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ORIGINAL PAPER

Screening for mutation site on the type I neurofibromatosis gene in a family

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Abstract
Purpose The purpose of the study was to determine the sites and types of mutations associated with type I neurofibromatosis (NF1) in the *NF1* gene in a family with NF1 patients.
Methods The blood samples obtained from this family (four patients and one normal healthy individual) were analyzed by performing polymerase chain reaction (PCR) and DNA sequencing for mutation screening.
Results We found synonymous mutations in exons 7, 38, 50, and 56 of the *NF1* gene. This implied that the third codon had a new SNP that did not lead to a change in the amino acid coding. The exon 19 mutation was CAG homozygous, while it was C/TAG heterozygous in normal individuals. The stop codon led to nonsense-codon-mediated decay of the mRNA (NMD), thus resulting in only one copy of the *NF1* gene that encodes the normal protein in individuals.
Conclusions The synonymous mutations in the *NF1* gene occur in exons 7, 38, 50, and 56. The CAG homozygous mutations may occur in exon 19, and the C/TAG heterozygous mutations may occur in the others. This mutation may be responsible for NF1 in patients in this family and may warrant extensive research on the *NF1* gene.

Keywords Gene · Neurofibromatosis · Hereditary diseases · Cafe au lait spots · Mutation · Exon

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Introduction

Neurofibromatosis (NF) is a common dominant genetic disease that is caused by an aberration in a dominant gene and manifests as tumors of neuroectodermal and mesodermal origin. It is characterized by multi-system and multi-organ involvement, especially involvement of the central nervous system, and a variety of tumors. Type I NF, also known as the Von Recklinghausen disease, is the most common type. The gene located on chromosome 17q11.2 is the main cause of multiple nervous system tumors characterized by pigmented skin lesions and multiple NF; it may also be associated with mental retardation, neurological

changes, and neurofibroma-like and sarcoma-like changes. NF1 is currently reported to be associated with a total of 728 mutations, including base substitutions, major mutations, insertions, deletions, nonsense mutations, missense mutations, and frameshift mutations. Most mutations can lead to the production of truncated proteins. By using polymerase chain reaction (PCR) and DNA sequencing, we screened members of a Chinese family for NF gene mutations and found that four members had NF1 and one was normal and healthy.

Materials and methods

Clinical data

We collected the dossiers on a family with patients having type I NF from the Shandong Cancer Hospital between 2009 and 2010. We had data on eight people in the family, of whom six were diagnosed with type I NF. All the patients were diagnosed on the basis of the US NIH diagnostic standard of the year 1987.

The clinical data of three generations of the family has been shown in Fig. 1. The proband (III1) was a 21-year-old woman who had undergone a surgery at the age of 9 years because of a swelling and pain in her left lower extremity along with coffee-with-milk spots (Fig. 2); the pathological diagnosis was NF (Fig. 3). Case I 1 was of a 69-year-old man who had lesions on his abdominal wall, arms, angle of the eyes, and other sites (Fig. 4), and case I 2 was of a healthy female family member. Case II 1 was of a 36-year-old woman with a 1-year history of NF, and case II 2 was of a 40-year-old man with dorsal neurofibroma (Fig. 5). Case II 1 was of a 40-year-old woman who was a healthy subject, and case II 2 was of a 7-year-old girl showing coffee-with-milk spots; she did not have NF. Case III 3 was of a 1-year-old female child with coffee-with-milk spots and no NF.

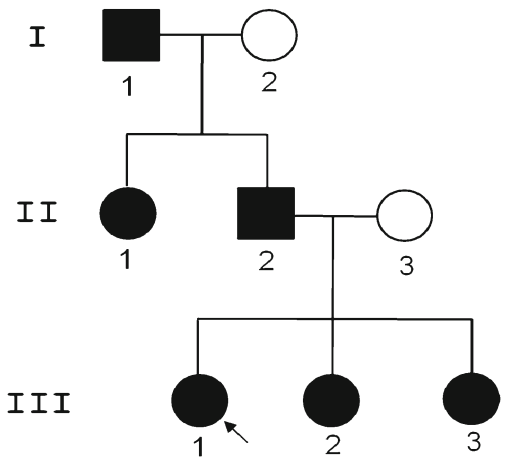

Fig. 1 A diagram showing the affected members of the family

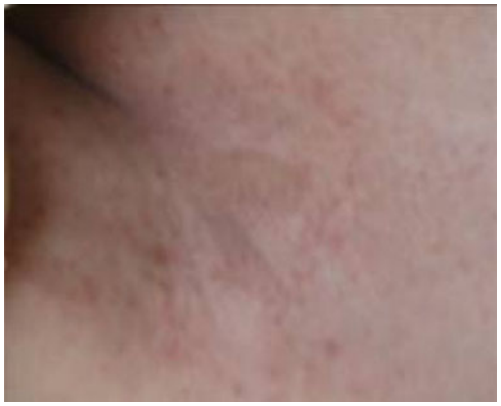

Fig. 2 Patient III 1. A diagram showing the affected members of the family axillary coffee-with-milk spots

DNA extraction

Our study was approved by the medical ethics committee of the hospital, and we obtained informed consents from the family members before collecting peripheral blood samples from four patients diagnosed with NF (I 1, II 1, II 2, III 1) and one normal healthy subject (II 3) of the same family. We stored the blood samples at −85°C in an ultra-low temperature freezer. We extracted the *NF1* gene DNA by using a modified salting-out and precipitation techniques and measured the DNA concentration by using a spectrophotometer.

Primer design

We used the NCBI database to obtain the *NF1* salting-out and precipitation techniques salting-out and precipitation techniques gene sequence salting-out and precipitation techniques and the Primer 5.0 software for designing the primers required to amplify the region coding for the *NF1* gene (Table 1).

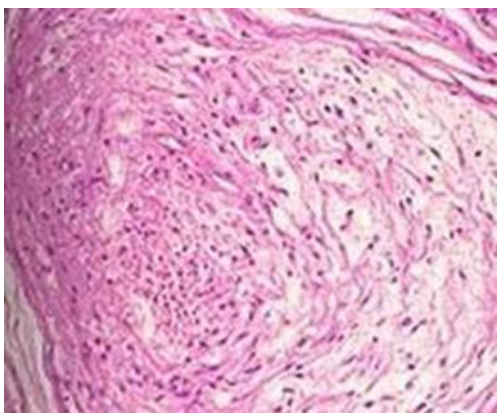

Fig. 3 Patient III 1. Postoperative pathological examination

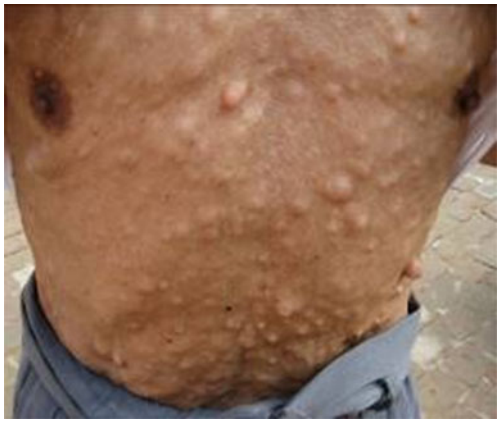

Fig. 4 Patient I 1. Neurofibroma on the anterior chest

PCR amplification

The PCR reaction setup has been shown in Table 2. This reaction was set up in the Biometra PCR instrument, and the reaction conditions were as follows: polymerization at 95°C for 5 min, followed by cooling in an ice bath, a quick spin, addition of 1 μl *Taq* DNA polymerase, and a final quick spin for mixing. The thermal cycling amplification conditions were as follows: pre-denaturation reaction at 96°C for 3 min, denaturation at 96°C for 30 s, annealing at 55°C for 55 s, extension at 72°C for 30 s, and lastly a final extension at 72°C for 10 min. The PCR amplification products were analyzed by performing agarose gel electrophoresis. We used Exon I and the shrimp alkaline phosphatase enzyme for purification.

DNA sequencing

DNA was sequenced by DNA dideoxy sequencing method by using Biomek FX Beckman fluid moving workstation and a NanoDrop 8000 spectrophotometer. We then transferred the reaction plate to the Applied Biosystems 3730 XL fully automatic DNA sequencing instrument and electrophoresed the samples. When we obtained abnormal DNA sequencing results, we used the TA cloning system kit (Invitrogen, San Diego, CA) and directly sequenced the PCR products; we then constructed a color map representing the DNA sequence.

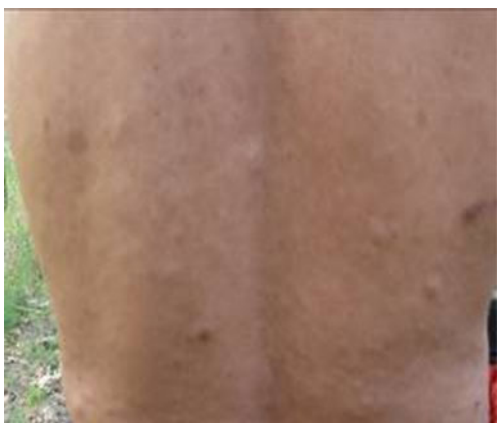

Fig. 5 Patient III 2. Dorsal neurofibroma surgery

Results

We detected *NF1* gene mutations in five blood samples from this family by direct sequencing and DNA cloning methods, and we found totally synonymous mutations in four *NF1* exons—exon 7, exon 38, exon 50, and exon 56. These mutations were new single-nucleotide polymorphisms (SNPs), located on the third codon position, and the amino acid coding did not change. We used a combination of specific primers and universal primers for sequencing, but the peaks were disordered, which indicated that this part of the exon possibly had an indel mutation and should be sequenced. On comparison of the forward and reverse sequencing results, we observed that this part of the exon had a more complex structure and had both an SNP and a deletion mutation, which should be sequenced.

Cloning and sequencing of exons 18, 19, 20, 22, 23, 26, 27, and 36 showed that these exons were completely normal and that there was a nonsense mutation in exon 19 (CAG [glutamine]>TAG [stop codon]) (see Fig. 6). The normal healthy individual was a CAG homozygote, and the patients were C/TAG heterozygotes. The stop codon led to nonsense-codon-mediated mRNA decay (NMD), which led to the formation of only one copy of the *NF1* gene that encodes the normal protein in individuals.

Discussion

NF is a common autosomal dominant genetic disease manifested as neuroectodermal abnormalities. There are approximately 15 million NF patients worldwide with a clear family history, often involving multiple systems and organs [3]. In Von Recklinghausen disease (or NF1-type), the aberrant gene is located on human chromosome 17q11.2 and has a length of 350 kb, with 60 exons; the transcription length is about 11–13 kb, also a 315-kb region of chromosome that is composed of three untranslated regions. Kang et al. [4] found that specific methylation can occur at the transcription-factor-specific binding sites on the *NF1* gene promoter, which is parallel and conformal to tumor cell genome hypomethylation.

NF1 gene mutations mainly include DNA replication errors, point mutation, and other mutations, with replication errors accounting for 20% to 30% gene mutations. The

Table 1 Primer sequences of the target gene

Exons	Primer sequence (5′→3′)	PCR product length (bp)
E1	GAGCCTGCACTCCACAGACCC	297
	TCCTCCAGAGCCTGAGGCAGC	
E 2	ATGGCAAGTAAGTTATTTATGGTC	331
	TATCCAAAGTCCACAGAAAATCA	
E 3	GAGGTAAAATGGAAGACTATTGTTG	363
	AAAATTGTCTGTCACCAGGTCAG	
E 4	TGGTAAGGATGGCAGGGGGATTG	465
	TCCCATGTGGATTTACACACTAACC	
E5	GATGTCTTGCTATGTTGCCCAGG	479
	ATTGCCAAGATTTAAAATGCTCA	
E6–7	AATCATTGATGTCCAAGGCATAT	723
	TTTCCTATTTGACACCAGTTGAC	
E8	CCAGGGATTTTGTTCCTATCTAA	483
	CAGAGGTATCGTTTAGTCTTCTCAGA	
E9	TTCTTTATAGTATGAGTTTTAGAGGC	402
	GAACTTATCAACGAAGAGTCAGA	
E10–11	GGGTGCTTTGTGCTTCTTCTGGC	651
	AAGAAATACGCAAAGAAAAGAAAGA	
E12	TGGAAATCATGGTGTGTGTTTGC	361
	AAAATAACCAAAGCAGCAGGAAT	
E13	AATACTGACCTTATGCTTACTATTGA	384
	TATCCTCAAGGTCTTGGCGTTTC	
E14	TTGAAGTTTCCTTTTTTTCCTTGCA	221
	AAACCACACACCAAAGGAACATCAT	
E15	AATGAAAGAGCTCAATTTCTTAGCA	315
	AAACCATAAAACCTTTGGAAGTGTA	
E16	CTCTAAACTTGTATTCATTATGGGAGA	288
	ATCTCTCACCATTACCATTCCAA	
E17	ATTCCTCTTGGTTGTCAGTGCTT	260
	AACAAACAGAGCACATAAAATGATACA	
E18	TTATACATAAAATTACCCAAGTTGCA	337
	ACTGAGTAAAAAAAACCACTATTCACA	
E19–20	TGGCAGGCAGGGCTCTAAGTG	473
	TAACAGACAAAAGTCAACTTTACAGA	
E21	TAAGGTTTAATTCATGCTTTGCA	516
	ATAGAGAAAGGTGAAAAATAAGAGAAC	
E22–23	CTCTAGGGGGTCTGTCTTCTGGG	518
	TGGACCATATTCCCAAGCACACG	
E24	TGTCACTTAGGTTATCTGGCAAA	453
	CTGTAGACTATTCTTCATAAACTGACAAC	
E25	TTTAAGGTAGCCATTTTGCCAAGAT	400
	TTGCTTTATGTTTTTTGGTGACT	
E26–27	TTGTTTTGGAATGTCTGGTTAGC	622
	ACTTTTCTTCCCCGCTTACTCTA	
E28–29	TTCCTACCTAAGAATAAAAATGGGA	502
	AACAGCGGTTCTATGTGAAAAGAT	
E30	CGTAAGCCATCCAGCCCTGTCAA	460
	CAATTCTCAATGTATTATTCATCCAAAC	
E31	TGTTGCTGTATGTAGTCGGTGCT	226

Table 1 (continued)

Exons	Primer sequence (5′→3′)	PCR product length (bp)
	TTTACAGTGAAGGTCAAATAGGC	
E32	GGACTGATTGATTCAGAGTTTTTATG	389
	GCACATAACTGAAAACCATAGGG	
E33	TATTTGGGAAGGTTAGAAACACT	305
	GAGCAACTGAGTAAGTGGCAAGA	
E34	ATAAGTCTGGGTGTATCTGGTGT	459
	AAAGAGCAAATCTGTGATTTCTT	
E35	GGGAAAAGTAGTGGACTGTGAAGC	452
	GTGGCAAACTCTCCTTCTCAACC	
E36	TACCCTTTAGAATGCCTGTTGCT	318
	AACTTGCCATCTCTCTATATTTGCTA	
E37	ATTCCCACTGTTTTCTTCCTTTC	776
	GAGGCCAGGATATAGTCTAGTTAGTCA	
E38	ATTCTTCTCCACTTCACCCCGTC	417
	ACCCCAAATCAAACTGAAGAGAA	
E39	AAGGGGTATTTTGGTTTTACTGTAG	622
	TAAAGTACCAAACTCTTGCCGCT	
E40	CAGGCCTGATTCTAGGTAATAGTCTT	432
	ACTGTGTTTTTTACAACTCTATCCCTA	
E41–42	ATCTCTTAATCTCTGAAGGAGTCAA	865
	TCAGGTGAAGTAAAATGGAGAAA	
E43	AATACTCAGTGCCAGTTGACCAT	676
	ATGCCTAAAAAAGGGGATACTCT	
E44–45	CAATATGTATTCAGAGTATCCCCTTT	384
	ATATTTGGGAGAAGTGAGGGCGG	
E46	TCCGAGATTCAGTTTAGGAGTTA	342
	CTAATATGACTACTTCAAACAACTAAA	
E47	GAAGAATCAACAAACCTTGGTGA	480
	GCAACAAGAAAAGATGGAAGAGTAC	
E48	AAGAAAGCTACTGTGTGAACCTCAT	511
	GCTCAAGCAATCCTTCCATCTAT	
E49	GTCAGGGAAGAAGACCTCAGCAG	333
	ACTGTGAACTTTCTGCTCTGCCA	
E50	GTTTCTCTACTCAGCAACACTTAGC	754
	AGCACAATCAGACTGGAAGAATAA	
E51	ACTTGGAAGGAGCAAACGATGGT	496
	AGCAAAGGCAAAACAAAATAAGG	
E52	TTAAACACTTTATGTCCAAACATTT	437
	TGGCTACCTATTTACAATGCTGT	
E53–54	GGTGAAGTGATTATCCAGGTGTT	515
	TTAACTTAAAGACAGGCACGAAG	
E55–56	AATGAAGAAATGCCCCAGAAAG	769
	CATTGTGTGTTCTTAAAGCAGGC	
E57	TATTTTTGGCTTCAGATGGGGAT	439
	AAAGTCAAGTCAGTTACAAGGTA	
E58	AATGTGTCCCCGTTGTTAAGCGA	310
	GGGCAAGGACAGGGAAGGGG	

Table 2 PCR reaction system setup

PCR reaction system	Volume (μl)
Phusion® high-fidelity DNA polymerase	0.5
Phusion® HF buffer pack (5×)	10
Deoxynucleotide solution mix (10 mM)	1
DMSO (100%)	1.5
F primer (2.5 μM)	1 1
R primer (2.5 μM)	1
DNA template	1
H_2O	34
Total volume	50

addition or deletion of a small direct repeat sequence in the open reading frame can result in deletion of a terminal inverted repeat sequence during replication, or when skipped reading phenomenon in gene sequence occurs and DNA hairpin-like structure is formed, which eventually leads to the deletions of some DNA sequences. The mechanism underlying these two mutations—in which a single-base substitution results in a missense or a nonsense mutation known as gene point mutation—is fairly complex and unclear. The point mutations are missense mutations with an incidence of about 5–10%, and nonsense mutations have an incidence of approximately 30–38%. The incidence of missense mutations or nonsense mutations that may affect RNA splicing is 20% to 35%. Our study showed that synonymous mutations can occur in the following exons in NF1: exon 7, exon 38, exon 50, and exon 56. Thomson et al. [9] reported the first new exon mutation in exon 26 of the *NF1* gene (A4435G, Ser1479Gly). Analysis of exons 24 to 29 showed that a new mutation in exon 26 affecting RNA splicing resulted in skipped reading phenomenon of the first 68 amino acids in exon 26, which caused a reading frameshift mutation, as analyzed by RT-PCR technique. Messiaen et al. [6] found that a mutation (A1466G) in the region of exon 10 also had a dormant splicing receptor, which can lead to frameshift mutations.

The effect of mutations on gene transcriptional levels has been reported in recent years [1, 7]. The rates of occurrence of other mutations, including large gene-fragment insertions and large gene-fragment deletions, are less than 5%. Chromosomal translocation and inversion are rare types of mutations. The rate of occurrence of mutations caused by large gene-fragment deletions, covering the whole *NF1* gene and the neighboring genes, is about 2% to 10%, and it mainly

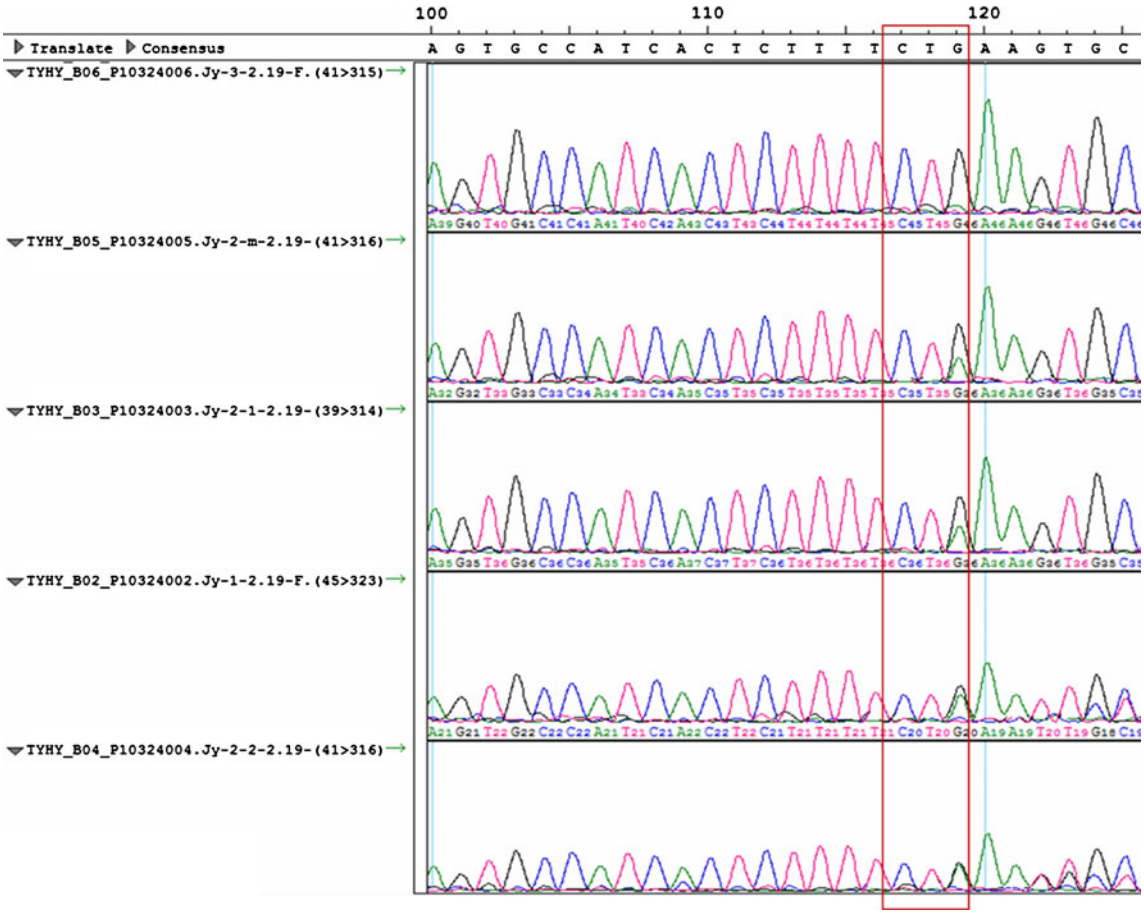

Fig. 6 Exon 19

leads to a deletion through simple sequence repeats [2, 8]. Studies confirmed that deletions of 112 Mb and 114 Mb are, presently, the most common types of deletions. Other researchers also reported one case in which 2-Mb fragments of the *NF1* gene was absent; this fragment was absent mainly in the paternal chromosome. The patient with deleted type of mutations may show disease manifestations at a relatively early age and will have malformations and mental retardation [5].

In this study, we detected point mutation in the exonic region of the *NF1* gene in four type I nerve fibroma patients and one normal healthy subject in a family by using DNA direct sequencing and cloning techniques. We found a new SNP in the *NF1* exon, which was located on the third codon with an unaltered amino acid sequence encoding for synonymous mutated exons, such as exons 7, 38, 50, and 56. Cloning and sequencing confirmed that the mutation in exon 19 is CAG homozygous, and that in other samples is C/TAG heterozygous. The presence of the terminal codon led to degradation of the *NF1* mRNA under the NMD mechanism, which in turn resulted in a unique copy of the *NF1* gene encoding for the normal NF1 protein. We believe this to probably be the first report on NF1 mutation, and our data will supplement genetic and clinical databases and further enrich NF1 pathogenic genetic mutation map. Our study has provided new data on patients with NF1 and will help understand the genetic mechanism underlying this condition. Our data will also provide a basis for future investigations involving NF1 genetic screening and subsequent diagnosis and treatment.

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Conflict of interest The authors declare that they have no conflict of interest.

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