. We used glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an endogenous reference gene, and the levels of *SMN* were expressed relative to those of *GAPDH*.

The primers for the FL-*SMN* and Δ7-*SMN* transcripts have been described previously [36]. For the amplification of total-*SMN* transcripts, the primers were designed to bind in *SMN* exon 1 (5′-GCT ATG GCG ATG AGC AGC GGC-3′) and *SMN* exon 2b (5′-GTT GTA AGG AAG CTG CAG TA-3′). *GAPDH* was amplified using primers in exon 2/3 (5′-GAG TCA ACG GAT TTG GTC GT-3′) and exon 4 (5′-GAC AAG CTT CCC GTT CTC AG-3′).

Conditions for all PCRs were optimized regarding the primer concentration, $MgCl_2$ concentration and annealing temperatures. A mastermix of the following reaction components was prepared at the indicated final concentration: 9.4 μl of water, 1.6 μl of $MgCl_2$ (3 mM), 1 μl of forward primer (0.5 μM), 1 μl of reverse primer (0.5 μM), 2 μl of Fast Start DNA Master SYBR Green I and 5 μl of cDNA (equivalent to 40 ng of total RNA). The LightCycler experimental run protocol was as follows: denaturation (95 °C for 10 min), 45 cycles of amplification (95 °C for 15 s, 58–64 °C for 10 s, 72 °C for 25 s with a single fluorescence measurement). Quantitation of the PCR products was performed with the second derivative maximum method of the LightCycler software, using the external standard curve method. qRT-PCR products levels, which correspond to the transcript levels were normalized to those of *GAPDH*. All sample measurements were repeated at least three times and the results were expressed as the mean ± SD.

2.6. Protein extraction and western blotting

Proteins were extracted from the fibroblast cultures by homogenization in lysis buffer containing 1 mM sodium orthovanadate, 1% sodium dodecyl sulfate (SDS) and 10 mM Tris (pH 7.4). Subsequently, the homogenized protein samples were subjected to western blotting. The protein samples were electrophoresed on 10% SDS polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA) by wet blotting. Then, the membranes

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were blocked in TBS-T buffer containing 5% dry-milk (ECL™ Blocking Agent; GE Healthcare, Little Chalfont, UK) overnight at 4 °C. Immunostaining of the membranes was performed using several antibodies, each according to the manufacturer's instructions. Detection of the signals with Amersham™ ECL Plus Western Blotting Detection Reagents (GE Healthcare) was carried out using an LAS mini 3000 (Perkin-Elmer Life Sciences, Oak Brook, IL).

The following antibodies were used: mouse anti-SMN (BD Transduction Laboratories™, Franklin Lakes, NJ; 1:1000), mouse anti-splicing factor-2 (SF2/ASF) (Invitrogen Camarillo, CA; 1 μg/ml), mouse monoclonal anti-hnRNPA1 [4B10] (Abcam®, Cambridge, MA; 1:1000), mouse monoclonal anti-beta-actin (Abcam® Cambridge, MA; 1:1000) and peroxidase-linked sheep anti-mouse IgG (ECL, Amersham Biosciences; 1:5000). The intensity of the signals was determined using ImageJ (National Institutes of Health, Bethesda, MD; downloaded from http://rsbweb.nih.gov/ij/). The protein levels were normalized to those of beta-actin. All protein measurements were repeated at least three times and the results were expressed as the mean ± SD.

2.7. Statistical analysis

Statistical analysis of the data was performed using Microsoft Excel 2003 software and Statistical Package for the Social Sciences (SPSS Inc, Chicago, USA). Student's *t* test was conducted to evaluate the differences between groups. A probability of less than 0.05 was considered statistically significant. All data were expressed as the mean ± SD. Analysis of variance test was used to examine the differences between data obtained from the mock and VPA-induced cell cultures.

3. Results

3.1. Cytotoxicity analysis of VPA using a fibroblast cell line

We used the MTS assay to analyze the cytotoxicity of 10 mM VPA in the OK11 cell line after 0, 2, 4, 8, 16 and 24 h incubation with 10 mM VPA. The MTS assay measures survival and/or proliferation of cells [42].

As shown in Fig. 1, the 16 h and 24 h incubation with 10 mM VPA decreased cell viability by 14% and 17%, respectively. This suggests that the 16 h incubation with VPA at this concentration was not particularly toxic to the cell line. Based on these data, we incubated the cells for 16 h in all subsequent experiments.

3.2. VPA induces SMN expression

To confirm the effect of VPA on *SMN* gene expression [33,34], we compared the levels of VPA-induced

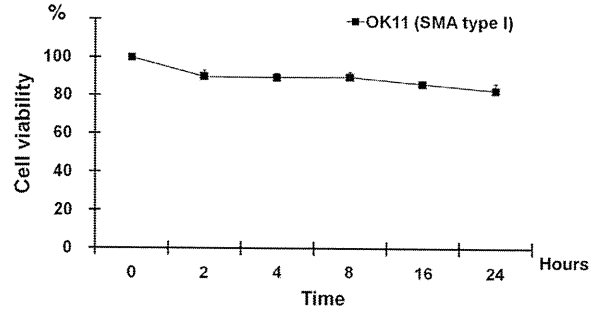

Fig. 1. Cytotoxicity analysis of VPA using the MTS assay. The cell viability of SMA type I fibroblast cell line (OK11) was measured after 0, 2, 4, 8, 16 and 24 h incubation with 10 mM VPA. The cell numbers decreased by only 14% and 17% after 16 h and 24 h incubation with VPA, respectively.

SMN transcripts and SMN protein levels with their respective baseline (mock status) levels in the cell lines, OK11, AM21 and CO31. The culture medium contained VPA at 0.05–10 mM. We measured the total-*SMN*, FL-*SMN* and Δ7-SMN transcript levels and SMN protein levels. Transcript levels were normalized to *GAPDH*, and protein levels were normalized to beta-actin.

The highest *VPA* concentration used in this study, 10 mM, induced the highest total-*SMN* and FL-*SMN* transcript levels in all three cell lines (Fig. 2a and b). The changes in total-SMN transcript levels from 0 to 10 mM of VPA were all statistically significant, at 0.29–0.75 in OK11, 0.44–0.78 in AM21 and 0.68–1.2 in CO31 (arbitrary units relative to GAPDH). The changes in FL-*SMN* transcript levels from 0 to 10 mM of VPA were also all statistically significant, at 0.21–0.51 in OK11, 0.38–0.83 in AM21 and 0.75–1.6 in CO31. Notably, the VPA-induced total-*SMN* and FL-*SMN* levels were similar between OK11 (SMA type I) and AM21 (SMA type II), but that the VPA-induced total-*SMN* and FL-*SMN* levels in CO31 (control) were significantly higher than those in the SMA cell lines. Δ7-*SMN* transcript levels were also significantly increased with 10 mM VPA treatment in cell lines AM21 and C031 (Fig. 2c). However, no change in the ratio of FL-*SMN* to Δ7-*SMN* (FL/Δ7 ratio) was observed in any cell lines (Fig. 2d).

The baseline (mock status) levels of the total-*SMN*, FL-*SMN* and Δ7-*SMN* transcripts in the SMA type I fibroblast cell line, OK11, were significantly lower than those in the SMA type II fibroblast cell line, AM21 (Fig. 2). These baseline *SMN* levels may reflect the differing copy number of the *SMN2* gene among the cell lines. However, during VPA treatment, the increases in the transcript levels in OK11 and AM21 were similar to each other, which does not reflect the copy number.

To determine the SMN protein levels, we performed western blotting with a monoclonal antibody directed against the amino-terminus of the SMN protein.

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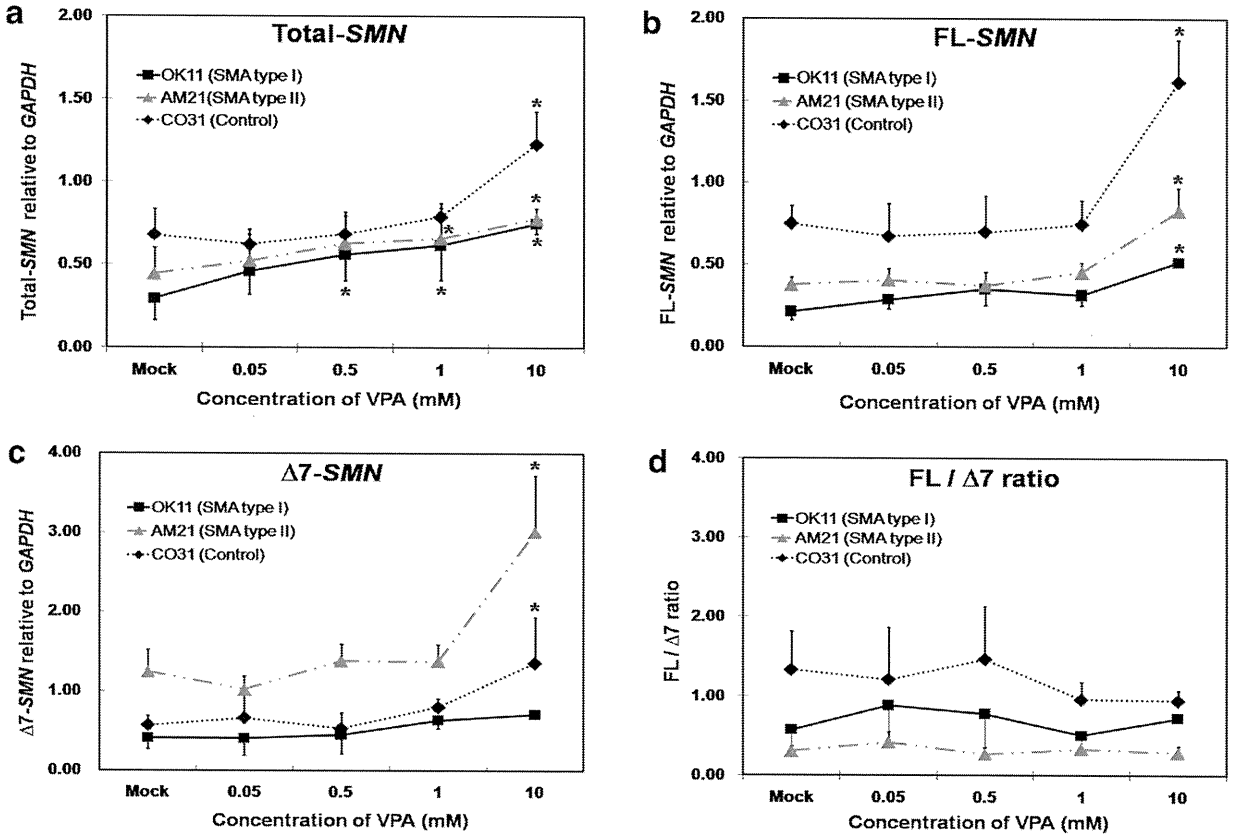

Fig. 2. Quantitative analysis of *SMN* transcripts. Upregulation of *SMN* transcript levels determined by quantitative real-time PCR in the cell lines, incubated for 16 h with different concentrations of VPA. All data are expressed as mean ± SD in arbitrary units relative to *GAPDH*. (a) Total-*SMN*. (b) FL-*SMN*. (c) Δ7-*SMN*. All SMN transcript levels reached their maximum at the highest VPA concentration, 10 mM. $^{*}p < 0.05$ *vs.* the baseline (mock). (d) The calculated FL/Δ7 ratios showed nearly unchanged levels, suggesting no effect on *SMN2* splicing after treatment with up to 10 mM VPA for 16 h.

Because the Δ7-SMN protein is essentially undetectable by western blotting [43], the SMN protein that we detected in this study was mainly FL-SMN protein. As shown in Fig. 3a, western-blotting revealed a slight increase in SMN protein expression in all three cell lines. The changes in SMN protein levels upon treatment with

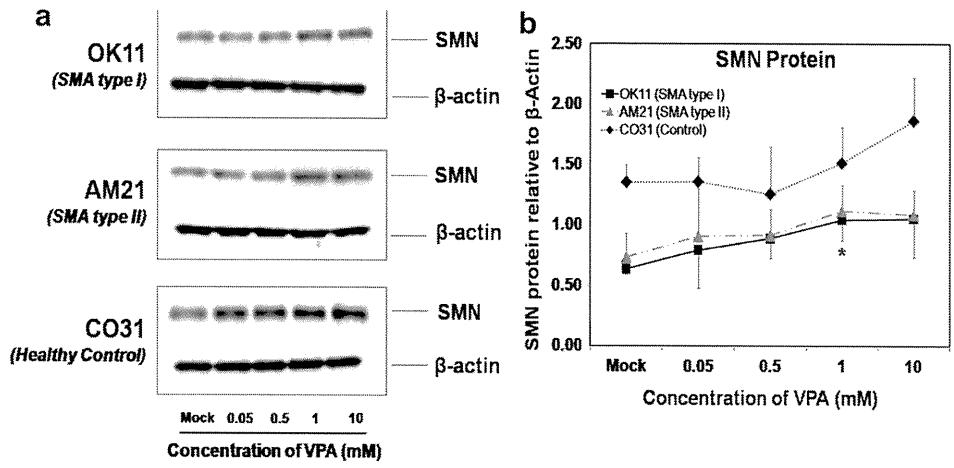

Fig. 3. Quantitative analysis of SMN protein. (a) Representative western blotting data illustrating the increase in SMN protein levels with VPA concentration. (b) Upregulation of SMN protein levels determined by western blotting in the cell lines, incubated for 16 h with different concentrations of VPA. The data are expressed as mean ± SD in arbitrary units relative to beta-actin. Densitometry revealed a 1.5-fold increase in SMN protein at 1 and 10 mM VPA relative to the baseline (mock) levels. $^{*}p < 0.05$ *vs.* the baseline (mock).

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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

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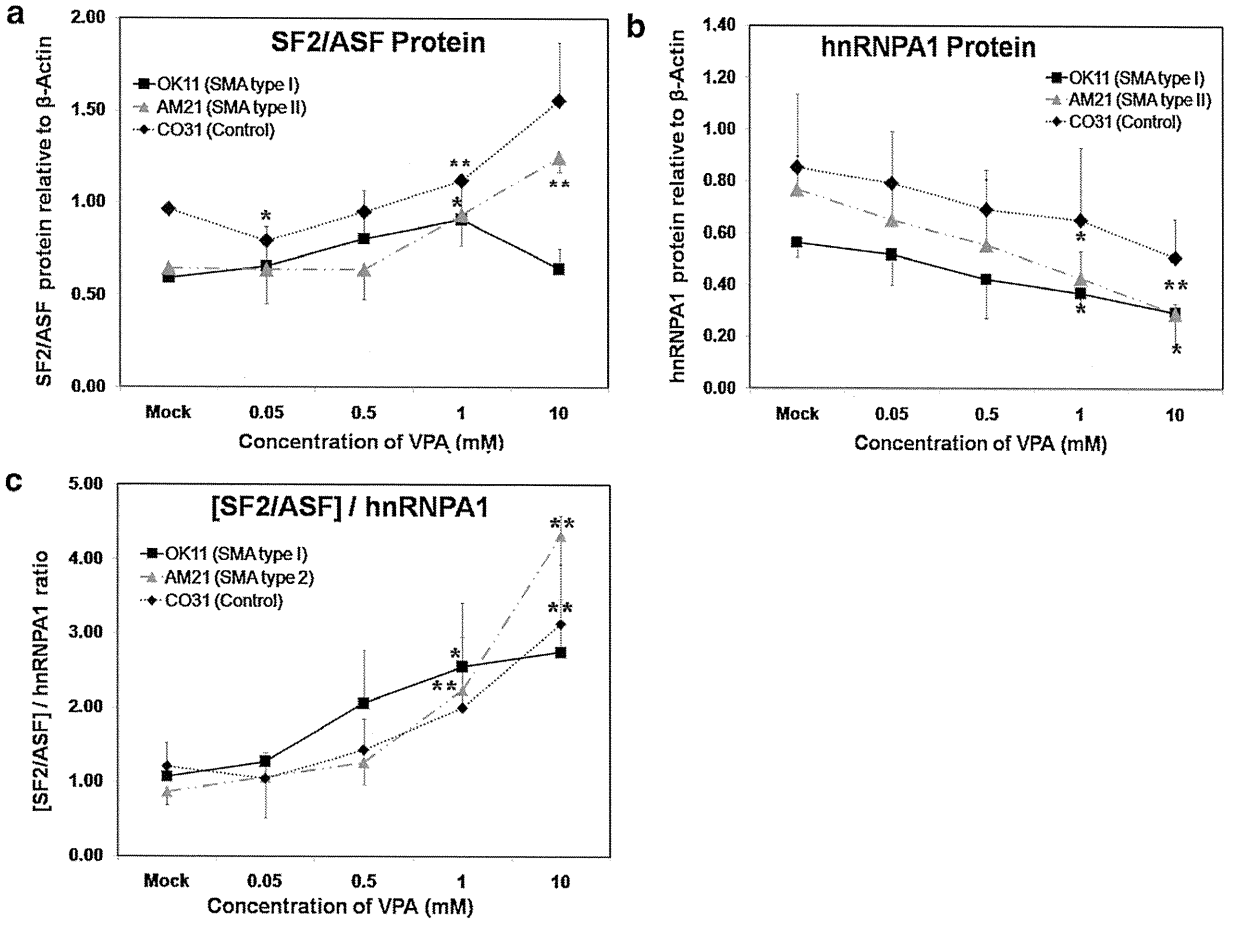

Fig. 4. Quantitative analysis of SF2/ASF protein. (a) Upregulation of SF2/ASF protein levels determined by western blotting in the cell lines, incubated for 16 h with different concentrations of VPA. The data are expressed as mean ± SD in arbitrary units relative to beta-actin. Densitometry revealed 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 *CYP11A1* and reduce CYP11A1 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

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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

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

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