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Nuclear localization of SMN and FUS is not altered in fibroblasts from patients with sporadic ALS

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Abstract

Background—Sporadic amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease with no established biological marker. Recent observation of a reduced number of gems (survival

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CONFLICT OF INTEREST DISCLOSURE

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motor neuron protein (SMN)-positive nuclear bodies) in cells from patients with familial ALS and the mouse models suggests an involvement of SMN in ALS pathology. At a molecular level, fused in sarcoma (FUS), one of the familial ALS-linked proteins, has been demonstrated to directly interact with SMN, while impaired nuclear localization of mutated FUS causes defective gem formation.

Objective—To determine whether gems and/or nuclear FUS levels in skin derived-fibroblasts from sporadic ALS patients are consistently reduced and thus could constitute a novel and readily-available biomarker of the disease.

Methods—Fibroblasts from 20 patients and 17 age-matched healthy controls were cultured and co-immunostained for SMN and FUS.

Results—No difference was detected between two groups in the number of gems and in expression pattern of FUS. The number of gems negatively correlated with the age at biopsy in both ALS and control subjects.

Conclusions—The expression pattern of SMN and FUS in fibroblasts cannot serve as a biomarker for sporadic ALS. Donor age-dependent gem reduction is a novel observation that links SMN with cellular senescence.

Keywords

Gem; SMN; FUS; Sporadic ALS; Fibroblast; Donor age

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a relentlessly progressive and fatal motor neuron disease. Approximately 90% of all cases are sporadic with no family history of the disease. Diagnosis of sporadic ALS is based primarily on clinical criteria with few satisfactory diagnostic or prognostic biomarkers that are both sensitive and specific (1). Therefore, a reliable and easily-accessible biomarker of the disease is needed.

Survival motor neuron (SMN) is an ubiquitously expressed protein involved in small nuclear ribonucleoprotein (snRNP) biogenesis (2). In cytoplasm, SMN catalyzes assembly of snRNAs and Sm proteins, the two major components of spliceosomal snRNPs. The snRNP-SMN complexes are then transported into the nucleus and accumulate at Cajal bodies to receive modification. Finally, the snRNPs translocate to spliceosome leaving SMN at Cajal bodies where gems are detected as SMN-positive nuclear bodies (3, 4). SMN deficiency due to mutations in the *SMN1* gene is a cause of the childhood-onset neuromuscular disorder, spinal muscular atrophy (SMA) (2). Because of low SMN levels, the number of gems is reduced within SMA cells (3). Recent observation of the reduced number of gems in neurons from several mouse models of ALS has raised a possibility that SMN is involved in ALS pathology (5–7). This phenomenon has also been shown to be present in fibroblasts from patients bearing ALS-related mutations in *fused in sarcoma* (*FUS*) and *TDP-43* (6). If gems are also reduced in fibroblasts from sporadic ALS patients, it could constitute a novel and easily-accessible biomarker of the disease. Furthermore, fibroblasts, propagated in a cell culture system, could be a live tool for investigating the disease pathology, as well as for

screening potential therapeutic candidates. Recent studies have revealed that direct interaction of FUS with SMN protein is required for gem formation (6, 8), while ALS-linked mutated FUS proteins abnormally accumulate in cytoplasm, aberrantly sequester SMN protein, and cause gem reduction (6, 9–13). These findings linking two distinct proteins, SMN and FUS, at the molecular mechanistic level (6, 8) have led us to consider whether distribution of FUS protein is altered and/or nuclear FUS level correlates with the number of gems in cells from patients with sporadic ALS.

Accordingly, we analyzed fibroblasts from patients with sporadic ALS and age-matched healthy individuals to determine whether gems, nuclear FUS levels, or both were reduced and thus could serve as novel and readily-available biomarkers of the disease. We also performed a cross-sectional study to estimate whether the immunocytochemical data correlated with any of patient clinical features.

MATERIALS AND METHODS

Study subjects

All individuals participating in this study were recruited after obtaining informed consent, and experiments were carried out in accordance with The Code of Ethics of the World Medical Association (Helsinki Declaration of 1975). All ALS patients (11 males and 9 females) were clinically diagnosed as sporadic cases based on pedigree analysis, and none of them showed signs of cognitive dysfunction or dementia at the time of skin biopsy. The mean age at symptom onset was 62.7 ± 8.3 (mean $\pm$ standard deviation) years old, average age at biopsy was 64.1 ± 8.3 years old, and mean duration of illness was 16.7 ± 9.1 months. The number of patients with initial symptoms in their arms, legs, and bulbar muscle were respectively 5, 6, and 9. Among 20 patients, 11 were taking riluzole (14). The mean age of biopsy of healthy control subjects (8 males and 9 females) was 60.3 ± 7.3 years old. Among control subjects, 15 were Caucasian and 2 were South Asian. Of the ALS subjects, 19 were Caucasian and one was East Asian. Clinical information is summarized in the Table.

Skin biopsy

The skin biopsy was performed using sterile technique in an unobtrusive area (axilla or upper thigh), under local anesthesia with 1% lidocaine. A 3 mm punch biopsy (AcuPunch, Ft. Lauderdale, FL) was used to remove two full thickness samples, which were placed in transport media. The wound was dressed with bacitracin, Steristrips (Nexcare) and an occlusive dressing. Most skin samples used in this study were acquired from Columbia University's ALS Center, while others came from the University of Kansas (Dr. Richard Barohn), Texas Neurology (Dr. Daragh Heitzman), and California Pacific Medical Center (Dr. Jonathan Katz).

Cell culture and immunocytochemistry

Skin samples were explanted on a dish, incubated under standard culture conditions in Medium 106 supplemented with LSGS (Low serum growth supplement: Life Technologies Corporation, Grand Island, NY) and antibiotics penicillin and streptomycin. Fibroblasts expanded from the skin were plated at a density of 1×10^4 cell/cm² on an uncoated round

cover-slip put on the bottom of 24-well plates. The entire medium was changed every 3 days after plating. On day 14 (~80% of confluency), the cells were fixed with 100% methanol (20 min at 4°C), incubated (24 h at 4°C) with a mouse monoclonal anti-SMN (7F3, 1:4, a generous gift from Dr. Livio Pellizzoni) and a rabbit polyclonal anti-FUS (H-76, 1:400, Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation (4h at room temperature) with Alexa Fluor 488 donkey anti-mouse and Alexa Fluor 594 donkey anti-rabbit antibodies (1:800, Invitrogen, Eugene, OR) and 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) for the visualization. In each sample, the mean number of gems per fibroblast was calculated by counting gems in ~300 cells under a Nikon Eclipse 80i fluorescence microscope. For estimation of mean nuclear FUS expression levels, images of 45 randomly selected cells were taken, and the fluorescence intensity was measured by using the ImageJ software (NIH, Bethesda, MD). To estimate the distribution pattern of nuclear FUS levels, the intensity was categorized into 8 groups (101–150, 151–200, 201–250, 251–300, 301–350, 351–400, 401–450, 451–500), and the number of cells in each group was counted. All the analysis was done with fibroblasts at the cell passage number two and in a blinded manner to avoid any bias.

Western blot

Cells were homogenized in a lysis buffer (150 mM NaCl, 50 mM Tris pH7.5, 0.1% NP-40, 0.1% SDS, and proteinase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), and then denatured for 10 min in a loading buffer (62.5 mM Tris pH6.8, 20% glycerol, 10% 2-mercaptoethanol, and 0.2% bromophenol blue). Proteins (20µg) were separated by 12% SDS-PAGE and subjected to immunoblot analysis with specific antibodies as follows: mouse monoclonal anti-SMN (1:2000, BD Biosciences, San Jose, CA), rabbit monoclonal Histone H3 (1:2000, Cell Signaling Technology, Danvers, MA). Protein bands were visualized using the ECL Detection Kit (RPN2109, GE Healthcare, Piscataway, NJ). Quantification of band intensities was performed by using the ImageJ software.

Quantitative PCR

The copy number of two *SMN* genes, *SMN1* and *SMN2*, was determined by a quantitative real-time PCR assay as previously described (15). Briefly, 50ng of genomic DNA was amplified in the presence of 300 nM of forward and reverse primers and 100 nM of FAM dye-labeled MGB probes (Applied Biosystems, Carlsbad, CA). The PCR was conducted as described previously (15), with an exception in using RNase P as the internal endogenous control (Applied Biosystems, Carlsbad, CA). The copy number of *SMN* genes was determined by using the ABI7500 SDS software and the CopyCaller v2.0 software from Applied Biosystems.

Statistical analysis

All the data were expressed as mean ± standard deviation, and $p < 0.05$ was considered to indicate significance in two-tailed tests. In linear regression analysis, $p < 0.05$ was considered significant.

RESULTS

All the fibroblasts analyzed in this study showed similar morphology whether they were derived from healthy control individuals or ALS patients (Figure 1A). The mean number of gems (Figure 1B) was 1.02 ± 0.33 in ALS samples ($N = 20$) and 1.20 ± 0.32 in control samples ($N = 17$) without significant difference ($p = 0.095$). The number of gems ranged from 0 to 5 in both ALS and control samples, and there was no significant difference between the two groups in the proportion of cells with each number 0–5 (0: $p = 0.165$; 1: $p = 0.349$; 2: $p = 0.016$; 3: $p = 0.560$; 4: $p = 0.651$; 5: $p = 0.655$) except for 2 (Supplemental figure 1). The expression level of nuclear FUS protein (Figures 1C) showed no difference ($p = 0.073$) between ALS (281.5 ± 15.6) and control samples (272.8 ± 13.4). The intensity distribution, as expressed by the histogram (Supplemental figure 2), manifested no significant difference between the two groups (101–150: $p = 0.831$; 151–200: $p = 0.883$; 201–250: $p = 0.422$; 251–300: $p = 0.050$; 301–350: $p = 0.634$; 351–400: $p = 0.215$; 401–450: $p = 0.006$; 451–500: $p = 0.704$) except for the FUS intensity of 401–450. None of the samples manifested abnormal staining pattern of cytoplasmic FUS protein (Figure 1A) as reported in cells from patients with familial ALS-linked *FUS* mutations (9, 11). The number of gems negatively correlated with the age at biopsy (Figure 1D) in both ALS ($p < 0.001$) and control samples ($p = 0.005$), but nuclear FUS levels did not vary with age in either group (Figure 1E) (ALS: $p = 0.89$; control: $p = 0.54$). There was no correlation between the number of gems and the nuclear FUS levels (Figure 1F) in both ALS ($p = 0.90$) and control samples ($p = 0.50$). As *SMN* gene copy number frequently varies among individuals and thus may affect gem quantity and/or nuclear FUS levels, copy numbers of *SMN1* and *SMN2* were assessed in each sample. Among 17 control subjects, 1 individual harbored 1 *SMN1* copy, 12 had 2 copies, and 4 had 3 copies. In 20 ALS subjects, 2 patients had 1 *SMN1* copy, 17 had 2 copies, and 1 had 3 copies (Supplemental table). There were no differences between ALS and control groups in mean copy numbers of *SMN1* (ALS: 1.95 ± 0.39 ; control: 2.17 ± 0.53 , $p = 0.156$) as well as *SMN2* (ALS: 1.50 ± 0.69 ; control: 1.35 ± 0.93 , $p = 0.595$). In the ALS group, the duration of illness did not correlate with the number of gems (Figure 2A, $p = 0.38$) or the nuclear FUS levels (Figure 2B, $p = 0.39$). These immunocytologic parameters showed no difference among patients with different initial symptoms (Figure 2C, upper limbs ($N = 5$) vs lower limbs ($N = 6$) vs bulbar muscles ($N = 9$) or between patients with ($N = 11$) and without ($N = 9$) riluzole (Figure 2D). Furthermore, western blot analysis revealed no difference ($p = 0.476$) in the total amount of SMN protein between fibroblasts harboring a high (Ctrl 5: 1.70, Ctrl 16: 1.60, ALS 3: 1.73, ALS 18: 1.61) and a low (Ctrl 3: 0.82, Ctrl 9: 0.61, ALS 20: 0.63, ALS 14: 0.50) number of gems (Figures 3, A and B).

DISCUSSION

Here we have clearly shown that the number of gems is not affected in fibroblasts from patients with sporadic ALS and thus it cannot be used as a biomarker of the disease. Gem reduction has been recently demonstrated in the neuronal cells from ALS model mice linked to mutations in *SOD1*, *TDP-43*, and *FUS* (5–7). Importantly, this phenomenon has also been detected in fibroblasts from familial ALS patients with mutations in *TDP-43* and *FUS* (6) as well as in spinal motor neurons from sporadic ALS patients (8). Although the pathogenic

significance of gem reduction in ALS remains unclear, these observations have introduced the following interesting concepts: (i) two distinct motor neuron diseases SMA and ALS are mechanistically linked (6, 8, 16); and (ii) gem reduction is a phenomenon shared by multiple forms of ALS (5–8). Provided that gem reduction is indeed related to ALS pathology, the lack of gem phenotypes in sporadic ALS fibroblasts suggests that, unlike familial cases where a disease-linked mutation may manifest itself in all cell types, the pathogenic mechanism underlying sporadic cases could be more cell-specific. In relation to this hypothesis and, in particular, to distinguish whether the cause is an intrinsic factor or not, it will be interesting to study in the future whether a gem phenotype emerges in motor neurons differentiated from the sporadic ALS fibroblasts such as those analyzed in this study.

We have also shown that nuclear FUS levels are not altered in cultured fibroblasts derived from sporadic ALS patient skins. A recent histological study on skin specimens from patients with sporadic ALS has demonstrated the increased level of nuclear FUS protein in keratinocytes, the predominant cell type constituting 90% of the epidermal cells (17). The difference between the two studies may simply be explained by the different cell types analyzed (keratinocyte vs fibroblast). It is also possible that, during artificial manipulation for cell culture, the purified fibroblasts analyzed in our *in vitro* study may have lost disease-relevant features gained *in vivo*. In addition to detailed histological quantification of FUS protein levels in dermal fibroblasts, *in vitro* and *in vivo* estimation of gems in ALS keratinocytes may provide intriguing insights for further understanding the SMN involvement in ALS pathology.

In the present study, we demonstrated for the first time the phenomenon of donor age-dependent gem reduction in human adult skin-derived fibroblasts cultured *in vitro*. A donor age-dependent alteration of cell features had been described in the past in various types of human cells: i.e., increased susceptibility to apoptosis in retinal pigment epithelial cells (18), shortening of telomere length in sperm and leukocytes (19). In fibroblasts, post-ultraviolet DNA repair capacity is known to decline in a donor age-dependent manner (20). Although there were differences in the cell types and the parameters analyzed, all the observation in these studies are linked to *in vivo* cellular senescence. In SMA, SMN protein levels are ubiquitously very low and thus gems are barely detectable in all the cell types including skin fibroblasts (3, 5); however, there is no report describing the abnormal *in vitro* phenotype of SMA fibroblasts. Clinically, the patients do not develop skin abnormalities, but rather progressive neuromuscular abnormalities, indicating specific requirements of the SMN protein for certain cell types. Together, it is most likely that the reduced gems detected in “aged fibroblasts” have no impact on the cellular function, and are not a cause but a consequence of cellular senescence. To see whether this phenomenon also appears in other cell types, such as spinal motor neurons whose functions are considered to be affected by gem reduction, it may be informative to collect *in vivo* data from mice at different ages.

To gain insights into the cause of gem reduction in aged fibroblasts, we compared the total amount of SMN protein in fibroblasts harboring high or low number of gems. Our semi-quantitative assay by western blot showed no difference among two groups, indicating that the age-dependent gem reduction is not a consequence of SMN reduction, but, instead, is due to impaired intracellular dynamics of the SMN protein.

In summary, there was no difference between cultured fibroblasts from patients with sporadic ALS and age-matched controls in the nuclear localization of SMN and FUS proteins, and thus they cannot serve as a biomarker for the disease. It may be interesting to see if any difference emerges after differentiating these cells into motor neurons. The donor age-dependent gem reduction is an observation that needs to be kept in mind for further understanding the physiologic spatiotemporal alteration of SMN function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SMN	survival motor neuron
ALS	amyotrophic lateral sclerosis
FUS	fused in sarcoma

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Figure 1.

(A) Representative images of fibroblasts co-immunostained for SMN and FUS. Arrow heads and insets indicate gems as visualized by SMN staining. Scale bar = 15 μ m (inset: bottom side = 3 μ m). (B) The mean number of gems in fibroblasts from ALS patients (N = 20) and age-matched controls (Ctrl, N = 17). Red dots represent a mean value. n.s.: not significant ($p = 0.095$). (C) The mean intensity of nuclear FUS in fibroblasts from ALS patients (N = 20) and age-matched controls (Ctrl, N = 17). Red dots represent a mean value. n.s.: not significant ($p = 0.073$). (D) Correlation of age at biopsy to the mean number of fibroblast gems. Black: control samples (N = 17, $Y = -0.029X + 2.9$), Red: ALS samples (N = 20, $Y = -0.027X + 2.7$). (E) Correlation of age at biopsy to the mean intensity of nuclear FUS. Black: control samples (N = 17), Red: ALS samples (N = 20). (F) Correlation between the mean number of gems and the mean intensity of nuclear FUS. Black: control samples (N = 17), Red: ALS samples (N = 20).

Figure 2.

(A) Correlation of duration of illness to the mean number of gems in ALS fibroblast (N = 20). (B) Correlation of duration of illness to the mean intensity of nuclear FUS in ALS fibroblasts (N = 20). (C) The mean number of gems and the mean intensity of nuclear FUS in fibroblasts from ALS patients with an initial symptom at their arms (A, N = 5), legs (L, N = 6), and bulbar muscles (B, N = 9). Data are indicated as means $\pm$ SD. n.s.: not significant ($p > 0.05$). (D) The mean number of gems and the mean intensity of nuclear FUS in fibroblasts from ALS patients with (N = 11) and without (N = 9) riluzole. Data are indicated as means $\pm$ SD. n.s.: not significant ($p > 0.05$).

Figure 3.

(A) Western blot showing the amount of SMN and Histone H3 proteins in the total protein extracted from fibroblasts. Ctrl 5, Ctrl 16, ALS 3, and ALS 18 contained the highest number of gems among 17 control samples and 20 ALS patient samples. Ctrl 3, Ctrl 9, ALS 20, and ALS 14 contained the lowest number of gems among 17 control samples and 20 ALS patient samples. The numbers on the bottom indicate the ratio of SMN to Histone H3 as an index for the total amount of SMN per cell. (B) The total SMN amount (Ratio of SMN to Histone H3) in fibroblasts containing a high (Ctrl 5, Ctrl 16, ALS 3, ALS 18) and a low (Ctrl 3, Ctrl 9, ALS 20, ALS 14) number of gems. Data are indicated as means $\pm$ SD. n.s.: not significant ($p > 0.05$).

Table 1

Clinical summary

Duration of illness = (Age at biopsy - Age at symptom onset), A: arms, L: legs, B: bulbar muscle.

ALS 0No.		Symptom onset (y.o.)	Initial symptom	Skin biopsy (y.o.)	Duration of illness (months)	Riluzole	Ctrl No.	Skin biopsy (y.o.)
1	M	64	A	65	10	(+)	1	65
2	M	63	A	64	9	(+)	2	64
3	F	56	A	57	9	(+)	3	57
4	F	53	B	55	22		4	55
5	M	69	B	70	12		5	52
6	F	65	B	67	24	(+)	6	72
7	M	71	L	72	12		7	62
8	F	66	L	66	5		8	67
9	M	62	B	64	24	(+)	9	69
10	M	57	B	58	14	(+)	10	73
11	F	65	A	67	25		11	64
12	F	40	L	41	7	(+)	12	57
13	F	72	B	74	18	(+)	13	54
14	F	64	L	67	34		14	58
15	M	62	B	64	24	(+)	15	52
16	M	55	A	55	10	(+)	16	53
17	M	71	L	73	25		17	51
18	M	53	B	56	34			
19	F	73	B	73	6	(+)		
20	M	72	L	73	10			