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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).

2.4.2. KRAS *abnormal protein modelling*

To find the appropriate homologue model to demonstrate the effects of the novel mutation in the *KRAS* gene (L53W), the Swiss-Model template library was searched with Blast [35] and HHBits [36] for evolutionary related structures matching the target sequence from which, the template with the highest quality and target-template alignment (4q21.1.A– CHRAS P21 PROTEIN CATALYTIC DOMAIN) is chosen. The template has 87.17% sequence identity with a resolution of 2.00 Å and 99% coverage of the target template. Models (Mutant and Normal) are also built on the target-template alignment method by using ProMod3 through Swiss-Model workspace [30]. In case the modelling process with ProMod3 fails, the workspace uses PROMOD-II [37] to build an alternative model. The Global and per-residue model quality has been also assessed by using the QMEAN scoring function [38].

3. Results

3.1. Phase 1 (traditional Sanger sequencing)

After performing PCR on all patients, one previously reported mutation in *PTPN11*A>G transition at position 922 in Exon 8, (p. N308D), was identified in three patients and also one new mutation was found in one patient (Patient Number 1) —*PTPN11*G>A transition at position 463 in Exon 4, (p.D155N).

3.2. Phase 2 (NGS-targeted resequencing)

NGS results showed an average of 45 variants per sample, of which, 29 (64.5%) were intronic, 14 (31%) exonic, and 2 (4.5%) 5′/3′-UTRs variants. Among the exonic variants, an average of one variant not previously reported was found. Coverage analysis revealed an average base coverage depth of 787.7× and an average target base coverage of 98% at 20×, with an average uniformity of base coverage of 94.4%. After applying sequencing, we identified one known mutation (*SOS1* p.R552G) in one patient and selected three previously unreported variants for Sanger validation from three different samples. All variants have been confirmed by Sanger sequencing, indicating the absence of any false positive result. Segregation study of one patient with a variant in *CBL* gene showed that it is inherited from an affected mother, who just had mild ptosis. Considering the variable expressivity for NS symptoms, the identified variant could have a causative role on the patient's features. The other mutations were a result of denovo origin. The details for all mutations found in phase 1 and 2 reviewed in Table 1.

3.3. Protein modelling

The model of two novel mutated proteins were also investigated in terms of stereochemical and geometrical parameters such as G-Factor, bond lengths, and bond angles, for which all the results met the criteria discussed. Moreover, most of the residues were in the favorable regions of the Ramachandran map. Fig. 1 demonstrates the structural effects caused by alterations (p.D155N, p. L53W) in the mutated regions of the *PTPN11* and *KRAS* proteins.

3.4. Genotype-phenotype correlations

Genotype, phenotype correlations were performed in patients with either known mutations (Table 2) or variants with possible causative roles and new 'pathogenic' mutations (Table 3). After performing genotype, phenotype correlation, a new pattern of the presence of intellectual disability in two patients was registered, which discussed in details with other patients features in the next section.

4. Discussion

In 2001, it was demonstrated that germline mutations in *PTPN11,* which encodes the protein tyrosine phosphatase SHP-2, are the main cause of NS [39]. Subsequent studies have highlighted the genetic heterogeneity in NS, in addition to *PTPN11* as the main constitutional mutated gene [40]. There are many other studies that emphasize variable expressivity and clinical heterogeneity in NS [5,7,41–43]. Also, some rare studies have shown chromosomal abnormalities in these patients [44]. But the turning point in comprehensive investigations on NS were those that used NGS techniques for mutational analysis and accurate molecular diagnosis. In most of them, which happened recently, NGS methods revealed some new mutations as well as a true diagnosis in atypical and complicated cases. [4,25]. For example, recent studies performed with NGS techniques identified gain-of-function mutations in *RIT1, RASA2* [2] *SOS2* and *LZTR1* genes in NS patients [45]. These studies proved that NGS methods would be the best choice for diagnostic testing for NS and other related disorders. Now NGS RASopathies panels for specific genes and targeted resequencing is available, which result in more efficient and rapid mutation detection than previous methods. Also, protein modelling to analyze and characterize the effects of protein structural changes on patient phenotypes signs and symptoms in many areas of research is a routine procedure [46,47]. As it is known, the novel alterations, *PTPN11* (p.D155N) and *KRAS* (p. L53W) are placed on the related residues, which are conserved and/or functional protein domains. These domains are commonly found in adaptor and signal transduction proteins that present in the receptor tyrosine kinase pathways [48,49]. As a result, the novel alterations (p.D155N, p.L53W) could have major impacts on changing the function of the relevant proteins by altering the binding sites of the protein that are located on the conserved and/or functional domains. According to American College of Medical Genetics (ACMG) guidelines for variant interpretation [50] and their functional data, to assist in determining the status of all the novel variants *PTPN11* (p.D155N), *KRAS* (p.L53W) and *SOS1* (p. I1302V), the identified changes are the 'pathogenic/likely pathogenic' mutations.

Table 1
List of mutations found in NS patients at phase 1 (traditional PCR methods) and phase 2 (targeted resequencing).

Phase	Case	Gene	Codon change	Amino acid substitution	Status	Segregation
1	1	*PTPN11*	c.463GAT>AAT	p.D155N	Validated	de novo, paternity confirmed
1	4	*PTPN11*	c.922AAT>GAT	p.N308D	Validated	de novo, paternity confirmed
1	5	*PTPN11*	c.922AAT>GAT	p.N308D	Validated	de novo, paternity confirmed
1	8	*PTPN11*	c.922AAT>GAT	p.N308D	Validated	de novo, paternity confirmed
2	2	*CBL*	c.2708GTA>ATA	p.V904I	Validated	Inherited from mother
2	3	*KRAS*	c.158TTG>TGG	p.L53W	Validated	de novo, paternity confirmed
2	6	*SOS1*	c.3904ATC>GTC	p.I1302V	Validated	de novo, paternity confirmed
2	7	*SOS1*	c.1654AGG>GGG	p.R552G	Validated	de novo, paternity confirmed

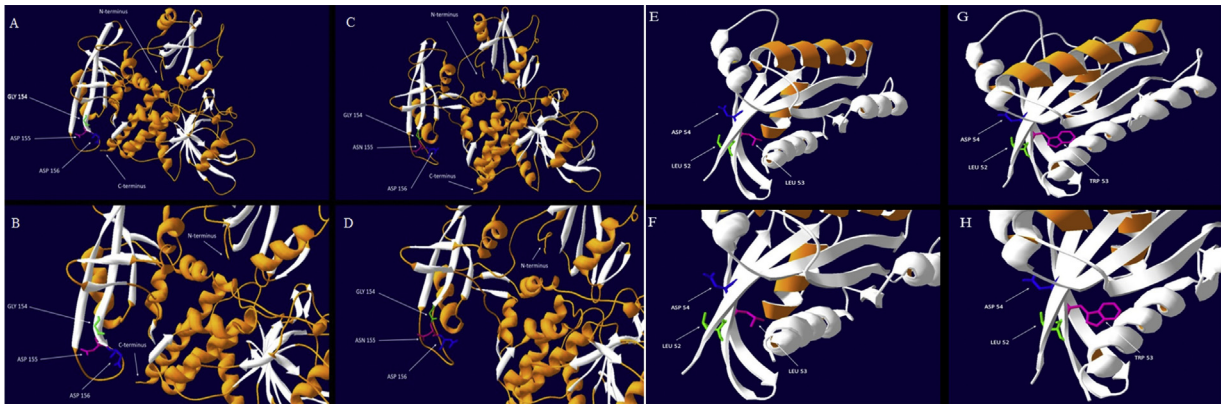

Fig 1. Whole view of the normal protein of *PTPN11* by the use of 4nwf structure (A), Close view of the normal protein of *PTPN11* (B), Whole view of the novel mutated protein of *PTPN11* (C), Close view of the location of novel mutated protein of *PTPN11* (green and blue: surrounding residues, pink: mutated residue) (D). Whole view of the normal protein of *KRAS* (E), Close view of the normal protein of *KRAS* (F), Whole view of the novel mutated protein of *KRAS* and the relevant residue (pink) (G), and Close view of the novel mutated protein of *KRAS*, showing disrupted and altered structure (H). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Genotype-phenotype correlations for patients with known mutations.

Patients code	Mutated Gene	Clinical features	Drug/Surgery history
2	*CBL* (V904I)	[a]HOCM, short stature, Intellectual disability, Hypertelorism, Petosis, Down-slanting palpebral fissures, Depressed nasal bridge, Micrognathia, Low set posteriorly ear.	–
4	*PTPN11* (N308D)	[b]P.S., [c](ASD), Broad thorax, Cryptorchidism, Hypertelorism, Epicanthic fold, Depressed nasal bridge, Micrognathia, High arc palate, Low set posteriorly rotated ear, Reduced auricle and Low posterior hairline.	Orchiopexy (×2)
5	*PTPN11* (N308D)	P.S., short stature, Hypertelorism, Ptosis, Down-slanting palpebral fissures, Epicanthic fold, Micrognathia, High arc palate, Low set posteriorly rotated ear and Reduced auricle.	–
7	*SOS1* (R552G)	P.S./HOCM Typical Echocardiographic for [d]NS, Intellectual disability, Hypertelorism.	–
8	*PTPN11* (N308D)	[e]VSD, short stature, Broad thorax, Hypertelorism, Petosis, Micrognathia, and Low set posteriorly ear, Reduced auricle, and webbed neck.	–

[a] HOCM: Hypertrophic Obstructive Cardiomyopathy.
[b] P.S: Pulmonary valve stenosis.
[c] ASD: Atrial Septal Defect.
[d] NS: Noonan Syndrome.
[e] VSD: Ventricular Septal Defect.

Table 3
Genotype-phenotype correlations for patients with new identified "pathogenic/likely pathogenic" mutations.

Patients code	Mutated Gene	Clinical features	Drug/Surgery history
1	*PTPN11* (D155N)	[a]P.S, short stature, Cryptorchidism, Broad thorax, Hypertelorism, Petosis, Low set posteriorly ear.	–
3	*KRAS* (L53W)	[b]VSD, short stature, Broad thorax, Hypertelorism, High arc palate, Low set posteriorly ear, Reduced auricle, low posterior hairline, webbed neck.	Growth hormone
6	*SOS1* (I1302V)	[c]HOCM, Broad thorax, Cryptorchidism, Intellectual disability	–

[a] P.S: Pulmonary valve stenosis.
[b] VSD: Ventricular Septal Defect.
[c] HOCM: Hypertrophic Obstructive Cardiomyopathy.

Until recently, NS was first considered by some clinical presentations like failure to thrive or typical facial dysmorphisms [5]. Therefore, early diagnosis was just based on clinical features and then investigators would search for mutations using complex and time-consuming approaches like PCR and Sanger sequencing. However, due to genetic heterogeneity, the molecular characterization can still be reached in approximately 75–90% of affected individuals [51]. In this study, the molecular basis of NS in some cases remained unrevealed as well. For this, more comprehensive approaches like whole exome sequencing are needed for detection. This, together with the clinical and genetic overlapping with other disorders, causes, diagnosis and genetic counselling to be very complicated and hard to manage. Now NGS methods have changed in diagnostic approaches for NS and other heterogeneous disorders, such as retinitis pigmentosa and hypertrophic cardiomyopathy. With NGS methods and ultrasonography, early diagnosis in clinics can be antedated with prenatal diagnostic options. Mutational test is a useful diagnostic method to investigate recognized normal karyotype and abnormal ultrasound findings in fetuses. Priority for testing genes with the aforementioned ultrasound findings are *PTPN11*, *KRAS*, *RAF1*, *BRAF,* and *MAP2K1* respectively [52]. As cited before, NS is a heterogeneous disease

Fig. 2. Clinical manifestations of all patients with identified molecular defects.

with different clinical manifestations, so it would require both clinicians and geneticists for accurate diagnosis. This mini-cohort study was the first to investigate Iranian patients with NS in both clinical and genetic features. Like other previous studies, the most mutated gene in patients was *PTPN11*, confirming exon eight as a hot spot for this gene [1,8,39,53,54]. After *PTPN11*, mutations in *SOS1* have been seen more in our patients, which was yet another confirmation regarding previous studies [13,25]. Two 'pathogenic' mutations in *PTPN11* and *KRAS* with one 'likely pathogenic' mutation was found in *SOS1*, which have all been novel. All variants were not registered in HGMD for NS. In the COSMIC (Catalogue of Somatic Mutation in Cancer) database; there was a report for the *PTPN11* p.D155N variant, although not confirmed somatic, and strikingly, the confirmed somatic p.D155Y, both from the large intestine carcinoma. Solid tumors for *PTPN11* mutations were mentioned before too [21,24,55]. Thus, there should be an increased awareness regarding cancer in NS patients with these kinds of mutations. *KRAS* and *SOS1* variants were not reported in COSMIC, but, interestingly, a confirmed somatic variant in the adjacent residue of *KRAS*, p.L52F, was described in skin carcinoma.

Finally, in order to better disease management, it is important to evaluate genotype, phenotype correlations in our patients. Patients with *PTPN11* mutations have symptoms such as pulmonic stenosis, short stature, cryptorchidism, broad thorax, hypertelorism, ptosis, and low set posterior ear. Clinical findings in our patients confirmed that too. *SOS1* mutations show a higher prevalence of ectodermal abnormalities, less intellectual disability, lower prevalence of short stature, and atrial septal defects. Our patients with this mutation showed all these features, too. Moreover, intellectual disability features were also found in a patient with a putative mutation in *SOS1*. Patients with *KRAS* variant showed the expected manifestations, too, with the exception of intellectual disability. Fig. 2 illustrates clinical manifestations of all the patients whom molecular defects have been found. The interesting point in our study of genotype, phenotype correlations was the pattern of the presence of intellectual disability in our NS patients, unlike previous studies. Most references mentioned that *KRAS* mutations are accompanied with intellectual disabilities while this is less associated with *SOS1* mutations [1,12–14,56–64], unlike patients described in this study. Although they just could be exceptional cases, these findings could be considerable for other specialists who want to work with Iranian patients with NS in the future. Also investigations indicate high intra-familial phenotypic variability in NS patients [65]. Such studies remind the intra-familial along the inter-familial genotypes diversities for NS, which have to be considered by physicians too. Other complications include the absence of large families with NS and more male than female patients in the most cohorts along the new unknown causative genes still remained [3]. In this study, we have found variants in only eight patients out of 15. This could be an emphasis on different corresponding genes causing NS in different populations, which may not be included in the present NGS panels for the disease. As mentioned earlier, 20–30% of all patients with NS have unknown gene mutations and this fact indicates more comprehensive genes analysis methods like whole genome/exome sequencing for identifying causative variants in the patients. Furthermore, clinical manifestations in mutation-negative patients could be re-evaluated for ensuring correct diagnosis.

5. Conclusion

Early diagnosis would be highly beneficial for treatment interventions or management of children with NS. Because of the other related/unrelated diseases with overlapping clinical features with NS, molecular testing is the best approach for

differential diagnosis in these patients. Also strong variable expressivity and high genetic heterogeneity along possible unknown other causative genes for NS, especially in distinct populations and ethnic groups make it an appropriate disease for more comprehensive and new molecular detection techniques. Possibly the cost- and time-saving aspects of NGS tests would make it the only way to deal with this type of common diseases. However, the establishment of a genetic basis of NS in specific ethnic groups would be necessary to interpret any acquired data in patients, genotype, phenotype correlation and disease management as well.

Conflict of interest

The authors declare there is no conflict of interest.

Financial disclosure

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Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This research project was approved by the ethics committee at the Mashhad University of Medical Sciences (MUMS).

Informed consent

Informed consent was obtained from all patients included in this study. The patients knowingly signed a consent form for participating in the research project.

The authors confirm that the all figures in the manuscript are original. All the patients are Dr. Peyman Eshraghi (our co-author) patients and collected in Department of Pediatrics, Imam Reza Hospital, Mashhad University of Medical Sciences, Mashhad, Iran. They signed a consent form for the exhibition under Mashhad University of Medical Sciences ethics committee's confirmation.

The authors confirm that all the tables in the manuscript are original.

Novel mutations Genbank accession numbers are include: PTPN11 (c.463GAT>AAT) Genbank accession #: MF547512 KRAS (c.158TTG>TGG) Genbank accession #: MF547513 SOS1 (c.3904ATC>GTC) Genbank accession #: MF547514.

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