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A novel evaluation method of survival motor neuron protein as a biomarker of spinal muscular atrophy by imaging flow cytometry

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ABSTRACT

Spinal muscular atrophy (SMA) is caused by mutations within the survival motor neuron 1 (*SMN1*) gene. These mutations result in the reduction of survival motor neuron (SMN) protein expression and SMN complex in spinal motor neurons and other tissues. SMN protein has been used as a therapeutic biomarker in recent SMA clinical studies using enzyme-linked immunosorbent assay (ELISA). Here, we investigated whether imaging flow cytometry can be a viable source of quantitative information on the SMN protein. Using a FlowSight imaging flow cytometer (Merck-Millipore, Germany), we demonstrated that imaging flow cytometry could successfully identify different expression patterns and subcellular localization of SMN protein in healthy human fibroblasts and SMA patient-derived fibroblasts. In addition, we could also evaluate the therapeutic effects of SMN protein expression by valproic acid treatment of SMA patient-derived cells *in vitro*. Therefore, we suggest that imaging flow cytometry technology has the potential for identifying SMN protein expression level and pattern as an evaluation tool of clinical studies.

1. Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive disorder caused by mutations of the survival motor neuron 1(*SMN1*) gene, leading to progressive limb and trunk muscle weakness associated with muscle atrophy [1,2]. SMN protein is ubiquitously expressed in mammalian tissues; it plays a critical role in RNA metabolism, participating in small ribonucleoproteins (sRNPs) biogenesis and in pre-mRNA splicing [2]. Two *SMN* genes translate SMN proteins: a telomeric copy (*SMN1*) and an inverted centromeric copy (*SMN2*). The *SMN2* gene is present in all patients, but is not able to compensate for *SMN1* gene defects completely, resulting in low levels of the full-length SMN protein in order to have a single point mutation in exon7 of the *SMN2* gene [3,4]. In SMA clinical specimens, reduction of SMN has been assessed by several methods. Typically, immunocytochemistry and Western blotting have been used on primary dermal fibroblasts and leukocyte cell lines, leading to a correlation between healthy human controls and SMA patients in preclinical studies [5,6]. However, this method is not sufficiently reliable for clinical and diagnostic use. Recently, enzyme-linked immunosorbent assay (ELISA) has also been used in preclinical and clinical studies of SMA [7–10]; nevertheless, SMN protein levels in human peripheral-blood mononuclear cells (PBMC) were not correlated between healthy controls, carriers, and SMA phenotypic severity in clinical trials [9,10]. These methods should be optimized to allow detection of SMN protein in human cells for SMA clinical studies.

In this study, we focus on the imaging flow cytometry technique as a new assay method of SMN protein evaluation. Usually, standard flow cytometry cannot be used to assess the localization of molecules within specific cellular compartments. However, imaging flow cytometry can evaluate intact proteins, using a digital microscope system, and immunological technologies [11,12]. SMN proteins are localized intracellularly throughout the cytoplasm and nucleus, as a multi-protein complex. Specifically, SMN proteins form SMN complexes in the nucleus, where they accumulate in structures called Gemini of Cajal bodies (Gems) that play an essential role in the assembly of spliceosomal snRNPs and biogenesis during mRNA processing [13,14]. The predicted outcome of decreased snRNPs assembly is an alteration in gene splicing, containing minor introns due to reduced snRNPs levels [2,15]. In SMA-derived cells, gems formation is clearly decreased compared to that of healthy controls [16].

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At present, there is no effective treatment for SMA. Some therapeutic approaches are recently under investigation; therapies aim at increasing the amount of full-length SMN protein levels produced by *SMN2* promoter activation, while reducing *SMN2* exon7 alternative splicing, using small molecules, and antisense oligonucleotides [6,17–20]. Therefore, a new method to accurately measure SMN protein levels is needed, to assess disease severity and response to treatment.

The aim of this study was to evaluate SMN protein expression and to qualitatively assess its cellular localization using imaging flow cytometry. We therefore explored the applicability of this new technology for evaluating SMN protein as a biomarker in SMA clinical trials.

2. Materials and methods

2.1. Materials

Human fetal dermal fibroblasts (from healthy controls) were obtained from Cell Applications, Inc., SMA patient-derived dermal fibroblasts were obtained from skin biopsies of SMA patients. The patient having SMA type I was a 7-month-old female who had not acquired head control with *SMN1* deletion and two copies of *SMN2*, as assessed by molecular diagnoses. Ethical approval for tissue collection was granted by the Institutional Review Board of Tokyo Women's Medical University, Japan. For immunocytochemical analyses, we used a mouse monoclonal FITC-conjugated anti-SMN, (clone 2B1, Merck Millipore, Germany), and a mouse monoclonal anti-SMN antibody (BD Transduction Laboratories, San Diego, USA).

2.2. Cell culture and valproic acid treatment

Human dermal fibroblasts (from healthy controls) and type I SMA patient-derived dermal fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) including 1.0 g/L glucose and supplemented with 20% fetal bovine serum (FBS) without antibiotics. These fibroblasts were cultured in six-well plates for 24 h and then treated with the histone deacetylase inhibitor, valproic acid (VPA: 0, 0.1, 1, 10 mM) diluted in PBS for 24 h at 37 °C with 5% CO_2.

2.3. Immunocytochemical staining

After cells were cultured for 48 h, 1.5×10^6 cells were rinsed twice with cold PBS, fixed with 4% paraformaldehyde in PBS for 10 min, and then rinsed three times for 5 min with PBS. The cells were then treated with 0.2% TritonX-100 in PBS for 10 min at room temperature. The cells were incubated in blocking buffer (10% normal goat serum in PBS) for 60 min at room temperature. Following blocking, the cells were incubated with a mouse monoclonal anti-SMN antibody (1:100, BD) at room temperature for 60 min and then cells were then visualized using an Alexa Fluor 488-conjugated goat anti-mouse (1:400, Molecular Probes) for 60 min at room temperature. The cells were treated with Hoechst 33342 (0.5 μg/mL) to stain the cell nuclei for 5 min at room temperature. Image photographs were taken using a Leica fluorescent microscope system.

2.4. Quantitative RT-PCR analysis

Cells were cultured for 24 h after VPA treatment, and total RNA was isolated using the RNeasy kit (QIAGEN Sciences, USA) according to the manufacturer's instructions. For reverse transcription reactions, 500 ng of total RNA was used with PrimeScripts RT Mix (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Aliquots of cDNA were mixed with SYBR Premix Ex Taq II (Takara Bio Inc., Shiga, Japan) each containing 400 nM primers. Quantitative PCR was performed on Thermal Cycler Dice Real Time Systems (Takara Bio Inc., Shiga, Japan). Primers used in this paper were as follows: glyceraldehyde-3-phasphate dehydrogenase (*GAPDH*): 5′-GCACCGTCAAGGCTGAGAAC-3′ for forward and 5′-TGGTGAAGACGCCAGTGGA-3′ for reverse; *SMN2*, 5′-AACCTG TGTTGTGGTTTACACTGGA-3′ for forward and 5′-CAGATTTGGGCTT GATGTTATCTGA-3′ for reverse. All samples were assayed in duplicate.

2.5. Western blotting

Cells were cultured for 24 h VPA untreatment or treatment and then washed twice with PBS. Cells were homogenized on ice in protein lysis buffer (ER4, Enzo Life Sciences, Farmingdale, NY). After incubation on ice for 15 min, the samples were centrifuged at 10,000 rpm for 10 min at 4 °C. Sample protein concentrations were determined by the BCA method (Pierce, Rockford, IL). The amount of total protein was adjusted to equal levels between samples with SDS sample buffer, and the samples (10 μg of protein) were subjected to electrophoresis on 10% SDS polyacrylamide gels. Proteins were transferred to a PVDF membrane (Millipore Corp., Billerica, MA) and treated with blocking buffer (5% skim milk in 0.1% Tween20 in TBS) for 1 h at room temperature. After blocking, the membrane was treated with monoclonal anti-SMN antibody (1:2000, BD) diluted in blocking buffer for 1 h at room temperature and then incubated with an HRP-conjugated anti-mouse IgG antibody (1:2000, DAKO) for 1 h at room temperature. The membrane was treated with a substrate (ECL plus substrate kit). To test for equal amounts of loaded protein, membranes were stripped and incubated with monoclonal anti-α-tubulin antibody (1:5000, Sigma) as described above. Proteins were visualized using a luminescent image analyzer ImageQuant LAS-1000 (Fuji Photo Film, Tokyo, Japan). All samples were assayed in duplicate.

2.6. ELISA

Cells were cultured for 24 h after VPA treatment, and then washed twice with PBS. Cells were homogenized in protein lysis buffer on ice as described above. Aliquots of protein extracts were diluted with lysis buffer. SMN ELISA kit (Enzo Life Sciences, Farmingdale, NY) was carried out according to the manufacturer's instructions. All samples were assayed in duplicate.

2.7. Immunostaining for the imaging flow cytometry

SMA patient-derived fibroblasts were rinsed twice with PBS and trypsinized. Cells were then washed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min on ice. After they were washed three times with PBS, we permeabilized the cells using chilled BD Phosflow Parm buffer II for 30 min on ice. Cells were washed with Stain buffer (BD) and counted; 10 μl FITC-conjugated human SMN antibody (Millipore) or normal mouse Ig (Sigma) was added in 1×10^6 cells/90 μl and incubated at room temperature for 45–60 min. After incubation, and a single wash with PBS, the cells were treated with Hoechst 33342 (5 μg/mL) in PBS for 5 min at room temperature.

2.8. Imaging flow cytometry analysis

Samples were analyzed on a FlowSight imaging flow cytometer (Merck-Millipore, German). Data from a minimum of 10,000 cells (counts) were acquired, utilizing the 405, 488, and 785-nm lasers to calculate cell granularity, at a 20× magnification, using INSPIRE

software. Compensation was performed using single-color staining for all channels. Single cells were first identified based on a scatter plot of bright field area versus the aspect ratio. A gate was drawn around the population containing putative single cells based on the criteria of the area being large enough to exclude debris, and the aspect ratio being greater than ~0.6, which eliminates debris and clusters. Focused-Single cells were plotted on the SMN-FITC and Hoechst dye intensity. Both double positive population was gated and confirmed by image gallery to determine correct gate. Cellular localization of SMN protein was analyzed using the Bright Detail Intensity (BDI) feature algorithm. Acquired data were analyzed using the IDEAS analysis software.

2.9. Statistical methods

Analysis of statistical significant between SMN levels between the VPA-treated groups was done by Student's *t*-test. Values are presented as mean ± standard deviation value (SD). Statistically significant differences were defined as $p < 0.05$.

3. Results

3.1. Detection of SMN protein levels in healthy human fibroblasts, and type I SMA patient-derived fibroblasts by immunocytochemistry and Western blotting

In control fibroblasts from healthy individuals, endogenous SMN protein is expressed in the cytosol and nucleus, and accumulates in discrete nuclear foci known as gems. In this study, we checked SMN protein expression in human controls by immunocytochemistry and Western blotting using a specific antibody (Fig. 1A and B). On the other hand, type I SMA patient-derived fibroblasts showed decreased SMN protein levels resulting from *SMN1* gene loss, compared to healthy controls (Fig. 1A and B).

3.2. Effects of valproic acid treatment on type I SMA patient-derived fibroblasts

In our preclinical studies, we investigated the effects of VPA (concentrations used were 0, 1, and 10 mM) on cell morphology, full-length *SMN2* mRNA transcription levels and changes in SMN protein levels in SMA patient-derived fibroblasts (VPA; 0, 0.1, 1, 10 mM) at 24 h after treatment.

Our results showed that 24 h VPA treatment did not cause any changes in cell morphology or any toxicity (Fig. 2A). Moreover, these treatments were dose-dependent. Thus, a 10 mM dosage of VPA significantly increased full-length *SMN2* mRNA transcription levels, detected by quantitative RT-PCR method, compared to non-treated SMA cells ($p < 0.05$, Fig. 2B). Quantification of full-length *SMN2* mRNA transcription levels was determined with respect to a standard curve constructed using serial dilutions of cDNA. We used mRNA transcription levels of a housekeeping gene, *GAPDH* as internal control. On the other hand, SMN protein levels were also increased when examined by two independent methods, Western blotting (Fig. 2C), and ELISA ($p < 0.05$, Fig. 2D), after VPA treatments.

3.3. Detection of SMN protein expression and cellular localization by imaging flow cytometry

In this study, we report for the first time the use of imaging flow cytometry to assess the intracellular expression and localization of SMN protein in fibroblasts from healthy controls and SMA patients. Cells were labeled with the same mouse monoclonal FITC-conjugated anti-SMN antibody (2B1), and Hoechst 33342 nucleic staining, as described in materials and methods. There were significant differences in fluorescence intensity in the number of FITC-labeled SMN-positive cells between SMA cells and healthy controls (Fig. 3A). Moreover, the expression of SMN protein in SMA cells was clearly lower, compared to healthy controls. Nonetheless, at least 20–30% of cells were strongly positive for SMN as identified by a plotted histogram of FITC-SMN-positive cells (Fig. 3A). Furthermore, in order to investigate the accumulation area of SMN protein in healthy control and SMA cells, we used the BDI modulation to measure the distribution of SMN protein. The BDI features compute the intensity of localized bright spots within a masked area in the image where the background has been removed around the spots. SMN proteins are generally known to localize both within the cytoplasm and within nucleus, especially in nuclei foci called gems, where the SMN complex is composed. Our results from immunocytochemical analysis showed that the SMN protein accumulated in the nucleus and cytoplasm, and appeared as cellular dots in healthy controls (Fig. 1A). However, we could not detect SMN complex like gems or accumulation in our SMA cells under the fluorescent microscopic observation (Fig. 1A). On the other hand, using imaging flow cytometry, we also

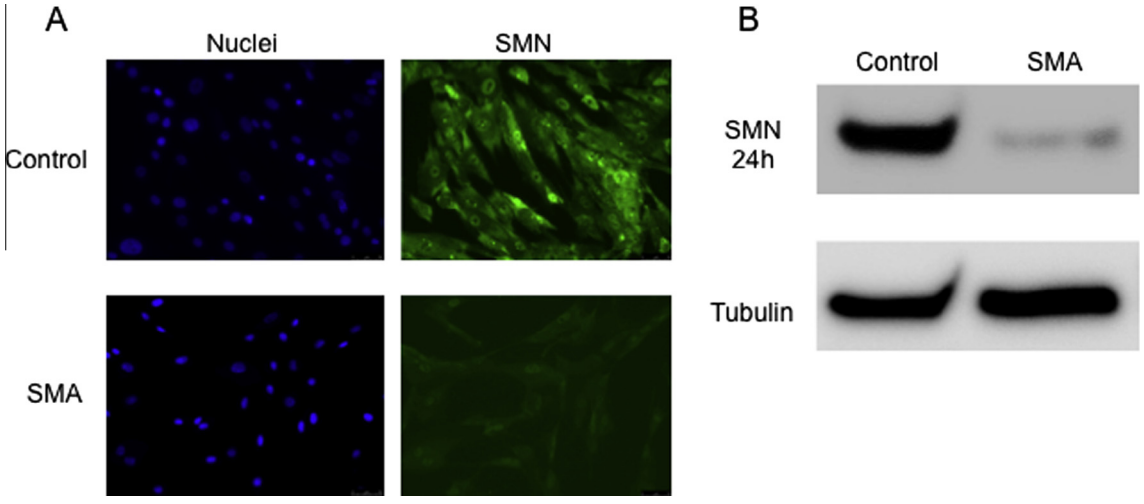

Fig. 1. Detection of SMN protein in human fibroblasts from healthy controls and type I SMA patient-derived fibroblasts. (A) Micrographs showing SMN protein immunocytochemistry. Cells were cultured for 24 h and then stained for SMN using a monoclonal anti-SMN antibody. SMN expression is reduced in SMA patient-derived fibroblasts when compared to healthy controls. (B) Detection of SMN protein by Western blotting analysis, using the same antibody as described above, shows reduced SMN expression in SMA patients.

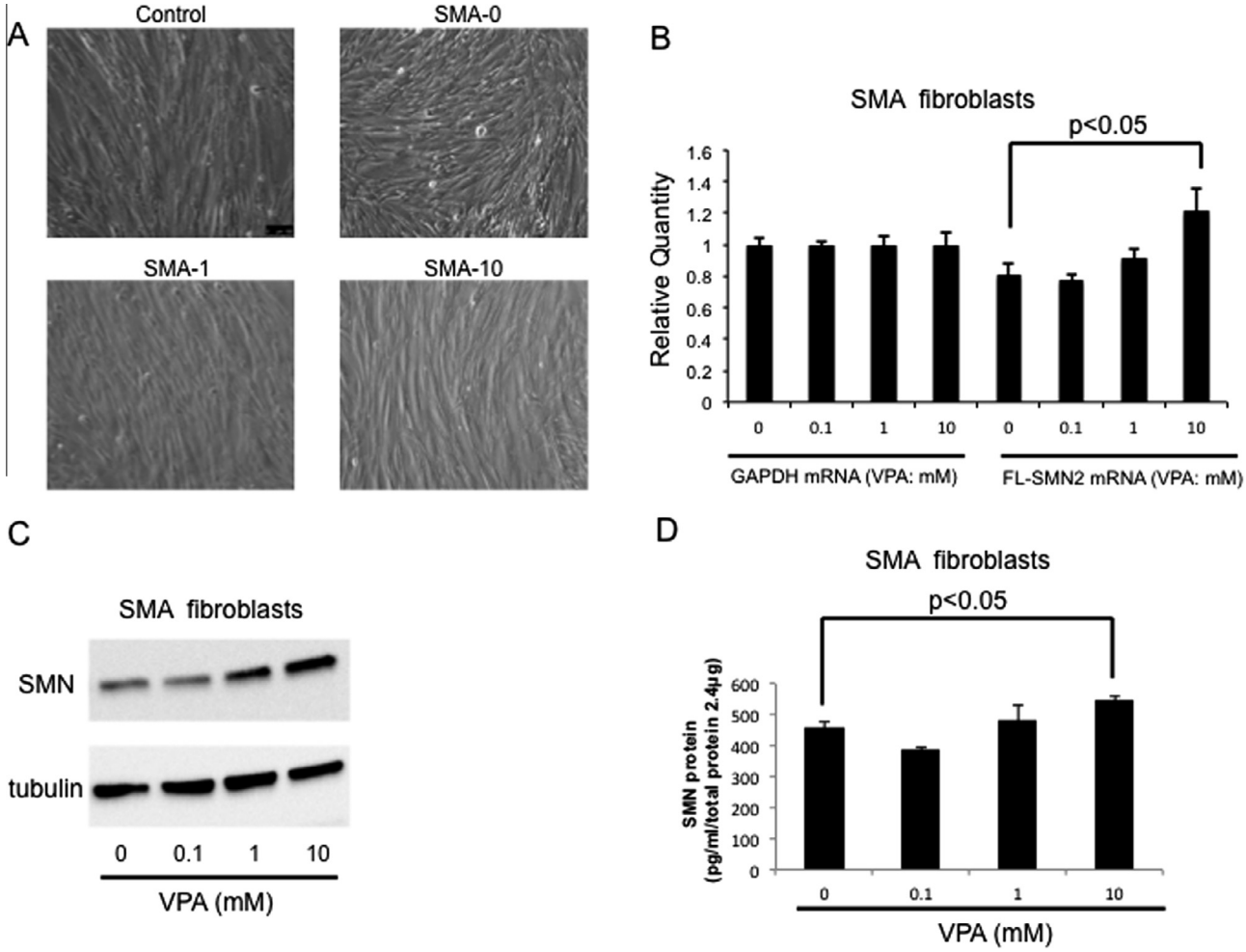

Fig. 2. Effects of VPA treatment on SMA patient-derived fibroblasts. (A) Effects on cellular morphology. Cells were treated with 0, 1, 10 mM VPA for 24 h. All treatments did not cause any morphological changes and any toxicity. (B) Real-time PCR analysis of *SMN2* mRNA expression after VPA treatment in SMA-derived fibroblasts. Cells were treated with 0, 0.1, 1, 10 mM VPA for 24 h, and then total RNA was extracted and subjected to real-time PCR. *GAPDH* was used as internal control gene. (C) Western blotting analysis of SMN protein expression after VPA treatment in SMA-derived fibroblasts. Cells were treated with 0, 0.1, 1, 10 mM VPA for 24 h and then total protein levels were analyzed by Western blotting. (D) ELISA data showing SMN protein expression in SMA-derived fibroblasts treated with VPA. Cells were treated with 0, 0.1, 1, 10 mM VPA for 24 h and then total protein was analyzed using an ELISA kit (Enzo Life Sciences). Error bars represent the mean ± S.D. obtained from three independent samples.

observed that the accumulation of SMN protein was significantly decreased in SMA cells (Fig. 4A and B).

3.4. Evaluation of SMN protein expression and cellular localization in VPA-treated SMA patient fibroblasts using imaging flow cytometry

To investigate the change of SMN protein expression and cellular localization in SMA patient-derived fibroblasts treated with VPA, we used imaging flow cytometry technology to detect SMN protein-positive cells by a specific anti-SMN antibody as described above. In this technology, data from SMA cells exhibited a significant increase in the total SMN protein amount, translated from each *SMN2* locus, under dose-dependent VPA-treatments (Fig. 3B–D). In addition, BDI analysis showed that VPA treated-cells were not only increasing SMN protein levels, but also SMN was accumulating in the nucleus and cytoplasm (Fig. 4C–E). Using imaging flow cytometry, we could first detect significant increases in SMN protein accumulation in discrete nuclear foci after VPA treatment (Fig. 4C–E).

4. Discussion

In this study, we developed a new method of SMN protein evaluation using imaging flow cytometry. This method can be easily and clearly detect SMN protein levels in healthy human fibroblasts and type I SMA patient-derived fibroblasts. In our preclinical studies 24 h after VPA treatment, the endogenous full-length *SMN2* mRNA and SMN proteins derived from full-length *SMN2* mRNA were significant increased in type I SMA patient-derived fibroblasts. VPA treatment may stimulate the transcriptional system of *SMN2*. The SMN protein expression, therefore, increased in VPA-treated SMA cells. These results indicate that VPA may serve as a promising therapeutic candidate for SMA.

Using imaging flow cytometry analysis, we found that SMA patient fibroblasts clearly expressed SMN protein at least at 20–30% of normal levels. In mammalian neural cells and tissues, SMN protein immunohistochemistry shows heterogeneous staining [21]. Therefore, the imaging flow cytometry analysis can be used to examine the population of SMN protein-positive cells acquired from SMA patients. Moreover, we first demonstrated that VPA-dependent SMN protein expression was also significantly increased, resulting in the accumulation of SMN to the cell nucleus as shown by BDI analysis. Our results suggest that the imaging flow cytometry system can play a role as a novel evaluation tool of SMN protein analysis for clinical studies in SMA.

The SMN protein is considered as the most suitable and sensitive molecular biomarker for SMA by many researchers. So far, several techniques have been used for SMN protein quantification.

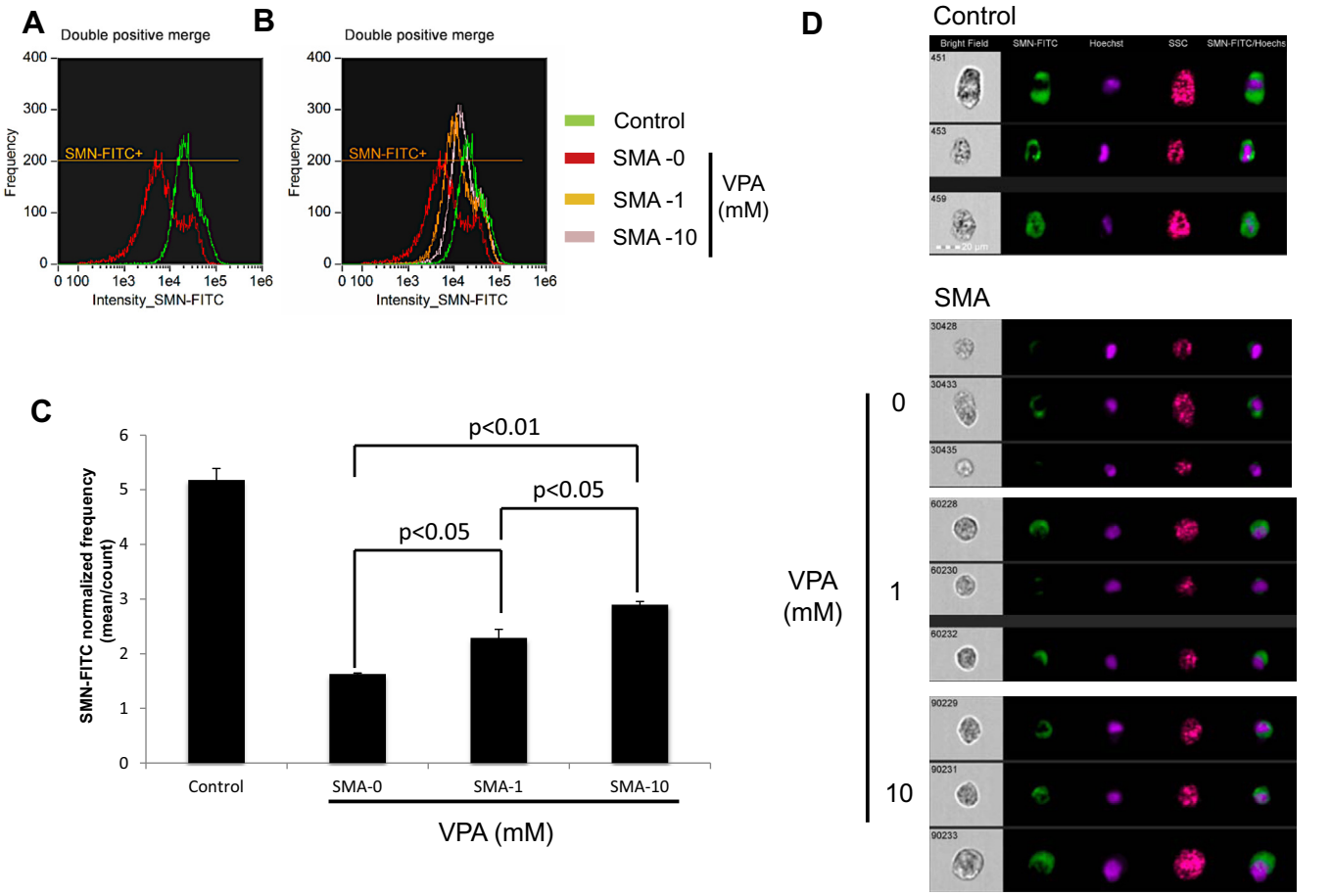

Fig. 3. SMN protein analysis by imaging flow cytometry. (A) Cells were cultured for 48 h, and then trypsinized and stained for SMN protein using a monoclonal FITC-conjugated anti-SMN antibody. Histogram represents the mean FITC intensity versus frequency (count). (B) Detection of SMN protein expression by imaging flow cytometry analysis after treatment with VPA (0, 1, 10 mM) for 24 h. The SMN protein was increasing in a dose-dependent manner. (C) Evaluation of SMN protein expression after VPA treatment. Values are represented as FITC-SMN normalized frequency (mean/count). (D) Cellular localization of SMN protein by imaging flow cytometry. FITC-SMN (green), Hoechst 33342 (blue), side scatter (red) and bright-field digital images are shown for human healthy controls and SMA-derived fibroblasts untreated or treated with VPA. SMN staining is clearly visible in the cytoplasm and nucleus. Error bars represent mean ± S.D. obtained from three independent samples.

Western blot analysis, which was used in *in vitro* and *in vivo* studies, mainly aimed at evaluating possible variations of SMN protein levels related to pharmacological treatments [6]. However, this assay has several limitations, related to its semi-quantitative nature, thus requiring normalization versus housekeeping proteins, whose levels are subject to wide variations.

Kolb et al. developed an immunoassay suitable for total SMN protein quantification in PBMC, through which they could demonstrate a correlation with the number of *SMN2* copies [22]. However, they found a reduction in SMN levels only in PBMC of type I SMA patients, and they could not find any correlation between protein levels and phenotypic severity [22]. These findings clearly question the meaning of quantifying SMN protein levels in clinical trials. Generally, ELISA is considered more sensitive and adequate for protein quantification since it does not require normalization to other proteins, given that SMN levels are quantified with respect to a standard curve constructed with serial dilutions of purified protein. To date, these assays have been developed and validated [7–10]. These authors showed that their assay is sufficiently sensitive to measure SMN variations, related to a candidate drug treatment, and found that SMN protein levels in PBMC of SMA patients show a tendency to be reduced, compared to healthy controls [7–10]. Although these results are promising, the small number of samples analyzed, the absence of age-matched controls, of a placebo arm, and of clinical-molecular correlations, do not allow firm conclusions to be drawn on the validity of SMN protein dosage in clinical trials. SMA is a phenotypically heterogeneous disorder with variable disease onset and severity, which creates a series of issues in the design of clinical trials. Sensitive and accurate biomarkers are, therefore, needed that can be used as predictive, prognostic, and surrogate endpoint measures.

SMN protein, as a biomarker or surrogate outcome measure, presents some technical issues that need to be taken under consideration in the context of clinical trials. For example, the acquisition of SMA patient fibroblasts is an invasive procedure. On the other hand, obtaining peripheral whole blood cells and PBMC is a less invasive process, and more suitable samples for imaging flow cytometry, although peripheral blood draws are often hard to obtain from very young patients.

Moreover, with the imaging flow cytometry analysis, quantification of SMN accumulation could be considerably evaluated in intact cells, by using an algorithm of bright detail intensity. Generally, increases in gem numbers related to SMN complexes, were counted as gems per 100 cell nuclei [16]. In fact, our analysis may not only be reliable and beneficial for the evaluation of SMN protein expression, but also for the quantification of gems without counting cell nuclei. Therefore, our results suggest that imaging flow cytometry analysis can play a role as a novel tool for the evaluation of intact protein expression and localization of biologically active molecules, like the SMN protein.

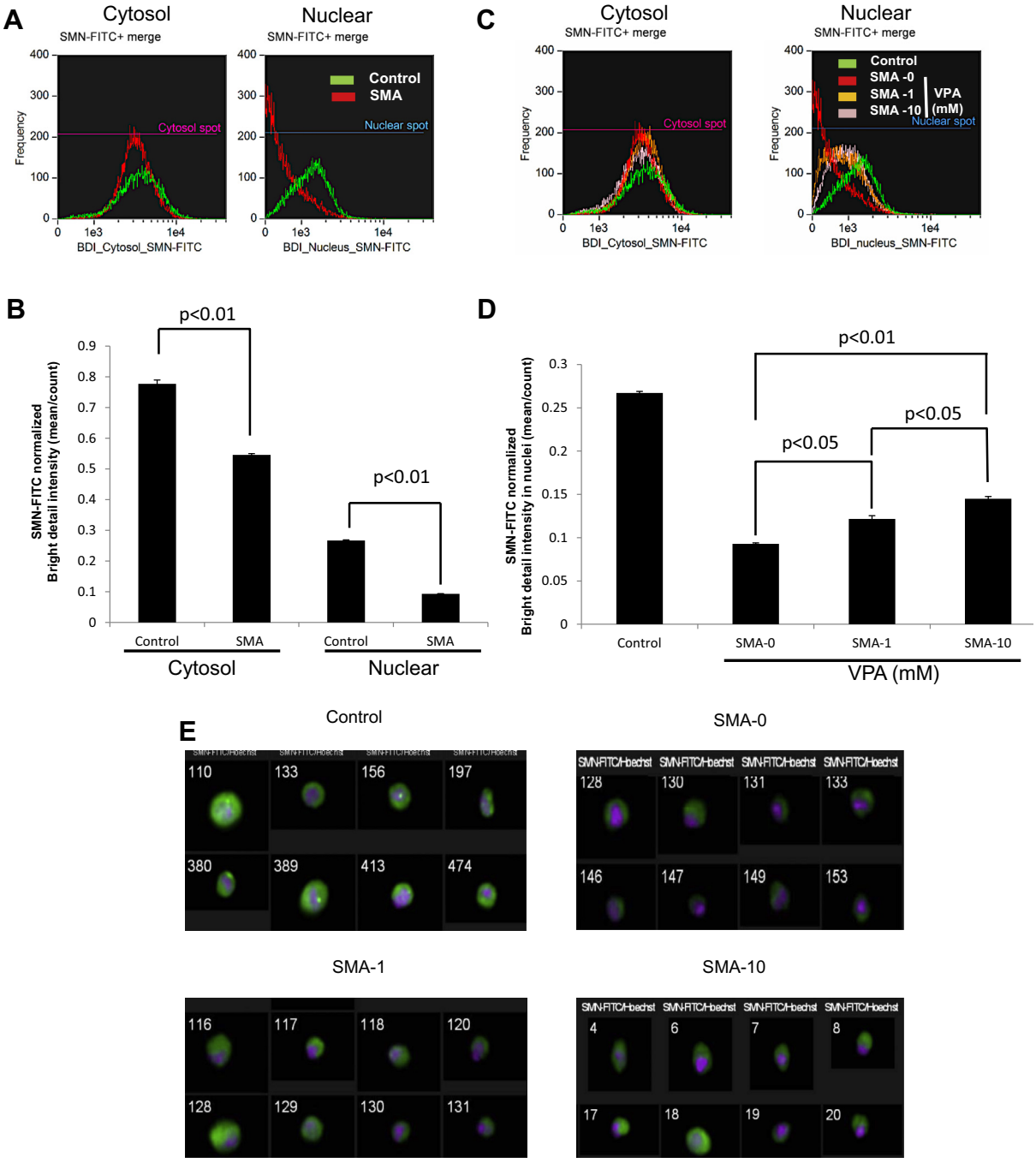

Fig. 4. Cytosolic and nuclear localization of SMN protein evaluated by imaging flow cytometry. (A) Comparison of bright detail intensity in the cytosol or nucleus of human healthy controls (green) and SMA patient-derived fibroblasts (red). Cells were cultured for 48 h and then subjected to imaging flow cytometry analysis. (B) Results show a significant decrease in SMN protein both in the cytosol and in nucleus in SMA patient-derived fibroblasts. (C) Effects of the SMN protein localization and accumulation on VPA-treated SMA patient-derived fibroblasts. Cells were treated with VPA (0, 1, 10 mM) for 24 h and then subjected to imaging flow cytometry analysis. Histogram represents cytosol spots or nuclear spots. (D) Accumulation of SMN protein in the nucleus of VPA-treated SMA patient-derived fibroblasts. Values are represented as FITC-SMN normalized bright detail intensity (mean/count). (E) Fluorescent micrographs showing localization of SMN protein by imaging flow cytometry. FITC-SMN (green) and Hoechst 33342 (blue) merged digital images are exhibited in human healthy control and SMA-derived fibroblasts treated with VPA (0, 1, 10 mM) respectively. Error bars represent mean ± S.D. obtained from three independent samples.

The imaging flow cytometry technique is a novel approach to qualitative and quantitative assessment of SMN protein expression in healthy human controls and SMA patient fibroblasts. The addition of digital images to standard quantitative and statistical measurements makes this the most sensitive flow cytometry method available for the assessment of cellular SMN accumulation and localization. We believe that imaging flow cytometry has a place as a first-line technique to assess the molecular genetic phenotype of cells acquired from SMA patients for clinical trials.

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