Identification of a Novel NF1 Frameshift Variant in a Chinese Family with Neurofibromatosis Type 1

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Received 25 August 2019; Revised 23 October 2019; Accepted 1 November 2019; Published 9 December 2019

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Neurofibromatosis type 1 (NF1) is progressive neurocutaneous disorder in humans, mainly characterized by café-au-lait macules (CALMs) and neurofibromas. NF1 is caused by variants of the neurofibromin 1 gene (NF1), which encodes a Ras-GTPase-activating protein called neurofibromin. NF1 variants may result in loss of neurofibromin function and elevation of cell proliferation and tumor formation. In this study, a Chinese NF1 family with an autosomal dominant inheritance pattern was recruited. Exome sequencing and Sanger sequencing were performed to discover the causative variant responsible for the family, followed by molecular analysis of effect of the mutated NF1 protein on Ras activity. A novel frameshift variant c.541dupC (p.(Gln181Profs∗20)) in the NF1 gene was identified in all three affected family members. The variant cosegregated with the disease phenotypes in the pedigree and was absent in 100 healthy controls. Bioinformatic analysis showed that the variant c.541dupC (p.(Gln181Profs∗20)) was pathogenic. Further molecular analysis verified the cells expressing NF1 variant p.(Gln181Profs∗20) partially enhanced Ras activity and elevated cell proliferation and tumor formation due to loss of neurofibromin function caused by the variant. Taken together, the data strongly advocate the c.541dupC (p.(Gln181Profs∗20)) variant as the underlying genetic cause of the Chinese family with NF1. Moreover, our findings broaden the spectrum of NF1 variants and provide molecular insights into the pathogenesis of NF1.

1. Introduction

Neurofibromatosis type 1 (NF1; OMIM 162200), also known as von Recklinghausen disease, is a progressive autosomal dominant disorder in humans, mainly characterized by café-au-lait macules (CALMs), neurofibromas, skinkold freckling, and Lisch nodules. NF1 is one of the most widespread genetic disorders worldwide with a prevalence of 1/3500 live births [1]. Currently, many studies show that NF1 is solely caused by variants in the neurofibromin 1 gene (NF1), which encodes a RAS-GTPase-activating protein called neurofibromin [2, 3]. The NF1 gene is located on 17q11.2, containing 60 exons and spanning 282,751 bp in length. Loss of neurofibromin function caused by NF1 variants may lead to enhanced Ras activity and uncontrolled cell proliferation [4]. So far, more than 2700 disease-causing NF1 variants have been reported in the Human Gene Mutation Database (http://www.hgmd.org), and these variants are distributed throughout the gene [5]. Owing to the large size and complexity of the NF1 gene, using conventional Sanger sequencing to identify NF1 variants is extremely time-consuming and expensive; in contrast, exome sequencing is a powerful and cost-effective tool which reveals the genetic basis of the disease [6]. In this study, we first performed a combination of exome sequencing and Sanger sequencing, and the results revealed a novel frameshift variant c.541dupC
(NM_001042492.3, p.(Gln181Profs*20)) in the NF1 gene in a Han Chinese family with NF1. Then, molecular analyses demonstrated the cells expressed the NF1 variant partially enhanced Ras activity and elevated cell proliferation and tumor formation.

2. Materials and Methods

2.1. Subjects. A Han Chinese NF1 family with autosomal dominant inheritance participated in the study. We obtained written informed consent from all participants and carried out this study according to the Declaration of Helsinki. This study was also approved by the Medical Ethics Committee of Hunan University of Medicine. Eight members (three affected; Figure 1(a)) from the family were enrolled and performed complete dermatological and physical examination. The diagnosis of neurofibromatosis followed the consensus criteria of the National Institutes of Health, while the proband (III1) was diagnosed with NF1 by excisional biopsy (Figure 1(d)). 100 unrelated ethnically matched controls were also recruited in the study for exome biopsy (Figure 1(d)).

2.2. Exome Sequencing. Genomic DNA (gDNA) was extracted from peripheral blood as described in the manufacturer’s instructions (Tiangen Biotech Co. Ltd, Beijing, China). Exome sequencing for the proband was performed by the GENEWIZ-Suzhou, China. According to the manufacturer’s instructions (Tiangen Biotech Co. Ltd, Beijing, China), the sequence reads were aligned to a human genome reference obtained from the UCSC database version hg19 (http://genome.ucsc.edu) using the Burrows-Wheeler Alignment tool. SAMtools was used to detect single nucleotide polymorphism (SNP) of the candidate variants.

2.3. Verification with Sanger Sequencing. After exome sequencing, Sanger sequencing was used to verify genetic defects. Sequences of primers for potential causative variants in the NF1 gene (NM_001042492.3) were designed and synthesized as follows: 5′-TCTTGTGGGGGAAATCTGTTGAA-3′ and 5′-CCTATAGCCACCCTTGAGAGA-3′. PCR was performed with 30 μL reaction mixtures containing 40 ng of genomic DNA, 1.0 μL of the forward and reverse primers for the final concentration 1.0 μM, and 15 μL of 2 × Taq Master Mix (Huiling Biotech Co. Ltd, Shanghai, China). Thermocycling was performed using the following program: initial denaturation at 95°C for 2 min, followed by 35 cycles of 94°C for 10 s, 59°C for 30 s, and 72°C for 1 min, and final extension at 72°C for 5 min. PCR products were purified with the Cycle-Pure Kit (OMEGA; Bio-Tek, Doraville, GA) and sequenced using an ABI PRISM 3730 automated sequencer (Applied Biosystems). Cosegregation analysis was subsequently performed with available DNA samples from family members.

2.4. Cell Culture and Transfection. The HEK293T were cultured in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 100 IU/ml penicillin-streptomycin (Sigma, USA). The cells were incubated at 37°C in 5% CO₂.

The NF1 expression constructs were generated using human NF1 cDNA ligated into the BamH 1 and Not 1 sites of the pcDNA3.1 vector. The PCR reaction for NF1 WT cDNA was performed. The c.541dupC variant was performed with PCR-based mutagenesis. The NF1 WT plasmid was used as a template, and the site of variant was covered with the internal primers containing Bbs1 recognition sequences. The c.541dupC variant cDNA was ligated to the pcDNA3.1 vector. All primers for constructed plasmids are shown in Table 1.

The plasmid DNA containing NF1 WT or c.541dupC variant was amplified in DH5α and purified using the Plasmid Mini Kit (OMEGA, USA). The constructed NF1 WT or c.541dupC variant plasmids were validated with sequence analysis. The constructed plasmids were transfected into HEK293T according to the protocol of Lipofectamine® 3000 reagent (Thermo, USA).

2.5. Apoptosis and Immunoblotting Analysis. The HEK239T cells were transfected with the NF1 WT or NF1 mutant constructed plasmids at 70–80% confluency. After being transfected for 24 h, the cells were harvested and stained with Annexin V-FITC for apoptosis analysis via flow cytometry according to the manual of an Annexin V-FITC Apoptosis Detection Kit (Dojindo, Japan).

After being transfected for 24 h, the cells were collected for immunoblotting. The cell lysates were collected according to our previously published protocol [7]. The membrane was incubated with anti-Ras or anti-β-actin at 4°C overnight and incubated with anti-rabbit antibodies at room temperature for 1 h. The results of immunoblotting were visualized by chemiluminescence.
2.6. Statistical Analysis. All data from 3 independent experiments were represented as the mean ± standard deviation and analyzed with GraphPad Prism 8 and SPSS 24.0 software. The ANOVA was used for analyzing the statistical differences. The $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Clinical Manifestation. Three patients in the pedigree were clinically diagnosed with neurofibromatosis type 1. The proband (III1) was a 32-year-old man born with CALMs on his back and thighs. He developed skinfold freckling all over the body at the age of 8 years. These skin pigmentation spots increased in number with age. At the age of 12 years, many subcutaneous soft nodules were found on the trunk of the proband, which gradually scattered over his whole body before adulthood. Dermatological examinations revealed nearly 100 subcutaneous neurofibromas in different size over his entire body covering his face, limbs, and particularly the trunk, with a diameter that varied widely from 1 to 3.5 cm (Figure 1(b)). Histopathology of the resected tumor demonstrated a neurofibroma (Figure 1(d)). The father of the proband (II2), a 58-year-old patient, was born with a large CALM on his back. With age, he developed hundreds of subcutaneous neurofibromas and an increased number of CALMs and skinfold freckling all over his body. The younger sister of the proband (III2) was a 29-year-old female with similar clinic manifestation to the proband, but the number of subcutaneous neurofibromas and CALMs was much fewer. At the age of 20, a tender mass was observed on the radial side of her left hand’s thenar. This mass was resected twice, but regenerated. The recurrent mass had a diameter of 5.5 cm (Figure 1(c)). Histopathological features of the lesion were in accordance with neurofibroma. Moreover, these patients were obviously shorter than normal. No abnormalities were found on ophthalmologic examination or magnetic resonance imaging (MRI) of the central nervous system in three affected individuals.

3.2. Exome Sequencing. The proband generated 103,982,266 raw reads with a mean read length of 150 bp according to exome sequencing; 98.26% (102,172,975) of these raw reads were aligned to the human reference genome. Synonymous variants and known common variants described in dbSNP137, 1000 Genomes data, HapMap8, and the YH1 project were excluded. Nonsynonymous variants were predicted using SIFT, PolyPhen-2, and MutationTaster to eliminate benign variants or tolerated variants. In the known disease-causing gene for NF1, a novel heterozygous variant, c.541dupC (NM_001042492.3) and p.(Gln181Profs*20) (NP_001035957.1), in exon 5 of NF1 gene was identified in the proband (Figure 2(a)). However, no variants of NF1 modifier genes were found in exome data. We proposed that the slight phenotypic variability in this family could be due to environmental factors.

3.3. Identification of Causative Variants. The heterozygous variant c.541dupC (p.(Gln181Profs*20)) in the NF1 gene (Figure 2(b)) was found in all three affected family members according to Sanger sequencing and was absent in the healthy family members and 100 ethnically matched normal controls. The variant in the NF1 gene cosegregated with the disease phenotype in this family, suggesting that the c.541dupC (p.(Gln181Profs*20)) variant was likely

![Figure 1: Pedigree and clinical photographs. (a) Affected individuals were indicated by solid squares (males) or circles (females). Normal individuals were indicated by open symbols. Arrow showed the proband. (b) Back of the proband (III1) covered in neurofibromas. (c) A large neurofibroma on the radial side of left hand’s thenar of the proband’s sister (III2). (d) Biopsy of the proband (III1) showed that spindle-shaped tumor cells with extended wavy nucleus were immersed in a collagen background.]

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<th>Table 1: The primer of NF1 for plasmids constructed.</th>
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<td>NF1 F</td>
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<td>NF1 R</td>
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<td>NF1 c.541dupC F</td>
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2.6. Statistical Analysis. All data from 3 independent experiments were represented as the mean ± standard deviation and analyzed with GraphPad Prism 8 and SPSS 24.0 software. The ANOVA was used for analyzing the statistical differences. The $p < 0.05$ was considered as statistically significant.
3.4. The p.(Gln181Profs*20) Variant Partly Abolished NF1-Induced Apoptosis. After being transfected for 24 h, the cells were harvested for apoptosis analysis. The results are shown in Figure 4(a), and the apoptotic rate of the cells with overexpressed NF1 WT was 16.7%, which was apparently higher than the cells with control vector (Ctrl) (p < 0.001). Meanwhile, the apoptotic rate of the cells with overexpressed NF1 p.(Gln181Profs*20) mutant (MT) was higher than that of the control group (p < 0.05), whereas the apoptotic rate of MT was significantly lower than that of the WT group (p < 0.001).

To delineate the molecular mechanism of NF1 MT in apoptosis, the western blotting was performed for monitoring expression of Ras regulated by NF1. As shown in Figure 4(b), the expression of Ras in the cells with overexpressed NF1 was significantly lower than that in the control cells (p < 0.01). However, cells overexpressed with NF1 MT were higher than with NF1 WT (p < 0.05), but markedly lower than that of control cells (p < 0.01), indicating NF1 MT attenuated capacity of NF1 downregulated Ras expression.

Taken together, the results demonstrate that NF1 p.(Gln181Profs*20) mutant partly abolished NF1-mediated apoptosis via inhibiting downregulated Ras expression.

4. Discussion

Neurofibromatosis type 1 is a rare neurocutaneous genetic disease with two major clinical symptoms, i.e., neurofibromata and the café-au-lait spots. The significant advances in the understanding of NF1 etiology are attributed to the discovery of the NF1 gene. Neurofibromin encoded by NF1 gene is a large multidomain protein consisting of 2818 amino acids, which contains a RAS-GTPase-activating protein-related domain that can inactivate p21-RAS by converting the active p21-RAS-GTP to the inactive p21-RAS-GDP [3, 8, 9]. RAS is a crucial component in the RAS-MAPK signaling pathway, in which neurofibromin

**Figure 2:** Sequence chromatograms of the variant. (a) Detection of a candidate variant c.541dupC (NM_001042492.3) in exon 5 of NF1 gene with whole exome sequencing. (b) Validation and cosegregation of NF1 variant in the family with NF1. The frameshift variant c.541dupC (p.(Gln181Profs*20)) was found in all three affected patients and was absent in the normal family members by Sanger sequencing. Sequence chromatograms of the proband (III1) and his healthy mother (II3) were shown. The arrow indicated the mutated site.

**Figure 3:** Multiple sequence alignment of NF1 from different species. The results indicated high conservation of the amino acid sequence around the Gln181 residue across 10 different species.
functions as a regulator of signals for cell proliferation and differentiation, while being short of neurofibromin, it will promote uncontrolled cell proliferation [10]. Therefore, neurofibromin is thought to act as a tumor suppressor. Variants in the \textit{NF1} gene lead to a loss in neurofibromin function, causing downstream cell growth activation [11, 12]. Up to now, many types of variants have been reported, including chromosome abnormalities, base substitutions, insertions, deletions, splice-site variants, 3’-untranslated region variants, and frameshift variants. However, no true variant hot spots have been found in \textit{NF1}, and variants identified so far are randomly scattered within the \textit{NF1} gene [13]. In this Chinese pedigree, a novel frameshift variant c.541dupC (p.(Gln181Profs*20)) in exon 5 identified brings about a premature stop codon at codon 200, which results in a truncated protein of 199-amino acid residues instead of full-length neurofibromin. This produces a variant neurofibromin which loses the entire functional domains. The novel variant is a loss of function mutation in the light of the classification guidelines of ACMG. Moreover, multiple sequence alignment from 10 different species reveals a high degree of conservation around the Gln181 residue of the neurofibromin, demonstrating its functional importance and the potential pathogenicity of the variant. The majority of disease-causing \textit{NF1} variants are truncating variants, which are predicted to generate haploinsufficiency of the neurofibromin owing to the nonsense-mediated mRNA decay (NMD), the shortage of functional domains, or the degradation of the truncated proteins [14]. This might interpret the occurrence of \textit{NF1} in the Chinese family owing to the c.541dupC frameshift variant of \textit{NF1} gene.

In addition, molecular mechanism of c.541dupC (p.(Gln181Profs*20)) frameshift variant of \textit{NF1} gene identified in the Chinese family was further studied. The results revealed that this variant partly abolished the function of \textit{NF1} protein, which might lead to increased activation of the RAS-MAPK pathway. It was likely that the RAS-GTPase activation domain was lost or this domain was unexposed due to the \textit{NF1} truncation variant [15, 16]. Therefore, the activity of RAS was not completely inhibited by the hydrolysis of the GTP; subsequently, the RAS-MAPK signaling pathway was partially activated and thereby promoted uncontrolled cell proliferation and tumor formation for the Chinese family.

5. Conclusion

In conclusion, by a combination of exome sequencing and Sanger sequencing, we found a novel disease-causing variant (c.541dupC) in the \textit{NF1} gene from a Chinese family with \textit{NF1}. Functional research implied that this novel variant may enhance Ras activity and elevate cell proliferation and tumor...
formation. The current study expands the spectrum of \textit{NF1} variants and provides further evidence that the loss or decreased function of the neurofibromin results in \textit{NF1}. In addition, whole exome sequencing can be used for exact and rapid identification of \textit{NF1} variants to establish the molecular diagnosis of \textit{NF1}.

**Data Availability**

The data used to support the findings of this study are included within the article.

**Conflicts of Interest**

The authors declare that they have no conflicts of interest.

**Authors’ Contributions**

Guoyao Xu and Ming Li contributed equally to this work.

**Acknowledgments**

The authors greatly thank the patients and their family members for their participation in this study. This study was supported by grants from the Natural Science Foundation of Hunan Province, China (2018JJ2278 and 2017JJ3224), and College Students Innovative Pilot Scheme of Hunan Province (2018-1225), China.

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