NRAS Mutations in Noonan Syndrome

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Key Words

Noonan syndrome · *NRAS* · RAS-MAPK pathway

Abstract

Noonan syndrome is a genetically heterogeneous disorder caused by mutations in *PTPN11*, *SOS1*, *RAF1* and less frequently in *KRAS*, *NRAS* or *SHOC2*. Here, we performed mutation analysis of *NRAS* and *SHOC2* in 115 *PTPN11*, *SOS1*, *RAF1*, and *KRAS* mutation-negative individuals. No *SHOC2* mutations were found, but we identified 3 *NRAS* mutations in 3 probands. One *NRAS* mutation was novel. The phenotype associated with germline *NRAS* mutations is variable. Our results confirm that a small proportion of Noonan syndrome patients carry germline *NRAS* mutations.

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Noonan syndrome (MIM 163950) is an autosomal dominant inherited disorder characterized by short stature, specific facial features, congenital heart defects, mild developmental delay and mildly increased tumour risk. It is a genetically heterogeneous disorder caused by mutations in *PTPN11* [Tartaglia et al., 2001], *KRAS* [Schubbert et al., 2006], *SOS1* [Roberts et al., 2007; Tartaglia et al., 2007], *RAF1* [Pandit et al., 2007; Razzaque et al., 2007], *SHOC2* [Cordeddu et al., 2009] or *NRAS* [Cirstea et al., 2010]. It belongs to a group of phenotypically overlapping

syndromes which are caused by mutations in genes of the RAS-MAPK cascade.

PTPN11 mutations are the most prevalent molecular defect found in Noonan syndrome (about 50% of patients), followed by *SOS1* (17–28%) and *RAF1* (3–17%). *KRAS* mutations account for less than 2% of individuals with Noonan syndrome. Mutations in *SHOC2* and *NRAS* have been described more recently in a small subset of patients and their incidence is still unknown. Previous studies have shown that there are some genotype-phenotype correlations in patients with Noonan syndrome. *PTPN11* mutations are significantly associated with the presence of pulmonic stenosis, short stature, easy bruising and thorax deformities [Zenker et al., 2004]. Patients with *SOS1* mutations often present with pulmonary valve disease and ectodermal abnormalities, including facial keratosis pilaris, sparse eyebrows and curly hair [Roberts et al., 2007; Tartaglia et al., 2007; Zenker et al., 2007a]. Hypertrophic cardiomyopathy and hyperpigmented cutaneous lesions like multiple naevi, lentigines or café-au-lait spots are frequently observed in association with *RAF1* mutations [Pandit et al., 2007; Razzaque et al., 2007]. The *SHOC2* Gly2Ser mutation underlies a specific phenotype, previously known as Noonan-like syndrome with loose anagen hair [Cordeddu et al., 2009]. In Noonan syndrome patients with *KRAS* mutations, variable phenotypes have been described, ranging from mild to severe, with features resembling Costello or cardio-facio-

cutaneous (CFC) syndrome. Since only 5 patients with *NRAS* mutations have been reported up to now, no specific genotype-phenotype correlation is obvious for this Noonan-associated gene [Cirstea et al., 2010; Runtuwene et al., 2011].

In this study we found 3 different *NRAS* mutations in a cohort of 115 *PTPN11*, *SOS1*, *RAF1* and *KRAS* mutation-negative individuals. Two mutations occurred de novo and one was familial. We describe phenotypic features in mutation-positive individuals.

Materials and Methods

Study Population

The initial cohort consisted of 146 unrelated patients with a clinical diagnosis of Noonan syndrome and who were negative for a *PTPN11* mutation. Only exons 2, 3, 4, 7, 8, 12 and 13 of the *PTPN11* gene were screened, since about 98% of mutations have been reported in these exons. The entire coding region of the *SOS1*, *RAF1*, *BRAF*, *MEK1*, *MEK2* and *KRAS* genes was sequenced. Previous mutation analysis detected 13 samples with a *SOS1* mutation, 8 with a *BRAF* mutation, 4 with a *KRAS* mutation, 4 with a *RAF1* mutation and 2 with a *MEK1* mutation. Mutation analysis of the *NRAS* and *SHOC2* genes was performed in the 115 remaining unrelated patients in whom no mutation had been found. The cohort consisted of patients suspected of having Noonan syndrome based on the clinical judgment of clinical geneticists or university hospital-associated pediatricians. DNA samples that had been sent for *PTPN11* analysis without any clinical information or with a clinical description not matching the Noonan phenotype were not included in this study. For mutation-positive patients the referring doctors were contacted and requested to fill out an extended clinical checklist.

Mutation Analysis

Genomic DNA was extracted from blood lymphocytes using standard procedures. Exon 2 of *SHOC2* and all coding exons and intron-exon boundaries of *NRAS* were sequenced bidirectionally using the Big Dye Terminator v.3.1 kit (Applied Biosystems, Foster City, Calif., USA) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Primers and PCR conditions are available on request. Each mutation was confirmed by re-amplification of a second PCR product and re-sequencing. DNA mutation numbering was based on the cDNA sequence with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (GenBank accession code: NM_002524.3 for *NRAS* and NM_007373.3 for *SHOC2*). For protein numbering the initiation codon is codon 1 (NP_002515.1 for *NRAS* and NP_031399.2 for *SHOC2*). Names of all variants were checked using the Mutalyzer program (<http://www.LOVD.nl/mutalyzer/>). Amino acid conservation for novel variants was evaluated with the ConSeq program (<http://conseq.tau.ac.il/>) which assigns a score ranging from 9 (conserved) to 1 (variable) to amino acid residues. Bioinformatical prediction of the possible impact of missense changes on the *NRAS* protein was performed with PolyPhen (<http://genetics.bwh.harvard.edu/pph/>).

Results

Mutation Analysis

In 3 probands 3 different *NRAS* mutations were identified. One *NRAS* mutation (p.Pro34Leu) had not been described before, while the other 2 had already been reported (p.Ile24Asn, p.Thr50Ile). The novel mutation affects a highly conserved residue. The p.Ile24Asn mutation was proven to be of de novo origin, the *NRAS* p.Pro34Leu mutation was inherited from the mother and was found in 3 other family members. An overview of the mutations is given in table 1 and shown in figure 1.

Case Reports

Proband 1 (fig. 2A) had feeding problems in infancy. He had slightly delayed motor milestones (sitting unsupported at 9, walking at 16 months of age). He performed well in normal school. Echocardiography was normal. He was treated for strabismus at the age of 7 years. Dysmorphic features included hypertelorism, downslanting palpebral fissures, low implanted ears and low posterior hairline. He had widely spaced nipples and pectus excavatum, unilateral cryptorchidism, a short stature (<P3) and macrocephaly (>P97).

Proband 2 (patient III:6; fig. 3) has been followed because of a short stature (P3), for which no growth hormone treatment was initiated. He had delayed pubertal development and reached an adult stature between the 10th and 25th centile. He had mild dysmorphic features including hypertelorism, epicanthal folds and short neck. Easy bruising was noticed. He followed normal education, but had learning difficulties. In addition visuo-spatial orientation skills were inadequate. The same mutation (p.Pro34Leu) was found in his mother (patient II:6; fig. 3) who had a normal stature and no dysmorphic or other features reminiscent of Noonan syndrome. Subsequently mutation analysis revealed the presence of the same mutation in the sister of the probands' mother and her 2 children (patients II:2, III:1 and III:2; fig. 3). Patient II:2 had a short stature below the 3rd centile. She suffered from asthma. Patient III:1 was followed during childhood by an endocrinologist because of short stature below the 3rd centile. He was treated with testosterone for 6 months because of delayed puberty. He followed vocational education and had attention problems. He had impulsive behaviour and was bullied as a child. He had typical Noonan facial dysmorphism. Patient III:2 had a stature between the 3rd and 10th centile. She had no typical Noonan-specific facial features, but had a long bleeding time. She had no learning difficulties. She suffered from

Fig. 1. *NRAS* gene and protein structure and position of Noonan syndrome-causing *NRAS* mutations. Top: exon-intron structure of the human *NRAS* gene showing untranslated regions as grey boxes and coding exons as green boxes. Middle: functional motifs of the *NRAS* protein, including the P-loop, switch I (Sw I), switch II (Sw II) and the hypervariable region (HVR), are indicated. Bottom: amino acid sequence with amino acids found to be mutated in this report indicated in red and mutated amino acids from other reports indicated in blue.

Fig. 2. Phenotypic features in patients with *NRAS* mutations. **A** Proband 1. **B, C** Proband 3.

Table 1. Overview of mutations and phenotypic features in the patients with *NRAS* mutations reported in this study

Patient	Gender/age	Mutation ^a	ConSeq score	PolyPhen	Stature	Heart defect	Intelligence/behavior	Dysmorphic features	Other
Proband 1	M/13 years	c.71T>A, p.Ile24Asn	8	possibly damaging	<P3	–	normal	+	unilateral cryptorchidism, pectus excavatum, low posterior hair line
Proband 2	M/19 years	c.101C>T, p.Pro34Leu*	9	probably damaging	P3, adult: P10–25	–	normal	+	delayed pubertal development, easy bruising, inguinal hernia
II:6, fig. 3	F/41 years				P25–50	–	normal	–	asthma
II:2, fig. 3	F/52 years				<P3	–	normal	–	asthma
III:1, fig. 3	M/27 years				<P3	–	ADHD	+	immature fine motor skills, delayed pubertal development, asthma
III:2, fig. 3	F/20 years				P3–10	–	normal	–	diabetes mellitus type 1, long bleeding time, asthma
Proband 3	M/2 years	c.149C>T, p.Thr50Ile	3	benign	P50	coarctation aortae, patent foramen ovale	normal development	+	left undescended testis

+ = yes; – = no.

^a Mutation numbering was based on the cDNA sequence with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (GenBank accession code: NM_002524.3). For protein numbering the initiation codon is codon 1 (NP_002515.1). * Novel mutation.

Fig. 3. Pedigree of the family of proband 2. Patients carrying the *NRAS* p.Pro34Leu mutation are indicated. The proband is indicated by the arrow.

diabetes mellitus type 1 which had been diagnosed at the age of 14 years.

Proband 3 (fig. 2B, C) was first examined at the age of 7 months because of congenital heart disease and facial dysmorphism. At that time the diagnosis of Noonan syndrome was considered because of many dysmorphic features including downslanting palpebral fissures, depressed and broad nasal root, thin upper lip, low implanted, posteriorly rotated ears with thick helices, short and mildly webbed neck, low posterior hair line, widely spaced nipples, pectus excavatum and left retractile testis. His family history was insignificant. His length was at the 50th centile and head circumference between the 25th and 50th centile. Cardiac examination revealed coarctation of the aorta, patent foramen ovale and minor aortic arch anomaly. At the age of 2.5 years his length was between the 10th and 25th centile. His psychomotor development and renal ultrasound findings were normal and he had no other medical problems.

Discussion

Noonan syndrome has a significant phenotypic overlap with Costello and CFC syndrome. These syndromes are all caused by mutations in genes of the RAS-MAPK pathway. Germline mutations in the proto-oncogene *RAS* are found in Costello (*HRAS*) [Aoki et al., 2005], Noonan (*KRAS*) [Schubbert et al., 2006] and CFC (*KRAS*) [Niihori et al., 2006] syndrome. Given the association between germline *HRAS* and *KRAS* mutations and RAS-MAPK syndromes, several authors screened the *NRAS* gene in cohorts of patients with Noonan, Costello or

CFC syndrome, but they failed to find any mutation [Aoki et al., 2005; Carta et al., 2006; Roberts et al., 2007]. Finally, in a screening of 917 individuals with a phenotype fitting with or suggestive for Noonan or CFC syndrome, 2 heterozygous germline *NRAS* mutations were found in 4 unrelated individuals [Cirstea et al., 2010]. An additional *NRAS* mutation in a patient with a typical Noonan phenotype was reported [Runtuwene et al., 2011], and here we found 3 *NRAS* mutations in a cohort of 115 patients with a clinical diagnosis of Noonan syndrome. The 3 patients presented with a relatively mild phenotype. The *NRAS* mutation p.Ile24Asn described in this study has been reported before [Runtuwene et al., 2011]. It is classified as pathogenic because of its occurrence in a highly conserved residue and de novo origin. It was shown to be a mildly activating mutation resulting in enhanced downstream signalling and causing developmental defects, including cell migration defects during gastrulation in zebrafish embryos [Runtuwene et al., 2011]. Another proband had a p.Pro34Leu mutation in *NRAS*, which had not been reported before. This mutation occurred familial and several mutation-positive individuals had very few Noonan-specific symptoms. Both mothers (patients II:2 and II:6) did not show a Noonan phenotype at an adult age. Also patient III:2 did not show a Noonan phenotype at a young adult age. The 2 boys (patients III:1 and III:6) had the typical Noonan facial morphology, also at a young adult age. The p.Pro34Leu mutation has been reported as a pathogenic mutation in *KRAS* in Noonan syndrome [Zenker et al., 2007b]. Pro34 is located in the Switch I region of RAS and another mutation in this residue (p.Pro34Arg) has been shown to accumulate in the GTP-bound conformation and to strongly activate the MAPK pathway [Schubbert et al., 2007]. This mutant protein was insensitive to p120 GAP and neurofibromin, but retained normal intrinsic GTPase activity [Schubbert et al., 2007]. Gremer et al. [2010] confirmed GAP insensitivity as the major mechanism leading to accumulation in the GTP-bound form for the p.Pro34Leu and p.Pro34Arg mutants. Finally, a Thr50Ile mutation in *NRAS* was found. This mutation has been shown to enhance signalling downstream of RAS without causing accumulation of RAS in the GTP-bound conformation. The mechanism is not fully understood [Cirstea et al., 2010].

In summary, we report phenotypic features in 3 probands with a clinical diagnosis of Noonan syndrome and a germline *NRAS* mutation. One novel *NRAS* mutation is reported.

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