Genotype and phenotype in patients with Noonan syndrome and a *RIT1* mutation

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Purpose: Noonan syndrome (NS) is an autosomal-dominant disorder characterized by craniofacial dysmorphism, growth retardation, cardiac abnormalities, and learning difficulties. It belongs to the RASopathies, which are caused by germ-line mutations in genes encoding components of the RAS mitogen-activated protein kinase (MAPK) pathway. *RIT1* was recently reported as a disease gene for NS, but the number of published cases is still limited.

Methods: We sequenced *RIT1* in 310 mutation-negative individuals with a suspected RASopathy and prospectively in individuals who underwent genetic testing for NS. Using a standardized form, we recorded clinical features of all *RIT1* mutation-positive patients. Clinical and genotype data from 36 individuals with *RIT1* mutation reported previously were reviewed.

Results: Eleven different *RIT1* missense mutations, three of which were novel, were identified in 33 subjects from 28 families;

codons 57, 82, and 95 represent mutation hotspots. In relation to NS of other genetic etiologies, prenatal abnormalities, cardiovascular disease, and lymphatic abnormalities were common in individuals with *RIT1* mutation, whereas short stature, intellectual problems, pectus anomalies, and ectodermal findings were less frequent.

Conclusion: *RIT1* is one of the major genes for NS. The *RIT1*-associated phenotype differs gradually from other NS subtypes, with a high prevalence of cardiovascular manifestations, especially hypertrophic cardiomyopathy, and lymphatic problems.

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Key Words: hypertrophic cardiomyopathy; Noonan syndrome; oncogenic mutations; RASopathies; RAS-MAPK signaling pathway

INTRODUCTION

Noonan syndrome (NS; MIM 163950) is a relatively common autosomal-dominant disorder characterized by distinct craniofacial dysmorphism, postnatal growth retardation, and congenital cardiac defects such as pulmonary valve stenosis, atrial septal defects, and hypertrophic cardiomyopathy. Craniofacial features include broad forehead, hypertelorism, downslanting palpebral fissures, ptosis, and low-set posteriorly rotated ears. Affected individuals can also have pectus deformities, mild developmental delay, and/or learning difficulties and bleeding disorders; they are at risk of developing cancer, for example, juvenile myelomonocytic leukemia.[1,2]

NS is a genetically heterogeneous disorder; to date, germ-line mutations in more than 10 genes have been discovered. The three major genes are *PTPN11*, mutated in ~50% of cases (MIM 176876),[3] *SOS1* (10–15%; MIM 182530),[4,5] and *RAF1* (5–10%; MIM 164760).[6,7] *RIT1* (MIM 609591) was more recently identified as another causative gene for NS.[8] The remaining causative genes for NS are *KRAS* (MIM 190070),[9] *NRAS* (MIM 164790),[10] *RRAS* (MIM 165090),[11] *CBL* (MIM 165360),[12] *SOS2* (MIM 601247), and *LZTR1* (MIM 600574),[13] which are rarely mutated in NS. Mutations of *SHOC2* (MIM 602775) account for the NS-like disorder with loose anagen hair (MIM 607721).[14] Two genes, *RASA2* (MIM 601589) and *A2ML1* (MIM 610627), have recently been reported with mutations in a few individuals with NS-like phenotypes,[15,16] but this finding has not yet been replicated by others. Mutations in *BRAF* and *MAP2K1* that are usually associated with cardio-facio-cutaneous syndrome (MIM 115150) have occasionally been reported in NS.[17–19]

All causative genes for NS, except *LZTR1* and *A2ML1*, encode components or regulators of the well-studied RAS mitogen-activated protein kinase (MAPK) signaling pathway. This pathway is critically involved in cell proliferation, differentiation, survival, and senescence.[20] *RAS* genes constitute a multigene superfamily that includes *HRAS*, *KRAS*, *NRAS*, *RRAS*, and *RIT1*. They code for monomeric G proteins, which cycle between a GTP-bound active state and a GDP-bound inactive state.[21] The RAS-MAPK signal transduction cascade is essential for normal mammalian development, implying that dysregulation of this pathway has severe consequences in embryonic development. NS and other clinically overlapping diseases such as cardio-facio-cutaneous syndrome, Costello syndrome (MIM 218040), Legius syndrome (MIM 611431), and neurofibromatosis type 1 (MIM 162200) constitute the group of RASopathies, a class of developmental disorders caused by increased signal flux through the RAS-MAPK pathway.[22]

So far, 11 different missense mutations in the *RIT1* gene have been reported in a total of 36 individuals with NS.[8,15,23–27] Enhanced transactivation of the transcription factor ELK1, which is activated by the kinase ERK2 of the MAPK cascade, has been shown upon overexpression of NS-associated RIT1 mutant proteins in NIH 3T3 cells.[8] Further evidence of a gain-of-function effect of the NS-related *RIT1* mutations came from MEK-ERK activation assays. The amino acid substitutions p.Ala57Gly and p.Met90Ile caused increased phosphorylation of the MAP kinases MEK and ERK when the respective RIT1 mutant was expressed in PC6 cells.[24] The phenotype displayed by *RIT1* mutation carriers is fitting of NS, with no obvious genotype–phenotype correlations identified to date.[8,23–25] Here, we report 33 subjects with a *RIT1* mutation and review clinical features of our and previously reported cases.

MATERIALS AND METHODS
Subjects and phenotyping
Study subjects were referred to two genetic centers (University Hospital of Magdeburg and University Medical Center Hamburg-Eppendorf) for molecular diagnosis of NS. The study cohort comprised 310 individuals who had previously tested negative for other NS-causative genes. It consisted of subjects exhibiting variable clinical features within the RASopathy phenotypic spectrum, including a considerable number of cases with an attenuated or atypical phenotype. Additionally, individuals with suspected NS who were newly referred to our center for diagnostic testing and turned out to have a *RIT1* mutation were included in the genotype–phenotype analysis. Clinical data and samples for all individuals were obtained with informed consent of the patients' parents/legal guardians or the patients themselves, including written consent to use photographs in this report, according to the Declaration of Helsinki and the national legal regulations (e.g., the German Genetic Diagnosis Act (GenDG)).

All affected individuals were personally examined by a physician who is experienced in clinical syndromology, and photos were reviewed by human geneticists with specific clinical expertise in RASopathies (M.Z., K. Kutsche, and S.S.). Standardized phenotypic data were collected using the electronic questionnaire of the NSEuroNet database (http://www.nseuronet.com; more details are provided in the legend to **Supplementary Table S4** online). Standard deviations (SD) of the height were calculated using the pediatric calculator ped(z) (https://www.pedz.de/en/welcome.html). For the majority of patients who were of German descent, the standard German curves were used as reference.[28] For individuals of non-German origin, the standards as published by the World Health Organization (birth to 2 years of age) and the Centers for Disease Control and Prevention (http://www.cdc.gov/growthcharts/) (2–18 years of age) were used as reference. The craniofacial phenotype was classified on a subjective basis as typical, suggestive, or atypical for Noonan syndrome.[29]

Molecular analysis
DNA was isolated from leukocytes by standard procedures. The coding region and exon–intron boundaries of the *RIT1* gene (six exons) (GenBank:NM_006912.5; encodes isoform 2 with a shorter N-terminus compared with isoform 1) was amplified from genomic DNA. Primer sequences are available on request. Amplicons were directly sequenced using the ABI BigDye Terminator Sequencing Kit (Applied Biosystems, Darmstadt, Germany) and an automated capillary sequencer (ABI 3500; Applied Biosystems). Sequence electropherograms were analyzed using the Sequence Pilot software SeqPatient (JSI medical systems, Ettenheim, Germany). Genotyping was performed with the AmpFLSTR SGM plus PCR Amplification Kit (Applied Biosystems) to confirm paternity and maternity. *RIT1* variants were described according to both the long and short transcript variants (mRNA RefSeqs NM_001256821.1 and NM_006912.5) and isoforms (protein RefSeqs NP_001243750.1 and NP_008843.1) in **Supplementary Table S1** online.

RESULTS
RIT1 mutations
We identified 11 different *RIT1* missense mutations in 28 unrelated patients (**Table 1**). Pathogenicity of *RIT1* variants was

Table 1 Clinical phenotype in 33 patients with a *RIT1* mutation

Patient ID	Age	Gender	RIT1 amino acid substitution	Inheritance	Prenatal findings	Premature birth (<37 weeks GA)	Feeding difficulties	Heart defects/anomalies	Lymphatic anomalies	Short stature (SD)	Motor developmental delay	Intellectual/learning disabilities	Cryptorchidism	Skin and hair abnormalities	Skeletal anomalies	Easy bruising	Ocular abnormalities	Other anomalies
1-1	8 y	F	p.G31R	Inherited	–	ND	–	PST	–	−(0.07)	–	–	NA	–	TH, SN	–	PT	–
1-2	47 y	F	p.G31R	Not known	ND	–	–	PST	–	−(−0.33)	–	–	NA	–	–	–	PT	–
2	11 y	F	p.A57G	Not known	–	–	ND	PST, HCM	aLE	+(−3.42)	–	–	NA	–	TH, SN	–	PT	Giant cell tumor of jaws
3	13 y	F	p.F82V	*De novo*	NE, PE, HF	+	–	PST, HCM	nLE, nCT	−(−1.18)	–	–	NA	–	SN	–	PT	–
4-1	8 y	M	p.G95A	Inherited	–	–	+	PST	–	−(−1.52)	+	ND	–	CH	TH	–	PT, RE	–
4-2	42 y	F	p.G95A	Not known	–	+	–	ASD, VSD	–	−(0.61)	–	–	NA	CH	SC	–	PT, RE	Depression
4-3	14 y	F	p.G95A	Inherited	–	–	–	PST	–	−(−0.90)	–	–	NA	CH	–	–	PT, RE	Sensorineural hearing deficit[b]
5	4 y	F	p.K23N	*De novo*	NE	–	+	PST, ASD	–	−(−0.27)	+	ND	NA	–	–	–	–	Minor renal anomalies
6	1 y	F	p.A57G	*De novo*	NE, PE, PH	+	+	PST, HCM	aCT, nAS	+(−3.15)	+	ND	NA	–	TH, SN	–	PT	–
7-1	6 y	M	p.A57G	Inherited	PH, HD	+	+	PST, ASD, VSD	–	−(−1.37)	+	+	+	–	TH	–	PT	Hydrocephalus
7-2	29 y	F	p.A57G	Not known	ND	ND	ND	PST	–	−(−0.80)	ND	ND	NA	–	–	+	–	Hypothyroidism
8	5 y	F	p.F82L	*De novo*	NE, HF, RA	–	+	HCM, ASD, MVA	nLE, aLE	+(−2.52)	+	–	NA	–	TH, SN	+	PT, RE	Benign seizures, minor renal anomalies
9	38 y	F	p.F82L	Not known	ND	–	ND	PST, MVA	aLE, aCT	−(−1.11)	–	–	NA	–	–	–	CT, glaucoma	Autoimmune thyroiditis, NET and GIST of the stomach
10	16 y	F	p.T83P	Not known	–	+	+	HCM	–	−(−0.37)	–	–	NA	KP, HA	–	+	RE, ST	HN
11	3 y	F	p.M90I	Not known	–	+	–	PST, ASD, MVA	nCT	−(−1.97)	+	ND	NA	HA	–	–	RE, CT	Hernia umbilicalis
12	1 y	F	p.S35T	*De novo*	–	+	+	PST	–	ND	ND	ND	NA	–	SN	ND	PT	–
13	5 y	F	p.S35T	*De novo*	PH	–	–	PST, HCM	–	−(−1.99)	+	–	NA	HA	SN	ND	–	–
14	5 y	F	p.F82L	Not known	PH	–	ND	PST, HCM	–	+(−2.96)	–	–	NA	CH	TH, SN	–	PT, RE	B-ALL
15	24 y	M	p.G95A	Not known	–	–	–	MVA	aLE	−(0.54)	–	+	+	HA	TH, SC, SN	+	PT, RE, ST	–
16	11 y	M	p.S35T	Not known	–	+	+	PST, HCM	–	−(−0.19)	–	+	–	–	TH, SN	+	PT, RE	–

RIT1 mutations are described in the one-letter code according to the short isoform (NM_006912.5 and NP_008843.1).

aTreated with growth hormones. bIndependently segregating trait in the paternal family. –, absent; +, present.

aCT, acquired chylothorax; aLE, acquired lymphedema (after newborn period); ASD, atrial septal defect; B-ALL, B-cell acute lymphoblastic leukemia; CH, curly hair; CT, cataract; F, female; GA, gestational age; GIST, gastrointestinal stromal tumor; HA, hemangioma; HCM, hypertrophic cardiomyopathy; HD, suspected fetal heart defect/anomaly; HF, hydrops fetalis; HN, hydronephrosis; IL, intestinal lymphangiectasis; KP, keratosis pilaris; M, male; MN, multiple nevi; mo, months; MVA, mitral valve anomalies; NA, not applicable; nAS, neonatal ascites; nCT, neonatal/congenital chylothorax; ND, no data; NE, fetal nuchal edema; NET, neuroendocrine tumor; nLE, neonatal lymphedema; PE, fetal pleural effusions; PH, polyhydramnios; PST, pulmonary valve stenosis; PT, ocular ptosis; RA, fetal renal anomaly; RE, refractive error; SC, scoliosis; SN, short, broad, or webbed neck; ST, strabismus; TH, thorax deformity; VSD, ventricular septal defect; y, year(s).

ORIGINAL RESEARCH ARTICLE

Table 1 Continued

Patient ID	Age	Gender	RIT1 amino acid substitution	Inheritance	Prenatal findings	Premature birth (<37 weeks GA)	Feeding difficulties	Heart defects/anomalies	Lymphatic anomalies	Short stature (SD)	Motor developmental delay	Intellectual/learning disabilities	Cryptorchidism	Skin and hair abnormalities	Skeletal anomalies	Easy bruising	Ocular abnormalities	Other anomalies
17	11 y	M	p.F82L	Not known	PH	+	ND	PST, HCM, ASD	aLE, aCT	–(–1.91)	+	+	+	–	TH	–	PT, RE, ST	Benign seizures, HN
18	13 y	F	p.F82L	Not known	–	–	–	PST	–	+[a]	–	–	NA	–	–	+	PT	–
19	2 mo	M	p.A57G	Not known	NE, HF, PH, HD	+	–	PST, HCM, VSD	nLE	+(–2.10)	ND	ND	–	CH	SN	–	PT	–
20	6 mo	F	p.A57G	Not known	–	–	+	PST, HCM, ASD, VSD	nLE	+(–2.31)	ND	ND	NA	–	SN	–	PT	–
21	3 y	F	p.A77T	Not known	PH	–	–	PST, ASD	ND	+(–2.21)	+	ND	NA	–	TH	ND	–	–
22	7 y	M	p.A57G	De novo	NE, PE, PH, HD	+	+	HCM, MVA	nLE, nCT	–(–0.03)	+	–	+	MN	TH	+	PT, ST	HN
23	1 y	F	p.F82L	Not known	NE, PE, HF	+	+	PST, HCM, ASD	aCT	–(–1.21)	ND	ND	NA	–	TH, SN	–	–	Nephrocalcinosis
24	15 y	F	p.G95A	Not known	NE, PH	+	+	ASD	IL	+(–2.11)	+	+	NA	HA	SN	–	RE	Submucosal cleft palate
25	11 y	M	p.G95A	Not known	NE, RA	+	–	PST, ASD	–	–(–1.16)	–	+	+	KP, MN	SN	–	PT, RE, CT	Minor renal anomalies, deafness, hypothyroidism
26-1	5 mo	M	p.A57G	Inherited	PH	–	–	PST, HCM	–	–(0.38)	ND	ND	+	–	–	–	–	–
26-2	32 y	F	p.A57G	Not known	ND	ND	ND	PST	–	–(–0.80)	–	–	NA	–	–	–	–	–
27	21 y	M	p.A77T	Not known	ND	ND	ND	PST, MVA	–	–(1.07)	–	–	–	MN	TH	ND	PT, RE, ST	Minor renal anomalies
28	29 y	M	p.F82S	Not known	–	+	+		–	+[a]	–	–	–	–	SN	–	PT, ST	Lipoma

RIT1 mutations are described in the one-letter code according to the short isoform (NM_006912.5 and NP_008843.1). [a]Treated with growth hormones. [b]Independently segregating trait in the paternal family. –, absent; +, present.

aCT, acquired chylothorax; aLE, acquired lymphedema (after newborn period); ASD, atrial septal defect; B-ALL, B-cell acute lymphoblastic leukemia; CH, curly hair; CT, cataract; F, female; GA, gestational age; GIST, gastrointestinal stromal tumor; HA, hemangioma; HCM, hypertrophic cardiomyopathy; HD, suspected fetal heart defect/anomaly; HF, hydrops fetalis; HN, hydronephrosis; IL, intestinal lymphangiectasis; KP, keratosis pilaris; M, male; MN, multiple nevi; mo, months; MVA, mitral valve anomalies; NA, not applicable; nAS, neonatal ascites; nCT, neonatal/congenital chylothorax; ND, no data; NE, fetal nuchal edema; NET, neuroendocrine tumor; nLE, neonatal lymphedema; PE, fetal pleural effusions; PH, polyhydramnios; PST, pulmonary valve stenosis; PT, ocular ptosis; RA, fetal renal anomaly; RE, refractive error; SC, scoliosis; SN, short, broad, or webbed neck; ST, strabismus; TH, thorax deformity; VSD, ventricular septal defect; y, year(s).
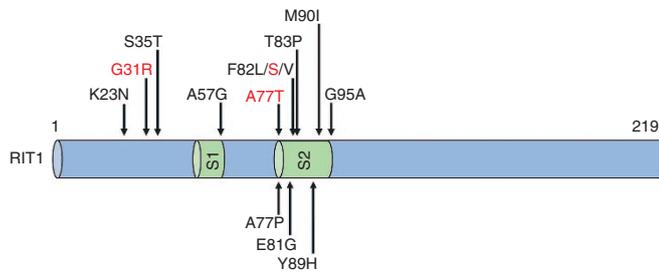
Figure 1 RIT1 domain structure and NS-associated amino acid substitutions. The first and the last amino acids of the small RIT1 isoform (according to protein Ref Seq NP_008843.1) are given above the domain structure. Switch I (S1) and switch II (S2) domains are indicated as green barrels. Amino acid substitutions identified in the two cohorts reported here are given in one-letter code above the structure and those reported previously and not found in this study are below the structure. Novel mutations are highlighted in red.

classified according to the guidelines of the American College of Medical Genetics and Genomics (**Supplementary Table S1** online).[30] Twenty-one index cases were identified in a retrospective cohort of 310 patients with NS who previously tested negative for other known NS genes, and 7 *RIT1* mutation-positive patients were prospectively recruited from individuals who underwent molecular diagnostic testing for NS since September 2013. Three of the missense changes have not been described previously: c.91G>A/p.(Gly31Arg), c.229G>A/p.(Ala77Thr), and c.245T>C/p.(Phe82Ser); however, a different *RIT1* mutation affecting codon 77 (c.229G>C/p.(Ala77Pro)) and two others changing codon 82 (c.244T>G/p.(Phe82Val) and c.246T>G/p.(Phe82Leu)) have already been reported (**Figure 1**).[8,15] The p.(Gly31Arg) variant is absent from the databases dbSNP, 1,000 Genomes Project, and ExAC Browser databases and is predicted to be damaging by the Combined Annotation Dependent Depletion scoring system (data not shown).[31] For seven mutations, *de novo* occurrence was demonstrated by parental testing. Four mutations were familial, with five additional affected family members being confirmed carriers of the respective mutation (patients 1-2, 4-2, 4-3, 7-2, and 26-2), including a mother and her daughter carrying the novel mutation p.(Gly31Arg) (**Table 1** and **Supplementary Table S1** online). These familial cases expand the number of *RIT1* mutation-positive individuals to 33. Segregation of the *RIT1* mutation could not be investigated in 21 individuals because DNA samples for one or both parents were not available (**Table 1** and **Supplementary Table S1** online).

Phenotype analysis

Clinical findings for 33 individuals with a *RIT1* mutation (22 females and 11 males) are summarized in **Table 1**. Overall, the clinical phenotype is characteristic of NS. In all subjects, the craniofacial phenotype was evaluated by experienced RASopathy specialists (M.Z., K.Kutsche, and S.S.) on the basis of personal examination or photographs. In all of the patients we found variable combinations of craniofacial dysmorphisms characteristic of NS, such as broad forehead, hypertelorism, downslanting palpebral fissures, ptosis, broad nasal bridge, low-set ears, and short neck; all faces were classified as typical or suggestive

of NS (**Figure 2** and data not shown). Prenatal abnormalities were recorded in 15/28 (54%) patients.

The most common findings were polyhydramnios (*n* = 10) and fetal nuchal edema (*n* = 9). Six affected fetuses had either isolated pleural effusions or hydrops fetalis. A fetal heart defect/anomaly was suspected in only three cases. Premature birth was common: 15/29 (52%) were born before the week 37 of gestation, and five of them were born before week 34 of gestation. In 13 of 27 individuals (48%), feeding difficulties in infancy were reported, and six patients required gavage feeding. In the latter case, infants were born prematurely, with one exception (data not shown). Two children had gastroesophageal reflux. Almost all patients had congenital heart defect (32/33; 97%), with pulmonary valve stenosis being the most common anomaly (26/33; 79%). A high rate of hypertrophic cardiomyopathy (HCM) was also observed (14/33; 42%); 12/33 (36%) had septal defects, mainly atrial septum defect. Twenty patients underwent either catheter intervention or open surgery for congenital heart defects (data not shown).

Postnatal lymphatic anomalies were noted in 13/32 (41%), including several cases with neonatal lymphedema (*n* = 5) and congenital chylothorax (*n* = 3). Notably, acquired lymphedema (occurring after the newborn period), such as lymphedema of lower limbs and genitalia, acquired chylothorax, or a combination of both, was found in 6/32 individuals, and another (patient 24) had intestinal lymphangiectasis with protein-losing enteropathy, resulting in 22% with late-onset lymphatic complications that lead to significant morbidity. Short stature (height SD below −2.00; third percentile) was found in only 10/32 individuals (31%), and another 5 patients had height below −1.25 SD (10th percentile). Two patients had undergone growth hormone treatment. None of the eight adult *RIT1* mutation carriers in our cohort had a body height below −1.25 SD (10th percentile). Motor developmental delay was observed in 10/27 subjects (37%) and was frequently associated with muscular hypotonia. In 5 of them, delay in the achievement of motor milestones was mild (unaided sitting between 9 and 12 months and/or unaided walking between 18 and 24 months) or not specified. Six individuals showed moderate delay in motor milestones (unaided sitting between 12 and 18 months and/or unaided walking between 24 and 36 months), but 5 of them had a history of premature birth.

Learning or intellectual disabilities were reported in 6/22 (27%) of our *RIT1* mutation-positive subjects aged 5 years or older. In four of six individuals, mental disabilities were mild (**Supplementary Table S2** online). Learning difficulties were associated with attention-deficit disorder in three individuals (**Supplementary Table S2** online). Six out of 11 male patients (55%) had cryptorchidism. Skin and hair abnormalities were found in 13/33 individuals (39%), with curly hair and cutaneous hemangioma as the most common findings in five individuals each. Keratosis pilaris was found in only 2 patients. A short, broad, and/or webbed neck was present in 16/33 (48%) individuals and pectus deformities in 14/33 (42%). In 6 out of 29 individuals (21%), easy bruising was reported.

Figure 2 Craniofacial phenotype in patients with *RIT1* mutation. Facial photographs of 23 individuals from our cohort displaying the spectrum of *RIT1* mutation–associated facial dysmorphism from infancy to adulthood. Patients 11, 1-1, and 13 are shown at different ages (infancy vs. 2.6, 8.5, and 4.7 years, respectively) to document evolution of the craniofacial phenotype. All affected individuals exhibit recognizable dysmorphic features of Noonan syndrome, although the expression is quite variable. Patient IDs refer to Table 1.

Ocular abnormalities were seen quite often (26/33 individuals; 79%), with ptosis as the most common feature observed in 22/33 subjects (67%). Ocular ptosis was significant in three individuals for whom surgical correction was either planned or performed. Refractive errors were present in 13/33 (39%). Three individuals had cataracts. Four patients developed benign or malignant neoplasias, including multiple giant cell tumors of the jaws in patient 2, B-cell acute lymphoblastic leukemia in patient 14, a gastrointestinal stromal tumor and a neuroendocrine tumor in patient 9, and a lipoma in patient 28. Three patients had hydronephrosis requiring surgery, and four others showed minor renal anomalies. Three individuals had documented hypothyroidism or autoimmune thyroiditis. Two subjects had deafness requiring hearing aids; however, in patient 4-3, this is possibly a different genetic trait.

DISCUSSION

Herein we present a large contiguous cohort of patients with NS caused by *RIT1* mutation. We identified 21 unrelated *RIT1* mutation-positive patients among 310 individuals with a clinical diagnosis of NS or a RASopathy-like disorder who were negative for a mutation in the previously identified NS-linked genes, providing a mutation detection rate of 6.8% in this cohort. An additional seven *RIT1* mutation-positive cases were identified prospectively among patients referred for molecular diagnostic testing. We identified *RIT1* mutations in individuals with a clinical diagnosis of NS; none of them had been suspected of having cardio-facio-cutaneous or Costello syndrome. Two previous studies by Aoki et al. (2013) and Bertola et al. (2014) reported a *RIT1* mutation detection rate of approximately 9% in cohorts of patients who had tested negative for mutations in the previously known genes. By contrast, Gos

et al. (2014) found that only 3.8% of their mutation-negative cases with NS carried a *RIT1* mutation. The differences in the detection rates, however, are likely to be explained by more or less strict clinical inclusion criteria, and these figures do not reflect the prevalence of *RIT1* alterations in an unselected cohort of NS-affected subjects. To provide a better estimate of the contribution of *RIT1* mutations to NS, we calculated the relative frequency of mutations in the NS-linked genes among 507 individuals who tested positive for NS at the University of Magdeburg since 2009: *PTPN11*, 54%; *SOS1*, 18%; *RAF1*, 11%; *RIT1*, 5%; *KRAS*, 3%; *CBL*, 2%; and *NRAS*, 1% (M.Z., unpublished data). This cohort is not free from any bias, and, compared with an unselected patient population, these figures may be an overestimation for mutations in *SOS1* and *RAF1*, but an underestimation for *RIT1* alterations. Nevertheless, these numbers indicate that *RIT1* mutations are quite common, and *RIT1* is among the four major genes for NS, accounting for at least 5% of molecularly confirmed NS-affected cases.

In the 28 *RIT1* mutation-positive index patients reported here, the majority of mutations were found to affect codon 82 (*n* = 8) (**Table 1**), giving rise to amino acid substitution of phenylalanine to valine, leucine, or serine (**Figure 1**). The two other most common *RIT1* codons mutated in NS-affected individuals are 57 (*n* = 7) and 95 (*n* = 4) (**Table 1**). Clustering of disease-causative variants at codons 57, 82, and 95 has already been observed.[8,23] By combining the published *RIT1* germ-line alleles with those reported here, we calculated that 23% of the substitutions occurred at codon 57, 22% occurred at codon 82, and 22% occurred at codon 95, yielding a total of 67% of all *RIT1* mutations affecting one of these three triplets, whereas alterations of the eight other codons (23, 31, 35, 77, 81, 83, 89, and 90) together account for 33% of identified mutations (this study and refs. [8,15,23–27]). These data are in line with gain-of-function mutations in other genes found in NS-affected individuals because they cluster at codons encoding highly conserved amino acids in functionally important domains.

The *RIT1* mutation p.(Gly31Arg) identified in this study is novel and alters a functionally relevant amino acid residue. Glycine 31 in *RIT1* corresponds to glycine 13 in the RAS GTPases. Glycine 13 of HRAS is mutated in individuals with Costello syndrome.[32] These findings, together with pathogenicity prediction and segregation of the variant with disease (**Supplementary Table S1** online), provide strong evidence for causality of the *RIT1* mutation p.(Gly31Arg).

To better define the phenotype associated with a *RIT1* mutation, we summarized the clinical features observed in our 33 patients and reviewed those of 36 individuals described in the literature (**Supplementary Table S3** online).[8,15,23–27] We also compared the frequency of phenotypic features in our *RIT1* mutation-positive cohort with patients harboring a *PTPN11*, *SOS1*, or *RAF1* mutation, whose phenotype data had been collected using the same standardized form (NSEuroNet database; **Supplementary Table S4** online). In more than half of the *RIT1* mutation-positive cases, prenatal abnormalities were observed, including polyhydramnios as the most common finding and

also fetal nuchal edema, fetal pleural effusions, and hydrops fetalis. The frequency of prenatal abnormalities was comparable between our cohort and previously published cases with *RIT1* mutation (**Supplementary Table S3** online). Compared with other NS genes, especially *PTPN11*, in which mutations account for the largest fraction of NS-affected cases, a significantly higher frequency of fetal nuchal edema (32%), fetal chylothorax, and/or fetal hydrops (21%) was observed (**Supplementary Table S4** online). Notably, *RIT1* mutation-positive individuals from our cohort also had a higher incidence (22%) of lymphatic disorders occurring later in life, which was significant in comparison to patients with *PTPN11* and *RAF1* mutations (**Supplementary Table S4** online). Three cases with *RIT1* mutation and acquired lymphatic disorders were also reported in the literature (**Supplementary Table S3** online).

A significant proportion of all patients with a *RIT1* germline mutation in our cohort (48%) and previously reported cases (59%) had feeding difficulties (**Supplementary Table S3** online); however, in our cohort we could relate the more severe feeding difficulties to premature birth (data not shown). Overall feeding issues appear to occur less frequently in individuals with a *RIT1* mutation compared with NS-affected individuals linked to other genes;[33] however, this difference does not reach statistical significance in our data set (**Supplementary Table S4** online). Cardiovascular abnormalities were seen in almost all individuals with a *RIT1* alteration (combined frequency 66/68; 97%; **Supplementary Table S3** online). This is significantly higher than in patients with *PTPN11* mutation (79%; **Supplementary Table S4** online), and also higher than the frequency reported for NS overall (81%; no patients with *RIT1* mutation included).[34] The most prevalent heart defect was pulmonary or pulmonary valve stenosis found in a total of 54 out of 68 *RIT1* mutation-positive individuals (79%) (**Supplementary Table S3** online) compared with a prevalence of 56% in patients with *PTPN11* mutation, 15% in those with *RAF1* mutation (**Supplementary Table S4** online), and 57% in molecularly confirmed patients with NS overall.[34] A high incidence of HCM in NS-affected subjects with *RIT1* mutation has already been noticed by Aoki et al.[8] There is a total prevalence of HCM of 52% (33/63) in individuals with a *RIT1* mutation (**Supplementary Table S3** online) compared with 16% in reported individuals with a molecularly confirmed diagnosis of NS in general (without *RIT1*).[34]

Although HCM was somewhat less frequent in our patient cohort with *RIT1* alteration (42%) (**Supplementary Table S4** online), we found significant differences compared with individuals with *PTPN11* or *SOS1* mutation (12% each) in our data set (**Supplementary Table S4** online), which is consistent with published data.[33] These findings corroborate that *RIT1* is the second most important NS gene associated with HCM; only *RAF1* germ-line mutations show a higher association with HCM (approximately 80%) (**Supplementary Table S4** online).[33] NS-affected individuals with HCM show significant early mortality (22% by the age of 1 year).[35] However, there was no instance of cardiac death related to HCM in our cohort of *RIT1*

mutation-positive patients or in other reported patients,[8,15,23–27] suggesting that HCM associated with *RIT1* mutations has a more benign course. Atrial or ventricular septal defects were found in a proportion of subjects with *RIT1* mutation, which is comparable to individuals with mutations in other genes (**Supplementary Table S4** online).[33] Short stature, which is a common feature in NS,[36] was consistently found at a relatively low frequency, affecting a total of only 24 of 64 individuals with *RIT1* alteration (38%) (**Supplementary Table S3** online). The frequency of short stature in *RIT1* mutation-positive individuals is significantly lower than in patients with an alteration in *PTPN11*, *SOS1*, and *RAF1* in our data set (**Supplementary Table S4** online). Accordingly, Kobayashi et al.[33] recorded a higher incidence of short stature, particularly in patients with a *RAF1* (82%) or *PTPN11* (56%) mutation.

Consistent with our data (**Supplementary Tables S2** and **S3** online), a relatively low percentage of subjects with *RIT1* mutation was recorded to have intellectual/learning disabilities (13/49; 27% in total; **Supplementary Table S3** online), which corresponds well to 21% of individuals with *SOS1* mutation in our cohort (**Supplementary Table S4** online) and 18% reported in the literature.[33] Conversely, in patients with *RAF1* and *PTPN11* mutation, frequencies of 43% and 40%, respectively, were recorded for intellectual/learning disabilities in our data (**Supplementary Table S4** online), which is consistent with published data.[33] However, differences in the overall prevalence of intellectual/learning disabilities between patients with *RIT1*, *PTPN11*, *SOS1*, and *RAF1* mutation in our cohort did not reach statistical significance, probably because of a limited sample size (**Supplementary Table S4** online). A more detailed analysis of the degree of intellectual impairment showed that the recorded deficits were usually mild in the *RIT1* mutation-positive patients described here. Specific learning disabilities were uncommon, but numbers were too small to achieve statistically significant differences (**Supplementary Table S2** online).

Ectodermal abnormalities such as curly hair and hyperkeratosis are relatively uncommon in *RIT1* mutation-positive subjects from our cohort as well as in previously published cases (**Supplementary Tables S3** and **S4** online). Significantly higher frequencies of ectodermal abnormalities were recorded in individuals with *SOS1* mutation in our data set (**Supplementary Table S4** online), which fits well with data from the literature.[33] Pectus deformities and a short, broad, or webbed neck are consistently observed in NS-affected individuals with mutations in different genes.[24,33] We observed significantly lower frequencies of these anomalies in patients with *RIT1* mutation compared with *SOS1* and *RAF1* (**Supplementary Table S4** online).

Bleeding diathesis was rarely observed in patients with *RIT1* mutation (21%) in our cohort, but it is not significantly different from the rate of patients carrying mutations in *PTPN11*, *SOS1*, and *RAF1* (33, 21, and 17%, respectively; **Supplementary Table S4** online). In our cohort, bleeding problems were rather mild and without any severe complications (data not shown). Ocular ptosis was seen quite often in our *RIT1* mutation-positive cohort (67%) (**Supplementary Table S4** online) and in a total of 36 out of 56 (64%) individuals with *RIT1* alteration (**Supplementary Table S3** online). This association is quite similar in all other patient groups with molecularly confirmed NS (60–78% in our cohort in **Supplementary Table S4** online and 62–79% reported in ref.[33]). However, severe ptosis requiring surgical interventions appears to be rare in *RIT1*-mutated patients (three individuals in our cohort and no documentation in previous studies).

Somatic mutations in *RIT1* have recently been identified in myeloid malignancies and lung adenocarcinomas.[37–39] The *RIT1* mutational spectrum found in human cancers significantly overlaps with *RIT1* germ-line alleles: all oncogenic mutations clustered around glutamine 79 in the switch II region (p.(Ala77Pro), p.(Glu81Gly), p.(Phe82Leu/Val), and p.(Met90Ile)) and induced cellular transformation.[37–39] A recent study of cancer spectrum and frequency in patients with RASopathies demonstrated an 8.1-fold increased risk of all childhood tumors in NS-affected children,[40] but because this study included only patients with a molecularly confirmed diagnosis of a RASopathy before 2013, no cases with a *RIT1* mutation were included. We report the second case of *RIT1* mutation–associated NS with acute lymphoblastic leukemia and multiple giant cell lesions of the jaws ([Table 1] and refs.[8,25]), suggesting that individuals carrying a *RIT1* germ-line mutation might be at increased risk for developing these types of neoplasia. Two other patients in our cohort developed malignant or benign tumors, thus bringing the total number of *RIT1* mutation-positive individuals with any type of neoplasia to 6/68 (9%) (**Supplementary Table S3** online), including three cases of malignancy. The three subjects with a malignant tumor had alterations affecting codons 81 and 82, which are common sites of somatic *RIT1* mutations.[37–39] The two patients reported with giant cell tumors of the jaws (our cohort and Bertola et al. (2014)) shared the *RIT1* mutation p.(A57G). In summary, these findings indicate that Noonan syndrome–associated *RIT1* germ-line mutations do confer susceptibility to neoplasia; however, further studies are needed to determine the risk for malignancies in this particular NS patient group.

Conclusion

We conclude that *RIT1* is one of the four most frequently mutated genes in patients with a clinical phenotype of NS. Using careful clinical evaluation of our patient cohort and reviewing previously reported cases, we delineated in more detail the clinical characteristics of *RIT1*-associated NS. We found a high frequency of cardiovascular abnormalities with particular risk of HCM and a predisposition to lymphatic problems, whereas short stature, developmental delay, bleeding diathesis, and ectodermal abnormalities occurred at lower frequencies compared with NS in general. We add the three novel amino acid substitutions p.(Gly31Arg), p.(Ala77Thr), and p.(Phe82Ser) to 11 already reported NS-associated *RIT1* alterations. The majority of *RIT1* germ-line mutations cluster at codons 57, 82, and 95, and the mutation spectrum in NS-affected individuals significantly overlaps with that of somatic mutations in tumors. We

found a possible hint of increased risk of neoplasia in subjects with *RIT1* mutation–associated NS.

REFERENCES
1. Romano AA, Allanson JE, Dahlgren J, et al. Noonan syndrome: clinical features, diagnosis, and management guidelines. *Pediatrics* 2010;126:746–759.
2. van der Burgt I. Noonan syndrome. *Orphanet J Rare Dis* 2007;2:4.
3. Tartaglia M, Mehler EL, Goldberg R, et al. Mutations in PTPN11, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome. *Nat Genet* 2001;29:465–468.
4. Roberts AE, Araki T, Swanson KD, et al. Germline gain-of-function mutations in SOS1 cause Noonan syndrome. *Nat Genet* 2007;39:70–74.
5. Tartaglia M, Pennacchio LA, Zhao C, et al. Gain-of-function SOS1 mutations cause a distinctive form of Noonan syndrome. *Nat Genet* 2007;39:75–79.
6. Pandit B, Sarkozy A, Pennacchio LA, et al. Gain-of-function RAF1 mutations cause Noonan and LEOPARD syndromes with hypertrophic cardiomyopathy. *Nat Genet* 2007;39:1007–1012.
7. Razzaque MA, Nishizawa T, Komoike Y, et al. Germline gain-of-function mutations in RAF1 cause Noonan syndrome. *Nat Genet* 2007;39:1013–1017.
8. Aoki Y, Niihori T, Banjo T, et al. Gain-of-function mutations in RIT1 cause Noonan syndrome, a RAS/MAPK pathway syndrome. *Am J Hum Genet* 2013;93:173–180.
9. Schubbert S, Zenker M, Rowe SL, et al. Germline KRAS mutations cause Noonan syndrome. *Nat Genet* 2006;38:331–336.
10. Cirstea IC, Kutsche K, Dvorsky R, et al. A restricted spectrum of NRAS mutations causes Noonan syndrome. *Nat Genet* 2010;42:27–29.
11. Flex E, Jaiswal M, Pantaleoni F, et al. Activating mutations in RRAS underlie a phenotype within the RASopathy spectrum and contribute to leukaemogenesis. *Hum Mol Genet* 2014;23:4315–4327.
12. Martinelli S, De Luca A, Stellacci E, et al. Heterozygous germline mutations in the CBL tumor-suppressor gene cause a Noonan syndrome-like phenotype. *Am J Hum Genet* 2010;87:250–257.
13. Yamamoto GL, Aguena M, Gos M, et al. Rare variants in SOS2 and LZTR1 are associated with Noonan syndrome. *J Med Genet* 2015;52:413–421.
14. Cordeddu V, Di Schiavi E, Pennacchio LA, et al. Mutation of SHOC2 promotes aberrant protein N-myristoylation and causes Noonan-like syndrome with loose anagen hair. *Nat Genet* 2009;41:1022–1026.
15. Chen PC, Yin J, Yu HW, et al. Next-generation sequencing identifies rare variants associated with Noonan syndrome. *Proc Natl Acad Sci USA* 2014;111:11473–11478.
16. Vissers LE, Bonetti M, Paardekooper Overman J, et al. Heterozygous germline mutations in A2ML1 are associated with a disorder clinically related to Noonan syndrome. *Eur J Hum Genet* 2015;23:317–324.
17. Nava C, Hanna N, Michot C, et al. Cardio-facio-cutaneous and Noonan syndromes due to mutations in the RAS/MAPK signalling pathway: genotype-phenotype relationships and overlap with Costello syndrome. *J Med Genet* 2007;44:763–771.
18. Nyström AM, Ekvall S, Berglund E, et al. Noonan and cardio-facio-cutaneous syndromes: two clinically and genetically overlapping disorders. *J Med Genet* 2008;45:500–506.
19. Sarkozy A, Carta C, Moretti S, et al. Germline BRAF mutations in Noonan, LEOPARD, and cardiofaciocutaneous syndromes: molecular diversity and associated phenotypic spectrum. *Hum Mutat* 2009;30:695–702.
20. Yoon S, Seger R. The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. *Growth Factors* 2006;24:21–44.
21. Colicelli J. Human RAS superfamily proteins and related GTPases. *Sci STKE* 2004;2004:RE13.
22. Rauen KA. The RASopathies. *Annu Rev Genomics Hum Genet* 2013;14:355–369.
23. Gos M, Fahiminiya S, Poznański J, et al. Contribution of RIT1 mutations to the pathogenesis of Noonan syndrome: four new cases and further evidence of heterogeneity. *Am J Med Genet A* 2014;164A:2310–2316.
24. Koenighofer M, Hung CY, McCauley JL, et al. Mutations in RIT1 cause Noonan syndrome - additional functional evidence and expanding the clinical phenotype. *Clin Genet* 2016;89:359–366.
25. Bertola DR, Yamamoto GL, Almeida TF, et al. Further evidence of the importance of RIT1 in Noonan syndrome. *Am J Med Genet A* 2014;164A:2952–2957.
26. Justino A, Dias P, João Pina M, et al. Comprehensive massive parallel DNA sequencing strategy for the genetic diagnosis of the neuro-cardio-facio-cutaneous syndromes. *Eur J Hum Genet* 2015;23:347–353.
27. Nemcikova M, Vejvalkova S, Fencl F, Sukova M, Krepelova A. A novel heterozygous RIT1 mutation in a patient with Noonan syndrome, leukopenia, and transient myeloproliferation-a review of the literature. *Eur J Pediatr* 2016;175:587–592.
28. Kromeyer-Hauschild K, Wabitsch M, Kunze D, et al. Perzentile für den Body-mass-Index für das Kindes- und Jugendalter unter Heranziehung verschiedener deutscher Stichproben. *Monatsschr Kinderheilkd* 2001;149:807–818.
29. Allanson JE, Bohring A, Dörr HG, et al. The face of Noonan syndrome: Does phenotype predict genotype. *Am J Med Genet A* 2010;152A:1960–1966.
30. Richards S, Aziz N, Bale S, et al.; ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* 2015;17:405–424.
31. Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet* 2014;46:310–315.
32. Gripp KW, Hopkins E, Sol-Church K, et al. Phenotypic analysis of individuals with Costello syndrome due to HRAS p.G13C. *Am J Med Genet A* 2011;155A:706–716.
33. Kobayashi T, Aoki Y, Niihori T, et al. Molecular and clinical analysis of RAF1 in Noonan syndrome and related disorders: dephosphorylation of serine 259 as the essential mechanism for mutant activation. *Hum Mutat* 2010;31:284–294.
34. Prendiville TW, Gauvreau K, Tworog-Dube E, et al. Cardiovascular disease in Noonan syndrome. *Arch Dis Child* 2014;99:629–634.
35. Wilkinson JD, Lowe AM, Salbert BA, et al. Outcomes in children with Noonan syndrome and hypertrophic cardiomyopathy: a study from the Pediatric Cardiomyopathy Registry. *Am Heart J* 2012;164:442–448.
36. van der Burgt I, Kupsky W, Stassou S, et al. Myopathy caused by HRAS germline mutations: implications for disturbed myogenic differentiation in the presence of constitutive HRas activation. *J Med Genet* 2007;44:459–462.
37. Berger AH, Imielinski M, Duke F, et al. Oncogenic RIT1 mutations in lung adenocarcinoma. *Oncogene* 2014;33:4418–4423.
38. Cancer Genome Atlas Research Network. Comprehensive molecular profiling of lung adenocarcinoma. *Nature* 2014;511:543–550.
39. Gómez-Seguí I, Makishima H, Jerez A, et al. Novel recurrent mutations in the RAS-like GTP-binding gene RIT1 in myeloid malignancies. *Leukemia* 2013;27:1943–1946.
40. Kratz CP, Franke L, Peters H, et al. Cancer spectrum and frequency among children with Noonan, Costello, and cardio-facio-cutaneous syndromes. *Br J Cancer* 2015;112:1392–1397.