# Molecular analysis of SMARD1 patient-derived cells demonstrates that nonsense-mediated mRNA decay is impaired

## INTRODUCTION

Biallelic mutations in the immunoglobulin $\mu$-binding protein 2 (IGHMBP2) gene lead to motor neuron (MN) degeneration in the brain stem and anterior horns of the spinal cord, causing fatal spinal muscular atrophy with respiratory distress type I (SMARD1). Patients exhibit a certain degree of phenotypic variability that has not been explained.[1] No effective therapy is currently available, and understanding the function of IGHMBP2 is crucial for identifying specific disease targets.

IGHMBP2 is a DNA/RNA helicase protein involved in different cellular processes, but its precise function is unknown. IGHMBP2 exhibits similarities to the human regulator of nonsense transcripts homolog UPF1,[2] which is part of the core complex required for nonsense-mediated mRNA decay (NMD), a translation-dependent RNA degradation pathway implicated in different subtypes of amyotrophic lateral sclerosis (ALS).[3]

We analysed fibroblasts, induced pluripotent stem cells (iPSCs), and their derived MNs from eight patients with SMARD1 carrying different IGHMBP2 mutations. All cell types exhibited a marked deficiency in IGHMBP2 protein but not mRNA. We further demonstrated that the IGHMBP2 transcript is regulated by the NMD pathway, which resulted inhibited in SMARD1 condition.

## RESULTS

Our eight SMARD1 patients are summarised in online supplemental table 1. The identified mutations included four missense and four nonsense mutations, three point deletions, one inversion and one insertion mutation (figure 1A). We collected peripheral blood mononuclear cells and/or fibroblasts from the patients and three unaffected subjects (online supplemental table 2). We successfully reprogrammed iPSCs from four patients (online supplemental figure 1A) and differentiated them into MNs (online supplemental figure 1B) that exhibited pathological features of increased apoptosis and decreased axon length (online supplemental figure 1C,D).

Western blot analysis of MNs, fibroblasts and iPSCs from patients and controls (online supplemental figure 2) showed three migration bands specific for IGHMBP2 (~110 kDa,~75 kDa and ~55 kDa). Online supplemental figure 3 summarises the data regarding protein isoforms. Only the ~110 kDa band corresponded to the full-length and functioning IGHMPB2 protein[4]; it was significantly reduced in all SMARD1 samples (figure 1B; online supplemental figure 2) and nearly absent in cell lines with nonsense mutations. Immunofluorescence confirmed the western blot data in MNs (figure 1C) and iPSCs (online supplemental figure 4), with no difference in localisation. Interestingly, our analysis suggested that the reduction in IGHMBP2 was not the result of decreased mRNA (figure 1C; online supplemental figure 5A).

To determine whether the upregulation of IGHMBP2 mRNA in SMARD1 was attributable to impaired mRNA turnover, we evaluated the efficacy of IGHMBP2 mRNA decay after transcriptional inhibition in iPSCs. The ratio of mRNA before and after actinomycin D treatment was increased in SMARD1 iPSCs (online supplemental figure 5B), suggesting an impairment of IGHMPB2 transcript degradation. The treatment of SHSY-5Y neuroblastoma cells and control iPSCs with cycloheximide (CHX), which indirectly inhibits NMD by blocking translation, induced an increase of IGHMBP2 mRNA levels suggesting NMD regulation of IGHMBP2 transcript (online supplemental figure 5C).

We observed an increase in the abundance of a set of NMD target genes in SMARD1 MNs (figure 1E) and iPSCs (online supplemental figure 6A) compared with controls. Remarkably, the NMD-activating compound tranilast significatively decreased IGHMBP2 expression in SMARD1 MNs (figure 1G), and iPSCs (online supplemental figure 5D) and rescued the mRNA accumulation of some NMD targets both in MNs (figure 1F) and in iPSCs (online supplemental figure 6B). Importantly, NMD reactivation was also able to significantly rescue pathological MN hallmarks (figure 1H1). Moreover, in control iPSCs, NMD inhibition by CHX induced a variable increase in NMD-sensitive transcript isoforms (hnRNPL and TRA2B), which was less steep in SMARD1 iPSCs (online supplemental figure 7).

## DISCUSSION

SMARD1 is a rare but fatal disease with onset in early childhood. It affects the lower MNs, causing distal limb paralysis and respiratory distress. In the present study, we described eight new SMARD1 cases and reported updated data for two previously described cases. Given the rarity of this disease, this represents a substantial cohort of SMARD1 patients. Our results confirmed reduced expression of full-length IGHMBP2 protein (to<5%) in all cell types. In cases involving nonsense mutations, IGHMBP2 was absent, whereas the protein was mildly reduced in the presence of a missense mutation.

We also demonstrated that very low IGHMBP2 protein generally predicts a severe phenotype. However, SMARD1 patients did not have significantly reduced IGHMBP2 mRNA levels, confirming previous findings.[1] We demonstrated that IGHMPB2 mRNA is regulated by NMD, a mechanism that eliminates mRNAs containing premature translation–termination codons, but also regulates the expression of a large number of genes and that NMD is impaired in SMARD1. Several mRNAs that are normally target of NMD were upregulated in SMARD1 iPSCs and MNs, and these changes were rescued by NMD pathway reactivation. Thus, NMD is emerging as a critical regulator of neuronal development, MN viability and axon growth. Insufficient NMD may indeed underlie neurodegeneration, such as in ALS.[3 5] Therefore, it is conceivable that NMD deficiency represents a pathogenic mechanism for SMARD1, causing accumulation of aberrant defective mRNA to which MNs are particularly sensitive. NMD rescue can reestablish the mRNA balance in MNs improving their pathological phenotype. Importantly, reactivation of the NMD pathway was able to rescue axon length and apoptosis in affected MNs, supporting the NMD pathway as a potential target, as previously suggested for other MN disorders.[3] Therefore, further investigations of drugs that can rescue NMD activity for potential therapeutic use in inherited motor neuropathies that share the same pathological molecular mechanism are warranted.

## METHODS

### Cell culture

Human samples were reprogrammed into iPSCs using the CytoTune-iPS V.2.0 Sendai Reprogramming Kit (Life Technologies). Spinal MNs were then obtained following a rapid multistage protocol.

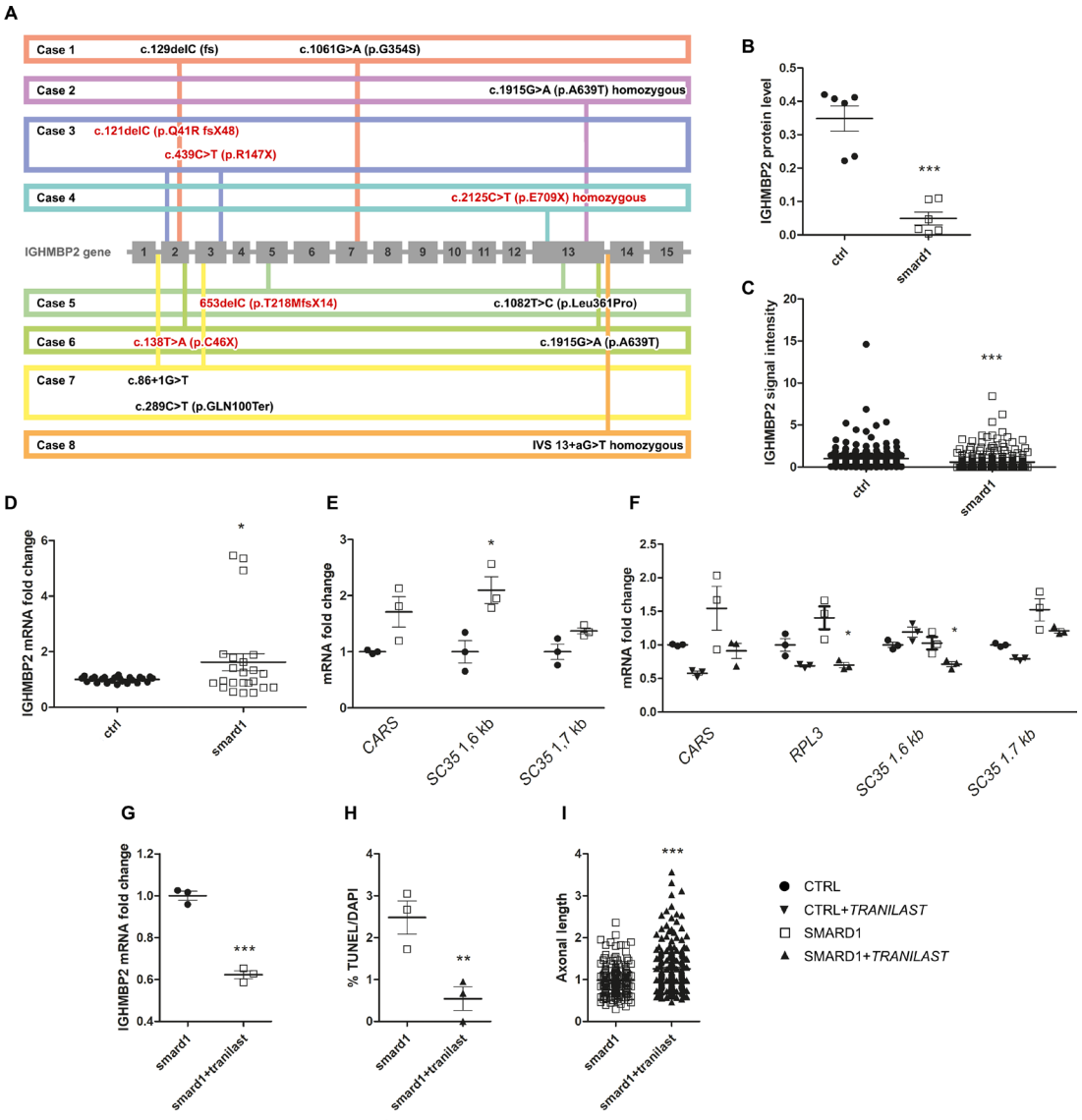


**Figure 1** IGHMBP2 levels and nonsense-mediated mRNA decay (NMD) decay in hiPSC-derived motor neurons (MNs). (A) Schematic representation of the distribution along the immunoglobulin μ-binding protein 2 (IGHMBP2) gene of mutations found in the patient cohort. STOP codon mutations are indicated in red. (B) 110 kDa IGHMBP2 protein, assessed by western blot, decrease in spinal muscular atrophy with respiratory distress type I (SMARD1) MNs versus ctrl (***p<0.001, Student's t-test). (C) Immunocytochemistry quantification confirmed lower levels of IGHMBP2 in affected MNs ***p<0.001, *p<0.05, Student's t-test, ctrl versus patients (smard1, case 2, 3 and 6). (D) qPCR analyses of IGHMBP2 mRNA levels in affected MNs showed no correlation with protein level reduction, increasing in SMARD1 lines,*p<0.05, Student's t-test, ctrl versus patients. (E) mRNA levels of NMD target genes were increased in SMARD1 MNs versus ctrl (**p<0.01, Student's t-test). (F) RNA levels of NMD target genes were decreased after tranilast treatment in SMARD1 MNs (smard1, case 2, 3 and 6) versus ctrl (*p<0.05, Student's t-test). (G) mRNA levels of IGHMBP2 were rescued after treatment with tranilast (5 μM) in SMARD1 MNs, ***p<0.001, Student's t-test. (H,I) Treatment with the activator of NMD tranilast (5 μM) rescued pathological hallmarks of SMARD1 MNs (smard1, case 2, 3 and 6), namely apoptosis evaluated by tunel assay (E, **p<0.01, Student's t-test) and axon length reduction (F, ***p<0.001, Student's t-test). In B, E–H, each data point represents the mean obtained from three technical replicates for each biological replicate (n=3, smard1: case 2, 3 and 6). In D, each data point represents a technical replicate (biological replicates n=3 for ctrl, n=4 for smard1, case 2, 3, 6 and 7). In C and I, each point represents data from a single cell. Values are presented±SEM. All the images are original and made by the authors.

The iPSCs and/or MNs were used in standard western blot analysis, and underwent immunohistochemistry for anti-human IGHMBP2 (Millipore) and SMI-32 (Millipore) and the terminal deoxynucleotidyl transferase dUTP nick end labelling system protocol (Promega). IGHMBP2 expression was evaluated by standard TaqMan qPCR assay. For transcripts known to be regulated by NMD, SYBR Green Real Time PCR was used.

The iPSCs and/or MNs were treated with transcription inhibitor actinomycin D at 2.5 μg/mL, with 100 μg/mL CHX for 6 hour and 5 μM tranilast (T0318-10MG) for 24 hours.

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