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The first *PTPN11* mutations in hotspot exons reported in Moroccan children with Noonan syndrome and comparison of mutation rate to previous studies

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Background/aim: Noonan syndrome is an autosomal dominant disorder with an incidence of 1/1000–2500. It results from protein-tyrosine phosphatase, nonreceptor type 11 (*PTPN11*) mutations in roughly 50% of cases. Mutational screening of *PTPN11* has been carried out in different populations. Thus, the aim of this study was to screen, for the first time, *PTPN11* mutations in a series of Moroccan Noonan syndrome patients.

Materials and methods: We used bidirectional sequencing of exons 3 and 8, considered as *PTPN11* mutation hot spots, and then compared the rate of mutational events of these exons between different populations using chi-square and Fisher's exact tests.

Results: We detected 3 heterozygous mutations (Asp61Gly, Tyr63Cys, and Asn308Ser) in 4 individuals of 16 sporadic patients (25%). The rate of mutation in our cohort did not differ from that of other populations. However, we found significant differences in the mutation rate of exon 8 between one Japanese cohort and some populations, which requires more investigations to be explained.

Conclusion: The present study allowed identification of mutations clustered in exons 3 and 8 of the *PTPN11* gene in a Moroccan Noonan syndrome cohort and enabled us to give appropriate genetic counseling to the mutation-positive patients.

Key words: Noonan syndrome, *PTPN11*, mutation analysis, SHP-2, mutation rate comparison

1. Introduction

Noonan syndrome (NS, OMIM 163950) is a common autosomal dominant disorder with an estimated incidence of 1 in 1000–2500 live births. Clinically, it is characterized by short stature, facial dysmorphia (e.g., hypertelorism, down-slanting palpebral fissures, low-set posteriorly rotated ear), skeletal defects (e.g., chest deformities), congenital heart defects (especially pulmonary valve stenosis) (1,2), and other features, occasionally observed, such mild mental retardation, cryptorchidism, and bleeding diathesis. However, there is no defined clinical diagnosis of this syndrome because of the high variation level of the phenotypic expression with age and between individuals (3).

Tartaglia et al. (4) demonstrated, using a positional candidacy approach, that heterozygous missense mutations in the protein-tyrosine phosphatase, nonreceptor type 11 (*PTPN11*) (MIM 176876) are responsible for Noonan syndrome in 50% of patients. The *PTPN11* gene mapped on chromosome 12q24 contains 15 exons and encodes the cytoplasmic nonreceptor protein tyrosine phosphatase (SHP-2) with 2 tandemly arranged amino-terminal src-homology 2 (SH2) domains (N-SH2 and C-SH2) and a protein-tyrosine phosphatase (PTP) domain (5,6).

The *PTPN11* gene is widely expressed in human tissues, especially in the heart, brain, and skeletal muscle. Indeed, it is involved in the regulation of the ubiquitous RAS–MAPK (mitogen activated protein kinase) signaling cascade, mediated by growth factors, cytokines, and hormones (7).

Crystallographic data on SHP-2 indicate that in the default inactive condition, the usual interaction between N-SH2 and PTP domains of SHP-2 hide the catalytic site. However, the missense mutations clustering in these domains cause changes in the SHP-2 conformation leading to the emergence of catalytic site. Thus, the SHP-2 active conformation keeps upregulating the RAS-MAPK pathway and generates NS features (4,8).

To date, several studies have been performed to describe *PTPN11* mutation patterns in specific populations, such as

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Italian (9), Japanese (10), Korean (11),Taiwanese (12), and Brazilian (13) populations, amongst others. Thus, in order to define, for the first time, the spectrum of mutations and clinical features in Moroccan children with Noonan syndrome, we carried out mutation screening of the most common *PTPN11* exons (and their intron boundaries) affected by mutations in 16 Moroccan patients with sporadic NS.

2. Materials and methods

2.1. Clinical evaluation

Noonan syndrome was diagnosed by the presence of the major clinical characteristics (typical facial dysmorphia, pulmonic stenosis or abnormal electrocardiogram pattern, pterygium colli, pectus carinatum/excavatum, short stature, and cryptorchidism in male subjects) and minor criteria (psychomotor retardation or speech delay, bleeding diathesis, family history of Noonan syndrome, and other additional features). According to the van der Burgt criteria (14), patients with at least typical facial dysmorphia associated with 1 major or 2 minor signs, or suggestive facial dysmorphism associated with 2 major or 3 minor signs, were enrolled from the Departments of Medical Genetics and Pediatrics between January 2009 and June 2013. Nineteen patients were identified as affected by NOONAN syndrome. Echocardiography and karyotyping were carried out routinely for affected individuals.

2.2. Mutational analysis

After obtaining informed consent, peripheral blood was obtained from each patient and genomic DNA samples were extracted from blood lymphocytes using the GeneCatcher Magnetic Beads Kit (Invitrogen). For each patient, both *PTPN11* exons 3 and 8, which are considered as mutation hot spots (4,11), were individually amplified by polymerase chain reaction (PCR) using 2 pairs of corresponding primers derived from published data (15).

PCR was performed in a 25-mL reaction volume containing 60 ng of genomic DNA, 5 U of Taq (Invitrogen), 20 pmol of each primer, 50 mM $MgCl_2$, 10 mM dNTP, and 10X PCR buffer (Invitrogen) in the Veriti 96-well Thermal Cycler 9902 (Applied Biosystems). The PCR cycling conditions were 94 °C for 8 min; 35 cycles of 94 °C for 45 s, 63 °C (exon 3) or 58 °C (exon 8) for 45 s, and 72 °C for 45 s; and 72 °C for 10 min.

Bidirectional direct sequencing of purified PCR products was performed using the BigDye Terminator V1.1 Cycle Sequencing Kit (ABI Prism) and an Applied Biosystems 3500Dx Genetic Analyzer. The chromatogram was analyzed by the Sequencing Analysis SeqA v.5.4 (Applied Biosystems). The sequences thus obtained underwent bioinformatics analysis using the "Nucleotide Blast" alignment program at http://blast.ncbi.nlm.nih.gov.

2.3. Statistical analysis

Comparisons between study cohorts were done using chi-square tests for >5 and Fisher's exact test for smaller cohort sizes.

3. Results

In this study, 19 individuals were identified to be affected by Noonan syndrome according to the van Der Burgt criteria. Of those, 16 subjects were screened for *PTPN11* mutations, while the 3 remaining could not be reached. All subjects had normal karyotypes with 46,XX for females and 46,XY for males.

3.1. Mutation analysis of the *PTPN11* gene

DNA from the 16 patients with Noonan syndrome were screened for *PTPN11* mutations. Exons 3 and 8 and their flanking intron sequences, which are considered as mutation hot spots, were PCR-amplified and bidirectionally sequenced. We identified 3 different heterozygous missense mutations in 4 subjects (25%). All the exonic mutations found in our series led to amino acid residue changes and were located in the N-SH2 and PTP functional domains (Figure 1). These mutations were previously reported as disease-causing mutations (4). Table 1 and Figure 1 summarize mutation-screening results. Moreover, 3 other subjects showed the same g.63899C > T substitution in intron 7 of their *PTPN11* sequences.

3.2. Clinical features

The total cohort includes 10 males (52.63%) and 9 females (47.37%) with age ranging from 3 days to 15 years. All cases were sporadic, and the patients' parents have normal phenotypes. Congenital heart disease was found in 17/19 (89.47%) patients, distributed as pulmonic stenosis in 10/17 (58.82%), pulmonic stenosis + septal defects in 3/17 (17.65%), pulmonic stenosis + hypertrophic cardiomyopathy in 2/17 (11.76%), and hypertrophic cardiomyopathy in 2/17 (11.76%). Among the 16 screened patients with Noonan syndrome, 4 subjects had mutation. Their clinical features are summarized in Table 1.

4. Discussion

PTPN11 mutations were demonstrated to be involved in Noonan syndrome by Tartaglia et al. (4). This evidence was proven afterward in the subsequent studies of different populations. The aim of the present study was to report, for the first time, the mutations of *PTPN11* in 16 Moroccan children using the bidirectional sequencing of the main exons affected by mutations (exons 3 and 8), and then to compare the frequency of mutational events of these exons between different populations.

4.1. Mutation analysis

The molecular analysis revealed 3 different missense mutations in 4 sporadic cases (25%). These substitutions

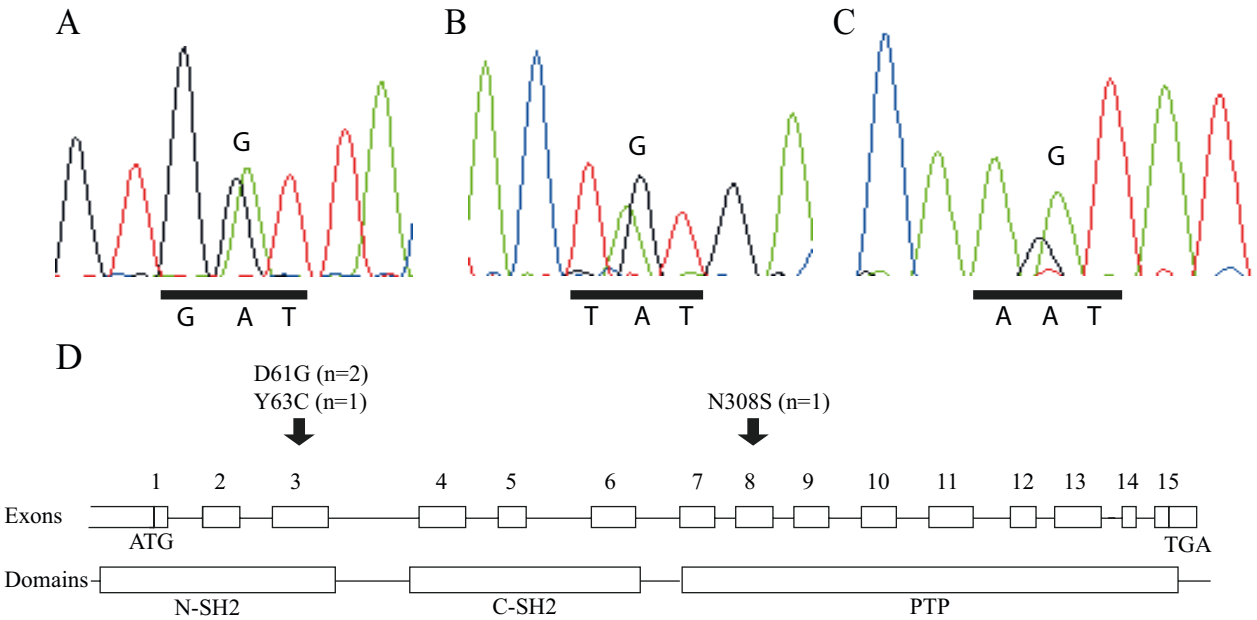

Figure 1. A, B, and C- Sequencing chromatogram of the detected *PTPN11* heterozygous mutations, respectively Asp61Gly, Tyr63Cys, and Asn308Ser; **D**- organization of *PTPN11* exons and domains and location of the 3 mutations.

were heterozygous and affected the highly conserved residues of the N-SH2 and PTP domains. The mutation A182G leading to the Asp61Gly substitution was observed in 2 patients. This mutation is located in exon 3, which encodes the N-SH2 domain. The second mutation, A188G, resulting in the Tyr63Cys substitution, was present in one patient. This is also located in exon 3. The third mutation was identified in one patient at position 923A→G in exon 8 and leads to the Asn308Ser substitution in the PTP domain. In several studies, Asn308Asp was the most prevalent mutation, affecting codon 308 (15,16). However, this mutation was not detected in our cohort.

The occurrence of these mutations had been proven to be pathogenic and responsible for Noonan syndrome manifestation. It was demonstrated (4,17) that, in the inactive conformation, residues located in or around the interactive surface of the N-SH2 and PTP domains interact with each other in order to hide the catalytic site, and their substitution disturbs the equilibrium between active and inactive conformation, leading to the gain of function effect and the increase of phosphatase activity in the RAS/MAPK pathway. Effectively, crystallographic data on inactive conformation of SHP-2 confirmed the direct involvement of Asp61 (among others) in the stabilization of inactive conformation (8), whereas Tyr63 and Asn308 are involved indirectly because of their closeness to the engaged residues.

It is worth mentioning that the substitution g.63899C > T (rs41279090:C>T) in intron 7 was identified in 3 other subjects. This change is present frequently in control subjects and considered as a polymorphism.

4.2. Comparison to previous studies

In this part of the study, we attempt to compare the mutation rate of exon 3 and exon 8 in our cohort to those of different studies, and also to compare mutation rates of different studies to each other, in order to reveal any putative differences.

The statistical analysis does not reveal any significant difference in mutation rates of exon 3 and exon 8 between our population and the other populations (Table 2).There was also no significant difference in exon 3 mutation rate between other populations (P = 0.1). These results suggest that the frequency of mutational events in exon 3 and 8 of *PTPN11* seems not to be affected by ethnicity.

It is important to mention that, when comparing the mutation rate of exon 8 between populations, we found significant differences between some populations: between American (4,15) and Japanese (18) populations (respectively P = 0.04 and P = 0.01) and between German (19) and Japanese (18) populations (P = 0.04), which could be due to the low rate of exon 8 mutations (2.4%) in the Japanese cohort studied by Niihori et al. (18). More investigations are required to explain these data.

4.3. Clinical features

In our study, pulmonic stenosis was observed in all mutated patients affected by congenital heart disease, which confirms the previous studies that demonstrated a significant correlation between *PTPN11* mutations

Table 1. *PTPN11* mutations and clinical features in mutated patients with Noonan syndrome.

Features	**Patient 1**	**Patient 2**	**Patient 3**	**Patient 4**
Nucleotide substitution	c.182A>G	c.182A>G	c.188A>G	c.923A>G
Amino acid substitu**tion**	Asp61Gly	Asp61Gly	Tyr63Cys	Asn308Ser
- Exon / domain	Exon 3 / N-SH2	Exon 3 / N-SH2	Exon 3 / N-SH2	Exon 8 / PTP
Age (years:months)	11:0	11:3	10:7	4:8
S**ex**	Male	Male	Female	Female
Growth (±SD): height (cm)	Short stature	117 (–4 SD)	127 (–2.5 SD)	105 (normal)
Weight (kg)	ND	19 (–3 SD)	27 (–1 SD)	17 (normal)
Congenital heart disease	Pulmonic stenosis, ASD	Pulmonic stenosis	Normal	Pulmonic stenosis, HCM
Dysmorphic facies				
Triangular face	ND	+	+	+
Micrognathia	ND	+	-	+
Hypertelorism	+	+	+	+
Ptosis	ND	-	+	-
Down-slanting palpebral fissures	+	+	+	+
Low-set posteriorly rotated ears	ND	+	+	+
Nerve deafness	ND	-	-	+
Epicanthic folds	+	+	-	+
Deeply grooved philtrum	ND	+	+	+
High arched palate	ND	+	+	+
Short and webbed neck	ND	+	+	+
Low posterior hairline	ND	+	+	+
Articulation and learning	Learning difficulties	Articulation and learning difficulties	Articulation difficulties	Articulation and learning difficulties
Chest	Chest deformity	Shield chest, pectus excavatum inferiorly	Shield chest, pectus excavatum inferiorly	Shield chest, pectus excavatum inferiorly
Skeletal	ND	Cubitus valgus, brachydactyly, kyphoscoliosis	Cubitus valgus	Cubitus valgus, brachydactyly, clinodactyly
Hematology	ND	Bleeding diathesis	Bleeding diathesis	ND
Genital	Cryptorchidism	Cryptorchidism	-	-

ASD: Atrial septal defect. HCM: Hypertrophic cardiomyopathy. ND: Not determined. SD: Standard deviation.

and pulmonic stenosis (15,23). This is especially true for the Asn308Ser mutation in patient 4 that was previously associated with pulmonic stenosis by Sarkozy et al. (9). This correlation was explained by the fact that SHP-2 is involved in the signal transduction pathway of the epidermal growth factor expressed in semilunar valve development (24,25). Generally, all clinical manifestations observed in mutated Noonan syndrome patients are attributed to the contribution of SHP-2/*PTPN11* in the RAS–MAPK molecular signaling cascade that is essential for cellular differentiation, proliferation, or migration processes in most tissues (26,27).

Moreover, there were several features shared by all subjects with *PTPN11* mutations. Facial dysmorphia, although slightly different from one patient to another, was present in all mutation-positive patients; short stature was seen in 75% of our mutation-positive patients; chest deformity was present in all mutation-positive subjects; and cryptorchidism was observed in all male patients. This could be due to the small sample size or may be explained by the fact that some features were initially among the main "major criteria" of selection.

It is important to differentiate the Noonan syndrome phenotype from the Noonan-like syndromes (Leopard

Table 2. Reported mutation rate in *PTPN11* exons 3 and 8 in different studies of patients with Noonan syndrome.

Reference study	Reference population	Exon 3	P-value[a]	Exon 8	P-value[a]
Tartaglia et al., 2001 (4)	American	5/22 (31.8%)	0.91	4/22 (18.2%)	**0.37**
Tartaglia et al., 2002 (15)	American	22/119 (18.5%)	1	21/119 (17.6%)	**0.46**
Sarkozy et al., 2003 (9)	Italian	8/71 (11.2%)	0.41	9/71 (12.6%)	**0.68**
Zenker et al., 2004 (19)	Germany	17/57 (29.8%)	0.53	9/57 (15.8%)	**0.44**
Yoshida et al., 2004 (20)	Japanese	12/45 (26.6%)	0.73	3/45 (6.6%)	**1**
Niihori et al., 2005 (18)	Japanese	10/41 (24.4%)	0.91	1/41 (2.4%)	**0.48**
Bertola et al., 2006 (13)	Brazilian	9/50 (18%)	1	4/50 (8%)	**1**
Hung et al., 2007 (12)	Taiwanese	6/34 (17.6%)	1	2/34 (5.8%)	**1**
Ferrero et al., 2008 (21)	Italian	4/40 (10%)	0.39	2/40 (5%)	**1**
Ko et al., 2008 (22)	Korean	6/59 (10.1%)	0.39	3/59 (5.1%)	**1**
Lee et al., 2011 (11)	Korean	9/59 (15.2%)	0.71	5/59 (8.4%)	**1**
Our study	**Moroccan**	**3/16 (18.75%)**	-	**1/16 (6.25%)**	-

[a]Compared to our study.

syndrome, cardiofaciocutaneous (CFC) syndrome, Costello syndrome, etc.) that show a number of the same traits. Some of those syndromes also share the genetic causes of Noonan syndrome, such as Leopard syndrome, which is considered as an allelic condition of Noonan syndrome (28). Previous studies established differential diagnosis guidelines that could be very useful to distinguish Noonan syndrome from the others (29).

4.4. Genetic counseling

Although Noonan syndrome is a dominant disorder caused by germline mutations, the fact that, on the one hand, it is genetically heterozygous and, on the other hand, it is almost always due to de novo mutations makes the quality of life much better for both the parents and the patient him/herself. Indeed, the heterozygosity of this disorder suggest that its inheritance from affected individuals to offspring remains limited at 50% for each pregnancy and could be easily avoid by preimplantation genetic diagnosis. Furthermore, de novo mutation implies that, for the parents, the risk of recurrence in an upcoming child remains theoretically very low, about 1%–5%.

Previous studies demonstrated that *PTPN11* germline mutations are present in 50% of Noonan syndrome cases, while *PTPN11* somatic mutations are observed in 34% of patients with nonsyndromic juvenile myelomonocytic leukemia (JMML). However, some Noonan syndrome cases associated with leukemia, especially JMML, were reported to be caused by somatic *PTPN11* mutations, but rarely as germline transmission (30).

Moreover, the mutation-positive subjects for whom diagnosis was confirmed by the present *PTPN11* mutations screening could receive appropriate genetic counseling and be followed by suitable multidisciplinary medical teams, even preventing likely subsequent complications.

The present study allowed, for the first time, the identification of exon 3 and 8 *PTPN11* mutations in a Moroccan cohort. We found 3 heterozygous mutations (Asp61Gly, Tyr63Cys, and Asn308Ser). The rate of mutation in our cohort did not differ from those of other populations. The mutation-positive patients got appropriate genetic counseling. The screening of the remaining exons is already underway in order to gain a full view about the spectrum of mutations in Moroccan children with Noonan syndrome.

Finally, it is important to mention that *PTPN11* is certainly the first genetic cause of Noonan syndrome manifestation, but not the last. Indeed, subsequent studies have proven the involvement of other genes from the same molecular signaling cascade (RAS–MAP kinase), although with less frequency, such as *SOS1* (31), the RAF family (32), and the RAS family (33), which will be surely interesting to explore in the Moroccan population.

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