Mutational spectrum of *NF1* gene in Korean patients with neurofibromatosis type 1

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Mutational spectrum of *NF1* gene in Korean patients with Neurofibromatosis type1

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ABSTRACT

Mutational spectrum of *NF1* gene in Korean patients with neurofibromatosis type 1

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Neurofibromatosis type 1 (NF1) is one of the most common autosomal dominant disorder with an incidence of 1 : 3,500 which is caused by mutations in the *NF1* gene. NF1 is characterized particularly by café-au-lait spots and fibromatous tumors of the skin. The *NF1* gene is located on the chromosome 17q11.2 and spans approximately 350 kb of genomic DNA. It consist of 60 exons which translates into neurofibromin.

Screening of mutations in *NF1* gene is complicated because of the large size of the gene, the presence of pseudogenes, the great variety of possible lesions, and the lack of significant mutational clustering.

We screened for mutations in 36 patients who are clinically diagnosed as neurofibromatosis type 1. The whole coding sequences and all splice sites were examined for mutations using DHPLC followed by

direct sequencing of PCR products. Disruptive mutations were identified in 31 individuals with an overall mutation detection rate of 86%. The mutations included one indel (nt.4079), three insertions (nt.1233, 4159, 4630), seven deletions (nt.953, 1017, 1418, 1541, 2679, 2816, 3525), sixteen missense / nonsense mutations (192, 384, 386, 465, 467, 489, 616, 1403, 1619, 2157, 2197, 2237, 2426, 2429, 2483, 2496 codon) and two splicing error (IVS 25, 34). Sixteen unclassified polymorphisms were also detected. Twenty one (72.4%) of the identified disruptive mutations are novel. Eight mutations have been previously reported. It appeared that mutational spectrum of *NF1* gene in patients is heterogeneous as previously shown in other populations. By using strategies for mutation screening in *NF1* gene used in this study can easily be applied for clinical purpose.

Key Words: NF1, Neurofibromatosis type1, neurofibromin, mutational analysis.

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I. INTRODUCTION

Neurofibromatosis type 1 (NF1; MIM# 162200), formerly known as Von Recklinghausen neurofibromatosis, is a common autosomal dominant disorder with an incidence of 1 : 3,500, characterised by cafe-au-lait spots, peripheral neurofibromas, Lisch nodules and flexural freckling. Other features found in a minority of patients include scoliosis, macrocephaly, pseudarthrosis, short stature, malignancies and learning disabilities.[1]

The mutation rate of the neurofibromatosis type 1 (*NF1*) gene is one of the highest in the human genome, with about 50 percent of cases being due to *de novo* mutations.[2]

The *NF1* gene is located on the chromosome 17q11.2 and spans approximately 350 kb of genomic DNA. It consist of 60 exons and

translates into neurofibromin[3,4,5](Fig 1.).

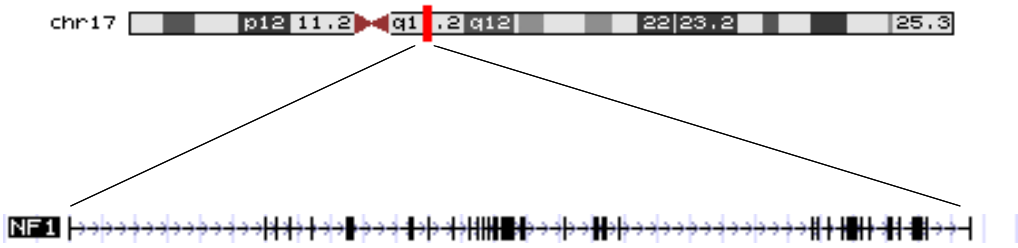


FIG 1. Genetic map of *NF1* gene. The *NF1* gene is located on the chromosome 17q11.2 and consist of 60 exons.

The neurofibromin comprises 2818 amino acids and has an estimated molecular weight of 327kDa. The central region of neurofibromin (encoded by exons 2127a) possesses marked homology to Ras-GTPase activation proteins (GAPs), or *NF1* GRD, that is able to down-regulate p21ras[6] by stimulating its intrinsic GTPase. GTP is a major regulator of growth and differentiation and mutant neurofibromins might interfere with ras signaling pathways and contribute to the development of tumors.[7,8]

Mutation screening in *NF1* gene has been made difficult because of the large size of the gene, the existence of a number of homologous pseudogene sequences spread throughout the genome,[9,10] and the lack of defined mutational hotspots. To overcome these problems, various techniques have been employed for screening mutations within the *NF1* gene. Most studies have been based on single strand conformation

polymorphism, heteroduplex analysis, temperature gradient gel electrophoresis and denaturing gradient gel electrophoresis. In the largest study to date, involving 500 patients used a protein truncation test, temperature gradient gel electrophoresis, and direct genomic sequencing to examine all of the individual exons, finding sequence variants in 301 patients. Within these variants 278 mutations were considered pathogenic.[11] In two more papers published recently, the same methodologies were used sequentially to raise mutation detection rates. In the other study, using cDNA single strand conformation polymorphism and heteroduplex analysis, a detection rate of 70-80% of mutations was achieved (22 of 80 patients).[12] Messiaen et al. used a protein truncation test, fluorescence in situ hybridization, southern blot and cytogenetic analysis with 67 patients, and reported a detection rate of 95%, including a high frequency of unusual splicing defects.[13] The sensitivity of each technique is hard to establish, as mutation analysis reports have either concentrated on groups of exons, small number of patients included in their studies, or used a combination of techniques. In reviews of known *NF1* mutations, several mutation types are described, but no correlation with phenotype was documented. Most of the fully characterised *NF1* mutations are either nonsense or frameshift mutations, which presumably lead to premature truncation of neurofibromin synthesis. Large deletions of the *NF1* gene are thought to account for less than 10% of cases. A relationship between whole gene deletions and a more severe NF1 phenotype has been reported.[14,15]

Denaturing high-performance liquid chromatography (DHPLC)[16], a

fast and highly sensitive technique based on the detection of heteroduplexes in PCR products by ion pair reverse-phase HPLC under partially denaturing conditions, is in many ways ideally suited to mutation detection in these conditions[17]. A preliminary study with a basic DHPLC system detected all known mutations within exon 16 of the *NF1* gene.[18] Recently, the sensitivity of DHPLC was evaluated in the retrospective study of a cohort of 111 unrelated NF1 patients with known germline mutations, which detected 97% of mutations.[17] In a subsequent prospective analysis of 50 unrelated NF1 individuals , germline mutations were identified in 34 (68%), including 22 novel alterations. This figure represents the highest rate of mutation detection in the *NF1* gene, so far reported using a single screening technique with genomic DNA as a target.[17]

We screened for mutations in 36 patients who are clinically diagnosed as neurofibromatosis. The whole coding sequences and all splice sites were examined for mutations using DHPLC followed by direct sequencing of PCR products. Disruptive mutations were identified in 31 individuals with an overall mutation detection rate of 86%.

II. MATERIALS AND METHODS

1. Subject

For 36 patients, the diagnosis of NF1 was based upon the

presence of two or more of the diagnostic criteria proposed by the NIH Consensus Statement in 1988 (Stumpf et al. 1988).

2. Polymerase Chain Reaction

Genomic DNA was extracted using phenol-chloroform extraction method from peripheral blood leukocytes.

The 60 exons of *NF1* gene were amplified in 60 PCR fragments. Sixty primer pairs were designed according to Han et al.[17] Genomic polymerase chain reaction (PCR) was carried out in 50 μl reaction volumes containing 100 ng genomic DNA, 20 ρM primers, 75 mM dNTP, 5 μl reaction buffer and 1U Ex-Taq Polymerase (TaKaRa Shuzo Co., Ltd., Otsu, Japan) with the following cycling profile; 5 min denaturation at 94℃ and 35 cycles of denaturation at 94℃ for 30 sec, specific annealing temperature for 30 sec, extension at 72℃ for 30 sec, followed by a 5 min final extension step at 72℃ (Table 1). All thermal cycles were run on a Takara PCR thermocycler (TaKaRa Shuzo Co., Ltd., Otsu, Japan). Amplicons were checked by DHPLC sizing method before DHPLC analysis.

An additional denaturation and re-annealing step was required after standard PCR amplification for heteroduplex formation prior to DHPLC analysis.[19] Samples were denatured at 95℃ for 10 minutes and then allowed to reanneal for over 30 min (-0.1℃/4sec).

3. DHPLC analysis

DHPLC was performed on a WAVE MD DNA fragment analysis system by using a DNASep column.[18] (Transgenomic Inc., Omaha, NE) DNASep columns contain non-porous alkylated polystyrene-divinylbenzene particles that are both electrically neutral and hydrophobic; thus, the negatively charged phosphate ions of DNA molecules cannot bind to the column unaided. Triethylammonium acetate (TEAA) is a positively charged reagent that facilitates interaction between the stationary matrix and DNA molecules. DNA fragments are eluted from the column by reducing the hydrophobic interaction between the alkyl chains of TEAA and the stationary phase of the column. This is achieved by altering the

Table 1. PCR Annealing Temperature for the *NF1* Amplicons.

Fragment	Annealing Temp.(℃)	Fragment	Annealing Temp.(℃).	Fragment	Annealing Temp.(℃)	Fragment	Annealing Temp.(℃)
1	65	11	60	23-2	58	36	62
2	58	12a	55	23a	62	37	58
3	60	12b	58	24	58	38	60
4a	55	13	64	25	53	39	62
4b	64	14	58	26	60	40	59
4c	58	15	65	27a	60	41	65
5	57	16	60	27b	53	42	60
6	58	17	56	28	64	43	53
7	64	18	58	29	57	44	64
8	62	19a	65	30	57	45	64
9	56	19b	56	31	64	46	60
9a	63	20	64	32	58	47	60
10a	58	21	58	33	64	48	66
10b	54	22	58	34	57	48a	65
10c	57	23-1	57	35	65	49	62

ratio of TEAA to acetonitrile. The DNA molecules eluted from the column are detected by scanning with a UV-C detector. The successful resolution of heteroduplexes from homoduplexes requires an elution gradient at partially denaturing temperature. At this temperature, only heteroduplexes are destabilised by the mismatched bases and are therefore slightly more melted than the homoduplexes, resulting in earlier elution than the homoduplexes. This special resolution temperature can be predicted by use of DHPLCMelt software (http://insertion.stanford.edu/melt.html).[20]

Re-annealed PCR products[19] after denaturation were injected onto the column and eluted with a linear acetonitrile gradient at a flow rate of 0.9ml/min, with a mobile phase consisting of a mixture of buffers A (0.1 mol/l TEAA and 1mM EDTA) and B (25% acetonitrile in 0.1 mol/l TEAA).

Table 2. Primer sequences, PCR product size and their fragment analysis conditions (DHPLC oven temperature; This resolution temperature can be predicted by use of DHPLCMelt software http://insertion.stanford.edu/melt.html). These primer pairs were designed according to Han et al. 2001.[17]

exon	Primer sequence (5'→3')	DHPLC oven temperature (℃)	PCR product size (bp)
1	CAGACCCTCTCCTTGCCTC	63.3	438
	GGATGGAGGGTCGGAGGC		
2	AAGCTGTTAACGTGTTTTTTTTT	55.6	228
	AAGAAAAGAAAGCAAATTCC		
3	TTTCACTTTTCAGATGTGTGT	55.6	245
	TGGTCCACATCTGTACTT		
4a	TTAAATCTAGGTGGTGT	53.6	517
	AAACTCATTTCTCTGG		
4b	GATGATGTCTTGCTATGTT	54.0	366
	TTGGTGTTCTAGTTCAGC		

exon	Primer sequence (5'→3')	DHPLC oven temperature (℃)	PCR product size (bp)
4c	TTTCCTAGCAGACAACTATC	54.8	308
	AGGATGCTAACAACAGCAA		
5	GAAGGAAGTTAGAAGTTTGTGA	55.2	172
	CAATCGTATCCTTACCAGCC		
6	CATGTTTATCTTTTAAAAATCTTG	55.0	301
	ATAATGGAAATAATTTTGCCCT		
7	ACATCTGGAATAGAAGAAACTT	53.9	377
	CAGTAACAACAAAAGCAAGT		
8	GGATTTTACTGCCATTTGTG	55.7	276
	TAACAGCATCAGTAAATATAGTTAGA		
9	TTGAAGTTCGTTTCAAGA	53.5	272
	ACGCAAAGAAAAGAAAGAA		
9a	CTGTGGCTCAGAACACTAA	54.1	308
	CACATGCAGTGCTCATTA		
10a	ACGTAATTTTGTACTTTTTCTT	57.0	222
	CAATAGAAAGGAGGTGAGAT		
10b	ATTATCCTGAGTCTTACG	54.4	229
	TAACTTAGTGTGATAATTTTGA		
10c	ATTGAAGTTTCCTTTTTTTCCTT	57.0	275
	GTATAGACATAAACATACCATTT		
11	CCAAAAATGTTTGAGTGAGT	52.9	256
	ACCATAAAACCTTTGGAAG		
12a	AAACCTTACAAGAAAACTAAG	53.7	303
	ATTACCATTCCAAATATTCTTC		
12b	CTCTTGGTTGTCAGTGCT	55.8	261
	CAGAAAACAAACAGAGCAC		
13	GTCTTCCACCCTTGACTC	57.2	387
	GCTACTTGAATTTCCCCT		
14	GCTCTTCCTACTCCTTTT	60.3	191
	TTTCTGTTGCTAAGGGCA		
15	ACTTGGCTGTAGCTGATT	56.6	247
	ACTTTACTGAGCGACTCTTG		
16	ACTTTACTGAGCGACTCTTG	56.2	549
	TAGAGAAAGGTGAAAAATAAG		
17	TCTCTAGGGGGTCTGTCT	55.6	326
	CACCCTAGTTTGTGTGCA		
18	AGAAGTTGTGTACGTTCTTTT	53.2	367
	CTCCTTTCTACCAATAACC		
19a	TCATGTCACTTAGGTTATCT	55.6	242
	TAAAACCCACTAATACTTGAA		
19b	TGAGGGGAAGTGAAAGAA	54.5	236
	GGCTTTATTTGCTTTTTG		

exon	Primer sequence (5'→3')	DHPLC oven temperature (℃)	PCR product size (bp)
20	CCACCCTGGCTGATTAT	57.0	402
	TAATTTTTGCTTCTCTTACAT		
21	TGGTCTCATGCACTCCA	55.6	474
	CATCTTTCTTCTGGCTCT		
22	TGCTACTCTTTAGCTTCCT	56.7	331
	CCTTAAAAGAAGACAATCAG		
23-1	TTTGTATCATTCATTTTGTGTG	56.6	282
	AAAAACACGGTTCTATGTGAAA		
23-2	CTTAATGTCTGTATAAGAGTC	53.3	268
	ACTTTAGATTAATAATGGTAATC		
23a	AGCCAGAAATAGTATACATGATTGG	54.0	446
	CTATTTTCTGCCAGAATTAGTA		
24	TTGAACTCTTTGTTTTCATGTC	54.1	266
	GGAATTTAAGATAGCTAGATTA		
25	CCTGTTTTATTGTGTAGATACTT	57.8	134
	TAAGTGGCAAGAAAATTAC		
26	AATTCTAATGACTTTGCATTTT	56.8	226
	ATCTAAATTTAAACGGAGAG		
27a	GTTACAAGTTAAAGAAATGTGT	56.6	298
	CTAACAAGTGGCCTGTGTGCAA		
27b	TTTATTTGTTTATCCAATTATAGAC	54.7	296
	TCCTGTTAAGTCAACTGGGAAAA		
28	TTTCCTTAGGTTCAAAACT	56.0	517
	CTAGGGAGGCCAGGATAT		
29	TCACCCCGTCACCACCACT	58.0	411
	GCAACAACCCCAAATCAAACT		
30	CAACTTCATTTGTGTTTTCTCCT	54.4	282
	CTTTGAATTCTCTTAGAATAATTGT		
31	ATAATTGTTGATGTGATTTTCAT	56.5	424
	AATTTTGAACCAGATGAAG		
32	ATCTAGTATTTTTGAGGCCTC	53.3	312
	CAGATATGCTATAGTACAGAA		
33	TCCTGCTTCTTTACAGGTTA	56.2	409
	AAGTAAAATGGAGAAAGGAACT		
34	TTTTCTGTCTTTACTTGTTCCTT	52.3	384
	CAGTCCATGCAAGTGTTT		
35	GCATGGACTGTGTTATTGG	52.2	319
	TGCAATTAAAAGATCCACA		
36	GTTCTGTGGATCTTTTAATT	55.1	238
	CATTGACCTCAAATTTAAA		
37	CATTCCGAGATTCAGTTTAGG	54.1	236
	AAGTAACATTCAACACTGATAC		

exon	Primer sequence (5'→3')	DHPLC oven temperature (℃)	PCR product size (bp)
38	CTATGTCATGATTCATCTTACTA	57.3	233
	CTAAATTTGAGTAATCTAGGAACC		
39	CTACTGTGTGAACCTCATCAA	53.4	284
	GTAAGACATAAGGGCTAACTTACT		
40	TCAGGGAAGAAGACCTCAGGAGAT	55.9	328
	TGAACTTTCTGCTCTGCCACGCAA		
41	GTGCACATTTAACAGGTACT	55.6	373
	CTTCCTAGGCCATCTCTAG		
42	CTTGGAAGGAGCAAACGATGGT	52.0	356
	CAAAAACTTTGCTACACTGACAT		
43	TTTTCTTTTTAGTGTATTCCCA	54.8	287
	GATTCTAAGAAATGGCTGG		
44	CACGTTAATTCCCTATCTT	56.1	268
	TGAGAAGTAGAAGACTGTAT		
45	CATGAATAGGATACAGTCTTCT	56.7	269
	CACATTACTGGGTAAGCATTTA		
46	AAATGTTCCTCTGTTGAC	56.0	211
	CATCAACCATCCTTCTCCA		
47	CTGTTACAATTAAAAGATACCTT	55.4	185
	TGTGTGTTCTTAAAGCAGGCAT		
48	TTTTGGCTTCAGATGGGGATTT	55.1	351
	AAGGGAATTCCTAATGTTGGTG		
48a	ATTCCTTCTGAAAACCAA	54.2	280
	AAGGCAGACTGAGCTTAC		
49	CTGGGAGAAACAGGCTAT	57.3	363
	AGCAAGCTTCACACGAT		

3. Sequencing

Those PCR products displaying a heterozygous pattern were purified and were directly sequenced in both directions using a BigDye 3.1 Terminator Cycle Sequencing Kit (PE Applied Biosystem, Foster City, CA) and an ABI-PRISM3100 Genetic Analyzer (PE Applied Biosystem), according to the manufacturer's instructions.

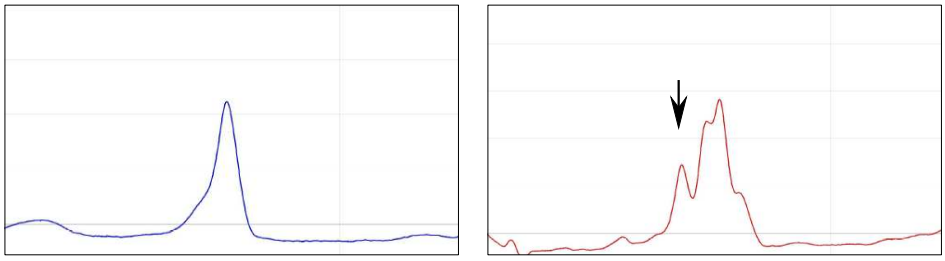
III. RESULTS

1. DHPLC analysis

We screened for mutations in 36 patients who were clinically diagnosed as neurofibromatosis type 1. The whole coding sequences and all splice sites were examined for mutations using DHPLC. PCR fragments

FIG 2. Different DHPLC chromatograms for *NF1* mutations identified. Arrow indicates chromatograms originating from a heteroduplex.

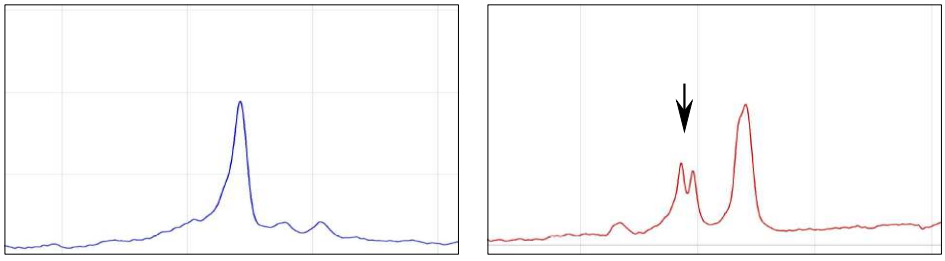
A. Exon 10b



Normal control 1418delCAAG

DHPLC oven temperature = 54.4℃

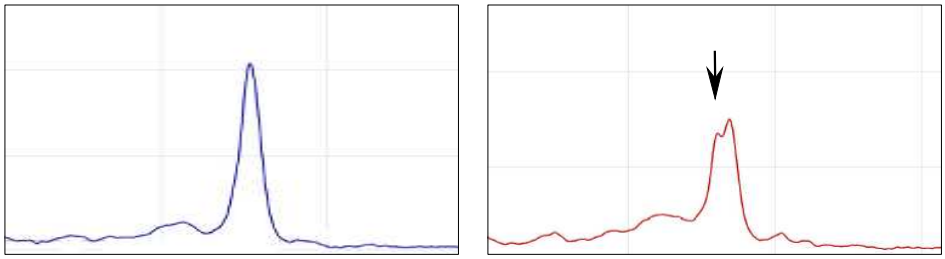
B. Exon 21



Normal control 3525delAA

DHPLC oven temperature = 55.6℃

C. Exon 27a



Normal control E1619K

DHPLC oven temperature = 56.6℃

that showed heteroduplex forms in DHPLC analysis appeared to show abnormal sequences in more than 98%. Fig 2. shows DHPLC chromatograms for *NF1* mutations identified in exons 10b, 21 and 27a.

2. DNA Sequencing analysis

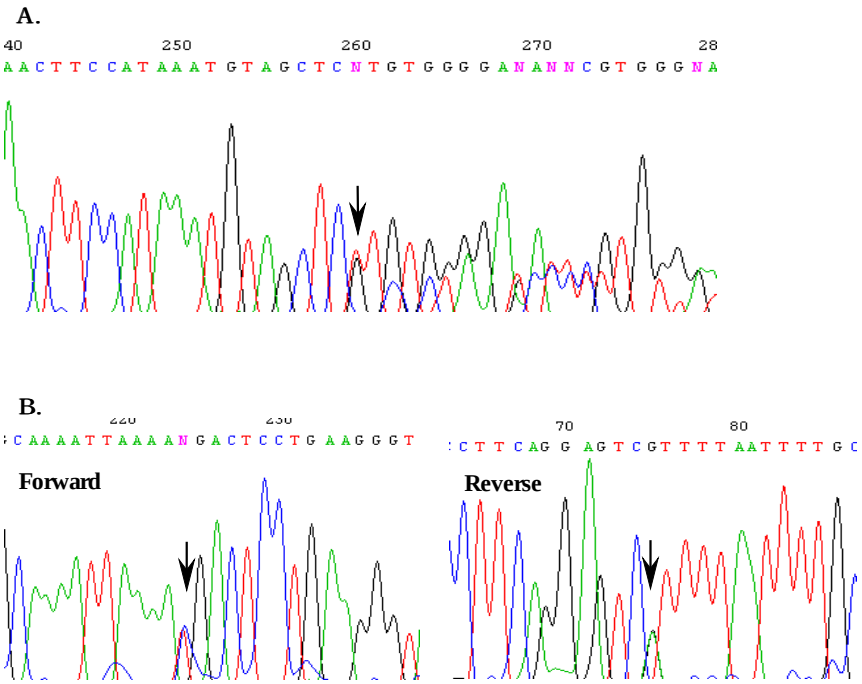
Could find twenty-nine different mutations in direct sequencing with PCR fragment that shows heteroduplex pattern in DHPLC analysis (Fig 3.).

3. Mutation spectrum

All 60 exons of the *NF1* gene were screened by optimised DHPLC analysis in 36 unrelated NF1 patients. Twenty nine different mutations were identified in thirty one unrelated individuals, including nine missense mutations (exons 8, 10b, 28, 34, 41, 42), seven nonsense mutations (exon 4b, 12b, 24, 35, 36, 41, 42), two splicing errors (intron 25, 34), one indel (exon 23-2), seven deletions (exon 7, 10b, 10c, 16, 21) and three insertions (exon 9, 24, 27a).

FIG 3. Results of direct sequencing

A: Electropherogram of exon21 from the patient showing the deletion of A(2816delA) **B**: Nonsense mutation of exon4b(R192X); reported by Fahsold et al.[11]



Two small deletions (2679 del C, 2816 del A) and one nonsense mutation (Gln611Term) were located within the putative cysteine/serine-rich domain (exons 11-17). Six different lesions in the *NF1* GRD region (exons 20-27a) were identified. All of these lesions are novel and together comprise one nonsense mutation (Tyr 1403 Term), one mutation in splice sites (IVS 25 -1 G>A) and four frameshift mutations (3525delAA, 4079 AA>T, 4159insT, 4630insA).

Two splicing errors were detected (IVS25 -1 G>A and IVS34 +45 T>A). These mutations are predicted to generate truncated neurofibromin protein. (Table 3.)

4. Polymorphisms

A total of sixteen polymorphisms detected, were novel (1458 A>C, 1461 A>G, 1393-9 A>C, 1641+117 G>A, 1721+88 A>C, 3315-106 C>T, 3496+37 A>G, 3871-96 A>G, 5379 C>T, 5886 C>T, 6459 T>G), while five were previously reported (288+41 G>A, 702 G>A, 1641+39 T>C, 5540+19 T>A, 7126+37 C>G ; Table 3). Six silent mutations were also identified. (Leu234Leu, Thr486Thr, Arg487Arg, His1793His, Ile1962Ile, Ala2153Ala). (Table 4.)

Table 3. Summary of Mutations identified in the *NF1* Gene. (Nucleotide numbering is based on GeneBank accession no. M82814.)

Location	Genomic mutation	Amino acid substitution	Mutation type	References
Exon 4b	574 C>T	R192X	Nonsense	Fahsold et al.[11]
Exon 7	953 del AAA		Frameshift	·
Exon 7	1017 del CT		Frameshift	Upadhyaya et al.[15]
Exon 8	1150 T>C	F384L	Missense	·
Exon 8	1156 A>G	I386V	Missense	·
Exon 9	1233 ins T		Frameshift	·
Exon 10b	1394 G>C	S465T	Missense	·
Exon 10b	1400 C>T	T467I	Missense	·
Exon 10b	1418 del CAAG		Frameshift	·
Exon 10b	1466 A>G	Y489C	Missense	·
Exon 10c	1541 del AG		Frameshift	Robinson et al.[23]
Exon 12b	1846 C>T	Q616X	Nonsense	·
Exon 16	2679 del C		Frameshift	·
Exon 16	2816 del A		Frameshift	·
Exon 21	3525 del AA		Frameshift	·
Exon 23-2	4079 AA>T		Frameshift	·
Exon 24	4159 ins T		Frameshift	·
Exon 24	4209 T>G	Y1403X	Nonsense	·
IVS 25	IVS25 -1 G>A		Miss splicing	·
Exon 27a	4630 ins A		Frameshift	·
Exon 28	4855 G>A	E1619K	Missense	·
Exon 34	6469 T>A	F2157I	Missense	·
IVS 34	IVS34 +45 T>A		Miss splicing	·
Exon 35	6589 A>T	R2197X	Nonsense	·
Exon 36	6709 C>T	R2237X	Nonsense	Fahsold et al.[11]
Exon 41	7276 G>C	E2426K	Missense	·
Exon 41	7286 C>T	R2429X	Nonsense	Fahsold et al.[11]
Exon 42	7447 C>T	L2483F	Missense	·
Exon 42	7486 C>T	R2496X	Nonsense	Purandare et al.[24]

Table 4. Summary of Polymorphisms Identified in the *NF1* gene.
(dbSNP ID : NCBI SNP database ID ; http://www.ncbi.nlm.nih.gov/projects/SNP/)

Location	Nucleotide change	dbSNP ID
IVS 3	288 +41 G>A	2952976
Exon 5	702 G>A	1801052
Exon 10b	1458 A>C	·
Exon 10b	1461 A>G	·
IVS 10b	1393 -9 A>C	·
IVS 10c	1641 +39 T>C	2905880
IVS 10c	1641 +117 G>A	·
IVS 11	1721 +88 A>C	·
IVS 19b	3315 -106 C>T	·
IVS 20	3496 +37 A>G	·
IVS 23-1	3871 -96 A>G	·
Exon 29	5379 C>T	·
IVS 29	5540 +19 T>A	2285894
Exon 31	5886 C>T	·
Exon 34	6459 T>G	·
IVS 39	7126 +37 C>G	7405740
Exon 5	702 G>A Leu234Leu	·
Exon 10b	1458 A>C Thr486Thr	·
Exon 10b	1461 A>G Arg487Arg	·
Exon 29	5379 C>T His1739His	·
Exon 31	5886 C>T Ile1962Ile	·
Exon 34	6459 T>G Ala2153Ala	·

IV. DISCUSSION

Neurofibromatosis type 1 (NF1), formerly known as Von Recklinghausen Neurofibromatosis, is a common genetic disorder affecting approximately 1 in 3,500 people. It is a fully penetrant autosomal dominant disorder. Strict diagnostic criteria that include café au lait spots, neurofibromas, plexiform neurofibromas, freckling in the axillary or inguinal regions, Lisch nodules, optic or chiasma glioma, pseudoarthrosis, and sphenoid dysplasia define NF1 according to NIH diagnostic criteria. Most disease features are present in more than 90% of patients at puberty.[1] Further manifestations are known to occur in this disorder, including macrocephaly, short stature, learning difficulties, scoliosis and certain malignancies. There is, however, great intra and interfamilial phenotypic variability. In addition a number of patients who have a clinical picture suspected to be NF1 do not fulfil the diagnostic criteria particularly in the younger age groups and large number of patients are asymptomatic in their lifes except café au lait spots. As a consequence, genetic testing would have a major impact on the diagnosis and management of patient's families. However, mutation detection in the *NF1* gene is laborious and complex due to the great variety of possible lesions. These may include chromosomal abnormalities, large deletions or insertions, microdeletions or insertions, splicing mutations, nonsense and missense mutations.[9,10] As previously shown , NFI is a very heterogeneous disease on the basis of phenotypes and genotypes. Experimented to observe whether this mutational heterogeneity exists in

Korean patients with neurofibromatosis type1.

The whole coding sequences and all splice sites were examined for mutations using DHPLC followed by direct sequencing of PCR products. Disruptive mutations were identified in 31 individuals with an overall mutation detection rate of 86%. This is the highest mutation detection rate reported so far for the *NF1* gene by using a single technique to screen patient genomic DNA samples. Previous to this report, Han et al.[17] and Alessandro et al.[21] have applied DHPLC method to the screening of the entire *NF1* gene for mutation, reporting a detection rate of 68% and 72%. DHPLC is unable to detect the multi-exonic and large deletions that comprise approximately 20% of all *NF1* gene mutations.[15,22] In addition, some splice site errors will also be missed by DHPLC as the change may be too far into the intronic or too near the end of the amplimers.[20] Therefore, splicing error was found two cases in this research though splicing error of *NF1* gene is very high than other genetic disorder that is different (26%).[12,13] However, we have confirmed the possibility for routine clinical diagnosis in NF1 by direct mutation detection using DHPLC. DHPLC analysis of the entire *NF1* gene in the present group of Korean patients disclosed a number of mutations well comparable with the previous studies. In terms of mutation detection, DHPLC based heteroduplex analysis appears to be the efficient method available. Its advantages include the low costs, its potential for automation and the speed and sensitivity of each analytical run, which permits the rapid screening of large numbers of patient samples. The use of DHPLC for mutation identification thus represents a

significant advancement in the clinical diagnosis of NF1. If run parallel experiment method such as PTT to overcome shortcoming of DHPLC, NF1 diagnosis will be more efficient.

In this study, we found twenty nine mutations. Twenty one (72.4%) of the identified pathologic mutations are novel, eight mutations have been previously reported. This is an evidence of the high level of mutational heterogeneity in the *NF1* gene. It has also been claimed that missense mutations and single amino acid deletions tend to cluster in two distinct region of *NF1* gene. The regions encompassing exons 11-17 (cysteine/serine-rich domain) and exon 21-27a (GAP-related domain). In contrast to the earlier study of Fahsold et al.[11], in which 17% of alterations occurred within a CpG dinucleotide, only two of the mutations reported here involved a CpG dinucleotide, the two being small deletions (2679delC, 2816delA).

As in the previous studies, it was not possible to correlate the presence or severity of clinical features, malignancy, and/or mental retardation with the type of the mutations[25]. In addition, mutational hot spot was not found in Korean patients with neurofibromatosis type 1. However, when the mutation caused protein truncations, such as frameshifts and nonsense mutations, the symptoms were found more severely.

V. CONCLUSION

1. Showed mutation detection ratio of high efficiency, but detection of splicing error is insufficient. If run parallel experiment method such as PTT to overcome shortcoming of DHPLC, NF1 diagnosis will be more efficient.

2. Mutation was found evenly from *NF1* gene. Two specification domains (cysteine/serine-rich domain and GAP-related domain) and relation of mutation could not be found, but the corelations should be more closely examined through research of more NF1 patients.

3. As in the previous studies, we were unable to correlate the presence or severity of clinical features, malignancy, and/or mental retardation with the site of the mutation[25] and mutational hot spot was not found in Korean patients with neurofibromatosis type 1(Fig 4.). However, when the mutation caused protein truncations, such as frameshifts and nonsense mutations, the symptoms were found more severely.

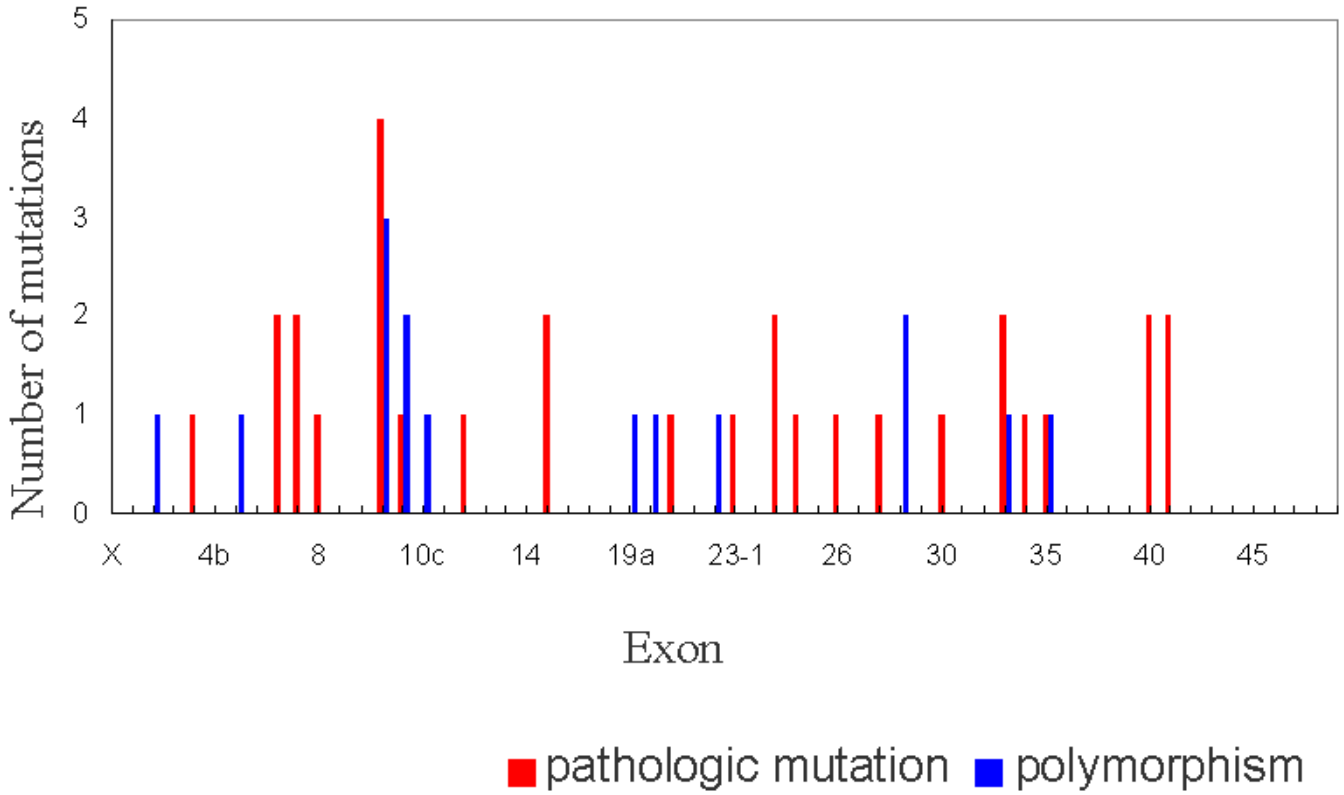


FIG 4. Histogram of number of mutations. As previously reported, no mutational hotspots within the *NF1* gene were identified in Korean NF1 patients.

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ABSTRACT(IN KOREAN)

한국인 제1형 신경섬유종(NF1) 환자의 돌연변이 분석

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제 1형 신경섬유종은 가장 흔한 유전성 질환 중의 하나로 *NF1* 유전자의 돌연변이에 의해 유발되며 상염색체 우성으로 유전되는 질환이다. 약 3,000명당 한명꼴로 나타나며 신경성섬유종, 커피색반점, axillary freckling, lisch nodule등의 증상을 동반하고, 한 가족 안에서도 임상적 증상이 매우 다양하게 나타난다고 알려져있다.

*NF1*은 17q11.2 에 위치한, 350kb 크기의 유전자로 60개의 엑손으로 구성되어있으며, 11-13kb의 mRNA로 전사된 후 2,818개의 아미노산으로 이루어진 neurofibromin을 합성한다. neurofibromin은 ras-specific GTPase activating protein(GAPs)과 기능과 구조가 매우 유사한 GAP-related domain(GRD, 360 a.a)을 가지고 있으며, 이는 ras의 활성조절을 저하시키는 역할을 한다.

제 1형 신경섬유종의 30~50%가 *NF1* 유전자의 자연발생적인 돌연변이에 의해 나타나며 어떠한 유전질환 보다도 높은 돌연변이 발생 빈도를 보인다(~1×10^{4}/gamete/generation). 그로인해 유전자가 규명된지 14년이 지났지만 돌연변이 위치에 대

한 정보는 매우 한정적이다. 그 밖에도 유전자의 크기가 매우 크다는 점, homologous pseudogene의 존재 (10), 특정 위치가 아닌 매우 다양한 위치에서 돌연변이가 발생하는 특징으로 인해 돌연변이의 위치를 찾아내는데 어려움을 겪고 있다.

본 연구에서는 PCR, DHPLC, direct sequencing 방법을 통하여 36명의 *NF*1환자의 유전자 돌연변이를 분석한 결과 31명에게서 발병 원인 유전자를 찾아낼 수 있었다(86%). 분석결과 하나의 indel, 세종류의 insertion, 일곱종류의 deletion, 열여섯종류의 nonsense/miss sense mutations, 두종류의 splicing error, 열여섯 종류의 polymorphsim이 발견되었으며, 그 중 스물한종류의 돌연변이는 보고되지 않은 새로운 것이었다.

핵심되는 말 : NF1, neurofibromatosis type1, 제1형 신경섬유종, neurofibromin.