ORIGINAL ARTICLE

Identification and characterization of *NF1* splicing mutations in Korean patients with neurofibromatosis type 1

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Neurofibromatosis type I (NF1) is an autosomal dominant genetic disorder caused by *NF1* mutations. Although mutations affecting mRNA splicing are the most common molecular defects in NF1, few studies have analyzed genomic DNA (gDNA)–mRNA correlations in Korean NF1 patients. In this study, we investigated 28 unrelated NF1 patients who showed splicing alterations in reverse transcription-PCR of *NF1* mRNA and identified 24 different *NF1* splicing mutations, 9 of which were novel. These mutations can be categorized into five groups: exon skipping resulting from mutations at authentic 5' and 3' splice sites (type I, 46%), cryptic exon inclusion caused by deep intronic mutations (type II, 8%), creation of new splice sites causing loss of exonic sequences (type III, 8%), activation of cryptic splice sites due to disruption of authentic splice sites (type IV, 25%) and exonic sequence alterations causing exon skipping (type V, 13%). In total, 42% of all splicing mutations did not involve the conserved AG/GT dinucleotides of the splice sites, making it difficult to identify the correct mutation sites at the gDNA level. These results add to the mutational spectrum of *NF1* and further elucidate the gDNA–mRNA correlations of *NF1* mutations.

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INTRODUCTION

Neurofibromatosis type 1 (NF1; Online Mendelian Inheritance in Man (MIM) 162200) is an autosomal dominant inherited disease that primarily affects the skin and nervous system.¹ NF1 is one of the most common genetic disorders in humans, with a birth incidence of ~1 in 2500 to 1 in 3000, half of which are sporadic cases.^{1,2} The main clinical features of NF1 are café au lait spots, Lisch nodules, inguinal and/or axillary freckling, cutaneous neurofibromas and a predisposition to develop benign and malignant nervous system tumors.¹

NF1 is caused by loss-of-function mutations in a tumor suppressor gene, *NF1* (*neurofibromin 1*; MIM 613113), located on chromosome 17q11.2, that consists of 60 constitutive and various alternative exons over 350 kb of genomic DNA (gDNA).¹ Previous studies have reported over 2000 different mutations in this gene. Many of these mutations arise *de novo*, as there is no clear hot spot.³

The majority of *NF1* mutations are predicted to produce a truncated form of the NF1 protein, and ~30–50% of these mutations cause splicing alterations affecting mRNA processing.^{4,5} The frequency of splicing errors in *NF1* is very high compared with other genetic disorders.² In Korea, a few studies have been conducted to determine the mutational spectrum of *NF1*, but there are few data available on

splicing alterations.^{6–9} In the present study, we performed mutation analysis of NF1 patients at both the gDNA and mRNA levels in order to reveal gDNA–mRNA correlations.

MATERIALS AND METHODS

Study subjects

We retrospectively analyzed *NF1* testing results in patients referred to a tertiary referral hospital in Korea between January 2011 and August 2015 with clinical suspicion or a differential diagnosis of NF1. We enrolled 28 unrelated NF1 patients who showed splicing alterations in reverse transcription-PCR of *NF1* mRNA. This study was approved by the institutional review board of Samsung Medical Center.

NF1 mutation analysis

Genomic DNA and RNA were extracted from peripheral blood leukocytes using standard protocols. Reverse transcription-PCR and complementary DNA sequencing were performed to screen for splicing alterations and coding region mutations of *NF1*. *NF1* complementary DNA was amplified in 24 overlapping fragments by PCR using primer sets designed by the authors (Supplementary Table S1). PCR was performed on a Thermal Cycler 9700 (Applied Biosystems, Foster City, CA, USA) as follows: denaturation at 94 °C for 5 min, 32 cycles at 94 °C for 30 s, at 60 °C for 30 s and at 72 °C for 30 s, and a final extension at 72 °C for 1 min. The size of the PCR products was verified by electrophoresis in 2% agarose gels. The PCR products were sequenced on an ABI 3730xl capillary

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sequencer (Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems). Sequences were analyzed using Sequencher software (Gene Codes, Ann Arbor, MI, USA) and were compared with the longest isoform of the reference sequence for *NF1* (NM_001042492.2). If a variant or a skipped exon was detected in complementary DNA analysis, the corresponding exonic and intronic regions of gDNA were sequenced to identify the variants that caused splicing alterations. All mutations and their putative effect at the protein level have been renamed according to the Human Genome Variation Society guidelines.

Classification criteria for identified *NF1* splicing mutations

We adopted the classification system of *NF1* splicing mutations proposed by Wimmer *et al.*⁵ The splicing variants were classified into five categories: type I, mutations in 5' and 3' splice sites resulting in exon skipping; type II, deep intronic mutations that result in inclusion of cryptic exons; type III, mutations within exons that create new splice sites and result in deletion of part of an exon; type IV, mutations inactivating the canonical 5' and 3' splice sites and promoting the use of cryptic exonic or intronic splice sites, leading to partial deletion of an exon or partial insertion of an intron, respectively; and type V, exonic mutations that cause exon skipping.

In silico prediction of *NF1* splicing mutations

The Human Splicing Finder (HSF) system is an online bioinformatics tool for predicting splicing signals (<http://www.umd.be/HSF/>).¹⁰ This software combines several different algorithms such as MaxEntScan (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html), ESEfinder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/ese_finder.cgi?process=home) and RESCUE-ESE (<http://genes.mit.edu/burgelab/rescue-ese/>) to identify and predict the mutation effect on splicing motifs including canonical splicing sites and auxiliary sequences known to either enhance (exonic splicing enhancers (ESEs)) or repress (exonic splicing silencers) splicing. We applied the HSF system to our

set of splicing mutations to determine the accuracy of the program for predicting experimentally detected mRNA effects.

RESULTS

NF1 mutations producing splicing alterations

As shown in Table 1, a total of 24 different *NF1* splicing mutations were identified in 28 unrelated patients, 9 of which have not been reported previously (38%, 9/24). Most mutations were unique (88%, 21/24), although 3 (13%, 3/24) were present in more than one patient: c.6855C>A (3 patients), c.4333-2A>G (2 patients) and c.1466A>G (2 patients). The majority of the splicing mutations involved the conserved AG/GT dinucleotides of the splice sites (58%, 14/24). The remaining mutations were found outside the intron–exon boundaries, such as deep intronic mutations and exonic mutations (42%, 10/24).

Mutation effect on mRNA processing

NF1 splicing mutations can be classified into five categories according to the observed effect on mRNA processing (Table 1). Several representative examples of type II–V splicing mechanisms are shown in Figure 1.

Mutations in intronic sequences (types I, II and IV)

A total of 19 splicing mutations were found in intronic sequences (79%, 19/24). Among them, exon skipping (type I) was the most common (58%, 11/19). Two deep intronic mutations (type II), which induce the insertion of a fragment of an intron that is recognized as a cryptic exon, were identified (11%, 2/19). The use of a cryptic splice site (type IV) was observed in six cases (32%, 6/19) that induced either the deletion of part of an exon (26%, 5/19) or the insertion of part

Table 1 Twenty-four *NF1* mutations and their effects on mRNA in 28 unrelated *NF1* patients

Class	Location	DNA mutation	Effect on mRNA	Predicted protein	Reference	Frequency ^a
Type I (N=11)	IVS6	c.655–2A>G	r.655_730del76	p.Ala219Asnfs*37	Disease database ^b	1
	IVS6	c.655–1G>A	r.655_730del76	p.Ala219Asnfs*37	Horn <i>et al.</i> ¹⁶	1
	IVS9	c.1062+1G>A	r.889_1062del174	p.Lys297_Lys354del	Eisenbarth <i>et al.</i> ¹⁷	1
	IVS13	c.1527+1G>T	r.1393_1527del135	p.Ser465_Cys509del	Pros <i>et al.</i> ¹³	1
	IVS13	c.1527+2T>C	r.1393_1527del135	p.Ser465_Cys509del	Disease database ^b	1
	IVS14	c.1642–2A>T	r.1642_1721del80	p.Ala548Leufs*13	This study	1
	IVS21	c.2851–6_2851-3del	r.2851_2990del140	p.Leu952Cysfs*22	Messiaen <i>et al.</i> ²	1
	IVS21	c.2851–1G>A	r.2851_2990del140	p.Leu952Cysfs*22	Sabbagh <i>et al.</i> ¹⁸	1
	IVS23	c.3113+1G>A	r.2991_3113del123	p.Tyr998_Arg1038del	Purandare <i>et al.</i> ¹⁹	1
	IVS32	c.4333–2A>G	r.4333_4430del98	p.Ile1445Valfs*4	Raygada <i>et al.</i> ²⁰	2
	IVS46	c.6921+4A>G	r.6820_6921del102	p.Ala2274_Lys2307del	This study	1
Type II (N=2)	IVS12	c.1392+754T>G	r.1392_1393ins1392+755_1392+824	Premature stop codon ^c	This study	1
	IVS38	c.5610–456G>T	r.5609_5610ins5610-538_5610-458	Premature stop codon ^c	This study	1
Type III (N=2)	E13	c.1466A>G	r.1466_1527del62	p.Tyr489*	Wimmer <i>et al.</i> ⁵	2
	E16	c.1748A>G	r.1722_1748del27	p.Ser574_Lys583delinsArg	Valero <i>et al.</i> ²¹	1
Type IV (N=6)	IVS2	c.204+1G>A	r.100_204del105	p.Val34_Met68del	Ars <i>et al.</i> ²²	1
	IVS2	c.204+1_204+4del	r.100_204del105	p.Val34_Met68del	This study	1
	IVS14	c.1642–10A>G	r.1641_1642ins1642-9_1642-1	p.Glu547_Ala548insLysIleGln	This study	1
	IVS21	c.2850+1G>C	r.2618_2850del233	p.Lys874Phefs*4	This study	1
	IVS36	c.4835+1G>A	r.4767_4835del69	p.Leu1590_Arg1612del	Wimmer <i>et al.</i> ⁵	1
	IVS36	c.4836–1G>A	r.4836delg	p.Arg1612Serfs*12	This study	1
Type V (N=3)	E46	c.6855C>A	r.6820_6921del102	p.Ala2274_Lys2307del	Robinson <i>et al.</i> ^{11,d}	3
	E46	c.6904C>T	r.6820_6921del102	p.Ala2274_Lys2307del	Upadhyaya <i>et al.</i> ^{12,d}	1
	E46	c.6921G>A (5' ss-1)	r.6820_6921del102	p.Ala2274_Lys2307del	This study	1

Abbreviations: E, exon; IVS, intervening sequence; *NF1*, neurofibromatosis type I; ss, splice site.

NF1 reference accession number: NM_001042492.2 (NP_001035957.1).

^aNumber of patients.

^bLeiden Open Variation Database https://grenada.lumc.nl/LOVD2/mendelian_genes/home.php?select_db=Nf1.

^cThe insertion of an intronic sequence leads to inclusion of an in-frame premature stop codon.

^dPreviously reported as a nonsense mutation.

of an intron (5%, 1/19). Except for two deep intronic mutations (type II), 53% of mutations were found at the 5' splice site (9/17), whereas 47% were found at the 3' splice site (8/17). The most common mutated nucleotides at the 5' and 3' splice sites were, in decreasing order, +1 (35%, 6/17), -1 (18%, 3/17) and -2 (18%, 3/17).

Mutations in exonic sequences (types III and V)

Five different mutations were identified in the coding region that affected mRNA splicing (type III, $n=2$; type V, $n=3$). Two of these exonic mutations were previously classified as nonsense mutations (c.6855C>A and c.6904C>T in type V),^{11,12} but actually altered the splicing of the exon itself.

In the two different type III mutations, the nucleotide change in the exonic region induced a new 5' splice site (c.1466A>G) or 3' splice site (c.1748A>G) that led to partial deletion of the exon. All type V mutations were identified in exon 46; among them, one mutation occurred in the last conserved G sequence of exon 46 (position -1 of the donor site), causing exon skipping (c.6921G>A). Of the two remaining mutations, c.6855C>A and c.6904C>T, neither altered the existing normal splice site nor created a novel site. The mutations may exert their effects through altered interactions between an ESE and mRNA splicing factor.

In silico prediction of *NF1* splicing mutations

In silico prediction of *NF1* splicing mutations by HSF and MaxEntScan programs are summarized in Table 2. We calculated the consensus values (CVs) of the splice sites involved in splicing alterations. In all cases where the mutations cause skipping of *NF1* exons, the CVs of the mutated splice sites were lower than those of the wild-type splice site sequences. The average reduction between wild- and mutant-type CV was 35 and 127% according to HSF and MaxEntScan, respectively. For all type II and III mutations where a novel splice site was created because of the mutation, the CV scores of the new acceptor or donor

site were higher than those for the wild-type splice site. In the cases with type IV mutations, almost all mutations revealed a significant decrease in the strength of the splice site compared with the wild-type sequence. When the HSF and MaxEntScan systems were used to score the strength of the cryptic splice sites, high scores for a potential splice site were obtained for only cryptic 3' splice sites (c.1642-10A>G and c.4836-1G>A), and not for cryptic 5' splice sites. Two type V mutations, c.6855C>A and c.6904C>T, were predicted by ESEfinder to decrease the score of an ESE element (Table 3). The RESCUE-ESE program predicted the creation of a novel ESE hexamer for the c.6855C>A mutation, but no changes for the c.6904C>T mutation.

DISCUSSION

Mutation analysis of *NF1* for diagnostic purposes is a difficult task owing to the large size of the gene, the presence of homologous pseudogenes and the lack of clear mutational hot spots.² Occurrence of unusual splice mutations outside of the conserved splice sites also makes the gene test challenging.^{2,4,13} In the present study, we observed that unusual splicing mutations are common, being present in 42% of the mutations (10/24) including intronic mutations outside the conserved AG/GT splice sites (21%, 5/24) and exonic mutations (21%, 5/24). Most of these unusual splicing mutations create novel splice sites or activate cryptic splice sites within exonic and intronic sequences. The proportion of unusual splicing mutations in our study was ~20% higher than those of published studies.¹³ Therefore, our results emphasize the importance of analyzing *NF1* at the mRNA level so as to clarify the primary effect of the mutation on mRNA processing. The mRNA-based assay could also be helpful in screening the complete *NF1* coding sequence and avoiding nonprocessed pseudogene amplification. It has been reported that whereas 50% of the mutations at the DNA level were predicted to disrupt splicing, this proportion increased significantly to 95% when the effect of the mutation was studied at the mRNA level.²

Figure 1 Schematic representations of the type II–V splicing mechanisms involved in some *neurofibromatosis type 1* (*NF1*) mutations. (a) Deep intronic mutation, c.1392+754T>G, activates a cryptic 3' splice site (ss) in intron 12 that results in the introduction of a supplementary exon 70 nucleotides (nt) in length in the mRNA between exons 12 and 13. (b) Exonic mutation, c.1466A>G, creates a new 5' ss within exon 13 that gives rise to a deletion of 62 nt from the mRNA. (c) Intronic mutation, c.1642-10A>G of intron 14, creates a new 3' ss that leads to a 9-nt insertion at exon 15. (d) Exonic mutation, c.6855C>A, disrupts an exonic splicing enhancer element that results in skipping of exon 46. Uppercase letters indicate exonic sequences, and lowercase letters indicate intronic sequences. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Table 2 *In silico* analysis of *NF1* splicing mutations by HSF and MaxEntScan prediction programs

Class	Mutation	Effect on mRNA	Predicted scores by HSF			Predicted scores by MaxEntScan		
			WT CV	Mut CV	Variation, %	WT score	Mut score	Variation, %
I	c.655–2A>G	Inact 3' ss, skip E7	85.99	57.05	–33.66	8.27	0.32	–96.13
I	c.655–1G>A	Inact 3' ss, skip E7	85.99	57.05	–33.66	8.27	–0.47	–105.68
I	c.1062+1G>A	Inact 5' ss, skip E9	84.7	57.86	–31.69	8.92	0.74	–91.7
I	c.1527+1G>T	Inact 5' ss, skip E13	82.29	55.46	–32.6	7.65	–0.85	–111.11
I	c.1527+2T>C	Inact 5' ss, skip E13	82.29	55.46	–32.6	7.65	–0.1	–101.31
I	c.1642–2A>T	Inact 3' ss, skip E15	79.57	50.63	–36.37	4.83	–3.53	–173.08
I	c.2851–6_2851–3del	Inact 3' ss, skip E22	89.92	19.95	–77.81	11.24	–17.93	–259.52
I	c.2851–1G>A	Inact 3' ss, skip E22	89.92	60.98	–32.18	11.24	2.49	–77.85
I	c.3113+1G>A	Inact 5' ss, skip E23	96.86	70.02	–27.71	8.83	0.65	–92.64
I	c.4333–2A>G	Inact 3' ss, skip E33	80.83	51.89	–35.8	7.92	–0.02	–100.25
I	c.6921+4A>G	Inact 5' ss, skip E46	86.73	78.39	–9.62	8.83	6.44	–27.07
II	c.1392+754T>G	New 3' ss, inclusion cryptic exon of 70 nt between E12 and E13	49.64	78.59	+58.32	–2.31	6.28	+371.86
II	c.5610–456G>T	New 5' ss, inclusion cryptic exon of 81 nt between E38 and E39	70.28	97.11	+38.18	2.87	10.51	+266.2
III	c.1466A>G	New 5' ss, deletion last 62 nt E13	55.79	82.62	+48.09	0.38	8.56	+2152.63
III	c.1748A>G	New 3' ss, deletion first 27 nt E16	62.26	91.2	+46.48	1.06	9.81	+825.47
IV	c.204+1G>A	Inact 5' ss, use cryptic 5' ss, deletion last 105 nt E2	93.14	66.3	–28.82	10.113	1.94	–80.85
IV	c.204+1_204+4del	Inact 5' ss, use cryptic 5' ss, deletion last 105 nt E2	93.14	73.17	–21.44	10.13	3.85	–61.99
IV	c.1642–10A>G	Inact 3' ss, use cryptic 3' ss, insertion 9 nt IVS14	79.57 (54.1)	79.69 (83.04)	+0.15 (+53.49) ^a	4.83 (3.58)	–2.2 (12.33)	–145.55 (+244.41) ^a
IV	c.2850+1G>C	Inact 5' ss, use cryptic 5' ss, deletion last 233 nt E21	85.9	59.07	–31.23	9.65	1.38	–85.7
IV	c.4835+1G>A	Inact 5' ss, use cryptic 5' ss, deletion last 69 nt E36	95.9	69.07	–27.98	10.06	1.88	–81.31
IV	c.4836–1G>A	Inact 3' ss, use cryptic 3' ss, deletion first 1 nt E37	84.57 (52.75)	55.62 (81.69)	–34.23 (+54.86) ^a	11.72 (–1.98)	2.97 (5.97)	–74.66 (+401.52) ^a
V	c.6921G>A (5' ss-1)	Inact 5' ss, skip E46	79.51	50.56	–36.41	3.04	–5.7	–287.5

Abbreviations: CV, consensus value; E, exon; HSF, Human Splicing Finder; Inact, inactivation; Mut, mutant type; nt, nucleotide; ss, splice site; WT, wild type.

NF1 reference accession number: NM_001042492.2 (NP_001035957.1).

^aThe consensus value of cryptic splice sites predicted by prediction programs.

Table 3 Two *NF1* mutations disrupting exonic splicing enhancer elements and their *in silico* analysis

Class	Mutation	Effect on mRNA	ESEfinder prediction			SR protein	RESCUE-ESE prediction
			WT motif	Mut motif	Variation, %		
V	c.6855C>A	ESE site broken, skip E46	89.64	80.24	–10.49	SRp40	New hexamer
V	c.6904C>T	ESE site broken, skip E46	93.05	78.80	–15.32	SRp40	No change
			83.08	Site broken	–100	SRp55	

Abbreviations: E, exon; ESE, exon splicing enhancer; Mut, mutant type; WT, wild type.

NF1 reference accession number: NM_001042492.2 (NP_001035957.1).

The scores of individual SR protein putative recognition sites calculated by ESEfinder and the presence or absence of hexamer sequences predicted by RESCUE-ESE to function as ESE elements are provided for wild-type and mutant sequences.

In this study, we report 24 different splicing mutations of *NF1* in 28 unrelated subjects suspected of having NF1. The mutations are scattered along the gene, with some exons having a higher number of mutations. It is of note that 4 splicing mutations of *NF1* were clustered in the exonic and intronic boundaries of exon 46, which is located in the carboxyl terminal domain of the NF1 protein, and were observed in 6 of 28 patients (21%). The importance of the carboxyl terminal domain and its potential role in tumor suppression are unclear; thus, the functional effect of this domain remains to be elucidated.¹⁴

In order to determine the putative mRNA effect of a given mutation, multiple software programs have been developed. In general, splice site prediction tools have higher sensitivity (~90–100%) than specificity (~60–80%) for predicting splice site abnormalities.¹⁵ To evaluate the efficiency of the prediction software for splicing mutations, we analyzed a set of 24 splicing mutations identified in our study cohort. HSF and MaxEntScan programs were able to correctly predict the disruption of the natural splice sites, and we confirmed that the mutation of the last nucleotide of an exon has a strong effect on the 5' splice site, with 36.41% CV reduction.

Furthermore, exonic mutations distant from the 5' and 3' splice sites can activate a cryptic splice site, leading to partial exonic deletion. ESE prediction programs indicate disruption of putative ESE elements for two *NF1* mutations, c.6855C>A and c.6904C>T. Thus, integrated analyses using bioinformatics tools were further applied to provide insight into the mechanisms of these splicing mutations caused by exonic variants, as well as other intronic variants at non-consensus splice sites.

This study has the following limitations. First, the lack of clinical information for most of the patients referred to our laboratory makes it impossible to provide genotype–phenotype correlations. In addition, large *NF1* deletion mutations detected only by fluorescence *in situ* hybridization or multiple ligation probe amplification were not considered in our study; however, they represented only 5% of the total *NF1* mutations.

Taken together, the results from our work show that the molecular basis of *NF1* splicing mutations is diverse. Therefore, molecular characterization at both the gDNA and mRNA levels allowed for a better understanding of gDNA–mRNA correlations of *NF1* mutations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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