Clinical and Molecular Findings of Tunisian Patients with RASopathies

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Key Words

BRAF · Cardiofaciocutaneous syndrome · Noonan syndrome · *PTPN11* · RASopathy · *RIT1* · *SOS1*

Abstract

Noonan syndrome (NS) and related disorders, which are now summarized under the term RASopathies, are caused by germline mutations in genes encoding protein components of the Ras/mitogen-activated protein kinase pathway. In this study, we evaluated the clinical and molecular spectrum of 21 Tunisian patients, recruited by a cardiology unit, for whom RASopathy diagnosis was suspected by clinical geneticists. Overall, 19 patients had a clinical diagnosis of NS and 2 were classified as having Cardiofaciocutaneous (CFC) syndrome. In 52% (n = 11) of patients, a RASopathy has been molecularly confirmed. Mutations in *PTPN11* and *SOS1* genes were found in patients with diagnosis of NS and *BRAF* gene mutations in patients with CFC syndrome. As reported from other cohorts, mutations in exons 3 and 8 of the *PTPN11* gene predominated in Tunisian NS patients. A very uncommon *PTPN11* mutation c.5C>T (p.T2I), the functional consequences of which have so far remained unclear, was identified in one patient. As biased by the mode of recruitment, all patients included in this study had a congenital heart defect,

with pulmonary valve stenosis being the most frequent one. Short stature and developmental abnormalities were present in mutation-positive cases. This is the first molecular study in patients from southern Tunisia with RASopathy diagnosis.

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Over the past 10 years, a series of molecular genetic discoveries have shown that mutations which alter the function of molecules interacting in a common signaling cascade, the RAS/mitogen-activated protein kinase pathway, are responsible for Noonan syndrome (NS) and the clinically related disorders Cardiofaciocutaneous (CFC) syndrome, LEOPARD syndrome (LS) and Costello syndrome (CS) [Aoki et al., 2008; Zenker, 2009; Tartaglia et al., 2010]. This pathway plays a pivotal role in the signal transduction from cell surface receptors to the nucleus, and it is involved in the control of cell cycle, differentiation, survival, and apoptosis, which are all essential for normal development.

NS (OMIM 163950) is clinically characterized by a combination of short stature, congenital heart defects with pulmonary valve stenosis (PVS) and hypertrophic cardiomyopathy, being the most typical ones, as well as a

distinct craniofacial appearance including hypertelorism with downslanting palpebral fissures, ocular ptosis, a broad forehead, low-set ears, and a broad or webbed neck [Noonan, 1968]. Additionally, chest deformities, lymphatic anomalies, cryptorchidism in males, bleeding diathesis, and variable developmental delay are frequent findings [Tartaglia and Gelb, 2010]. The major gene responsible for this disorder is *PTPN11* (40–50%) followed by *SOS1* (10–20%) and *RAF1* (3–17%). Mutations in *KRAS*, *NRAS* and *SHOC2* account for less than 5% of cases each, and mutations in *CBL*, *MEK1* and *BRAF* are occasionally associated with a NS phenotype [Tartaglia and Gelb, 2010].

CFC syndrome (OMIM 115150) shares many features with NS, but it is usually associated with more severe learning disabilities and ectodermal (skin and hair) abnormalities [Aoki et al., 2008; Tartaglia et al., 2011; Roberts et al., 2013]. It is also predominantly associated with mutations occurring in the *BRAF* gene, followed by *MEK1*, *MEK2* and *KRAS* [Niihori et al., 2006; Sarkozy et al., 2009; Tartaglia and Gelb, 2010; Zenker, 2011; Roberts et al., 2013].

LS (OMIM 151100) is an acronymic name for an NS-like condition with multiple lentiginos as the most distinguishing feature. It is mainly caused by distinct *PTPN11* mutations [Digilio et al., 2002; Leguis et al., 2002; Sarkozy et al., 2009].

CS (OMIM 218040) also overlaps with NS regarding the 3 cardinal features (short stature, heart defects and craniofacial dysmorphism), but it can usually be distinguished clinically. Notably, this disease is associated with a significantly increased risk of malignancy [Kerr et al., 2008; Hasle, 2009]. *HRAS* mutations are present in all cases with CS [Aoki et al., 2005; Kerr et al., 2008]. Disease-causing mutations in various genes lead to a dysregulation, mostly overactivation, of the RAS/mitogen-activated protein kinase pathway. They are predominantly (NS and LS) or exclusively (CFC syndrome and CS) de novo mutations clustering to certain regions of the respective genes, and the mutation spectrum appears to be independent of the ethnic background.

The aforementioned conditions are now collectively called RASopathies. Because of the broad spectrum of phenotypic expression, the existing clinical overlap between those entities, and the absence of selective clinical criteria, molecular genetic analysis has become a useful tool for diagnostic testing and differential diagnosis.

This study aimed to investigate the clinical and molecular spectrum in Tunisian patients with NS and related disorders.

Material and Methods

Patients

The Institutional Review Board of the Medical University of Sfax, Tunisia, approved the study, and informed consent was obtained from all patients or their parents.

From March 2006 to March 2011, a total number of 62 patients from southern Tunisia attended the Department of Histology, Faculty of Medicine at the University of Sfax, for genetic counseling and chromosomal and/or molecular analysis for congenital PVS associated with or without another cardiac defect. All patients were ascertained through the Department of Cardiology, Hedi Chaker University Hospital of Sfax.

The diagnoses of NS and CFC syndrome were based on cardiac diseases mainly pulmonary stenosis and other extra cardiac features, facial dysmorphism or developmental and cognitive delay. Patients with PVS associated with minor or major dysmorphic features of NS were enrolled in the present study. The NS scoring system by van der Burgt et al. [1994] and the CFC syndrome criteria outlined by Roberts et al. [2006] were applied to classify the phenotype.

Twenty-one unrelated patients were selected by a clinical geneticist and a pediatric cardiologist for genetic testing within this study. Cardiac evaluations were assessed by echocardiography. A hematologic test, including total blood count, was carried out for all patients, but it showed no abnormalities. Phenotypic features were also evaluated in the index patients' parents when available.

Methods

Molecular Analysis

Routine karyotyping was performed. Genomic DNA from the index patients and their parents (when available) was extracted from peripheral blood leukocytes using standard protocols.

The National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>) was used to obtain gene information.

All exons harboring known mutations in NS, CFC syndrome, CS and LS were analyzed for each studied gene. Initially the genes *PTPN11*, *SOS1*, *SHOC2*, *KRAS*, and *RAF1* were analyzed in patients with RASopathy diagnosis. For this purpose, exons and flanking intronic regions of the genes, including *PTPN11* exons 2–4, 7, 8, 11–14 (NM_002834); *RAF1* exons 7, 12, 14, 17 (NM_002880); *KRAS* exons 2, 3, 5 (NM_004985); *SOS1* exons 3–11, 13, 14, 16 (NM_005633), and *SHOC2* exon 2 (NM_007373), were amplified by PCR and analyzed by high resolution melting on a Light Cycler (Roche, LC-480; Roche Diagnostics, Grenzach-Wyhlen, Germany). Oligonucleotide primer sequences and PCR protocols are available on request. Melting curves were analyzed using the Light Cycler 480 Gene Scanning Software (Roche). For samples producing curves differing from the wild type, the respective PCR products were then purified and sequenced using an automated capillary sequencer (3500xl Genetic Analyzer, Applied Biosystems, Foster City, Calif., USA). We performed a mutation analysis of the genes *BRAF* (NM_004333), *MEK1* (NM_002755) and *MEK2* (NM_030662) in patients with a clinical diagnosis of CFC syndrome as well as in patients for whom no *PTPN11* mutations were identified. *HRAS* (NM_005343) was analyzed in one patient with a phenotype suggestive of CS. Patients with negative results upon high resolution melting screening and targeted sequencing, were subsequently sequenced for the genes *NRAS* exons 2 and 3 (NM_002524), *CBL* exons 7–9 (NM_005188), *RIT1* (NM_001256821.1) and the remaining exons of *PTPN11*.

Table 1. Major clinical and molecular features of RASopathy patients

Patients	Gender/ age, years	Diagnosis	Heart defect	Short stature (<3rd centile)	Developmental abnormalities	Facial features	Thorax deformities	Cryptorchidism	Genotype (protein)	Domain	Exon	Gene affected
1	M/5	NS	PVS	+	learning difficulties	broad forehead, short neck, low-set ears	pectus carinatum	+	c.188A>G (p.Y63C)	NH2	3	PTPN11
2	M/62	NS	PVS	+	intellectual disability	triangular face, hypertelorism, posteriorly rotated ears, low posterior hair line	pectus excavatum	-	c.922A>G (p.N308D)	PTP	8	PTPN11
3	M/4	NS	PVS	+	motor delay	short neck, low-set ears, pterygium colli	pectus excavatum	-	c.922A>G (p.N308D)	PTP	8	PTPN11
4	M/8	NS	PVS	+	learning difficulties	triangular face, pterygium colli, low posterior hair line	-	-	c.57T>C (p.Thr21le)	NH	1	PTPN11
5	F/2	NS	PVS, ASD	-	feeding difficulties, motor delay	hypertelorism, ptosis, short neck	-	NA	c.184T>G (p.Y62D)	NH2	3	PTPN11
6	F/3	NS	PVS	+	none	hypertelorism, high forehead, short neck	-	NA	c.1294T>C (p.W432R)	DH	10	SOS1
7	F/8	NS	PVS	+	motor and speech delay, failure in school	triangular face, pterygium colli, hypertelorism, curly hair, low-set ears	-	NA	c.1655G>A (p.R552K)	DH	10	SOS1
8a	M/6	NS	PVS, ASD	+	none	triangular face, hypertelorism, single palmar crease, low-set ears	-	+	c.188A>G (p.T63C)	NH2	3	PTPN11
8b	F/-	NS	PVS	+	-	triangular face, hypertelorism, short neck	-	-	c.188A>G (p.T63C)	NH2	3	PTPN11
9a	M/7	NS	PVS	+	learning difficulties	triangular face, high forehead, low-rotated ears	-	-	c.923A>G (p.N308S)	PTP	8	PTPN11
9b	M/-	NS	no data	+	-	triangular face, high forehead, low-rotated ears	-	-	c.923A>G (p.N308S)	PTP	8	PTPN11
10	F/4	CFCFS	PVS	-	speech difficulties	curly hair, sparse curly eyebrows, short neck	-	NA	c.736G>C (p.A246P)	phorbiter/ DAG-type protein kinase	6	BRAF
11	F/5	CFCFS	HCM	+	speech delay	curly hair, sparse eyebrows, strabismus	-	NA	c.1501G>A (p.E501K)		12	BRAF

CFCFS = Cardiofaciocutaneous syndrome; ASD = autism spectrum disorder; OII = ostium secundum; HCM = hypertrophic cardiomyopathy; a = index case; b = affected parent; NA = not applicable; + = present; - = absent.

Results

Clinical Data

A summary of clinical findings in mutation-positive cases is given in table 1. A total of 21 patients with a suspected RASopathy diagnosis were enrolled in this study. Among them, 19 received a clinical diagnosis of NS and 2 had a diagnosis of CFC syndrome. Two patients were suspected clinically to have LS and CS, but molecular analyses did not confirm this diagnosis. All patients represented sporadic cases, except for 2 cases with NS who had a parent with a suggestive Noonan phenotype. The ages at diagnosis in the index cases ranged from 3 to 12 years, except for one adult. Dysmorphic features of our patients are shown in figure 1.

Dysmorphic features were typical in mutation-positive patients (10/11). Cardiac defect (100%), short stature and learning disability or developmental delay were common in mutation-positive cases. Table 2 provides the frequencies of the main clinical features in the entire study cohort.

Mutational Screening

Our mutation screening protocol, including the genes *PTPN11*, *SOS1*, *SHOC2*, *RAF1*, *KRAS*, *BRAF*, *MEK1*, *MEK2*, *HRAS*, *NRAS*, *RIT1*, and *CBL* revealed heterozygous missense mutations in 11 out of 21 patients. Nine of them were sporadic cases, while 2 patients had an affected parent who was subsequently confirmed to carry the same mutation. The observed mutation rate was 52%. All mutations observed in this cohort (table 1) had been described previously as causative mutations responsible for NS or CFC syndrome [Tartaglia et al., 2002, 2007; Rodriguez-Viciano et al., 2006; Roberts et al., 2007; Hanna et al., 2009]. The remaining 10 patients did not exhibit a mutation in the investigated genes.

Discussion

In this study, we characterized the molecular and clinical spectrum in a cohort of 21 Tunisian patients with the clinical diagnosis of a RASopathy. Germline missense mutations in one of the known genes were identified in 11 out of 21 index patients. Two of them had a positive family history of NS, and the same mutation could be confirmed in the affected relative. The clinical diagnosis was confirmed in 52% of the index patients with suspected NS as well as in 2 out of 3 patients with a presumed diagnosis of CFC syndrome. Our molecular analysis re-

Fig. 1. Phenotypic features of affected patients and relatives with a RASopathy diagnosis. Index cases 1–4, 8a and 9a carry *PTPN11* mutations. Parents 8b and 9b are affected. Patients 10 and 11 with CFC syndrome carry *BRAF* mutations. Numbering follows table 1.

Table 2. Frequencies of main clinical features in the patients with a RASopathy diagnosis (n = 21)

	Patients with mutation	Patients without mutation
Heart defect	11/11	10/10
Short stature	9/11	7/10
Learning disabilities	7/11	3/10
Typical face	10/11	1/10
Thorax	3/11	2/10
Cryptorchidism (males)	2/6	2/3

vealed mutations in the genes *PTPN11*, *SOS1* and *BRAF* which are known to be the most commonly mutated genes in NS and CFC syndrome, respectively. The lack of mutations in other genes is probably due to the small size of the cohort. It is well documented that *PTPN11* is the major gene for NS [Tartaglia et al., 2001; Zenker et al., 2004]. The mutation frequency in this gene was reported in literature between 33 and 60% of patients with a clinical diagnosis of NS. In line with this, our analysis revealed heterozygous point mutations in the *PTPN11* gene in 9 of the 21 NS patients (43%). Mutations were mainly observed in the known hotspots in exons 3 and 8 which are consistent with previous data [Zenker et al., 2004; Tartaglia et al., 2006]. The following codons were previously

described as major hotspots for recurrent mutations: asparagine 308 (25%) followed by tyrosine 63 (10%). [Lee et al., 2005; Tartaglia and Gelb, 2005; Takahashi et al., 2006] In other studied cohorts, however, asparagine 308 was not replicated as the most common mutation site [Bertola et al., 2006; Simşek-Kiper et al., 2013]. It is not clear whether these discrepancies reflect true differences that might be related to the population background. In the present cohort from the Tunisian population, the distribution of mutations was not obviously different from mixed European populations in other studies [Tartaglia et al., 2002]. In the study by Tartaglia et al. [2006], molecular dynamics simulations were performed to determine the structural consequences of RASopathy-associated mutations and cluster in specific domains of the selected gene. These causative mutations have been shown to cause gain of function.

We identified a de novo missense mutation in exon 1 of *PTPN11* (p.T2I) in one patient displaying a typical NS phenotype including pterygium colli, mild developmental delay, learning difficulties, valvular pulmonary stenosis, and short stature. This rare mutation was initially reported in a patient with NS [Sarkozy et al., 2003]. Subsequently, the same change was described as a de novo mutation in a patient with clinical signs of NS and neurofibromatosis type 1 and who independently harbored an

inherited *NF1* mutation [Thiel et al., 2009]. The functional consequences of this mutation located at the very N-terminal end of the SHP2 protein and outside the known hotspots have previously been unclear.

After *PTPN11*, *SOS1* was found to be the second major gene causing NS [Roberts et al., 2007; Tartaglia et al., 2007]. Mutations were present in approximately 20% of affected patients without a *PTPN11* mutation [Zenker et al., 2007]. Patients with *SOS1* mutations were found to have more prominent ectodermal features and less impairment of growth and intelligence [Roberts et al., 2007; Tartaglia et al., 2007; Zenker et al., 2007]. In our cohort, we found 2 sporadic cases (10%) with *SOS1* alterations which were already reported as pathogenic mutations in NS. The small number of cases does not allow any genotype-phenotype correlation in our cohort. However, a strict relationship was found between heart defects, short stature, developmental delay and the disease [Zenker et al., 2004]. Individuals with CFC syndrome share many clinical features with NS, but despite significant overlap, these conditions can be distinguished both phenotypically and genotypically [Tartaglia et al., 2010]. Genes involved in this syndrome are mostly *BRAF*, followed by *MEK1*, *MEK2* and *KRAS*. In our cohort, we found 2 mutations in the *BRAF* gene which were previously reported [Niihori et al., 2006]. Clinically, these patients were presented with short stature, significant delay in cognitive and speech development as well as poor hair growth. The third patient of our cohort with a clinical diagnosis of CFC syndrome was found negative for the CFC syndrome genes, and subsequent analysis of the other known RASopathy genes turned out negative as well.

Recently, the *RIT1* gene has been demonstrated as a new candidate gene for NS with confirmed clinical diagnosis [Aoki et al., 2013]. In our cohort, we performed *RIT1* analysis in the *PTPN11*, *SOS1* and *BRAF* mutation-negative cases (10/21). Our result did not show any mutation.

Congenital heart defects, notably pulmonary stenosis, are often present as a major clinical sign in NS and re-

lated disorders. PVS was a predominant feature in our probands (with and without mutation) (table 2), whereas the rate of PVS in NS was around 70% in other cohorts [Tartaglia et al., 2002; Sznajder et al., 2007]. The predominance of PVS in our cohort reflects a selection bias, since all our patients were recruited from a cardiology department. Other clinical criteria such as typical facial anomalies, short stature and thorax deformities were common in mutated cases. Previous studies underlined a significant association between the type of heart defects and mutated gene [Sarkozy et al., 2003; Zenker et al., 2004]. While PVS is more prevalent in patients with *PTPN11* and *SOS1* mutations, hypertrophic cardiomyopathy is significantly associated with *RAF1* mutations. In our cohort, only one patient with a diagnosis of CFC syndrome had a hypertrophic cardiomyopathy.

Our study could not confirm the clinical diagnosis in 10 patients classified as NS nor could it confirm it in one patient with CFC syndrome, one patient with LS and one patient with CS. All mutation-negative cases had a heart defect but, on average, they fulfilled fewer clinical criteria of NS (table 2). These findings are consistent with the assumption of further genetic heterogeneity of the RASopathies, but in some of those individuals, the diagnosis of a RASopathy also remained uncertain.

Despite the relatively small size of our cohort, we could confirm the usefulness of a molecular genetic screening for the diagnosis and differential diagnosis of RASopathies. The obtained results have implications for genetic counseling of the families as well as prenatal diagnosis.

Acknowledgements

We would like to thank the technicians of the Department of Histology/Embryology of the Faculty of Medicine, Medical University of Sfax for their help. We also express our gratitude to the staff of the Department of Human Genetics, University Hospital Erlangen and Magdeburg, Germany, for their help and technical assistance, and to the families of the patients for their kind cooperation.

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