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Research article

Evaluation of the effect of c.2946 + 1G > T mutation on splicing in the *SCN1A* gene

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ABSTRACT

Mutations in the *SCN1A* gene have commonly been associated with a wide range of mild to severe epileptic syndromes. They generate a wide spectrum of phenotypes ranging from the relatively mild generalized epilepsy with febrile seizures plus (GEFS+) to other severe epileptic encephalopathies, including myoclonic epilepsy in infancy (SMEI), cryptogenic focal epilepsy (CFE), cryptogenic generalized epilepsy (CGE) and a distinctive subgroup termed as severe infantile multifocal epilepsy (SIMFE). The present study was undertaken to investigate the potential effects of a transition in the first nucleotide at the donor splice site of intron 15 of the *SCN1A* gene leading to CGES. Functional analyses using site-directed mutagenesis by PCR and subsequent *ex-vivo* splicing assays, revealed that the c.2946 + 1G > T mutation lead to a total skipping of exon 15. The exclusion of this exon did not alter the reading frame but induced the deletion of the amino acids (853 Leu –971 Val) which are a major part in the fourth, fifth and sixth transmembrane segments of the SCN1A protein. The theoretical implications of the splice site mutations predicted with the bioinformatic tool human splice finder were investigated and compared with the results obtained by the cellular assay.

1. Introduction

Mutations that affect splicing have been shown to account for up to half of disease-causing gene alterations (Ars et al., 2000). Since longer proteins are more likely to be involved in genetic disorders than shorter proteins and disease genes have, on average, a longer coding sequence and a higher number of introns than genes not causing recognizable phenotypes, it was hypothesized that splicing mutations may represent the most frequent cause of hereditary disease (Lopez-Bigas et al., 2005). In addition, mutations elsewhere in the consensus sequences or in splicing enhancers or silencers, affect splicing with various degrees, it is now recognized that a wide range of mutations, which can be localized not only in introns but also in exons, may affect these signals either directly (disruption of constitutive splice sites) or indirectly (creation of cryptic splice sites). Many examples have been reported of nucleotide substitutions being a cause of aberrant splicing in human *SCN1A* gene (Harkin et al., 2007; Depienne et al., 2006; Gennaro et al., 2006). Several links have recently been made between epileptic phenotypes and mutations in voltage-gated sodium channel genes. Mutations in the *SCN1A* gene have, for instance, been associated with a wide spectrum of epileptic phenotypes ranging from relatively mild phenotypes such GEFS+ to more severe forms such as CGE.

Organized into 26 exons, the Nav1.1 co-exists in three isoforms, two of them differ by 33 or 84 base pairs in the 3′ end of exon 11, resulting in 11 or 28 amino acid difference compared to full-length isoform that encodes for a 2009 amino acid protein. The variance in length stems from alternative splice junctions at the end of exon 11 that produce a full-length isoform or two shortened versions based on the number of base pairs deleted referred to as Nav1.1 [−33] and Nav1.1[−84] which omit residues 671–681 or 654–681, respectively (Lossin et al., 2002). The protein sequence published by Escayg et al. (2000) contains an additional 11 amino acids at the 3′ end of exon 11 in the larger isoform, which encode part of the

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cytoplasmatic loop between DI and DII. The relative expression of each isoform in neural tissue is not known. Recently, the complete cDNA and protein sequence of *SCN1A* was submitted to GenBank (accession numbers AF225985 and AAK00217). Splice variant 1 (GenBank accession number AB093548) refers to the full-length isoform encoding for a 2009 amino acid protein, and splice variant 2 (GenBank accession number NM_006920) refers to the shorter isoform encoding for a protein of 1998 amino acids. Different *SCN1A* mutations seem to alter sodium-channel kinetics in different ways (Sugawara et al., 2003). Recent studies, using human *SCN1A* mutant channels transfected into HEK293 cells, showed that the mutant sodium channels bearing SMEI missense or nonsense mutations were markedly deficient in passing sodium current, suggesting a loss of function in the aberrant channels. Whereas the pathogenic effect of a truncating mutation is clear, the postulated pathogenicity of splice-site mutations falling within these segments needs functional demonstration. This study aimed to use a site-directed mutagenesis by PCR and *ex-vivo* splicing assays to investigate the effect of the c.2946 + 1G > T mutation in the *SCN1A* gene previously associated with cryptogenic generalized epilepsy (Harkin et al., 2007).

2. Materials and methods

2.1. Mutagenesis

The mutant *SCN1A* (exon 15) and its intron boundaries were constructed by PCR mutagenesis using a series of mutagenic primers under standard conditions. Sequence mismatches were introduced as to create *Bst*EII and *Nhe*I restriction sites, respectively (underlined). Two non-mutagenic external primers, SCN1AE15F: 5′AGACGGTTACCGCAGATCAG3′ and SCN1AE15R: 5′ACTGCTAGCTTGCTGTGTTG3′, and two complementary internal primers containing the desired mutation, SCN1AMF15: 5′CCTAGTGTTATGTACCCACT3′, and the reverse primer, SCN1AMR15: 5′AGTGGGTACATAACACTAGG3′, were also constructed (the substitution nucleotides are underlined). For each mutation, two separate PCRs were performed and the resulting fragments were extracted separately. A third amplification was then carried out with a mixture containing those fragments in the presence of the external primers. The final PCR was performed in 50 μl with 0.1 μg DNA, 5 μl of 10× buffer (50 mM Tris–HCl, pH 9.2, 160 mM $(NH_4)_2SO_4$, 22.5 mM $MgCl_2$, 2% DMSO, and 1% Tween 20), 10 mM dNTP, 20 pmol of each primer and 0.5 unit of Go Taq DNA polymerase (Go Taq Promega). PCR conditions were as follows: 5 min at 95 °C followed by 35 cycles, each consisting of: 45 s at 95 °C, 45 s at 58 °C and 45 s at 72 °C, then a final elongation at 72 °C for 10 min was required. The final PCR product (715 bp) was purified using a gel band purification kit (Promega). The presence of mutations was confirmed by DNA sequencing.

2.2. Cloning of wild type and mutant mini-genes

The PCR products were digested with *Bst*EII and *Nhe*I restriction endonucleases, and inserted at the *Bst*EII/*Nhe*I site of the splicing cassette p(13,17)/CMV, that has been designed to contain the 2 adjacent constitutive exons 13 and 17 of human 4.1R gene with their downstream and upstream flanking intron sequences, respectively (Deguillien et al., 2001). After the subcloning of the mutant and wild type constructs, transformation was performed with TOP 10 competent cells (Invitrogen). The cells were grown to an optical density of 0.8 at 37 °C. The cassette and the WT and mutant inserts were fully sequenced to ensure the absence of additional mismatches resulting from PCR or cloning errors. The size of recombinant cassette was 1255 bp, a feature that was used to distinguish the recombinant from the native plasmids.

2.3. Cell transfection and cDNA generation

Total RNA was extracted from the Hela cells using the same protocol previously described by Ben Mahmoud et al. (2013).

2.4. Bioinformatics prediction

The effects of the c.2946 + 1G > T mutation on pre mRNA splicing was first assessed using the consensus score calculation method to quantify the influence of this mutation on the formation of splicing loops (Carmel et al., 2004) using the corresponding software available online at (http://www.umd.be/HSF/). The consensus sequences are (AG | ***GU***RAGU and Y***AG*** | RNNN for the donor splice site and the acceptor splice site, respectively, R = purine, Y = pyrimidine, N = nucleotide (Parent and Bisaillon, 2006). The mutation designations are based on SCN1A transcript ENST00000303395 from Ensembl. A in the ATG start codon is defined as c.1. The relative strength of the splice sites obtained from the bioinformatics tool is given as the consensus value (CV), which ranges from 0 to 100. Splice sites with CVs higher than 80 are strong splice sites; less strong sites have CVs that range from 70 to 80. Splice sites with CVs of 65 to 70 are weak, as only a few of these sites are active (Desmet et al., 2009). Hence, splice sites with CVs

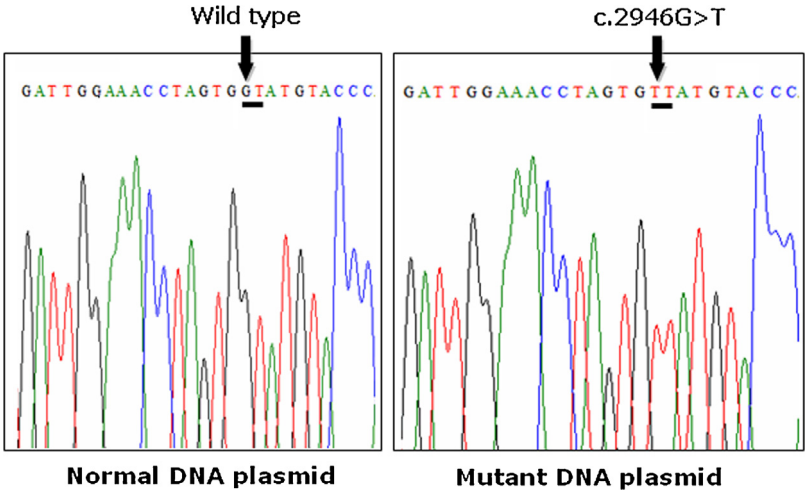

Fig. 1. Sequencing chromatograms showing the presence of the c.2946G > T mutation in the mutant DNA plasmid and its absence in a wild type.