Molecular and Clinical Profile of patients referred as Noonan or Noonan-like Syndrome in Greece: a cohort of 86 patients

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Research Article

Keywords: Noonan Syndrome, Rasopathies, PTPN11, Ras/MAPK pathway, Heart Defects, Craniofacial Dysmorphism

Posted Date: June 7th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1267516/v2>

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Abstract

Noonan syndrome (NS) is an autosomal dominant disorder characterized by clinical and genetic heterogeneity. It belongs to a wider group of pathologies, Rasopathies, due to the implication of mutations in genes encoding components of the Ras/MAPK signalling pathway. Recording the genetic alterations across populations helps assessing specific features to specific genes which is essential for better disease's recognition, prognosis and monitoring. Herein, we report the clinical and molecular data of a Greek cohort comprising of 86 NS or NS-like patients admitted at a single tertiary Centre in Athens, Greece. The analysis was performed using Sanger and Next-Generation-Sequencing, comprising 14 different genes. The mutational rates of the confirmed NS-associated genes in the Greek NS population are: *PTPN11* 32.5%; *RIT1* 5.8%; *SOS1* 4.7%; *BRAF* 1.2%; *CBL* 1.2%; *KRAS* 1.2%; *MAP2K1* 1.2%; *RAF1* 1.2%; *SHOC2* 1.2%, corresponding to 50% of positivity in total NS population. The genotype-phenotype analysis showed statistically significant differences regarding craniofacial dysmorphisms ($p=0.005$) and pulmonary valve stenosis (PS) ($p<0.001$) between patients harboring a mutation in any of the tested genes and mutation-free patients. Patients with at least one mutation had 6.71 times greater odds to develop PS compared to mutation-negative patients [OR=6.71, 95%; CI= (2.61, 17.27)]. *PTPN11* positive patients showed higher frequency of epicanthal folds ($p=0.004$), ptosis ($p=0.001$) and coarseness ($p=0.001$) and lower frequency of neurological findings ($p=0.006$), compared to patients with carrying mutations in other genes.

Conclusion: Craniofacial dysmorphisms and PS prevail among mutation positive compared to mutation negative NS and NS-like patients while neurological defects are less common in *PTPN11* mutated NS patients compared to patients with mutations in other genes. The significant prevalence of the Ras/MAPK mutations (17.4%), other than *PTPN11*, in Greek patients, highlights the necessity of a wider spectrum of molecular diagnosis.

What Is Known

- Noonan Syndrome (NS) has been associated with mutations in molecules-components of the Ras/MAPK pathway.
- Clinical and genetic description of NS patients worldwide helps establishing personalized monitoring.

What Is New

- NS and NS-like mutational rate in Greece reaches 50% with mutations identified mostly in *PTPN11* (32.5%), *RIT1* (6%) and *SOS1* (4.7%) genes.

The risk for pulmonary stenosis increases 6.71-fold in NS mutation-positive patients compared to mutation-negative ones.

Introduction

Noonan syndrome (NS) (OMIM 163950) is an autosomal dominant disorder with marked variability either during one patient's lifetime. The estimated worldwide frequency ranges between 1/1,000–2,500 births, although milder expression is thought to occur in 1/100 live births [1]. The syndrome has a multi-system involvement with key features including typical craniofacial dysmorphic characteristics and congenital heart defects, most commonly pulmonary valve stenosis (PS) [2]. Skin pigmentation, lymphatic abnormalities, cryptorchidism, ocular findings, skeletal defects, failure to thrive, developmental delay, and coagulation defects may also be present. Increased risk of developing haematological cancers and cases of rhabdomyosarcoma have also been reported [2; 3].

NS development has been associated with dysregulation of the Ras/MAPK signalling pathway, one of the central molecular pathways regulating proliferation, differentiation and cell's survival [4]. Alterations resulting in instability of its molecule-components, favour their permanent activation, thus, leading to uncontrolled signalling downstream, affecting multiple organs and contribute to the development of cell proliferative disorders, such as cancer. Pathologies associated with germline mutations in genes encoding members of Ras/MAPK pathway present overlapping phenotypes and are grouped under the term of "Rasopathies"[5], with NS being the most common.

Among the members of this molecular network, heterozygous mutations in the *PTPN11* gene, account for the majority of all NS cases [6]. NS patients negative for *PTPN11* mutations, may exhibit a pathogenic variant in a subset of genes encoding downstream regulators and effectors of Ras/MAPK pathway, while the role of other downstream genes such as *A2ML1* [7-9] is still under investigation [10; 11]. Variable mutational rates have been recorded, depending on the number of participants and on the method of patients' recruitment and evaluation [12; 13].

Extended use of Next Generation Sequencing (NGS) techniques during the last decade helps unfolding the heterogeneous genetic basis of NS. The discovery of new variants and new altered genes in NS/NS-like patients highlights potential therapeutic targets [14], and, in combination with specific features, contributes to establish accurate diagnosis and monitoring. Thus, germline mutations in *HRAS* have been associated with Costello syndrome [MIM 218040][15]; in *BRAF*, *KRAS*, *MAP2K1*, *MAP2K2* with Cardiofacio-cutaneous(CFC) syndrome [MIM 115150][16] and in *SPRED1* with Legius syndrome [MIM 611431] [17]. In clinical practice, however, it is often complicated to distinguish syndromes with overlapping phenotypes, especially in young age, as both severity and clinical heterogeneity make the diagnosis challenging.

Our group had previously published *PTPN11* analysis by Sanger sequencing in 60 individuals clinically characterized as NS/NS-like Greek patients[18]. The current study expands the genetic analysis in 14 Rasopathy-associated genes using NGS and presents a more comprehensive molecular and clinical profile of this cohort with inclusion of 26 more patients, along with genotype–phenotype correlations. The genetic testing of the additional genes increases the mutational rate in the NS Greek population confirming the necessity of molecular diagnosis with accurate diagnostic tools.

Materials And Methods

Patients

Study's cohort consists of 86 Greek neonates, children and teenagers recruited during 2005-2018, with the clinical suspicion of NS or NLS. Among them, 48 patients had been previously found negative for *PTPN11* mutations, and 16 had no molecular testing. The clinical and molecular characteristics of 22 *PTPN11* patients (presented in our previous work [18] and recruited during the same period) were also presented and included in statistical analysis of the total Greek NS population. Patients with phenotypic similarities to NS were referred by pediatricians (38/86, 44%), cardiologists (29/86, 34%), endocrinologists (12/86,14%), neurologists (5/86 6%) and dermatologists (2/86, 2%) to our outpatient clinics and were further assessed by a clinical geneticist at the Department of Medical Genetics of Athens University Medical School for evidence of major or minor anomalies. All phenotypes were systematically evaluated and registered by clinical geneticists at the Department of Medical Genetics of the National and Kapodistrian University of Athens. In every patient referred all organic system's impairment, which are involved in NS have been checked as cardiological, endocrine, hematology, urinary,skeletal, skin, hearing, vision and dental. Dysmorphic features were thoroughly itemized and detailed family history as well as a karyotype was undertaken in each case. Patients that fulfilled the criteria developed by Van der Burgt et al [19] (Table 1) as well as individuals with a highly suggestive phenotype who lacked a definitive NS diagnosis were referred consecutively to the Research Lab of the 3rd Department of Peadiatrics, of the National and Kapodistrian University Athens for molecular investigation. Major and minor criteria of NS are depicted in Table 1. In this scoring system patients are first classified according to the facial features (typical or suggestive); and if "typical", the diagnosis of NS is established in the presence of any other major sign (or two minor signs), while in cases with "suggestive" NS face there is need of two major (or three minor) criteria to confirm the NS diagnosis.

Table 1. Noonan syndrome minor and minor criteria-scoring system (van der Birgt et al., [19])

Findings	A major	B minor
Facial	Typical face	Suggestive face
Cardiac	Pulmonary valve stenosis and/or typical ECG	Other defect
Height	<3 rd centile	<10 th centile
Chest wall	Pectus carinatum/excavatum	Broad thorax
Family history	First-degree-relative definite NS	First-degree-relative suggestive NS
Other	All three (males): mental retardation, cryptorchidism, and lymphatic dysplasia	One of three: mental retardation, cryptorchidism, and lymphatic dysplasia

Definite NS: 1A plus one of 2A-6A or two 2B-6B; 1B plus two of 2A-6A or three of 2B-6B

Molecular study

Genomic DNA was isolated from peripheral blood lymphocytes (NucleoSpin Blood, Macherey-Nagel, Duren, Germany). All samples were tested for 14 RASopathy-associated genes using NGS except from the 22 patients previously found positive for *PTPN11* mutations. Targeted analysis referred to *PTPN11*(NM_002834.5), *SHOC2*(NM_007373.4), *RAF1*(NM_002880.4), *BRAF* (NM_001374258.1), *KRAS* (NM_004985.5), *NRAS*(NM_002524.5), *SOS1* (NM_005633.4), *SPRED1*(NM_152594.3), *A2ML1*(NM_144670.6), *MAP2K1*(NM_002755.4), *MAP2K2* (NM_030662.4), *CBL*(NM_005188.4), *RIT1*(NM_006912.6), and *HRAS*(NM_005343.4). NGS was performed using the Ion S5 system (Thermo Fisher Scientific). Sequence reads were aligned to the reference sequence(GRCh38) Ion reporter, followed by variant calling using Noonan workflow version 5.10. Sanger sequencing was used to confirm all clinically significant variants and fill in regions with insufficient coverage(horizontal<100%; vertical<30%).

Statistical Analysis

Clinical and molecular description of NS Greek population, as well as statistical analyses was conducted for all 86 NS patients referred to our hospital. Initial statistical analysis was based on descriptive statistics. Categorical variables including mutations and polymorphisms were described by absolute and relative frequencies, while for continues variables mean, standard deviation, median, minimum, and maximum were provided. The association between clinical features and mutations/polymorphisms was assessed by Fisher's Exact test or Chi square test where the assumptions were met. Multiple comparisons adjustment was necessary due to the various clinical features to be tested across several mutations and polymorphisms. Each mutation/polymorphism was tested for association with 25 clinical features, thus statistical significance level was set to $\alpha = 0.05/25 = 0.002$ (Bonferroni correction). Statistically significant association between mutations and polymorphisms was not found. Entire statistical analysis was conducted in STATA 15.1 and all tests were two-sided

Results

Molecular Study

In the total Greek NS cohort, 50/86 cases (58%) were positive for mutations in, at least, one of the Rasopathy-associated genes tested. Among them, 28 patients (32.5%) were *PTPN11* positive presenting 13 different *PTPN11* mutations, with p.Tyr63Cys (6/28 cases; 22%); p.Asn308Asp(5/28 cases; 17%) and p.Met504Val (4/28 cases; 14%) being the most frequent. Among the *PTPN11* negative patients (58/86), targeted sequencing of all 14 RASopathy-associated genes included in NGS panel revealed 22 more patients (25.8%) with pathogenic or likely pathogenic variants. The most frequently altered reported genes were *RIT1* (7 patients;5 different mutations), *A2ML1* (3 patients;3 different mutations), *SOS1* (3 patients;3 different mutations) and *SPRED1* (2 patients;2 different mutations). Two patients were compound heterozygotes; one (N022) carried a mutation both in

PTPN11 and *SPRED1* (p.Glu139Asp and p.Arg334Cys; respectively); and the second (N030) carried one mutation in *A2ML1* and another one in *SOS1* (p.Arg657His and p.Met269Thr; respectively).

Mutations in *BRAF*, *CBL*, *KRAS*, *MAP2K1*, *RAF1* and *SHOC2* were found in 6 different patients while no mutation was identified in *MAP2K2*, *NRAS* or *HRAS*. *Table 1* resumes molecular and clinical data of each patient with pathogenic, likely pathogenic mutations or variants of unknown clinical significance(VUS) in all genes. All mutations were heterozygous missense changes, except for c.1096-4_1096-1del in the *CBL* gene. One novel mutation was identified in *RIT1* gene (c.235C>G; p.Gln79Glu) (N013), which has been recently reported in public databases (dbSNP138, ExAC and 1000 genomes), however, no clinical symptoms have been provided. According to PolyPhen analysis (PolyPhen-2 v2) this variant is predicted to be damaging (score 1.00). N002 carried the c.4076G>A (p.Arg1359His) in the *A2ML1* gene which is characterized as VUS or likely benign. The patient presented with craniofacial dysmorphisms, short stature and cryptorchidism. However, as *A2ML1* has not been confirmed as a NS causative gene we excluded *A2ML1* positive samples from further statistical analysis, as well as patients with a *SPRED1* mutations which have been associated with Legius syndrome. We also excluded from statistical analysis the patient that was positive for a *RIT1* mutation characterized as VUS and the patient with the novel *RIT1* mutation. Thus, the mutational rates of the confirmed NS-associated genes in the Greek NS population is: *PTPN11* 32.5%; *RIT1* 5.8%; *SOS1* 4.7%; *BRAF* 1.2%; *CBL* 1.2%; *KRAS* 1.2%; *MAP2K1* 1.2%; *RAF1* 1.2%; *SHOC2* 1.2%, corresponding to 50% of positivity in total NS population.

Presenting symptoms and clinical features of patients

Table 2 shows patients’ clinical features. In the total population mean age at diagnosis was 4.4±4.74 years (1 month to 19 years) and male patients prevailed to female (56 vs.30). In all patients, variable combinations of craniofacial abnormalities were recorded such as microcephaly, epicanthal folds, ptosis, low nasal bridge, hypertelorism, down slanting palpebral fissures, low set/posteriorly rotated ears, papillomas, coarseness, and webbed/thick neck. Craniofacial dysmorphisms (98.8%) and short stature (67.4%) were the most frequent characteristics of the patients, while PS was the most prevalent among congenital heart defects (47.7%). Neurological and skeletal defects were observed in 39.5% and 46.5% of patients, respectively and cryptorchidism in 25%. No patient presented with seizures, lymphatic dysplasia, bleeding diathesis, cancer or macrocephaly, while sensorineural hearing loss, lymphedema or kidney disorders were rarely reported. Seven patients had a positive family history. As shown in Table 3, most patients presented with typical face and heart defect (49%).

Table 2: Demographics and clinical features of study population

Patients' characteristics	Totaopulation (N=86)	Mutation <i>positive</i> patients (N=43)	Mutation <i>negative</i> patients (N=43)	<i>PTPN11</i> mutation <i>positive</i> patients (N=28)	<i>Other</i> mutation <i>positive</i> patients (N=15)
Age at diagnosis; years (mean ± SD)	4.4±4.7	4.61±5.0	4.21±4.4	5.34±5.7	3.2±3.1
Sex; N (male vs female)	56/30	23/20 <i>p</i> =0.024	33/10	17/11	6/9
Craniofacial dysmorphism; N (%)	85 (98.8)	43 (100)	42 (97.7)	28 (100)	15 (100)
Microcephaly; N (%)	8 (9.3)	5 (11.7)	3 (7.0)	3 (10.7)	2 (13.3)
Epicanthal folds; N (%)	33 (38.4)	30 (69.8) <i>p</i> <0.001	3 (7.0)	24 (85.7) <i>p</i> =0.004	6 (40.0)
Ptosis; N (%)	30 (34.8)	29 (67.4) <i>p</i> <0.001	1 (2.3)	24 (85.7) <i>p</i> =0.001	5 (33.3)
Low nasal bridge; N (%)	34 (39.5)	31 (72.1) <i>p</i> <0.001	3 (7.0)	23 (82.1)	8 (53.3)
Hypertelorism; N (%)	36 (41.9)	31 (72.1) <i>p</i> <0.001	5 (11.6)	21 (75)	10 (66.7)
Down slanting palpebral fissures; N (%)	28 (32.6)	25 (58.1) <i>p</i> <0.001	3 (7.0)	20 (71.4) <i>p</i> =0.024	5 (33.3)
Low set/posteriorly rotated ears; N (%)	36 (41.9)	32 (74.4) <i>p</i> <0.001	4 (9.3)	23 (82.1)	9 (60.0)
Papillomas; N (%)	1 (1.2)	1 (2.3)	0	1 (3.6)	0
Coarseness; N (%)	46 (53.5)	31 (72.1) <i>p</i> =0.001	15 (34.9)	25 (89.3) <i>p</i> =0.001	6 (37.5)
Webbed/thick neck; N (%)	36 (41.9)	32 (74.4) <i>p</i> =0.001	4 (9.3)	23 (82.1)	9 (60.0)
Heart disease; N (%)	62 (72.1)	36 (83.7) <i>p</i> =0.016	26 (60.5)	23 (82.1)	13 (86.7)
Pulmonary stenosis; N (%)	41 (47.7)	30 (69.8) <i>p</i> <0.001	11 (25.6)	20 (71.4)	10 (66.7)
Hypertrophic myopathy; N (%)	5 (5.8)	3 (7.0)	2 (4.7)	0 <i>p</i> =0.037	3 (20.0)
Other cardiological	17 (19.8)	9 (20.9)	8 (18.6)	5 (17.9)	4 (26.7)

problems; N (%)					
Skeletal features; N (%)	40 (46.5)	18 (41.9)	22 (51.2)	11 (39.3)	7 (46.7)
Low height; N (%)	58 (67.4)	31 (72.1)	27 (62.8)	19 (67.9)	12 (80.0)
Skin manifestations; N (%)	7 (8.1)	4 (9.3)	3 (7.0)	1 (3.6)	3 (20.0)
Neurological features; N (%)	34 (39.5)	14 (32.6)	20 (46.5)	5 (17.9)	9 (60.0)
			<i>p=0.008</i>		
Others					
Hearing loss; N (%)	1 (1.2)	1 (2.3)	0	1 (3.6)	0
Cryptorchidism; N (%)	14 (25)	7 (30.4)	7 (21.2)	2 (11.8)	5 (83.3)
Kidney disorders; N (%)	4 (4.7)	2 (4.7)	2 (4.7)	0	2 (13.3)
Lymphedema; N (%)	4 (4.7)	3 (4.7)	1 (2.3)	3 (10.7)	0
Family history; N (%)	7 (8.1)	7 (14)	0	5 (17.9)	2 (13.3)

The comparison between mutation-*positive* and-*negative* patients revealed a higher frequency of heart disease ($p=0.016$) and especially for PS ($p<0.001$) (only 3 patients had hypertrophic cardiomyopathy, HCM) in patients who had a mutation in any of the tested genes compared to mutation-free patients. Specifically, patients with at least one mutation had 6.71 times greater odds to develop PS compared to mutation-negative patients [OR=6.71, 95% CI= (2.61, 17.27)]. Epicanthal folds, ptosis, low nasal bridge hypertelorism, down slanting palpebral fissures, low set rotated ears, coarseness and webbed neck were more frequently observed in mutation positive patients compared to negative ones ($p<0.001$ for all features). Additionally, the phenotypical combination of typical face and heart disease (1A+2A, Table 3) was found in 65% of mutation-positive patients and in 33% of mutation-negative patients. No other significant correlation was found with respect to presence of growth and skeletal anomalies.

Table 3: Patients' scores according van der Burgt criteria

Scoring	Total population (N=86)		Mutation positive patients (N=43)		Mutation negative patients (N=43)		PTPN11 mutation positive patients (N=28)		Other mutation positive patients (N=15)	
	n	%	n	%	n	%	n	%	n	%
1A+2A	42	48.84	28	65.12	14	32.56	19	67.86	9	60.00
1A+3A	9	10.47	5	11.63	4	9.30	5	17.86	0	0.00
1A+4A	8	9.30	3	6.98	5	11.63	2	7.14	1	6.67
1A+2B+3B	3	3.49	1	2.33	2	4.65	1	3.57	0	0.00
1A+2B+6B	5	5.81	1	2.33	4	9.30	0	0.00	1	6.67
1A+3B+5B	1	1.16	1	2.33	0	0.00	0	0.00	1	6.67
1A+3B+6B	6	6.98	0	0.00	6	13.95	0	0.00	0	0.00
1B+2A+3A	2	2.33	0	0.00	2	4.65	0	0.00	0	0.00
1B+2A+4A	2	2.33	1	2.33	1	2.33	0	0.00	1	6.67
1B+2B+3B+4B	1	1.16	0	0.00	1	2.33	0	0.00	0	0.00
1B+2B+3B+6B	1	1.16	1	2.33	0	0.00	1	3.57	0	0.00
1B+3A+4A	5	5.81	1	2.33	4	9.30	0	0.00	1	6.67
1B+3A+6A	1	1.16	1	2.33	0	0.00	0	0.00	1	6.67

Regarding genotype-phenotypes associations, we compared patients' phenotypes harboring a mutation in a specific gene with patients harboring a mutation in any other gene. We observed a higher frequency of epicanthal folds ($p=0.004$); ptosis ($p=0.001$); down slanting palpebral fissures ($p=0.024$) and coarseness ($p=0.001$) in *PTPN11* mutation positive patients compared to mutation-positive patients in other genes. On the other hand, intellectual disability of variable degree was less frequent in *PTPN11* mutation positive patients compared with mutation-positive patients in other genes. ($p=0.008$). The detailed description of the phenotypical features and the genetic findings for all patients is presented in the Supplementary table (Table_S1). Due to the small size of mutation-subgroups, further statistical analysis was not allowed..

The record of single nucleotide polymorphisms (SNPs) showed 27 different exonic SNPs, with *A2ML1* accounting for most of them (Table 4). Their frequencies varied from 1.6% to 100% in the total population. Variant c.660C>A in *MAP2K2* gene was observed in patients without neurological features or downward eye slant [OR = 0.083, 95% CI = (0.02, 0.34); OR = 0.11, 95% CI = (0.02, 0.45), respectively] (data not shown). Thirty-three (33/36) mutation-negative patients vs 17(17/50) mutation-positive patients carried this polymorphism. No other correlation was found between a specific SNP and any clinical feature or mutation. It should be noted that all polymorphisms were also detected in patients who did not fulfilled NS criteria.

Table 4: Single nucleotide polymorphisms identified in study population

Polymorphism	Frequency (%)	Polymorphism	Frequency (%)
<i>NRAS</i> _c.360G>A	1.6	<i>SPRED1</i> _c.291G>A	95.3
<i>RIT1</i> _c.31G>C	98.4	<i>SPRED1</i> _c.1044T>C	98.4
<i>SHOC2</i> _c.457C>T	1.6	<i>MAP2K1</i> _c.-2A>G	1.6
<i>KRAS</i> _c.483G>A	96.9	<i>MAP2K1</i> _c.255C>T	1.6
<i>KRAS</i> _c.643T>C	32.8	<i>MAP2K1</i> _c.711G>A	1.6
<i>A2ML1</i> _c.186C>T	9.4	<i>MAP2K1</i> _c.804C>G	1.6
<i>A2ML1</i> _c.196C>T	1.6	<i>MAP2K2</i> _c.192C>T	12.5
<i>A2ML1</i> _c.619G>C	1.6	<i>MAP2K2</i> _c.453C>T	39.1
<i>A2ML1</i> _c.1101T>C	1.6	<i>MAP2K2</i> _c.660C>A	78.1
<i>A2ML1</i> _c.1109T>C	1.6	<i>MAP2K2</i> _c.1211C>T	1.6
<i>A2ML1</i> _c.1275A>G	17.5	<i>MAP2K2</i> _c.1212G>A	3.1
<i>A2ML1</i> _c.2367G>A	98.4	<i>MAP2K2</i> _c.1222C>T	3.1
<i>A2ML1</i> _c.2550C>A	98.4	<i>MAP2K2</i> _c.1227C>T	25
<i>A2ML1</i> _c.2868C>T	6.3	<i>SOS1</i> _c.553A>G	1.6
<i>A2ML1</i> _c.2909G>A	40.6	<i>SOS1</i> _c.570C>T	1.6
<i>A2ML1</i> _c.3237G>A	50	<i>SOS1</i> _c.984C>T	1.6
<i>A2ML1</i> _c.3364C>T	57.8	<i>SOS1</i> _c.1964C>T	3.2
<i>A2ML1</i> _c.3569C>T	15.6	<i>SOS1</i> _c.2376A>G	100
<i>A2ML1</i> _c.3676_3677del	17.5	<i>RAF1</i> _c.171T>C	1.6
<i>A2ML1</i> _c.3686A>G	98.4	<i>BRAF</i> _c.723G>A	1.6
<i>A2ML1</i> _c.3769A>G	92.2	<i>BRAF</i> _c.1462A>C	1.6
<i>A2ML1</i> _c.3843T>C	8.1	<i>BRAF</i> _c.1929A>G	46.9
<i>A2ML1</i> _c.4020A>G	95.3	<i>BRAF</i> _c.2128-2A>TA	1.6
		<i>BRAF</i> _c.2128-4TG	4.8

Discussion

The phenotype of Noonan and Noonan-like syndromes are recognised, or suspected, at birth, by experienced paediatricians and cardiologists and, later in life, by other specialties, such as neurologists, endocrinologists or dermatologists. Due to the overlapping phenotypes with other entities and because of the subsequent changing craniofacial features during life, it is often difficult to obtain a definite diagnosis based only on clinical criteria. The genetic analysis may help in ending the diagnostic odyssey of the patient and, sometimes, guides medical decisions for monitoring and therapy. In the case of NS and NS-like patients, the recording of mutational detection rates in different cohorts worldwide shows a great variation, depending on the clinical inclusion criteria stringency, the number of participants and the genetic testing applied[2; 11]. According to the published results, *PTPN11* mutations account for ~50% of cases, whereas the analysis of other Ras/MAPK related genes increases the mutational rate up to 70-80%[11; 20-22].

In Greece, the genetic analysis depicted 52 heterozygous pathogenic/likely pathogenic variants in 11 out of 14 genes tested in 86 NS patients, with *PTPN11*, *RIT1* and *SOS1* presenting the higher frequency. The current knowledge allows the recognition of

the genetic basis of more NS patients. The screening of more Ras/MAPK related genes increased the NS and NS-like mutational rate in Greece from 28%, reported in our previous work [18] including only *PTPN11*, to 51.4% in the current paper. Taking into consideration that *A2ML1* gene has not been confirmed as a NS gene and that *SPRED1* has been associated with Legius syndrome, the mutational rate of NS patients in Greece is formed as of 50%. Phenotypically, our findings confirmed those observed in other cohorts with craniofacial dysmorphisms, and pulmonary stenosis (PS) being significantly associated with the presence of mutations in any of the tested genes[2; 20].

PTPN11 mutations were observed in 32.5% of our population. The risk for pulmonary stenosis increased 7.85-fold in NS mutation-positive patients compared to mutation-negative ones while intellectual disability of variable degree was negatively associated with *PTPN11* mutations compared to the presence of mutations in other genes. All mutations found were missense and favour the open configuration of the enzyme allowing its permanent catalytic function[6; 23]. Among the more frequent variants, p.Asn308Asp has been also recorded as the most prevalent in other populations, reaching ~20% in Europe, USA, Argentina[21; 24], Brazil[25] and 11% in South India[12].

It has been suggested that *RIT1* mutated NS patients present higher rates of HCM compared to other NS patients[13; 26]. Although *RIT1* was the second most frequently altered gene in our cohort, detected in 6% of total NS population, we could not confirm the above clinical relevance because of the rarity of patients with HCM in our population. The detected mutational rate is similar to previous studies[13; 27; 28] worldwide (4%-9%). All but one mutations detected have already been described in the literature and cluster at codons encoding highly conserved amino acids in functionally important domains. However, the most commonly observed mutations in other cohorts -at positions 57 and 82 of the protein-[13; 28; 29] were absent from our population. The most frequent variant was p.Ser35Thr (3/5 of *RIT1* patients) which has been associated with activated MAPK signalling[13]. The identified novel variant p.Gln79Glu corresponds to an oncogenic RAS alteration p.Gln61Glu, implicated in the activation of MAPK signalling in a cell-specific manner[13]. These findings, together with pathogenicity prediction and clinical signs provide strong evidence for the causality of p.Gln79Glu mutation. Phenotypically, this patient (N0880) presented craniofacial dysmorphisms, webbed/thick neck, intellectual disability, cryptorchidism and short stature.

Four patients (4.7%) of our cohort presented 4 different mutations in the *SOS1* gene[2], the second-most-common genetic cause of NS worldwide, accounting for ~15% of NS cases[29-31]. All variants observed in our patients have been previously described[30; 32] and reside in regions that may disrupt the autoinhibited protein's conformation[30]. The associated phenotypes of our patients were clearly in NS spectrum, without the specific features attributed to *SOS1* mutations such as ectodermal features and macrocephaly[30].

Among the other genes tested, mutations were observed in *BRAF*, *CBL*, *KRAS*, *MAP2K1(MEK1)*, *RAF1* and *SHOC2* at a lower frequency (1.2%). Germline mutations in *KRAS* and *BRAF* genes have been associated with NS and CFC with a variable clinical phenotype depending on the affected residue, while somatic mutations are closely related to myeloid malignancies (*KRAS*), melanoma (*BRAF*) and other cancers[33-35]. Our patients with heterozygous either *KRAS* or *BRAF* mutations showed typical NS features. *KRAS* p.Asp153Val variant has already been published as *de novo* alteration associated with NS and CFC [33; 35; 36] and it is characterized as pathogenic. On the other hand, it has been shown that the observed *BRAF* mutation p.Glu501Gly, does not enhance ELK-dependent transcription similar to other mutations identified in the kinase domain of the protein[35]. *RAF1*, *SHOC2* and *CBL* are downstream effectors of the Ras/MAPK pathway which have been associated with specific clinical features in NS patients; *RAF1* has been suggested to cause cardiomyopathy[37], *SHOC2* a rare subset of NS with a unique phenotypic feature of loose anagen hair[38; 39] and *CBL* has been linked to an entity that resembles NS and predispose to juvenile myelomonocytic leukaemia that may follow a variable course[40; 41]. Although pathogenicity has been attributed to all variants observed in our study, these patients presented craniofacial defects, PS but no other specific features.

According to our results, small-scale recording of NS genetic variants may deviate the genotype-phenotype associations that have been proposed for the NS or NLS associated genes, such as the presence of HCM in *RIT1* carriers, or macrocephaly and ectodermal manifestations attributed to *SOS1* mutation positive patients. What complicated more the definite diagnosis and the extraction of solid associations was the detection of genetic variants classified as of VUS in patients fulfilling the van der Burgt criteria. For instance, a patient with craniofacial dysmorphisms, carrier of the *RIT1* p.Lys23Arg mutation (VUS) and a patient

with typical NS facial characteristics and heart defects but without skin manifestations revealed the *SPRED1* p.Thr196Ile mutation (VUS). In line with this, four of our patients with a rather mild phenotype and typical NS face, revealed 4 different rare and non-synonymous variants in *A2ML1* gene classified as of VUS. Functional studies and detailed clinical information on *A2ML1* mutated NS patients reported in previous studies have put up the question of the driver role of *A2ML1* in RASopathies[8] as *de novo* variants have been identified in patients that hardly met the diagnostic criteria[7; 42]. Furthermore, results from *in vitro* analysis have failed to show an impact of *A2ML1* variants on RAS/MAPK activation[7; 42].

For the significant patients' number with unknown genetic cause or VUS in our cohort paediatricians should inform the patients for a long follow up and future reanalysis for mutations in other genes, recently associated with NS, like *LTRZ1*, or even genes from other molecular pathways that may collaborate with the Ras/MAPK pathway, such as mTOR[43]. Moreover, in some cases, functional studies have shown that the mutation detected does not stimulate the Ras/MAPK pathway, although the respective patient fulfills the diagnostic criteria. It would be possible that other genes or polymorphisms may have an additive or modifying effect on the phenotype, as the c.660C>A in *MAP2K2* which was observed in our patients without neurological features. Thus, frequent follow-up of patients with typical clinical NS diagnosis is essential for their appropriate and successful management. For patients with NS phenotype, the presence of a genetic variant in one of the Noonan genes, facilitates the paediatrician's planning for precise and multidisciplinary clinical guidance and also allows the referral for proper genetic counseling for the family.

It is certain that unravelling NS molecular basis, may help discovering new therapeutic targets and personalizing RASopathy monitoring. In particular, drugs that inhibit components of the Ras/MAPK pathway could be useful for treating RASopathy patients. *In vivo* experiments have shown that MEK inhibitor treatment during the early life stages may reduce or reverse NS defects in Raf1 or Sos1 mutant mice[44; 45]. In humans, it has recently been reported that trametinib, a highly selective reversible allosteric inhibitor of MEK1/2 activity might limit myocardial hypertrophy[14; 46] in NS patients with *RIT1* mutations, while a complete remodelling of the entire central lymphatic system in a girl with NS and a *SOS1* mutation has recently been reported[47].

Abbreviations

Cardiofacio- cutaneous syndrome (CFC), Hypertrophic Cardiomyopathy (HCM), Noonan syndrome (NS), Pulmonary valve stenosis (PS), Next Generation Sequencing (NGS), Single Nucleotide Polymorphisms (SNPs), Variants of Unknown clinical Significance (VUS)

Declarations

Acknowledgments

We cordially thank the families for their cooperation.

Funding

No financial assistance was received in support of the study

Conflict of Interests

The authors have no relevant financial or non-financial interests to disclose.

Data availability

Raw data are available upon request.

Code of availability

Not applicable

Author Contributions

Authors' contributions: G.P., A.Papadopoulou. and E.B. conducted material preparation, and wrote of the manuscript. Patient's stratification was performed by S.K-Tz., K.K., A.Papadimitriou., C. K-G. and V.P. A.P. wrote the first draft of the manuscript and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Ethical Approval

This study was performed in line with the principles of the Declaration of Helsinki and its later amendments. Medical ethical approval was granted by the local medical ethics committee of Attikon University general hospital with the file number (4492/16.07.2019).

Consent to participate

Written informed consent was obtained from the participants or/and the parents of the participating children, as well as patients' assess where applicable.

Consent to publish

Not applicable.

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