NF1 Mutations Analysis Using Whole Exome Sequencing Technique in 11 Unrelated Iranian Families with Neurofibromatosis Type 1

Samira Foji[1], Saeed Dorgaleleh[2], *Morteza Oladnabi[3, 4], Leila Jouybari[5]

[1]PhD Candidate in Nursing, Student Research Committee, Golestan University of Medical Sciences, Gorgan, Iran. [2]MSc Student Research Committee, Golestan University of Medical Sciences, Gorgan, Iran. [3]PhD, Gorgan Congenital Malformations Research Center, Golestan University of Medical Sciences, Gorgan, Iran. [4]Ischemic Disorders Research Center, Golestan University of Medical Sciences, Gorgan, Iran. [5]PhD, Nursing Research Center, Goletsan University of Medical Sciences, Gorgan, Iran.

Abstract

Background
Neurofibromatosis is an autosomal dominant disease. It affects one in 2,700 to 3,300 people. The main gene mutated in the disease is a tumor suppressor protein called neurofibromin. There are several categories, the most important of which is divided into two types of type I and type 2 neurofibromatosis. Here, we aimed to identify the underlying genetic defect in eleven Iranian families with Neurofibromatosis type 1.

Materials and Methods
In this cross-sectional descriptive study, 18 patients were studied in 11 Iranian families. After clinical examination by the relevant specialist, DNA extraction was performed on the affected individuals, and then whole exome sequencing was used for accurate diagnosis.

Results
11 individuals (4 males and 7 females) with average age 26 ± 1.18 year participated in the study. Precise diagnosis of type 1 neurofibromatosis was made. The location of the gene and even the type of mutation was also determined. These mutations, reported in eleven families include 4 deletions (c.747_75 del ATTTG, c.1458.1459delAA, c.1186-13delT, c.2804_2804delA), 3 nonsense mutations (Arg1306x, R1276X, L276X,), 2 splice site mutation (c.1261-2A>G, c.1185+1G>T), a silent mutation (c.3395G>A), and an Insertion mutation (c.4446_4447insT).

Conclusion
In conclusion, owing to the complexity of the diagnosis and, in some cases and the need to better-understand the molecular mechanisms of the disease, determining the genetic mutation profile of the disease may be of great help in better understanding the disease and Whole Exome sequencing is an extremely efficient method to identify possible disease-causing mutations.

Key Words: Cancer, Family, Iran, Neurofibromatosis Type 1, Tumor.

---

*Corresponding Author:

Dr. Morteza Oladnabi, Department of Medical Genetics, School of Advanced Technologies in Medicine, Golestan University of Medical Sciences, Gorgan, Iran.

Email: oladnabidozin@yahoo.com

1- INTRODUCTION

Neurofibromatosis type 1 (NF1) is an autosomal dominant, multi-system tumor-prone disorder with incidence of 1 in 2,700 to 1 in 3,300 (1). Past research shows that children suffering from type 1 neurofibromatosis are shorter in growth than their unaffected same age peers. For example, children with type 1 neurofibromatosis also have a wide range of non-neoplastic features, including (cafe´-au-lait spots. Layered spots, skin patches, and leish nodules), skeletal abnormalities (2), and neurobehavioural disorders (inability to learn, Learning difficulties and cognitive impairments) are also common symptoms of NF1. NF1 also tends to have lower IQ, whereas findings related to verbal IQ are mixed predictors (ADHD), Autism spectrum disorder and macrocephaly are common features of this disorder; however, the pathophysiology of abnormal growth, NF1, is still not fully understood. Not all cafe´-au-lait macules are type 1 neurofibromatosis (3).

In 1982, Ricciardi classified heterogeneous neurofibromatosis disorders into eight types; but these are not universally accepted. Other categories include neurofibromatosis 1, neurofibromatosis 2. The most common form is neurofibromatosis 1 (96%), followed by neurofibromatosis 2 (3%), and more recently a lesser-known form of schwannomatosis is a tumor suppressor syndrome that is susceptible to neurofibroma gliomas, café au lait macules, and bony. It affects all groups, races and genders equally. This disease and other phenotypic-related diseases such as schwannomatosis in childhood and adulthood often lead to severe complications. Until recently it was believed that there was no cure for NF1 confirmation of diagnosis in people who are out of the diagnostic criteria or when using prenatal diagnosis. Early genetic diagnosis of this particular malformation has led to the hope that recent therapies, especially molecular targeted therapies, may be a more effective treatment approach (1). A serious inhibitor of clinical trials for NF1-dependent plexiform neurofibromas was the absence of explicit objective measures to evaluate agent efficacy (4). Neurofibromatosis is a heterogeneous group of hereditary cancer syndromes that lead to central and peripheral nervous system tumors as well as other organs. Most plexiform neurofibromas and gliomas are serious complications in these patients (5). NF1 also occurs in mosaics, which are classified as segmental, generalized, or sex glands. Patients with segmental involvement may have areas of pigmentation, tumor growth, or both manifestations confined to one or more parts of the body (1). According to the type of inheritance pattern of the disorder, which has an almost identical split between inheritance and spontaneous, on average 50% of parents have inherited the NF1 mutant allele (6).

Therefore, a lack of family history does not eliminate the possibility of NF1 because 42% of patients have new mutations that are born to healthy parents. Symptoms of NF1 are very different in patients, so timely and accurate diagnosis can be difficult. Therefore, the clinical diagnosis that may exist at an early age may prevent heart problems at an older age. About 30% of NF1 patients up to 1 year of age develop diagnostic symptoms, 97% of patients under 2 years of age have diagnostic criteria, and in a retrospective study of NF1 patients, all patients up to 20 years of age developed diagnostic criteria (7). In some cases, the impact of the mutation at age 20 is close to 100% (1). The NF1 gene was identified in 1990 and was found to be one of the largest genes in the human genome containing 280 kbp of DNA. The NF1 gene is located on chromosome 17q11.2, which encodes a

protein known as neurofibromin. Neurofibromin is a GTPase activator family of tumor suppressor proteins that plays a role in RAS/MAPK signaling and regulates the mechanical target of rapamycin pathways. Even among family members with the same mutation. This is important for genetic counseling because a person with mild clinical findings may have a severe childhood phenotype or vice versa (Neurofibromatosis type 1). This large exon 60 and 300 kb gene has one of the highest spontaneous mutation rates in the entire human genome. It is also one of the largest human genes with a genome size of 282 kb, comprising 57 exons forming three alternate exons. It can also be added that due to the large size of the NF1 gene, its homologous distribution is scattered on other chromosomes, the diversity of mutation positions and the high diversity of lesions make traditional NF1 mutation screening a complex process (8). About 1,500 different mutations have been identified from the NF1 gene and these mutations have been found throughout the large gene. Genetic tests are usually performed on individuals with a family whose pathological mutation has been identified, so a precise mutation in their surroundings can be investigated (5).

Thus loss of neurofibromin function may disrupt this regulation and lead to uncontrolled cell proliferation (9). NF1 molecular testing is currently clinically available. Multi-stage detection protocol is preferred due to the large NF1 gene and the lack of hot spots of mutation. A comprehensive screening procedure for the NF1 gene was performed in 95% of the subjects tested (including both spontaneous and hereditary mutations) based on NIH diagnostic criteria. This holistic approach is optimized by a protein cleavage test followed by fluorescence in situ hybridization analysis, this method identifies about 95% of the mutations that cause the disease, but in people with type 1

segmental neurofibromatosis, examination of damaged tissues is necessary because NF1 mutations are not usually detected in the blood (1). The clinical presentation of patients may also vary. Power, speed, and accuracy, next-generation sequencing techniques offset any deficiencies in the Sanger method. Because there is no single cure for this disease so far, current strategies include proper clinical management, timely detection of disease phenotype (risk assessment), and targeted therapies, which improve the quality of life of patients. New genome sequencing techniques, despite the relatively high cost, have greatly helped early detection of diseases (10). This study aimed to identify NF1 mutations using whole exome sequencing technique in 11 unrelated Iranian families with neurofibromatosis type 1.

2- MATERIALS AND METHODS

2-1. Subjects

According to the announcement from the Iranian Supportive Neurofibromatosis Association in 2020 (as a part of larger project), the genetic tests of 11 families from four provinces were studied (**Table.1**). The study included 11 Iranian families with one to three patients with neurofibromatosis. Given the inherent pattern of autosomal dominant disease as well as the emergence of a new mutation and the complexity of the disease, it may be difficult for the specialist to diagnose the disease.

2-2. DNA Extraction

After obtaining consent form and adhering to ethical principles, we collected 2-5 ml of blood in EDTA anticoagulant tubes and then DNA samples were isolated from Microliter (µl) of mononuclear blood cells using a Favorgen DNA kit according to the manufacturer's instructions and then genomic DNA (gDNA) was controlled using a spectrophotometer and about 2 µg

gDNA was used for constructing deep sequencing libraries. Workflow of this study is shown in **Figure.1.**
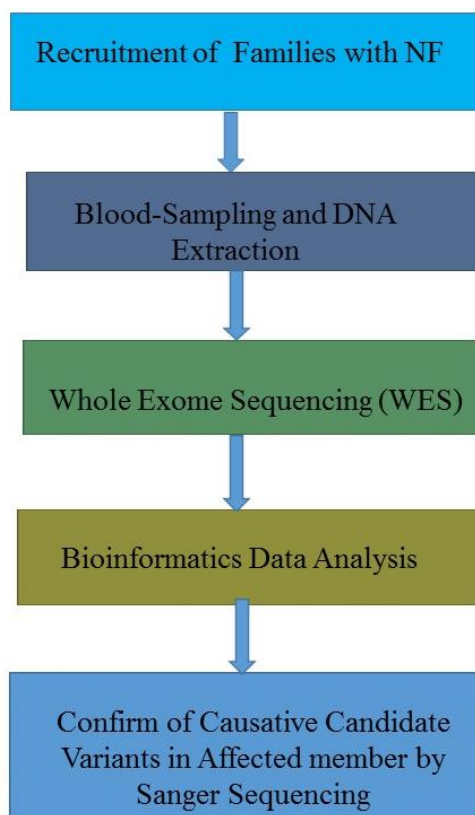
Fig1: Study flowchart.

2-3. Whole exome sequencing

All 11 patients of Iranian Families DNA samples underwent exome enrichment using Agilent SureSelect DNA enrichment kit (Agilent Technologies Inc Santa Clara, CA) to capture 60Mb of human genome. The coverage of the coding exons with at least 40X was call variant at 98% of the coding regions; then exome sequencing technique was performed with Illumina platform. Illumina Pipeline software was used to obtain the raw data needed for bioinformatics analysis. Using pre-set filter criteria, clean reading was extracted from the raw material. Subsequently, at least 90 bp long purified reads were selectively aligned to the human reference genome from the National Center for Biotechnology Information (NCBI) (https: // www.ncbi.nlm.nih.gov/grc) using BWA balance. BWA is a multifunctional software package that produces the output file in BAM format. Then, by analyzing the Bam data, the surface area of the target region, the depth of the sequence, the SNP / InDel / CNV identification were analyzed. SOAPsnp software, Bioinformatic, pileup software and computational framework, were used to identify single nucleotide polymorphisms (and SNPs), insertions / deletions (InsDels), and copy number variations (CNVs) and other modifications. Filter criteria are set for SNP or InDel with at least 10 times more than 20% of total reads. If the frequency is less than 0.05 in dbSNP (www.ncbi.nlm. nih. gov / projes / SNP/), Hapmap (https://www.genome. gov), filtered genome project (SNP) (www.1000genomes.org), healthy reference sample sequencing in this study,

it is considered as a causative mutation of the disease.

2-4. Ethical consideration

This study is a part of a research proposal approved by Golestan University of Medical Sciences by Ethics Committee with ID-code: IR.GOUMS.REC.1398.363. All required ethical issues including the principle of confidentiality and informed consent were included in this study.

3- RESULTS

In this study, 18 patients suspected of having NF of one of the 11 Iranian families were studied. These individuals, who were not the result of kinship marriage, showed the disease according to the autosomal dominant pattern of inheritance (**Table.1**). Of these patients, there were a number of mutations in the VUS that showed excess NF1 mutations (**Table.2**). Since this disease has overlap with other diseases and because of VUS, we have used the WES technique to arrive at a precise and definitive diagnosis.

Table-1: Baseline and clinical characteristics of 11 of patients.

Family ID	Age(Year)	Gender		Severity of the disease according to Riccardi Scale	Time of first signs and symptoms
		Male	Female		
1	41	*		Severe	At birth
2	3	*		Brief	2 months after birth
3	31		*	Mild	At birth
4	25		*	Moderate	from16 years old
5	32		*	Mild	from 15 years old
6	26		*	Moderate	from 12 years old
7	21		*	Severe	At birth
8	6	*		Brief	1 month after birth
9	42		*	Moderate	from 4 years old
10	29		*	Mild	At birth
11	31	*		Moderate	At birth

Table-2: NF1 mutations spectrum in eleven Iranian Families with Neurofibromatosis Type 1.

Code	NAF*	Ref Seq**	Nucleic Acid Alternation	ACMG Criteria	Mutation Type
1	1	NM-001042492.2	c.747_751 del ATTTG P.leu249leu fsx8	Likely Pathogenic	Frameshift
2	1	NM-001042492.2	c.3916C>T P.(Arg1306)	Pathogenic	Nonsense
3	1	NM_00267	c.1458.1459delAA(p.Arg487Lys)	Pathogenic	Frameshift
4	1	NM-001128147	c.827T>A p.L276X	Pathogenic	Nonsense
5	3	NM_001042492	c.1261-2A>G	Pathogenic	Splice

6	1	NM_000267	c.1185+1G>T	Pathogenic	Splice
7	3	NM_000267	c.4446_4447insT	Pathogenic	Frameshift
8	1	NM_000267	c.C3826T p.R1276X	Pathogenic	Nonsense
9	2	NM_001042492	c.3395G>A	Pathogenic	Silent
1	2	NM_001042492	c.1186-13delT	Pathogenic	Frameshift
11	2	NM-000267	c.2804_2804delA Asn935Thr fs	Pathogenic	Frameshift

*Number of affected in family. **Reference Sequence.

3-1. Family NF001

One heterozygous mutation c.747_751delATTTG on NF1 gene has been detected. Although the mutation c.747_751delATTTG (P.lUE249Lue249LuefsX8: Het) has not been reported for pathogenicity, the frequencies of this mutation in normal population are very low. The frameshift mutation leads to early termination of the amino acid coding, which is expected to affect the protein's function. NF1-related neurofibromatosis, type 1 is inherited in an autosomal dominant manner. The mutation c.747_751delATTTG (P.lUE249Lue249LuefsX8: Het) on NF1 gene is possibly a pathogenic mutation in the patient, which is consistent with the clinical diagnosis.

3-2. Family NF002

The NF1 variant c.3916C>T P. (Arg1306) creates a premature stop codon, the variant has been confirmed by Sanger sequencing. According to HGMD professional 2017.3, this variant has previously been described as disease causing neurofibromatosis type 1. ClinVar lists this variant as pathogenic. It is classified as pathogenic (class 1) according to the recommendations of Centogene and ACMG (The American College of Medical Genetics and Genomics).

3-3. Family NF003

A heterozygous mutation c.1458_1459ndelAA (p.Arg487Lysfs*3) on gene NF1 has been detected. Although this mutation has not been reported, the frequencies of it in 1000 genome database and BGI database are very low. The mutation leads to early termination of the amino acid coding, which is expected to affect the protein's function. The mutation c.1458_1459del AA on gene NF1 is possibly the pathogenic mutation of the sample that is consistent with the clinical diagnosis.

3-4. Family NF0004

This sample is heterozygous for a pathogenic variant in NF1 gene. This variant, c.827T>A, p.L276X, has not been reported for its pathogenicity in ClinVar, the frequency of it in public population database is very low. Mutation in this gene is associated with neurofibromatosis type 1. This sample is also heterozygous for a variant in MUTYH gene. Trhis variant c.884C>A, p.P295L has been reported in ClinVar. Mutation in this gene is associated with colorectal adenomatous polyposis, with pilomaricomas.

3-5. Family NF0005

Patients' family history was positive. Her father and some other members in the pedigree have the disease. Heterozygous mutation was detected on NF1 gene of the sample of this patient by Sanger

sequencing, which is the result of next generation sequencing. One likely pathogenic mutation (c.1261-2A>G, Het) on NF1 gene of the sample has been detected. Although this mutation has not been reported, the frequencies of it in normal population are very low, and the splice mutation is expected to affect the mRNA's splicing. The splice mutation is c.1261-2A>G on NF1 gene. Therefore, the possibility exists that this mutation compatible with the disease phenotype within the family

3-6. Family NF0006

A heterozygous splice site mutation was detected in NF1 gene causing neurofibromatosis type 1. The detected mutation was not reported in any of the population databases (ExAC, 1000g, dbSNP). Based on ACMG guidelines the detected mutation can be classified as a pathogenic variant.

3-7. Family NF0007

Next generation sequencing technique was used for every three suspected individuals in this family. The conclusion is that three of them are affected by neurofibromatosis.

3-8. Family NF0008

Regarding the clinical manifestations, one heterozygous pathogenic variant in NF1 gene was found in this patient. Mutation in this gene causes neurofibromatosis type I with autosomal inheritance. Furthermore, four pathogenic/likely pathogenic variants were found in this patient, which are worthy of being studied in the patient and family members at risk: in MUTYH gene causing Adenomas, multiple colorectal (AR) colorectal adenomatous polyposis, autosomal recessive (in clinvar / likely pathogenic ACMG guideline).In the CD36 gene that causes coronary heart disease (pathogenic according to ACMG2015 guidelines). In the BMPR1A gene that causes Juvenline polyposis syndrome (presumably pathogenicity based on the

ACMG 2015 guidelines). In ATM gene causing Ataxia-telangiectasia (AR); Breast cancer (likely pathogenic based on ACMG 2015 guideline).

3-9. Family NF0009

In this family, one patient was identified by the exome sequencing technique, and the rest of the family were tested for variants causing trench disease. A heterozygous c.3395G> A mutation was found in the NF1 gene, which is a pathogenic variant based on taster mutator software (www.mutationtaster.org), and polyphenol software (genetics.bwh.harvard.edu). The basis is HGMD (WWW.HGMD.CF.AC.UK).

3-10. Family NF0010

In this family, on the base of HGMD (www.hgmd.cf.ac.uk) the heterozygote mutation c.1186-13delT is pathogenic variant.

3-11. Family0011

Based on Mutation Taster that this is an online free application the pathogenicity of detected variants (c.2804_2804delA) is confirmed.

4- DISCUSSION

Neurofibromatosis abnormalities 1 and 2 have common features but are in fact two distinct diseases and are examined separately. Thus, it can be said that neurofibrosis is a complex disease that is associated with another disorder (11). Type 2 neurofibromatosis, although less common than type 1, has symptoms similar to those of type 1 (12). In this study, 18 individuals from 11 families with exome sequencing technique were able to precisely identify the causative agent gene. By this technique, we found that in addition to the NF1 gene, there were mutations in several other genes, each of which plays an important role in certain cancers. Thus, accurate and timely diagnosis made through the new

generation sequencing technique can effectively and efficiently manage many untreated diseases and increase their life expectancy. This technique is also used to investigate unknown variants that somehow contribute to the disease (13-17). The aim of this study was to evaluate early mutations or variants of NF1 material that can lead to appropriate clinical management by early detection of the disease. Based on the data obtained, we find that patients with neurofibromatosis are at risk for other cancers, so timely and rapid detection with the help of new generation sequencing techniques can help prevent early death in these patients as secondary tumors are prevented. Although numerous studies have suggested that the diagnosis of complex diseases, such as neurofibromatosis, which overlaps with other diseases and makes it difficult to diagnose (1), precise detection has also been performed. Thus, the interpretation of the data reveals the importance of new generation sequencing techniques and that neurofibromatosis patients are at risk for other cancers.

4-1. Limitations of the study

The limited sample size can be considered as the limitation of this study since the test is very expensive and patients are not covered by insurance. Although in our study the technique of WES was very effective, this technique has some limitations. First, exome sequencing does not target all of the genes in the complete set of nucleic acid sequences for humans; about ninety to seventy percent of exons are targeted. However, approximately ten percent of coding sections may not be covered at adequate levels to reliably call heterozygous variants. Each individual may have small differences in coverage efficiency distributions across the entire exons. Clinical test of any personal exome sequencing is not calculated. Rare variants at the oligonucleotide target site of protein coding genes may affect clinical analytical sensitivity.

5- CONCLUSION

Collectively, we report in this study that WES is an effective diagnostic method for complicated and multi-system involved rare diseases; also, this method is cost-effective and timesaving to determine mutations in patients. This research actually provided a bright future for researchers using the technique. In addition, our research extends the NF1 mutation spectrum in the Iranian population. We hope to eradicate congenital diseases by managing sequelae, by managing genetic counseling before pregnancy and by performing prenatal diagnostic tests in the first and second trimesters of pregnancy, as well as by managing born patients and increase life expectancy in these patients.

6- ACKNOWLEDGMENT

7- CONFLICT OF INTEREST: None.

8- REFERENCES

1. Boyd KP, Korf BR, Theos A. Neurofibromatosis type 1. J Am Acad Dermatol. 2009; 61(1):1-4.

2. Soucy EA, Van Oppen D, Nejedly NL, Gao F, Gutmann DH, Hollander AS. Height assessments in children with neurofibromatosis type 1. J Child Neurol. 2013; 28(3):303-7.

3. Ali A, Haki C, Guven Gumustas O, Yalcin R. Neurofibromatosis type 1 case that has macrocephaly and skeleton anomalies, "Neuropsychological evaluation." Gen Med Open.2018; 2(5): 1-4.

4. Gutmann DH, Blakeley JO, Korf BR, Packer RJ. Optimizing biologically targeted clinical trials for neurofibromatosis. Expert Opin Investig Drugs. 2013; 22(4):443-62.

genetic counseling practice: recommendations of the National Society of Genetic Counselors. J Genet Counsel. 2007; 16(4):387-407.

7. Bergqvist C, Servy A, Valeyrie-Allanore L, Ferkal S, Combemale P, Wolkenstein P. Neurofibromatosis 1 French national guidelines based on an extensive literature review since 1966. Orphanet J Rare Dis. 2020; 15(1):1-23.

8. Yap YS, McPherson JR, Ong CK, Rozen SG, Teh BT, Lee AS, et al. The NF1 gene revisited–from bench to bedside. Oncotarget. 2014; 5(15):5873.

9. Zhang H, Hudson FZ, Xu Z, Tritz R, Rojas M, Patel C, et al. Neurofibromin deficiency induces endothelial cell proliferation and retinal neovascularization. Invest Ophthalmol Vis Sci.2018; 59(6):2520-28.

10. Lohmann K, Klein C. Next generation sequencing and the future of genetic diagnosis. Neurotherapeutics. 2014;11(4):699-707.

11. Ghalayani P, Saberi Z, Sardari F. Neurofibromatosis type I (von Recklinghausen's disease): A family case report and literature review. Dent Res J (Isfahan). 2012; 9(4):483.

12. Asthagiri AR, Parry DM, Butman JA, Kim HJ, Tsilou ET, Zhuang Z,et al.

5. Kresak JL, Walsh M. Neurofibromatosis: a review of NF1, NF2, and schwannomatosis. J Pediatr Genet. 2016; 5(02):098-104.

6. Radtke HB, Sebold CD, Allison C, Haidle JL, Schneider G. Neurofibromatosis type 1 in

Neurofibromatosis type 2. The Lancet. 2009; 373(9679):1974-86.

13. Di Resta C, Galbiati S, Carrera P, Ferrari M. Next-generation sequencing approach for the diagnosis of human diseases: open challenges and new opportunities. EJIFCC. 2018; 29(1):4.

14. Dong YY, Zhang YH, Li HW, Chen LZ, Wang TM, Hu W, et al. A Novel Frameshift Mutation in Neurofibromin 1 Gene in a Chinese Family with Neurofibromatosis Type 1. Chin Med J 2017;130(5):629.

15. Oladnabi M, Haddadi T, Kianmehr A, Mansour Samaei N, Mehri M. A neurofibromatosis type 1 family report with multiple cases in 3 consecutive Generations. J Gorgan Univ Med Sci. 2017;19(2):104-8.

16. Fattahi Z, Beheshtian M, Mohseni M, Poustchi H, Sellars E, Nezhadi SH, et al. Iranome: A catalog of genomic variations in the Iranian population. Hum Mutat. 2019; 40(11):1968-84.

17. Mehrjoo Z, Fattahi Z, Beheshtian M, Mohseni M, Poustchi H, Ardalani F, et al. Distinct genetic variation and heterogeneity of the Iranian population. PLoS Genet. 2019; 15(9)1-29.