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Original research article

Novel mutations and their genotype-phenotype correlations in patients with Noonan syndrome, using next-generation sequencing

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ABSTRACT

Purpose: Noonan Syndrome (NS) is an autosomal dominant disorder with many variable and heterogeneous conditions. The genetic basis for 20–30% of cases is still unknown. This study evaluates Iranian Noonan patients both clinically and genetically for the first time.
Materials/methods: Mutational analysis of *PTPN11* gene was performed in 15 Iranian patients, using PCR and Sanger sequencing at phase one. Then, as phase two, Next Generation Sequencing (NGS) in the form of targeted resequencing was utilized for analysis of exons from other related genes. Homology modelling for the novel founded mutations was performed as well. The genotype, phenotype correlation was done according to the molecular findings and clinical features.
Results: Previously reported mutation (p.N308D) in some patients and a novel mutation (p.D155N) in one of the patients were identified in phase one. After applying NGS methods, known and new variants were found in four patients in other genes, including: *CBL* (p. V904I), *KRAS* (p. L53W), *SOS1* (p. I1302V), and *SOS1* (p. R552G). Structural studies of two deduced novel mutations in related genes revealed deficiencies in the mutated proteins. Following genotype, phenotype correlation, a new pattern of the presence of intellectual disability in two patients was registered.
Conclusions: NS shows strong variable expressivity along the high genetic heterogeneity especially in distinct populations and ethnic groups. Also possibly unknown other causative genes may be exist. Obviously, more comprehensive and new technologies like NGS methods are the best choice for detection of molecular defects in patients for genotype, phenotype correlation and disease management.

1. Introduction

Noonan Syndrome (NS) (OMIM 163950) is a relatively common autosomal dominant, variably expressed, developmental, and complex multisystem disorder [1]. This disease occurs both in familial and sporadic forms [2]. The main clinical features in patients with NS are short stature, facial anomalies, cognitive impairment, and congenital heart defects. Although no causative mutations have been detected in 20–30% of all cases so far, NS etiology is associated with germline mutations affecting the RAS-MAPK (mitogen-activated protein kinase) signal transduction pathway [3]. The pathway is responsible for the regulation of cell proliferation, differentiation, and survival. It involves the activity of a number of proteins that include the RAS GTPase family [4]. NS has a wide range of features, both clinical and genetic, in common with a set of other related, autosomal dominant developmental disorders, also called RASopathies [5]. NS, the most common RASopathy, shows age-dependent clinical features, especially facial dysmorphology, which tends to be more subtle and difficult to detect at higher ages [5,6]. Moreover, genotype, phenotype correlation studies have demonstrated a specific association

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between gene mutated/type of mutation and clinical trait. [7]. Also, some types of cancers have been frequently reported in NS patients with special types of mutations [8,9]. Generally, patients with mutations in specific genes have their specific signs and symptoms too [10–18]. *PTPN11* (most common gene affected in NS), and other genes such as *KRAS*, *HRAS*, *NRAS,* and *BRAF* play essential roles in the RAS-MAPK signal transduction pathway. They also control several developmental processes. Thus, it is not surprising that patients with RASopathies are predisposed to benign or malignant cell proliferative disease [19–24]. Molecular genetic testing is one of the best diagnosis methods for genetic counselling and management [6]. High genetic heterogeneity of NS and related disorders, which affect genes that altogether span over 30 kb of genomic DNA, requires an accurate and fast diagnostic testing protocol. Nowadays, Next Generation Sequencing (NGS) methods—and, more specifically, targeted sequencing—have been shown to be the best choice for early detection. They also save time and costs in detecting molecular characterization of patients [25].

2. Materials and methods

2.1. Sampling and DNA extraction

Fifteen Iranian patients with clinical features suspected of NS or other RASopathies were referred to the Pediatrics section of Imam Reza Hospital, Mashhad, Iran. After the exact clinical assessment using Van der Burgt diagnostic criteria [26], six patients with consanguineous parents and nine patients with sporadic mutations were confirmed. All patients were informed and knowingly signed a consent form for participating in the research project. This research project was approved by the ethics committee at the Mashhad University of Medical Sciences (MUMS). The clinical symptoms—along with the drug/surgery history for each patient—were recorded and pedigrees of patients with consanguineous marriage were drawn using PED6® software. Then 10 ml blood sample in EDTA tubes was collected from all patients. DNA extraction was performed by the standard salting out protocol. Owing to the low quantity of blood samples in some cases, the Genet Bio® kit was used for the extraction process, according to the manufacturer's protocol.

2.2. PCR-based methods and Sanger sequencing

In Phase 1, mutational analysis of the *PTPN11* gene in all patients was performed by PCR and the Sanger sequencing method. Specific primers were designed for each of the 15 coding exons using online softwares, including Batch Primer® and Primer 3 (sequences of all primers are available upon request). According to previous studies, three exons of the *PTPN11* gene were introduced as hotspot regions for mutations (exon 3, 8, 13) [27–29]. Therefore, an amplification of these three exons was performed first. After the Sanger sequencing, analysis of the identified variations was conducted using Sequencher 5.1® software. For new identified mutations, ARMS-PCR was applied with mutation specified primer in 100 healthy people to check and rule out any non-functional polymorphisms. All 100 control individuals were also of the same Iranian ancestral background as the proband. ARMS-PCR consisted of two complementary reactions that utilized three primers in each reaction (sequences of all primers are available upon request). PCR products have been visualized by electrophoresis in 2% agarose gel. There was no product band for the mutation specific primers in all 100 healthy people. Segregation studies were carried out with parental DNAs.

2.3. NGS methods

In Phase 2, a targeted resequencing approach was applied to analyze the coding sequences of the majority of the known RASopathies disease genes with the Personal Genome Machine (PGM) Ion Torrent instrument (ThermoFisher Scientific). By means of the Ion AmpliSeq™ Noonan Research Panel (a Community Panel from ThermoFisher), 14 known disease genes—*A2ML1, BRAF, CBL, HRAS, KRAS, MAP2K1, MAP2K2, NRAS, PTPN11, RAF1, RIT1, SHOC2, SOS1, SPRED1*—were targeted at patients. The panel design spans 47,724 bp over 181 exons and 5 bp padding, with a total of 268 amplicons. Library preparation was performed with the Ion AmpliSeq Library Kit 2.0 (ThermoFisher Scientific). Emulsion PCRs were performed on an Ion OneTouch System (Ion OT2 instrument, ThermoFisher Scientific) with the Ion PGM Template OT2 200 kit. Enrichment of template-positive Ion Sphere Particles (ISPs) was carried out on an OneTouch ES System (ThermoFisher Scientific). ISPs were subsequently loaded onto 318 v2 chip (ThermoFisher Scientific) and sequencing was completed on the PGM, with the use of the Ion PGM Sequencing 200 kit v2. All steps were performed in accordance with the manufacturer's instructions. Data generated by the PGM were transferred to the Ion Torrent server for base calling, alignment to NCBI37/hg19 genome reference and coverage analysis by means of the Torrent Suite Software (Version 4.2, ThermoFisher Scientific). To obtain variants annotation, data was uploaded to the Ion Reporter Software (Version 4.2, Thermofisher Scientific). Variant selection was based, among others, on annotation in clinical databases (OMIM, CliVar), population databases (dbSNP, 5000Exomes), functional effect prediction (SIFT, Polyphen), and conservation (Grantham score). Exonic missense variants not present in population databases and with a predicted damaging effect on protein function were considered for Sanger validation. For the variants found by NGS methods Sanger sequencing confirmation was done. Segregation studies were carried out when parental DNAs were available. Clinical features for both known and novel pathogenic/likely pathogenic mutation-bearing patients were collected before.

2.4. Protein modelling for novel mutations

Also, as part of an in-silico study, one novel discovered mutation from each phase selected and has been modelled using an online Swiss-Prot server for automated modelling and enhanced by the use of the latest structural analyses software and techniques.

2.4.1. PTPN11 *abnormal protein modelling*

To model the deduced novel mutation found in the altered amino acid sequence from *PTPN11* (p.D155N), at first the template most similar to the target mutated protein with 99.63% sequence identity (Accession Code: 4NWF) were chosen for the purpose of modelling. The structure has been solved at a resolution of 2.1 Å. The relevant accession code template was then considered for the homology modelling job using the online Swiss-Prot server for automated modelling [30]. The resulting modelled structure has been adjusted for the purpose of energy minimization using ZMM software. The ZMM software makes use of the Amber all-atom force field with a cutoff distance of 10 Å to minimize the conformational energy in the space of generalized coordinates including torsions and bond angles [31]. Low-energy conformation was reached by the Monte Carlo Minimization Method [32] after 100 sequential minimization tasks failed to improve the lowest-energy conformation. The essential accuracy of the model was then evaluated using PROCHECK [33] and WHAT-IF [34] web servers from the online server at http://nihserver.mbi.ucla.edu/SAVES and the results confirmed that the abnormalities in protein functionality had been caused by structural deficiency that occurred as a result of the alteration in the novel mutated protein *PTPN11* (p.D155N).