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Mutation spectrum of *NF1* gene in Korean unrelated patients with neurofibromatosis 1: Six novel pathogenic variants

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Purpose: Neurofibromatosis 1 (NF1) is one of the most common autosomal dominant diseases caused by heterozygous mutation in the *NF1* gene. Mutation detection is complex owing to the large size of the *NF1* gene, the presence of a high number of partial pseudogenes, and the great variety of mutations. We aimed to study the mutation spectrum of *NF1* gene in Korean patients with NF1.

Materials and Methods: We have analyzed total 69 unrelated patients who were clinically diagnosed with NF1. PCR and sequencing of the *NF1* gene was performed in all unrelated index patients. Additionally, multiplex ligation-dependent probe amplification (MLPA) test of the *NF1* and *SPRED1* gene analysis (sequencing and MLPA test) were performed in patients with negative results from *NF1* gene sequencing analysis.

Results: Fifty-five different variants were identified in 60 individuals, including six novel variants. The mutations included 36 single base substitutions (15 missense and 21 nonsense), eight splicing mutations, 13 small insertion or deletions, and three gross deletions. Most pathogenic variants were unique. The mutations were evenly distributed across exon one through 58 of *NF1*, and no mutational hot spots were found. When fulfilling the National Institutes of Health criterion for the clinical diagnosis of NF1, the detection rate was 84.1%. Cafe-au-lait macules were observed in all patients with *NF1* mutations. There is no clear relationship between specific mutations and clinical features.

Conclusion: This study revealed a wide spectrum and genetic basis of patients with NF1 in Korea. Our results aim to contribute genetic management and counseling.

Key words: Neurofibromatosis 1, *SPRED1*, Mutations, Genotype-phenotype correlation.

Introduction

Neurofibromatosis type 1 (NF1; OMIM #162200), inherited in an autosomal dominant pattern, is characterized by multiple café-au-lait macules (CALMs), skinfold freckling, iris Lisch nodules, tumors of the nervous system, and other features. Other complications include learning disabilities, mental retardation, optic gliomas, certain bone abnormalities, and an increased risk for certain malignancies [1,2]. The diagnosis of NF1 is based on clinical criteria established by the National Institutes of Health (NIH) Consensus Development Conference in 1987 and recently updated [3,4]. NF1-related clinical manifestations are age related

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and can differ within families, which makes genetic counseling difficult [5].

NF1 is caused by mutations in *NF1* gene (Neurofibromin 1; MIM #613113), which encodes neurofibromin, a large guanosine triphosphate GTPase-activating protein (GAP), which acts as a negative regulator of the cellular Ras/MAPK (mitogen-activated protein kinases) signaling pathway by stimulating the GTPase activity of the RAS protein [6,7]. So far, more than 3,000 *NF1* mutations have been reported in the Human Genome Mutation Database (HGMD), and the types of mutations range from a single amino acid missense mutation to an entire exon deletion, and a typical intronic location (canonical splice). Approximately half of cases are familial, while the remainder are due to de novo variants of the *NF1* gene [8]. Single nucleotide variations and small deletions (20 bp or less) account for around 80% to 90% of currently known mutations. Only 5% to 11% of NF1 patients have deletions of the entire *NF1* gene including contiguous genes [9,10]

In most cases, a definitive clinical diagnosis can be performed. However, many clinical features of NF1 increase in frequency with age, and some individuals who have unequivocal NF1 as adults cannot be diagnosed in early childhood, before these features become apparent. Furthermore, in 2007 a clinically overlapping disorder, Legius syndrome, characterized by the presence of multiple CALMs, freckling and macrocephalia, was described [11]. In a large database of individuals that met NIH criteria for NF1 diagnosis 1.9% had a molecular diagnosis of Legius syndrome (OMIM #611431) and 8% of cases aged 0 to 20 years with CAL but without non-pigmentary criteria for *NF1* had *SPRED1* (MIM #613113) mutations [11,12]. In present study, the *SPRED1* gene was also analyzed by gene sequencing and multiplex ligation-dependent probe amplification (MLPA) as part of the differential diagnosis with Legius syndrome.

The large size of the *NF1* gene, presence of multiple pseudogenes, and lack of mutation hotspots make mutation screening challenging [13]. However, *NF1* genetic testing has become clinically available with a high detection rate [9]. More recently, molecular genetic testing was added to the list of the revised diagnostic criteria for NF1 [4]. In this study, we planned one of the mutation analysis studies of Korean NF1 patients to investigate the *NF1* mutation spectrum.

Materials and Methods

1. Patients

A total of 69 unrelated patients who were clinically diagnosed with NF1 were referred to our molecular genetic center from different located hospitals in Korea between 2021 and 2022. The diagnosis of NF1 was made based on clinical features requiring the presence of at least two of the following NIH criteria [3]: six or more CALMs, axillary or inguinal freckling, two or more cutaneous neurofibromas, one plexiform neurofibroma, characteristic bony defects, optic glioma, two or more iris Lisch nodules, or a first-degree relative with NF1. Clinical data, including diagnostic criteria and various associated complications, were collected by a retrospective review of medical records. The study protocol was reviewed and approved by the Institutional Review Board of our lab center (IBC 2023-0503), and written informed consent was obtained from all subjects or from their parents.

2. Molecular genetic testings

The genomic DNA was isolated from peripheral blood leukocytes using Wizard Genomic DNA Purification kits according to the manufacturer's instructions (Promega). Fragments containing exons one to 58 and exon-intron boundaries of the *NF1* gene were amplified by PCR using primers designed by the authors. PCR was performed with a thermal cycler model GeneAmp PCR system 9700 (Applied Biosystems) as follows: 32 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Sanger sequencing was performed for all coding 58 exons and exon-intron boundaries of *NF1* (NM_000267.3). Cycle sequencing was performed with Big Dye Terminator Cycle Sequencing Ready Reaction kits (Applied Biosystems) on an ABI 3130xl Genetic Analyzer (Applied Biosystems). *NF1* cDNA nucleotides were numbered according to the reference sequence (GenBank accession number NM_001042492.3).

As part of genetic analysis, the MLPA test of the *NF1* gene and *SPRED1* gene analysis (sequencing and MLPA test) in the context of differential diagnosis were performed in patients with negative results from *NF1* gene sequencing analysis. (in a sequential way). MLPA analyses were carried out using SALSA MLPA Probemix P081 and P082 (MRC Holland) to detect the exon deletion and duplication of *NF1*. DNA was annealed by adding a probe, amplified by PCR, analyzed with a 3500xL Genetic Analyzer (Thermo Fisher Scientific), and read with GeneMarker version 1.91 (SoftGenetics). Each peak was compared with the normal control group, and if the ratio was less than 0.7, it was evaluated as deletion, and if it exceeded 1.3, it was evaluated as duplication [14]. When the ratio of the deletion was 0, it was evaluated as a homozygous deletion and a heterozygous deletion when 0.40-0.65. Additionally, the *SPRED1* gene was also analyzed by gene sequencing and MLPA as part of the differential diagno-

Table 1. Variant spectrum of *NF1* in 60 unrelated Korean patients with NF1

No. of patient	Exon/Intron	Nucleotide change	Aminoacid change	Mutation type	Classification	Age at diagnosis (yr)	Sex	Family H(x)	Novelty	Protein domain
1	49	c.7352delC	p.Pro2451fs	Frameshift	P	8	F	+		
2	36	c.5137delG	p.Glu1713fs	Frameshift	P	1	F	+	+	Sec14-PH
3	2	c.134A>G	p.Asn45Ser	Missense	LP	3	F	+		
4	44	c.6709C>T	p.Arg2237*	Nonsense	P	29	F	–		HLR
5	27	c.3587T>C	p.Leu1196Pro	Missense	LP	1	F	–	+	GRD
6	Intron 22	c.2990+1G>T	-	Splicing	P	1	F	+		
7	Exon 1-58	-	-	Whole-gene deletion	P	4	M	–		
8	26	c.3318C>G	p.Tyr1106*	Nonsense	P	1	M	–		GRD
9	18	c.2041C>T	p.Arg681*	Nonsense	P	37	F	–		CSRD
10	26	c.3445A>G	p.Met1149Val	Missense	LP	12	F	+		GRD
11	9	c.1039C>T	p.Gln347*	Nonsense	P	1	F	+		
12	20	c.2342A>C	p.His781Pro	Missense	LP	5	M	+		CSRD
13	2	c.154delT	p.Ser52fs	Frameshift	P	1	F	–		
14	Intron 21	c.2410-1G>A	-	Splicing	P	9	F	–		CSRD
15	27	c.3525_3526delAA	p.Arg1176fs	Frameshift	P	59	F	–		GRD
16	9	c.998dupA	p.Tyr333*	Nonsense	P	1	M	+		
17	15	c.1667_1670delATAG	p.Asp556fs	Frameshift	P	9	F	–		CSRD
18	54	c.7897_7900delTTTC	p.Phe2633fs	Frameshift	P	1	M	+		SBR
19	44	c.6611G>A	p.Trp2204*	Nonsense	P	1	M	+		HLR
20	28	c.3721C>T	p.Arg1241*	Nonsense	P	1	M	–		GRD
21	36	c.4829T>G	p.Leu1610*	Nonsense	P	1	M	+		Sec14-PH
22	47	c.7095dupT	p.Asn2366*	Nonsense	P	14	F	–		HLR
23	45	c.6857A>G	p.Lys2286Arg	Missense	VUS	3	M	–	+	HLR
24	45	c.6789_6792delTTAC	p.Tyr2264fs	Frameshift	P	65	F	–		HLR
25	21	c.2537C>A	p.Ala846Asp	Missense	P	3	M	–		CSRD
26	48	c.7202_7205delAACA	p.Lys2401fs	Frameshift	P	1	F	+		HLR
27	12	c.1381C>T	p.Arg461*	Nonsense	P	41	F	–		
28	34	c.4537C>T	p.Arg1513*	Nonsense	P	8	F	+		GRD
29	34	c.4537C>T	p.Arg1513*	Nonsense	P	6	M	–		GRD
30	31	c.4267A>G	p.Lys1423Glu	Missense	LP	4	M	–		GRD
31	28	c.3847A>T	p.Lys1283*	Nonsense	P	43	M	–	+	GRD
32	12	c.1307C>A	p.Ser436*	Nonsense	P	33	M	+		
33	Intron 13	c.1527+1G>A	-	Splicing	P	1	F	+		
34	Intron 30	c.4110+1G>A	-	Splicing	P	5	M	+		GRD
35	Exon 1-58	-	-	Whole-gene deletion	P	4	M	–		
36	28	c.3847A>T	p.Lys1283*	Nonsense	P	65	M	+	+	GRD
37	29	c.3916C>T	p.Arg1306*	Nonsense	P	65	M	–		GRD
38	14	c.1639G>T	p.Glu547*	Nonsense	P	10	F	+		CSRD
39	36	c.5083G>C	p.Ala1695Pro	Missense	VUS	1	F	–		Sec14-PH
40	28	c.3739_3742delTTTG	p.Phe1247fs	Frameshift	P	4	F	+		GRD
41	Intron 44	c.6756+2T>G	-	Splicing	P	46	M	–		HLR
42	33	c.4402A>G	p.Ser1468Gly	Missense	LP	25	M	–		GRD
43	20	c.2329T>G	p.Trp777Gly	Missense	LP	1	M	–		CSRD
44	Intron 1	c.61-2A>G	-	Splicing	P	1	F	+		
45	18	c.2033dupC	p.Ile679fs	Frameshift	P	1	M	–		CSRD
46	18	c.2072T>C	p.Leu691Pro	Missense	LP	18	M	+		CSRD
47	45	c.6789_6792delTTAC	p.Tyr2264fs	Frameshift	P	7	F	+		HLR

Table 1. Continued

No. of patient	Exon/Intron	Nucleotide change	Aminoacid change	Mutation type	Classification	Age at diagnosis (yr)	Sex	Family H(x)	Novelty	Protein domain
48	4	c.479G>A	p.Arg160Lys	Missense	LP	14	M	–		
49	Intron 5	c.587-2A>G	-	Splicing	P	28	M	+		
50	13	c.1466A>G	p.Tyr489Cys	Missense	LP	10	M	–		CSRD
51	12	c.1372_1373delinsTA	p.Pro458*	Nonsense	P	1	M	+	+	
52	Exon 1-58	-	-	Whole-gene deletion	P	7	M	–		
53	51	c.7581_7582delAT	p.Ser2528fs	Frameshift	P	14	F	–		
54	3	c.278G>A	p.Cys93Tyr	Missense	LP	1	F	+		
55	11	c.1232dupC	p.His415fs	Frameshift	P	60	M	–		
56	10	c.1094C>G	p.Ser365*	Nonsense	P	10	F	–		
57	27	c.3579_3588dup	p.Ala1197*	Nonsense	P	2	M	–	+	TBD
58	18	c.2072T>C	p.Leu691Pro	Missense	LP	20	F	+		CSRD
59	Intron 6	c.655-1G>C	-	Splicing	P	30	M	–		
60	34	c.4537C>T	p.Arg1513*	Nonsense	P	49	M	–		GRD

CSRD, cysteine-serine rich domain; CTD, C-terminal domain; F, female; GRD, GAP-related domain; HLR, HEAT-like regions repeat; M, male; SBR, Syndecan-Binding Region; Sec14-PH, Sec14 homologous domain and Pleckstrin Homology domain; TBD, tubulin-binding domain.

sis with Legius syndrome. Amplification and sequencing were performed as mentioned above. The MLPA test was performed using SALSA MLPA Probemix P295 SPRED1 (MRC Holland).

NF1 and *SPRED1* gene variants were classified into pathogenic, likely pathogenic, variant of uncertain significance (VUS), likely benign, and benign in accordance with the recommendation of the American College of Medical Genetics and Genomics (ACMG) [15]. Different aspects regarding pathogenicity were considered, such as predicted impact, result of computational and predictive data (Polymorphism Phenotyping v2, sorting intolerant from tolerant, likelihood ratio test, Mutation Taster), conservation, and segregation analysis. Publicly available databases of variants annotated on the disease: ClinVar (www.ncbi.nlm.nih.gov/clinvar), HGMD (www.hgmd.cf.ac.uk) and Leiden Open Variation Database (LOVD; databases.lovd.nl/shared/genes).

Results

1. *NF1* mutation spectrum

In the cohort of 69 unrelated patients during two-year period, 58 were positive for *NF1* pathogenic variants (84.1%) (Table 1). Mutation analysis revealed a wide spectrum of *NF1* mutations. Fifty-five different *NF1* variants were identified in 69 unrelated NF1 patients. Most pathogenic variants were unique; only four mutations were observed more than once: c.6789_6792del (p.Tyr2264fs), c.4537C>T (p.Arg1513*) (three times), c.3847A>T (p.Lys1283*), and c.2072T>C (p.Leu691Pro). Six (6/55, 10.9%) mutations were novel, and 49 (49/55, 89.1%) mutations have been previously reported. Each newly identified mutation was indicated in Table 1. Among six novel mutations, three were nonsense mutations including c.3847A>T (p.Lys1283*) detected in 2 unrelated patients.

The mutation spots were spread out along the coding region of the *NF1* gene. Many mutations (45/58, 77.6%) resulted in a truncated or a shortened protein product, including nonsense, frameshift mutations (insertion, deletion, and indels), and splicing mutations. The mutations included 36 (34/58, 58.6%) single base substitutions (13 missense and 21 nonsense), eight (8/58, 13.8%) splicing mutations, 13 (13/58, 22.4%) frameshift, and three (3/58, 5.2%) whole-gene deletions. Splicing mutations were observed in eight patients, which were previously reported as disease-related mutations, and they occurred in the splicing donor and recipient sites. Fifteen missense mutations were detected in this study, including pathogenic mutations (1/15, 6.7%) and likely pathogenic mutations (12/15, 80.0%) reported as disease-related mutations, and VUS (2/15, 13.3%). Two VUSs in this study were missense mutations including one novel variant. The largest deletion was extended at least from exon one to 58, which was identified by the MLPA test (chr17:29413855-29709444 in hg19 coordinates). Depending on the analysis method, large deletion spanning more than one exon, which were detectable only in MLPA, were observed in three patients. In nine patients with normal results for *NF1* molecular genetic testing (sequencing and MLPA), the *SPRED1* gene analysis was performed (sequencing and MLPA), and none of the analyzed

patients had *SPRED1* pathogenic variants. A total of 15 patients had *NF1* mutations involving the GAP-related domain (GRD), 11 patients involving the cysteine-serine rich domain (CSRD), and eight patients involving HEAT-like regions repeat (HLR) domain (Table 1).

2. Clinical features in NF1 patients

Clinical characteristics delineation of patients according to the presence of *NF1* pathogenic variants was performed and is summarized in Table 2. Of the 58 *NF1* patients with pathogenic variants in our study, 43 were sporadic and 15 had a positive family history. Among the diagnostic criteria, CALMs were observed in all patients. Skin freckling, cutaneous neurofibromas, and Lisch nodules on the iris were commonly observed in 77.6%, 84.5%, and 77.6% of patients, respectively. Intracranial lesions were accompanied in 22 (37.9%) patients based on brain magnetic resonance image findings. High signal lesions were observed in 14 (24.1%) patients and were particularly common in children and young adults. Plexiform neurofibromas were accompanied in 14 (24.1%) patients. Lesions of plexiform neurofibromas were distributed throughout the entire body, from the scalp to the foot. Scoliosis affected 19 (32.8%) patients, while other bony lesions were observed in four patients. One patient showing facial asymmetry was found to have sphenoid bone dysplasia. Malignancies were identified in five (8.6%) patients. Among them, malignant peripheral nerve sheath tumor occurred in 3 (5.2%) patients of ages between 25 and 65 years. All three patients underwent repeated tumor resections or were treated by adjuvant chemotherapy. One child suffered from acute myeloid leukemia, and an infant had juvenile myelomonocytic leukemia. These two patients with hematologic malignancies received hematopoietic stem cell transplantation. A genotype-phenotype analysis suggests that there is no clear relationship between specific mutations and clinical features. Compared to patients with *NF1* pathogenic variants, the nine patients with undetected *NF1* pathogenic variants mostly had only CALMs and cutaneous neurofibromas and no family history (Table 2).

Table 2. Clinical characteristics of NF1 patients with and without *NF1* pathogenic variants

Pathogenic variant	Detected	Not detected
No. of patients	58	11
Age at diagnosis (yr)	14.8±19.1	23.1±13.3
Sex (male/female)	32/26	8/3
Sporadic/Familial cases	43 (74.1)/15 (25.9)	11 (100) /0 (0)
Clinical findings		
Café-au-lait macules	58 (100)	11 (100)
Cutaneous neurofibroma	49 (84.5)	9 (81.8)
Freckling (axillary or inguinal)	45 (77.6)	1 (9.1)
Lisch nodules	45 (77.6)	0 (0)
Plexiform neurofibroma	14 (24.1)	0 (0)
Juvenile xanthogranuloma	2 (3.4)	0 (0)
Intellectual diability	3 (5.2)	1 (9.1)
Learning difficulties	12 (20.7)	0 (0)
Behavior issues	4 (6.9)	0 (0)
Seizures	2 (3.4)	0 (0)
Hypertension	1 (1.7)	1 (9.1)
Intracranial lesions	22 (37.9)	0 (0)
Optic pathway glioma	1 (1.7)	0 (0)
Cerebral glioma	4 (6.9)	0 (0)
High signal lesion	14 (24.1)	0 (0)
Cerebral vascular abnormlities	3 (5.2)	0 (0)
Lacunar infarction	1 (1.7)	0 (0)
Bone lesions	23 (39.7)	1 (9.1)
Scoliosis	19 (32.8)	1 (9.1)
Sphenoid dysplasia	1 (1.7)	0 (0)
Tibial pseudoarthrosis	1 (1.7)	0 (0)
Pubic bone dysplasia	1 (1.7)	0 (0)
Osteoporosis	1 (1.7)	0 (0)
Malignancy	5 (8.6)	0 (0)
Malignant peripheral nerve sheath tumor	3 (5.2)	0 (0)
Juvenile myelomonocytic leukemia	1 (1.7)	0 (0)
Acute myeloid leukemia	1 (1.7)	0 (0)

Values are presented as mean±standard deviation or number (%).

Discussion

NF1 can show various phenotypes of the disease, and even if the criteria presented by the NIH are not met at the time of initial examination, new clinical features suitable for the diagnostic criteria may appear as the age increases. Diagnosis of NF1 is usually based on clinical findings according to NIH diagnostic criteria, nevertheless, owing to the extreme variability in clinical expression and age dependency of most clinical manifestations, molecular testing could represent a simple and effective strategy for early and differential diagnosis [5]. In this study, we investigated the spectrum of *NF1* mutations in Korean NF1 patients and detected 55 different *NF1* mutations, six of which were novel mutations. It did not occur intensively in some regions, but was detected in various regions of the *NF1* gene. Pathogenic variants were detected in 84.1% of 69 patients with two or more clinical features suitable for the NIH diagnostic criteria. This finding is consistent with previous reports indicating that mutations in the NF1 gene were observed in 80.9% to 92.4% of

NF1 patients [16,17].

If the phenotypic findings suggest the diagnosis of NF1, single-gene testing may be considered. Sequence analysis of NF1 genomic DNA (gDNA) and/or cDNA (complementary DNA, copied from mRNA) is performed in association with gene-targeted deletion analysis. In present study, pathogenic variants were not detected in nine patients with clinically diagnosed NF1. Because of the frequency of pathogenic variants that affect splicing, which are not detected by gDNA sequencing of protein-coding regions, methods that include cDNA sequencing have higher detection rates than methods based solely on analysis of gDNA. If cDNA analysis had also been performed in this study, the detection rate would have been higher. However, negative *NF1* molecular testing does not rule out a diagnosis of NF1 [18]. Some individuals diagnosed with NF1 based on clinical criteria do not have a pathogenic variant detectable by current technology. Also, parental somatic and germline mosaicism may be present even if there are no clinical signs of NF1 and no evidence of the proband's disease-causing *NF1* variant on standard molecular testing of either parent's leukocyte DNA [19,20]. Furthermore, *NF1* mutations could detected in some patients, even when clinically identifiable symptoms were limited to just one, failing to meet the NIH diagnostic criteria. This underscores the importance of genetic testing, even in the early stages of a disease with limited symptoms, as it facilitates a prompt and accurate diagnosis [10,21]

Since most *NF1* mutations were unique to a single individual or family without mutation hot spots, the correlations were assessed mainly based on the mutation types. The types of *NF1* mutations vary by race and country, but mostly truncating mutations, such as nonsense mutations and frameshift mutations, have a high rate, followed by splicing mutations, missense mutations, and large deletions over exons. Although the methods used in each study varied, the mutation types were similar [22-25]. In this study, the pathogenic variants included nonsense mutations (36.2%), frameshift mutations (22.4%), missense mutations (22.4%), splicing mutations (13.8%), and large deletions beyond the exon level (5.2%). There was no significant difference when compared to previous reports. Also, there were no significant correlations according to the functional domains, same as in a previous study [26].

This study attempted to identify recurrent mutations of the *NF1* gene in Korean patients, which would allow rapid and economical screening of certain selected exons. However, only four recurrent mutations were found in 60 Korean NF1 patients, and none was found in more than three. This finding indicates the widespread distribution of mutations and a lack of a mutational hot spot in NF1. Some previous studies proposed certain exons of *NF1* as more mutation-prone regions [9,23]. In this study, recurrent mutations were contained in exons 18, 28, 34, and 45. These data suggest that different populations have different exon sets that contain recurrent mutations. Four mutations were observed more than once: c.6789_6792del (p.Tyr2264fs), c.4537C>T (p.Arg1513*), c.3847A>T (p.Lys1283*), and c.2072T>C (p.Leu691Pro). Especially, c.6789_6792del (p.Tyr2264fs) and c.4537C>T (p.Arg1513*) were also recurrently detected in previous studies for Korean NF1 patients, which could be hot spot mutations in Korean population [22,27,28]. We found six (6/55, 10.9%) novel variants including three nonsense mutations, two missense mutations, and one frameshift mutation. Among three novel nonsense mutations, c.3847A>T (p.Lys1283*) was detected in two unrelated patients (No. 31 and 36). This variant is located in the GRD functional domain. The GRD is known to be the most important functional domain of the *NF1* gene (14/60, 23.3%). The predicted GRD in neurofibromin shares homology with other GAP family proteins [29]. *NF1* mutations involving the GRD were most common in present study. Among novel variants, three nonsense mutations and one frameshift mutation were considered pathogenic based on predictive impact, population data, and computational data, etc. One of six novel variants was missense mutation: c.6857A>G (p.Lys2286Arg), which were considered VUS. Allele frequency of this variant is 0.000008 (ExAC) and ClinVar classifies it as VUS and in silico analysis shows conflicting results. Another VUS, c.5083G>C (p.Ala1695Pro), shows allele frequency of 0.000012 (GnomAD) and ClinVar classifies the variant as VUS and in silico analysis predicts conflicting results. Although it could not be implemented at this time according to the ACMG guideline, it would be important to conduct additional tests (parental study, functional study, segregation study) to provide a basis for determining the risk of mutation.

In the current study, NF1 was confirmed in patients (58/69, 84.1%) by genetic testing, which was lower than the results of cDNA with MLPA analysis (~95%). A higher detection rate could be achieved by cDNA analysis to detect deep intronic variations affecting the splicing process [9,24]. In NF1, the frequency of splicing alterations affecting mRNA processing is high compared with other genetic disorders [23-25]. Also, previous studies for Korean NF1 patients [21,30] emphasize the importance of analyzing *NF1* at the mRNA level to clarify the effect of the mutation on mRNA processing. The present study using the only gDNA sequencing approach could result in mis-

classification of the mutation type in missense and nonsense mutations, since some missense and nonsense mutations lead to missplicing [9,13,24]. In addition, nine patients did not have a pathogenic *NF1* mutation. These patients have a possibility of Legius syndrome caused by *SPRED1* mutations, and none of the analyzed patients in this study had *SPRED1* pathogenic variants. In the future, more data on *SPRED1* gene study will be needed to diagnose Legius syndrome in Korean patients showing NF1-like clinical features. In this study, multiple exon deletions other than whole-gene deletions were not detected in MLPA. Patients with whole-gene deletions may have contiguous gene deletions in the 17q11.2 region as well as the *NF1* gene. To confirm this, it will be necessary to perform chromosomal microarray analysis as an additional test [10].

The prevalence of most clinical features of patients with *NF1* pathogenic variants in our study was like previous reports [5,31,32]. Several allele-phenotype correlations have been observed in NF1. Deletion of the entire *NF1* gene is associated with larger numbers and earlier appearance of cutaneous and plexiform neurofibromas, a higher risk of developing malignant peripheral nerve sheath tumor, more frequent and more severe cognitive abnormalities, somatic overgrowth, and large hands and feet [33,34]. A recurrent pattern of dysmorphic features that includes coarse facial appearance, flat forehead, ocular hypertelorism, broad nasal tip, low-set ears, and broad neck is often observed among adolescents and adults [34]. In this study, deletion of the entire *NF1* gene was present in three patients (No. 7, 35, and 52) with large number of neurofibroma and dystrophic scoliosis in addition to NIH criteria (CALMs, Lisch nodules, and inguinal freckling) and there was no family history. An unusually severe phenotype with frequent plexiform or spinal neurofibromas, optic pathway gliomas, malignant neoplasms, and skeletal abnormalities has been observed in adults with missense variants of one of five codons between 844 and 848 that code for the CSRD of neurofibromin [33,35]. In this study, a three-year old male patient (No. 25) with p.Ala846Asp showed multiple CALMs and axillary freckling but no other manifestations and no family history. Missense variants affecting p.Met1149 have been associated with a mild phenotype characterized by pigmentary features, frequent learning problems and features of NF1-Noonan syndrome [36]. The p.Met1149 mutation was identified in 12-year-old female patient (No. 10) who had CALMs, widespread cutaneous neurofibromas, scoliosis, and learning disability.

Our study had some limitations. Given the retrospective nature of this study, there were missing or incomplete data. In addition, we could have a higher detection rate achieved by cDNA analysis to detect deep intronic variations affecting the splicing process. Nevertheless, the findings of this study will help to improve our overall understanding of the correlation between genotype phenotype in patients with NF1. In conclusion, we revealed both the mutation spectrum with 6 novel mutations in this study. The present study will contribute to a better understanding of the distinct molecular genetic characteristics of patients with NF1. In the future, it would be necessary to expand the types of NF1-causing genes using next-generation sequencing and to make efforts to identify the clinical meaning of newly detected gene mutations.

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Authors' Contributions

Conception and design: SHH. Acquisition of data: SHH. Analysis and interpretation of data: EJK, SHH. Drafting the article: SHH, MY, SK, SGL, EHL. Critical revision of the article: SHH, MY, SK. Final approval of the version to be published: SHH.

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