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Expanding the clinical phenotype of RASopathies in 38 Turkish patients, including the rare *LZTR1*, *RAF1*, *RIT1* variants, and large deletion in *NF1*

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Abstract

RASopathies are a group of disorders caused by pathogenic variants in the genes encoding Ras/mitogen-activated protein kinase pathway and share overlapping clinical and molecular features. This study is aimed to describe the clinical and molecular features of 38 patients with RASopathies. Sanger or targeted next-generation sequencing of related genes and multiplex ligation-dependent-probe amplification analysis for *NF1* were performed. The pathogenic variant detection rate was 94.4%. While *PTPN11* was responsible for 50% of 18 patients with Noonan syndrome (NS), *SOS1*, *LZTR1*, *RIT1*, and *RAF1* were responsible for the remaining 27.8%, 11.1%, 5.5%, and 5.5%, respectively. Three variants in *LZTR1* were novel, of which two were identified in the compound heterozygous state in a patient with intellectual disability and hypertrophic cardiomyopathy, whereas the third variant was found in the heterozygous state in a patient with pulmonary stenosis and normal intelligence. We described pyloric stenosis, knee dislocation, and cleft palate in patients with *SOS1*, *RIT1*, and *RAF1* variants, respectively, that was not previously reported. We detected a *PTPN11* variant in three patients from same family with NS with multiple lentigines. *BRAF* and *MAP2K2* variants were found in eight patients with Cardiofaciocutaneous syndrome. Two variants in *HRAS* were detected in two Costello syndrome patients, one with a mild and the other with a severe phenotype. While large *NF1* deletions were identified in four Neurofibromatosis-NS patients with intellectual disability, intelligence was normal in one patient with missense variant. In conclusion, this study provided three novel variants in *LZTR1* and expanded the clinical phenotype of rare RASopathies.

KEYWORDS
genotype, MLPA, phenotype, RASopathies, targeted gene panel

1 | INTRODUCTION

RASopathies are a group of disorders caused by the pathogenic variants in genes encoding the Ras/mitogen-activated protein kinase (RAS/MAPK) pathway. Noonan syndrome (NS) is the most common form of RASopathies with the incidence of 1/1000 to 1/2500 newborns (Mendez & Opitz, 1985). Cardiofaciocutaneous syndrome (CFCS), Costello syndrome (CS), Legius syndrome, Neurofibromatosis-Noonan syndrome (NF-NS), Noonan-like syndrome with loose anagen hair (NS/LAH), and Noonan syndrome with multiple lentigines

syndrome (NS-MLS) represent overlapping clinical and molecular features with NS and classified as NS-related disorders (Tartaglia et al., 2011; Zenker, 2009). The patients share similar clinical features consisting of typical facial dysmorphism, congenital heart defect, short stature, ectodermal abnormalities, and variable degree of intellectual disability. Cardiac defects are present in 80% of patients, and the most common cardiac defects are pulmonary stenosis, atrial septal defect, and hypertrophic cardiomyopathy (HCM), respectively (Jhang et al., 2016; Tajan et al., 2018).

So far, several genes involved in RAS/MAPK pathway have been found to cause Noonan syndrome and related disorders; these genes are *PTPN11*, *SOS1*, *SOS2*, *RAF1*, *RIT1*, *LZTR1*, *MAP2K1*, *MAP2K2*, *BRAF*, *KRAS*, *NRAS*, *HRAS*, *SHOC2*, *CBL*, *SPRED1 NF1*, *RRAS2*, *RASA2*, *PPP1CB*, *MAPK1*, and *MRAS* (Motta et al., 2020; Tajan et al., 2018). RAS/MAPK pathway is highly conserved and responsible for signal transduction, cell cycle regulation, differentiation, and development (Tajan et al., 2018). *LZTR1*, *RIT1*, *PPP1CB*, and *MAPK1* are the recently discovered genes responsible for NS and related disorders (Abe et al., 2019; Gripp et al., 2016; Motta et al., 2020; Takahara et al., 2019). *LZTR1* affects the degradation of RAS and inhibits RAS/MAPK signaling (Abe et al., 2019). *RIT1* has sequence similarity with other RAS proteins and belongs to the RAS subfamily (Takahara et al., 2019). *PPP1CB* controls the phosphorylation of RAS/MAPK signaling proteins (Gripp et al., 2016). *MAPK1* encodes a serine/threonine kinase and activating germline variants in *MAPK1* have recently been associated with a new RASopathy syndrome (Motta et al., 2020).

In this study, we aim to describe the clinical and molecular findings of NS and related disorders in 38 Turkish patients, including the rare *LZTR1*, *RIT1*, *RAF1* variants, and large *NF1* deletions.

2 | METHODS

2.1 | Patients

Thirty-eight patients from 36 families who were clinically diagnosed with NS and related disorders admitted between the years 1997 and 2020 were enrolled in this study. The patients were clinically divided into four subgroups: NS, CFCS, CS, and NF-NS. The diagnosis of NS was considered in patients with characteristic facial findings (hypertelorism, downslanting palpebral fissures, low set ears, sparse eyebrows, ptosis, pterygium colli) and the other features including congenital heart defects, short stature, pectus deformity, cubitus valgus, cryptorchidism, and intellectual disability (Roberts et al., 2013). CFCS diagnosis was based on the facial features (coarse face, high forehead, bitemporal narrowing, hypertelorism, downslanting palpebral fissure, low set ears, sparse hair, and eyebrow), congenital heart defect, ectodermal findings (hyperkeratosis, hemangiomas), deep palmar creases, short stature, and intellectual disability (Roberts et al., 2006). CS was considered in patients with coarse face, full lips, large mouth, sparse and curly hair, anteverted nostrils, intellectual disability, deep palmar/plantar creases, and the papilloma of the skin (Quezada & Gripp, 2007). NF-NS diagnosis was considered in patients who share typical craniofacial and skeletal features of NS and NF1 such as café au lait macules and freckling (Hüffmeier et al., 2006).

Clinical data of patients were obtained from follow-up charts retrospectively. Thirty-three patients were followed up for 1–17 years. Growth and neuromotor development and other systemic examinations were assessed in every year. All patients were screened for malignancy risk by complete blood count, and abdominal ultrasonography at diagnosis and in the follow-up. The standard deviation score of height was calculated by using a national pediatric calculator (https://www.ceddcozum.com/).

The study was carried out in accordance with the Declaration of Helsinki of the World Medical Association. Informed consent and permission for the publications of photos of children were obtained from all patients/parents. The data presented in this study were retrieved from the routine clinical care facilities of Cerrahpasa Medical Faculty, Istanbul, Turkey.

2.2 | Genetic studies

Genomic DNA was extracted from peripheral blood samples by using standard procedures. In patients who were admitted before the usage of next-generation sequencing (NGS) for routine diagnostics, the most commonly mutated genes including *PTPN11*, *SOS1*, *RAF1*, *BRAF*, *MAP2K1*, *MAP2K2*, and *HRAS* were analyzed by Sanger sequencing. The patients without identified molecular etiology in Sanger sequencing and the patients who were admitted after the year 2014, 18 RASopathy genes including *PTPN11*, *SOS1*, *SOS2*, *RAF1*, *KRAS*, *BRAF*, *HRAS*, *NRAS*, *CBL*, *MAP2K1*, *MAP2K2*, *RIT1*, *RRAS2*, *RASA2*, *LZTR1*, *SPRED1*, *SHOC2*, and *NF1* were analyzed by targeted NGS on Illumina Mi-Seq platform. The variants identified by NGS were also confirmed by Sanger sequencing. *NF1* multiplex ligation-dependent probe amplification (MLPA) (SALSA P081/P082 *NF1* MLPA kit, MRC Holland, Amsterdam, The Netherlands) analysis was performed in patients clinically diagnosed with NF-NS. The variants were classified according to The American College of Medical Genetics and Genomics (ACMG) guidelines and ClinVar database (Richards et al., 2015). Parental studies were conducted on the clinically affected parents and the parents of patients with novel variants. RNA analysis was performed for a novel synonymous variant in exon 4 of *LZTR1*. Briefly, total RNA was extracted from blood using the PAXgene Blood RNA Kit (PreAnalytiX GmbH), and RNA was subsequently reverse transcribed (LunaScript RT SuperMix Kit, New England Biolabs) following the manufacturers' protocols. Reverse transcription polymerase chain reaction (RT-PCR) was performed with primers located in exon 3 (5′-GAAAGACTGCTCCTGGTGCAG-3) and exon 6 (5′-CCACAGCTTGTCACTGTACACC-3′), using the OneTaq Quick-Load 2X Master Mix (New England Biolabs) and a standard PCR protocol. RT-PCR products were separated by agarose gel electrophoresis and their sequence was confirmed after cloning into the pMiniT 2.0 Vector (New England Biolabs) and colony PCR by Sanger sequencing.

3 | RESULTS

3.1 | Molecular results

The pathologic variant detection rate in the whole group was 94.4% (34 of 36 families). Twelve patients from 10 families had a pathogenic variant in *PTPN11*. *BRAF* variants were found in six patients, and *SOS1* and *NF1* variants in five patients. Other rarely identified variants in the group were in *LZTR1*, *RIT1*, *RAF1*, *MAP2K2*, and *HRAS*. Three identified variants in *LZTR1* were novel (Table S1). One patient had de novo missense *LZTR1* c.271A>G variant, which was classified as likely pathogenic according to the ACMG guidelines. The second patient had c.[372C>T];[509G>A] variants in *LZTR1*. The c.509G>A variant was inherited from the unaffected father. It has a very low frequency in the gnomAD database (5/250,908 alleles). This variant was previously reported in schwannomatosis (Smith et al., 2015), and has a likely pathogenic annotation in ClinVar (Accession: VCV000420175.2). It affects the last nucleotide in exon 5 and is predicted to destroy the natural donor site of intron 5, thereby leading to abnormal splicing. The synonymous variant c.372C>T was inherited from the unaffected mother. This variant also has a very low frequency in the gnomAD database (10/251,242 alleles). It has not been reported in LZTR1-related schwannomatosis or Noonan syndrome before, but has been observed in two additional cases of Noonan syndrome in compound heterozygosity with different truncating variants in a large European cohort of LZTR1-related Noonan syndrome (M. Zenker, personal communication). Transcript analysis performed on RNA samples from the mother of our patient as well as RNA samples from the two other patients demonstrated an abundance of an alternative *LZTR1* transcript lacking exon 4, resulting in a premature stop codon p.Ala108Glyfs*11. The splicing effect may result from alteration of an exonic splice enhancer and appears to be incomplete, thus suggesting that c.372C>T is a hypomorphic loss-of-function allele (M. Zenker, personal communication). Together these findings are compatible with the inheritance pattern of autosomal recessive LZTR1-related Noonan syndrome.

NF1 variants were identified in five patients with NF-NS; among these variants, a missense variant was found in a patient and large deletions were detected by MLPA analysis in four patients. We did not identify any pathogenic variants in the screened genes in two unrelated patients with CFCS (Table S1).

3.2 | Clinical features

The mean age of the patients at admission was 5.6 years (0.1–34 years). Twenty of the patients were males, and 18 were females. Thirty-three patients were followed up 1–17 years (mean 6.1 years). The clinical features in the whole group and different gene groups are demonstrated in Table 1. In the whole group, cardiac defects were present in 81.5%, intellectual disability in 68.4%, ectodermal findings in 63.1%, and short stature in 55.3%.

Cardiac and ectodermal findings were present in all patients with *SOS1*, *LZTR1*, *RAF1*, *RIT1*, *BRAF*, *MAP2K2*, and *HRAS* variants. Short stature was most common in patients with *BRAF* and *PTPN11* variants. Intellectual disability was present in all patients with *BRAF* and *MAP2K2*, and 80% of patients with NF-NS. Scoliosis was present in two patients with NS, one patient with NS-MLS, and one patient with CFCS. The renal anomaly was present in four patients with NS, and two patients with CFCS. Central nervous system malformation was found in two patients with NS, two patients with CFCS, one patient with NS-MLS, and one patient with CS. Epilepsy was present in three patients with CFCS (Table 1).

In the follow-up, endocrinological abnormalities were detected in seven patients with NS (delayed puberty in two, hypothyroidism in two, growth hormone (GH) deficiency in two, hypothyroidism and GH deficiency in one patient). Two patients with CFCS also had GH deficiency. None of the patients had a history of bleeding problems or lymphatic abnormality and malignancy (Table 1).

3.2.1 | Noonan syndrome and Noonan syndrome with multiple lentigines

Eighteen patients were clinically diagnosed with NS (Table 1). Molecular diagnosis was confirmed in all patients with NS; 50% of them had pathogenic variants in *PTPN11* and 27.8% in *SOS1*. The rare gene variants in *RIT1* and *RAF1* were detected in one patient each, and *LZTR1* in two patients. NS patients with *PTPN11* (Figure 1a,b), *SOS1* (Figure 1c–g), *RAF1* (Figure 1h), *RIT1* (Figure 1i), and *LZTR1* (Figure 1j, k) variants showing the typical facial findings, pterygium colli, short neck, pectus excavatum, cubitus valgus, hyperplastic nipple, and deep palmar and plantar creases. Rare clinical findings were identified in patients with NS, including pyloric stenosis and Chiari malformation type 1 in the patients with *SOS1* variants, cleft palate in the patient with *RAF1* variant, and dislocation of the knee in the patient with *RIT1* variant (Table 1). Both patients with *LZTR1* variants had pulmonary stenosis, pterygium colli, cubitus valgus, and normal stature. The patient with de novo heterozygous *LZTR1* variant was mildly affected, whereas the patient with compound heterozygous variants in *LZTR1* had severe HCM and moderate intellectual disability. Two children and their father with the *PTPN11* variant were diagnosed with NS-MLS (Figure 1l). One of the siblings had severe scoliosis, and Chiari malformation type 1 (Table 1).

3.2.2 | Cardiofaciocutaneous syndrome

BRAF variants were identified in 60% and *MAP2K2* variants in 20% of the patients (Table S1). The patients with CFCS had typical facial findings, cubitus valgus, and deep palmar creases (Figure 2a–d). Two patients with *BRAF* variants had facial hemangioma and one patient with *MAP2K2* variant had congenital cataract and hip dislocation. We did not identify any pathogenic variants in two patients with CFCS. One of these patients had typical facial findings, pterygium colli,

TABLE 1 Distribution of clinical features and affected genes in all patients with Noonan syndrome and related disorders

Patients	Clinical diagnosis	Affected gene	Age at diagnosis (years)	Age at last examination	Sex	Height SDS at last examination	Coarse face at birth	Downslanting palpebral fissure	Hypertelorism	Epicanthal folds	Ptosis	Low set ears	Heart defect	Pectus deformity	Pterigium colli	Cubitus valgus	Sparse hair	Sparse eyebrow	Deep palmer creases	Hyperplastic nipples	Café au laits	Intellectual disability	Cryptorchidism	Other
1	NS	*PTPN11*	13.7	21.3	M	−2.5	−	+	+	+	−	+	ASD	+	+	+	−	−	−	−	−	−	+	Kyphosis, X bain deformity, delayed puberty
2	NS	*PTPN11*	14.9	14.9	F	−4.1	−	+	+	−	−	+	PS	−	+	+	−	−	−	−	−	Mild		Scoliosis, renal anomaly, delayed puberty
3	NS	*PTPN11*	0.4	8.7	M	−1.6	−	+	+	+	−	+	ASD, PS	+	+	+	−	−	−	−	−	−	+	Hypothyroidism
4	NS	*PTPN11*	0.3	8.8	M	−2.8	−	+	+	+	+	+	ASD, VSD, PS	+	+	+	−	−	−	−	−	−	+	Renal anomaly, GH deficiency, hypothyroidism
5	NS	*PTPN11*	6.6	8	F	−2.7	−	+	+	+	−	+	PS	+	+	+	−	−	−	−	+	Mild		Scoliosis
6	NS	*PTPN11*	12.4	12.4	M	−2.85	−	+	+	−	−	+	ASD, PS	+	+	+	−	+	−	−	−	Mild	+	
7	NS	*PTPN11*	9	15	M	−2.56	−	−	−	+	−	+	PS, PI	−	−	−	−	−	−	−	−	Mild	+	GH deficiency
8	NS	*PTPN11*	5.4	6.4	M	−1.42	−	+	−	−	+	+	PS	+	−	+	−	−	−	−	−	−	−	Hypothyroidism
9	NS	*PTPN11*	11.3	12.8	F	−3.04	−	+	−	+	+	+	AI	+	+	+	−	−	−	−	+	−		
10	NS	*SOS1*	0.6	14	M	−0.81	+	+	+	+	+	+	PS	+	−	+	−	+	+	+	−	−	+	Inguinal hernia, choroid plexus cyst, renal anomaly
11	NS	*SOS1*	0.1	4.3	F	−1.94	+	+	+	+	+	+	AVSD, VSD, PDA	+	−	−	+	+	+	+	−	Mild		Pyloric stenosis
12	NS	*SOS1*	2.2	10	M	−0.24	+	+	−	−	−	+	PS,AS	+	−	+	+	+	+	+	−	−	+	Syringomyelia, Chiari malformation type 1
13	NS	*SOS1*	1.0	10.5	M	−3.0	+	+	+	+	+	+	PS	+	−	+	+	+	−	+	−	−	+	GH deficiency, speech delay
14	NS	*SOS1*	26	26.6	F	−2.2		+	+	+	−	+	PS,AVSD, PDA	+	+	+	−	+	−	−	−	Mild		Frequent pneumonia, Guillian Barre syndrome history
15	NS	*RAF1*	0.1	2.5	F	−3.9	+	+	+	+	−	+	PS, VSD	−	−	−	+	+	+	−	−	Moderate		Renal anomaly, cleft palate, exitus due to heart failure
16	NS	*RIT1*	1.4	8.8	F	−0.7	−	+	+	+	−	+	PS, HCMP, PFO	+	−	+	+	+	+	−	−	−		Dislocation of the knee
17	NS	*LZTR1*	2.8	5.8	F	−0.5	−	−	+	+	−	+	PS	−	+	+	−	+	−	−	−	−		Clinodactyly
18	NS	*LZTR1*	0.3	6.2	F	−1.7	+	+	−	+	−	+	PS, HCMP (severe)	−	+	+	−	−	−	−	−	Moderate		Broad chest, coarse hair
19	NS-MLS	*PTPN11*	8.0	11.5	M	−2.8	−	+	+	+	+	+	−	+	−	+	−	−	−	−	+	Moderate	−	Lentigines, scoliosis, vertebral fusion, syringomyelia, Chiari malformation type 1
20	NS-MLS	*PTPN11*	6.5	10	M	−0.5	−	+	+	+	+	+	−	+	−	−	−	−	−	−	+	Mild	−	Lentigines
21	NS-MLS	*PTPN11*	34	37	M	−2.1	−	+	+	+	+	+	−	−	−	−	−	−	−	−	+	Mild	−	Lentigines
22	CFCS	*BRAF*	0.5	13	M	−4.6	+	+	+	−	−	+	PS, HCMP, MI, TI	−	−	−	+	+	+	+	−	Severe	+	Hypoplastic corpus callosum, epilepsy
23	CFCS	*BRAF*	0.4	13.8	F	−2.3	+	+	+	+	+	+	PFO	−	−	+	+	+	+	+	−	Mild		GH deficiency

TABLE 1 (Continued)

Patients	Clinical diagnosis	Affected gene	Age at diagnosis (years)	Age at last examination	Sex	Height SDS at last examination	Coarse face at birth	Downslanting palpebral fissure	Hypertelorism	Epicanthal folds	Ptosis	Low set ears	Heart defect	Pectus deformity	Pterigium colli	Cubitus valgus	Sparse hair	Sparse eyebrow	Deep palmer creases	Hyperplastic nipples	Café au laits	Intellectual disability	Cryptorchidism	Other
24	CFCS	*BRAF*	0.3	5	F	−2.5	+	+	+	+	−	+	PS, HCMP, PFO	+	−	+	+	+	+	+	−	Mild		Nystagmus, hemangioma, GH deficiency
25	CFCS	*BRAF*	1.1	18	M	−3.8	+	+	+	+	−	+	HCMP	+	−	−	+	+	+	+	−	Severe	+	Epilepsy
26	CFCS	*BRAF*	3.7	7.4	M	−5.4	+	−	−	−	+	+	HCMP	+	−	−	+	+	−	−	−	Mild	−	
27	CFCS	*BRAF*	0.4	5.8	F	−1.8	+	+	+	+	−	+	PS, HCMP, MI	+	−	+	+	+	+	+	+	Severe		Hemangioma, scoliosis, epilepsy
28	CFCS	*MAP2K2*	2.7	3.8	F	−0.9	−	+	−	−	−	+	HCMP	−	−	−	+	+	−	−	−	Moderate		Congenital cataract, hip dislocation
29	CFCS	*MAP2K2*	7.6	13	M	−1.3	+	+	+	−	−	+	AS, Bicuspid aorta	+	+	+	+	+	−	−	−	Mild	−	Renal anomaly, multiple nevus
30	CFCS	−	4.3	9.5	F	−0.3	−	−	+	−	−	+	HCMP	+	+	+	+	+	−	−	+	Severe		Epilepsy, arachnoid cyst
31	CFCS	−	1.5	8.2	F	−2.4	+	−	−	−	−	+	PS, ASD, Ebstein anomaly	−	−	−	+	+	+	−	−	Severe		Renal anomaly
32	CS	*HRAS*	11.7	20	F	−1.1	+	−	+	+	+	+	HCMP, PS	−	−	−	+	+	+	−	+	−		Papillomatosis, gallbladder polyp
33	CS	*HRAS*	0.1	4.2	E	−4.4	+	−	+	+	+	+	HCMP	−	−	−	+	+	+	−	−	Mild	+	Papillomatosis, laringomalasia, cortical dysplasia, Chiari malformation type 1
34	NF-NS	*NF-1*	1.4	8	F	−2.2	−	−	−	−	+	−	−	+	−	+	−	−	−	−	+	−		Freckling, neurofibroma, hamartoma, no Lisch nodule
35	NF-NS	*NF-1*	6.5	10	F	−0.4	−	+	+	+	+	+	ASD, PDA	+	+	+	−	−	−	−	+	Moderate		Freckling, no Lisch nodule, coarse face
36	NF-NS	*NF-1*	3.2	3.4	M	1.7	−	−	−	−	−	+	−	+	−	+	+	+	−	−	+	Moderate	−	Dolicocephaly, macrocephaly, no Lisch nodule, coarse face
37	NF-NS	*NF-1*	4.5	12	M	+2.0	−	−	−	−	−	−	−	+	−	+	+	+	−	−	+	Mild	−	Freckling, hamartoma, no Lisch nodule
38	NF-NS	*NF-1*	7.2	7.2	M	−3.0	−	−	+	−	−	+	−	+	+	−	−	−	−	−	+	Moderate	−	Freckling, hamartoma, no Lisch nodule

Abbreviations: (−), not found; AI, aortic insufficiency; ASD, atrial septal defect; AVSD, atrioventricular septal defect; CFCS, Cardiofaciocutaneous syndrome; CS, Costello syndrome; HCMP, hypertrophic cardiomyopathy; GH, growth hormone; MI, mitral insufficiency; NF-NS, Neurofibromatosis-Noonan syndrome; NS, Noonan syndrome; NS-MLS, Noonan syndrome with multiple lentigines; PDA, patent ductus arteriosus; PFO, patent foramen ovale; PI, pulmonary insufficiency; PS, pulmonary stenosis; SDS, standard deviation score; TI, tricuspid insufficiency; VSD, ventricular septal defect.

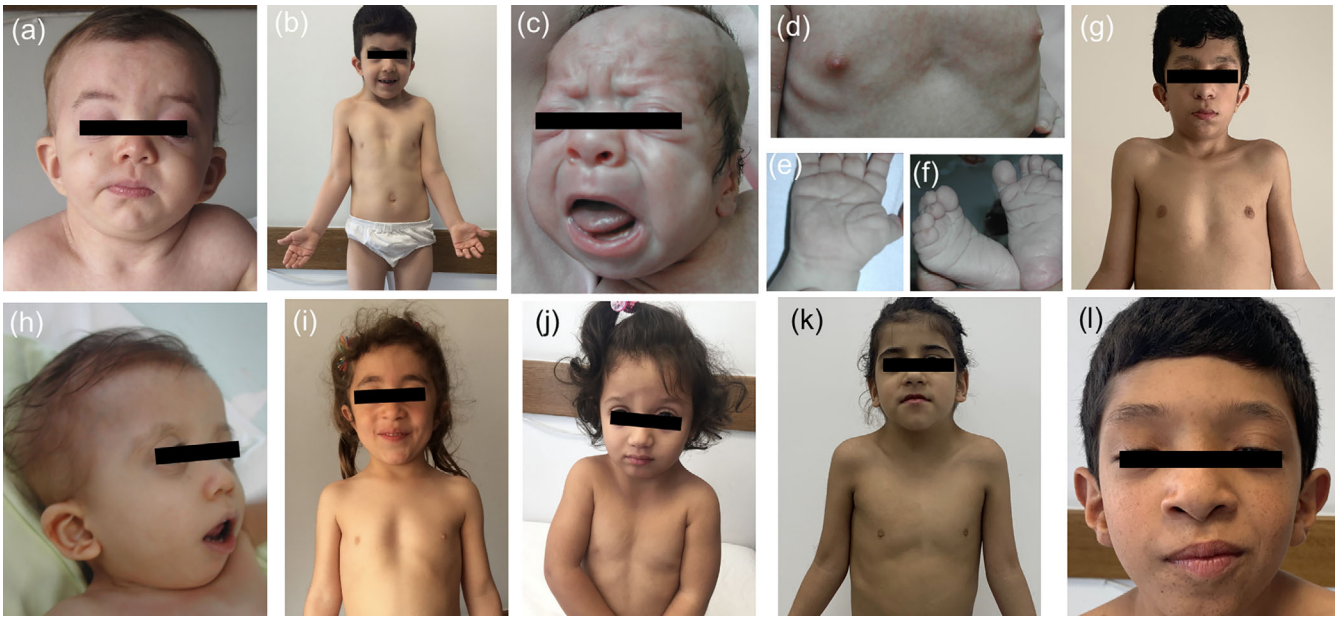

FIGURE 1 Photographs of patients with Noonan syndrome and Noonan syndrome with multiple lentigines syndrome (NS-MLS). Patient 4 with *PTPN11* variant at the age of 11 months. Note that downslanting palpebral fissure, epicanthal folds, ptosis, and low set ears (a). Patient 3 with *PTPN11* variant at the age of 7 years. Note that low set ears, pterygium colli, cubitus valgus, and pectus excavatum (b). Patient 11 with *SOS1* variant in the newborn period. Note that coarse face, sparse hair and eyebrow, hypertelorism, low set ears (c), hyperplastic nipple (d), and deep palmar and plantar creases (e, f). Patient 10 with *SOS1* variant at the age of 14 years. Note sparse eyebrow, low set ears, cubitus valgus, and short neck (g). Patient 15 with *RAF1* variant at the age of 4 months. Note that sparse hair and eyebrow, low set ears, anteriorly rotated ear lobe, and micrognathia (h). Patient 16 with *RIT1* variant at the age of 6 years. Note that low set ears, curly hair, short neck, and pectus excavatum (i). Patient 17 with de novo *LZTR1* variant at the age of 4 years. Note that sparse curly hair and sparse eyebrow (j). Patient 18 with biallelic *LZTR1* variants at the age of 7 years. Note that low set ears, short neck, pterygium colli, broad chest, and cubitus valgus (k). Patient 19 with *PTPN11* variant related with NS-MLS at the age of 10 years. Note that multiple lentigines of the face, downslanting palpebral fissure, ptosis, hypertelorism, and low set ears (l)

HCM, epilepsy, and severe intellectual disability (Figure 2e) and the other had short stature, sparse, curly hair and eyebrow, upturned nostrils, low set ears, congenital cardiac anomaly (pulmonary stenosis, Ebstein anomaly, atrial septal defect), renal anomaly, and severe intellectual disability (Figure 2f; Table 1).

3.2.3 | Costello syndrome

HRAS variants were found in two patients with CS. Both patients had sparse hair and eyebrow, low set ears, papilloma of skin, deep palmar creases, and HCM (Figure 2g,h). However, one of them had normal intelligence and stature whereas the other had intellectual disability, short stature, Chiari malformation type 1, and cortical dysplasia (Table 1).

3.2.4 | Neurofibromatosis-Noonan syndrome

Three whole gene deletions, one multiexon deletion, and one missense variant in *NF1* were identified in five families (Table S1). The photographs of the patients with NF-NS are revealed coarse face, ptosis, sparse eyebrows, hypertelorism, low set ears, pectus excavatum, cubitus valgus, short neck, and pterygium colli (Figure 3a–c). All patients had multiple cafe au lait spots (Figure 3d–f) and none of them had Lisch nodules, pulmonary stenosis, nor HCM. While there was no intellectual disability in the patient with missense variant, four patients with large deletions had mild to moderate intellectual disability (Table 1).

4 | DISCUSSION

Pathogenic variant detection rate (71%–94%) in RASopathies increases with the incorporation of newly discovered genes into the screened genes in recent years (Chinton et al., 2019, 2020). In this study, a high pathogenic variant detection rate (94.4%) was due to the usage of MLPA in combination with NGS. Targeted NGS analysis did not include the recently discovered genes *PPP1CB*, *MAPK1*, and *MRAS* in this study. This may be one reason for two patients without identified molecular etiology. Also, noncoding variants in the deep intronic or regulatory regions might be responsible for the phenotype. Most of the variants in RASopathies are reported to be missense, gain of function variants that lead to the activation of the RAS/MAPK pathway (Tidyman & Rauen, 2016). In this study, we identified 27 different intragenic variants, 24 of which were missense (Table S1). Three whole gene and one large multiexon deletion were also described in

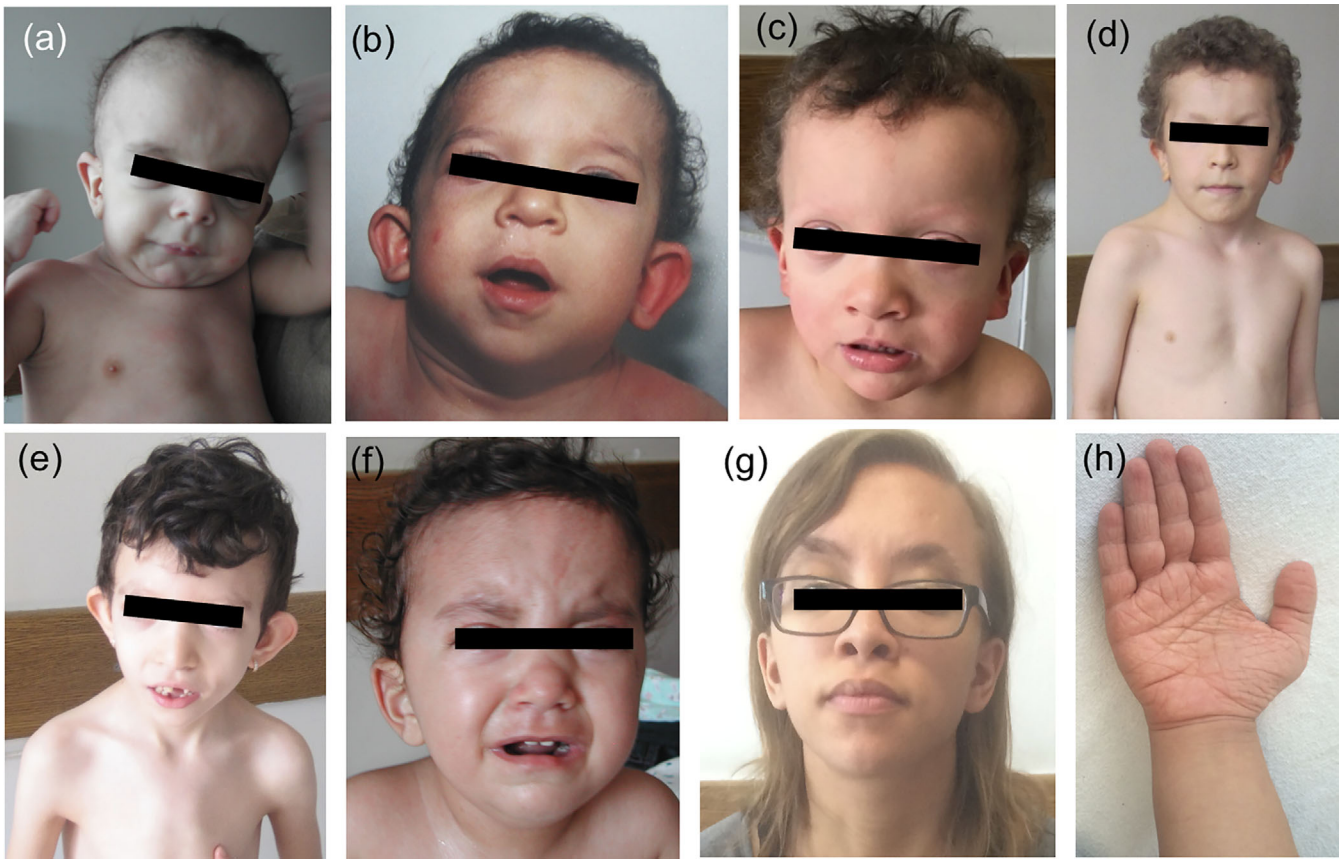

FIGURE 2 Photographs of patients with Cardiofaciocutaneous syndrome and Costello syndrome. Patients 27 and 22 with *BRAF* variant at the age of 8 months and 12 months, respectively (a, b), Patients 28 and 29 with *MAP2K2* variant at the age of 2.5 years and 8 years, respectively (c, d). Note that sparse hair and eyebrow, bitemporal narrowing, hypertelorism, broad nasal bridge, and low set ears. Patients 30 and 31 without any pathogenic variants at the age of 4.5 years and 1.5 years, respectively (e, f). Note that curly and sparse hair, high forehead, hypertelorism, low set ears, micrognathia, and pectus carinatum (e), curly and sparse hair, sparse eyebrow, and low set ears (f). Patient 32 with Costello syndrome at the age of 18 years. Note that sparse hair and eyebrow, anteverted nostrils, full lip, low set ears, papilloma of the lip (g), and deep palmar creases (h)

NF1 by MLPA analysis. Unlike other RASopathy genes, *NF1* gene regulates RAS/MAPK pathway negatively, and loss of function variants are related to the clinical phenotype (Tidyman & Rauen, 2016).

PTPN11 and *SOS1* variants are reported in 50% and 10%–15% of NS patients, respectively (Tartaglia et al., 2007, 2011). *PTPN11* and *SOS1* were responsible for 77.8% of our patients with NS. It was reported that *PTPN11* variants are related to more frequent short stature, intellectual disability, and pectus deformity, whereas *SOS1* variants are related to more frequent ectodermal abnormalities (Chinton et al., 2019; Roberts et al., 2013; Tartaglia et al., 2002; Zenker et al., 2007). We found that the prevalence of pulmonary stenosis, pectus deformity, cubitus valgus, mild intellectual disability, and cryptorchidism were similar in both groups (Table 1). While *PTPN11* variants were associated with a higher frequency of short stature and pterygium colli, *SOS1* variants were associated with a higher prevalence of coarse face at birth, ectodermal findings, and normal stature (Table 1). Pyloric stenosis and Arnold Chiari malformation were reported rarely in NS and were described in two patients with *SOS1* variant in our cohort (Ejarque et al., 2015).

RIT1, *RAF1*, and *LZTR1* were reported in 8%–10% of patients with NS (El Bouchikhi et al., 2016; Yamamoto et al., 2015). *RIT1*-related NS was associated with less frequent short stature and intellectual disability (Kouz et al., 2016). Compatible with this data, the patient presented here with the *RIT1* variant had normal intelligence and normal stature. She also had dislocation of the knee, which has not been described before. *RAF1* gene variants are strongly related to HCM (Razzaque et al., 2007). However, our patient with *RAF1* variant had pulmonary stenosis and ventricular septal defect. In addition, she had cleft palate, which has not been reported previously in *RAF1* gene variants.

To date, a few patients have been reported with *LZTR1* variants with autosomal recessive or autosomal dominant inheritance patterns (Gripp et al., 2020; Jacquinet et al., 2020). We identified three novel variants in *LZTR1* gene in two patients (Table S1). While the patient with de novo heterozygous variant in *LZTR1* gene had normal intelligence and pulmonary stenosis, other patient with compound heterozygous variants had moderate intellectual disability and severe HCM. Autosomal recessive *LZTR1* related Noonan syndrome has variable expressivity including

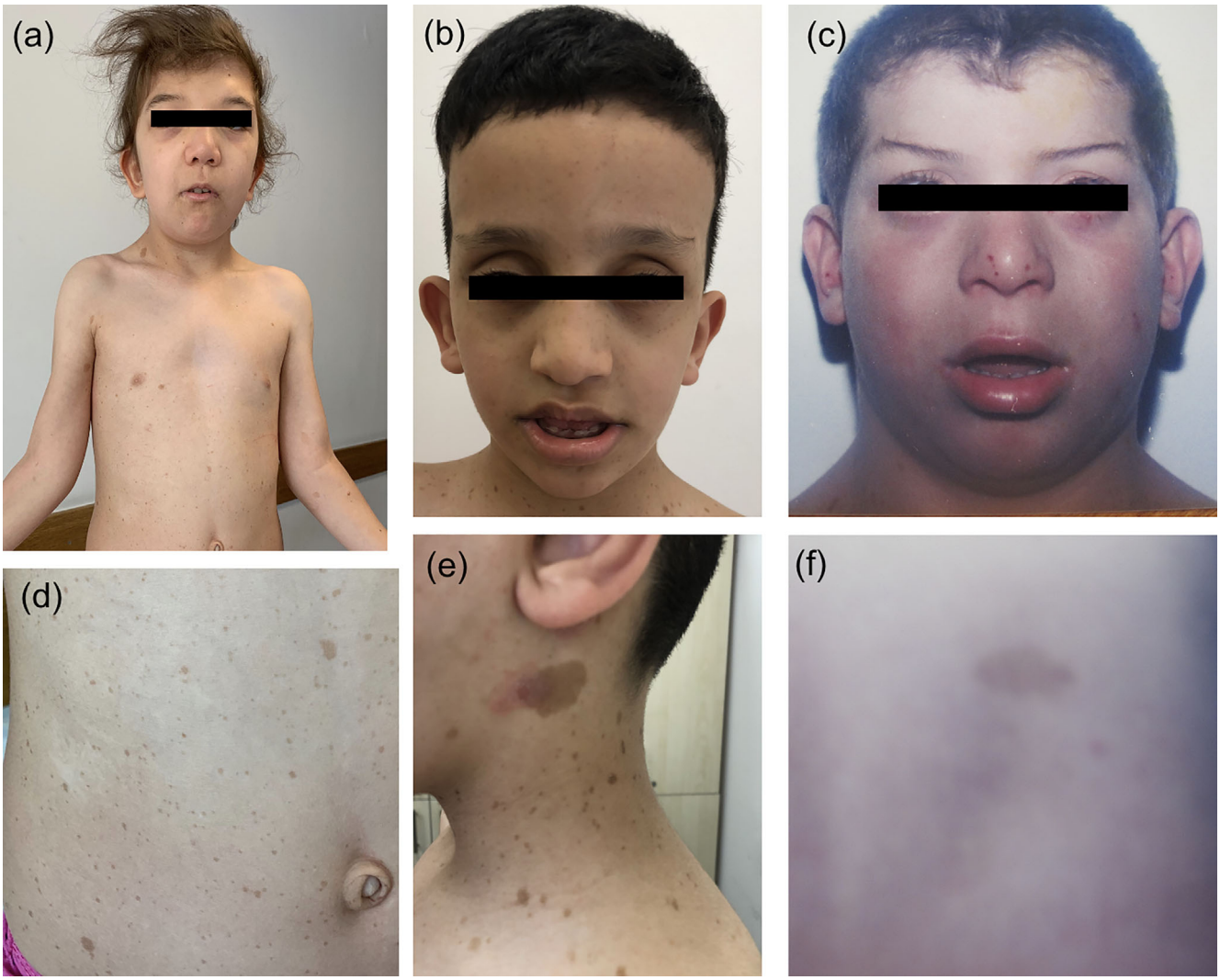

FIGURE 3 Photographs of patients with Neurofibromatosis-Noonan syndrome. Patient 35 with whole *NF1* deletion at the age of 10 years. Note coarse face, downslanting palpebral fissure, ptosis, low set ears, pectus excavatum, cubitus valgus, pterygium colli, café au lait spots, and neurofibroma (a, d). Patient 38 with multi-exon *NF1* deletion at 4.5 years of age (b, e). Note sparse eyebrow, low set ears, and café au lait spots. Patient 37 with whole *NF1* deletion at the age of 10 years. Note that hypertelorism, low set ears, pterygium colli, short neck, and café au lait spots (c, f)

mildly affected patients to severe forms with prenatal lethal types due to congenital cardiac defects (Johnston et al., 2018).

We also presented two affected brothers and their father with NS-MLS, who had *PTPN11* variant. They had typical facial features of NS, café au lait spots, lentigines, and intellectual disability. Cardiac defects were reported in 71% of NS-MLS patients; however, none of our patients with NS-MLS had cardiac defects (Sarkozy et al., 2004).

BRAF variants were identified in 60% and *MAP2K2* in 20% of patients with CFCS similar to the reported patients (Allanson et al., 2011). The coarse face at birth, sparse hair and eyebrow, deep palmar creases, and hyperplastic nipple in CFCS resemble *SOS1*-related NS (Zenker et al., 2007) (Table 1). Short stature was reported in 63% of patients with both *BRAF* and *MAP2K2* variants. PS was reported in 50% and 37% whereas HCM was reported in 38% and 24% of patients with *BRAF* and *MAP2K2* variants, respectively (Allanson et al., 2011). However, in our study, HCM was the most common cardiac defect and reported in 70% of the patients. Interestingly, unlike patients with *BRAF* variants, two patients with *MAP2K2* variants did not have hyperplastic nipple, deep palmar creases, or short stature (Table 1). The rare findings of cataract and hip dysplasia in the patient with *MAP2K2* variant were also reported previously (Reinker et al., 2011; Rodriguez-Viciana et al., 2006).

In CS, the phenotype–genotype correlation of specific variants was well established. Similar to the literature, we found p.Gly12Val variant in *HRAS* in the patient with severe phenotype and p.Gly13Cys variant in the patient with the mild phenotype (Gripp et al., 2019). Malignancy risk is known as 13% in CS (Aoki & Matsubara, 2013). However, our patient with mild phenotype was followed up until 20 years of age and did not develop any malignancy.

NF1 and NS exhibit overlapping features and some NS patients do not fulfill the criteria for NS or NF1. In recently published studies, it has been observed that 10%–15% of patients with NS and related disorders have pathogenic variants in *NF1* (Bertola et al., 2020; Castellanos et al., 2020; Witkowski et al., 2020). Compatible with the literature, we found *NF1* variants in 13.8% of our families. Large deletions have been reported less than 10% of all *NF1* variants and associated with more severe phenotypes consisting of intellectual disability, cardiac defects, dysmorphic feature, and overgrowth (De Luca et al., 2005; Kehrer-Sawatzki et al., 2017; Pinna et al., 2019). Unlike reported in the literature, we found that large gene deletions were responsible for 80% of the families with NF-NS. While there was no intellectual disability in the patient with the missense variant, four patients with large deletions had a mild–moderate intellectual disability. Our results strongly suggest that *NF1* sequencing and copy number variation analysis should be considered in patients with overlapping features of NF1 and NS.

Hypothyroidism was reported slightly higher than the normal population, and GH deficiency was reported in 37.7% of the patients with NS and related disorders (Quaio et al., 2012; Tamburrino et al., 2015). We detected both hypothyroidism and GH deficiency in 16.6% of patients with NS. GH deficiency was also identified in 33.3% of patients with CFCS. Scoliosis was reported in 13% of patients with NS and NS-MLS, 33% of patients with CFCS, and 17% of patients with CS (Stevenson & Yang, 2011). In this study, scoliosis was present in 11.1% of patients with NS, 33.3% of patients with NS-MLS, and 16.6% of patients with CFCS. Factor XI deficiency was reported in 9% and abnormal platelet aggregation was reported in 15% of patients with NS and related disorder, but none of the patients had bleeding problems in our cohort (Bertola et al., 2020). Malignancy risk is reported to be increased eightfold; however, none of our patients developed malignancy (Bertola et al., 2020).

5 | CONCLUSION

This study pointed out that the most commonly mutated genes in NS were *PTPN11* and *SOS1* that were responsible for 77.8% of the patients with NS. *RIT1* and *RAF1*, which are rare gene variants, were detected in one patient each, and *LZTR1* in two patients with NS. Novel findings in NS, including Chiari malformation type 1 and pyloric stenosis in patients with *SOS1*, knee dislocation in *RIT1*, and cleft palate in *RAF1* variants, were described in our study for the first time. Three variants in *LZTR1* were novel and exhibited autosomal recessive inheritance pattern in a patient with moderate intellectual disability, severe HCM, and dominant inheritance pattern in a patient with pulmonary stenosis and normal intelligence suggesting that biallelic variants in *LZTR1* cause severe phenotype. *BRAF* and *MEK2* genes were responsible for 60% and 20% of patients with CFCS, respectively. Our CS patient with mild phenotype was followed up until the age of 20, but malignancy, which was frequently seen in CS, did not develop. Four large deletions and one missense variant in *NF1* were identified in five patients with Neurofibromatosis-NS. While the patient with missense variant had normal intelligence, mild to moderate intellectual disability was observed in the patients with large deletions. This study expanded the clinical phenotype of rare RASopathies and reports three novel variants in *LZTR1* gene.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS

Dilek Uludağ Alkaya: conceptualization, methodology, formal analysis, writing—original draft. Christina Lissewski: software, validation, formal analysis, resources, data curation. Gözde Yeşil: conceptualization, methodology, investigation, resources. Martin Zenker: molecular analysis, resources, data curation, Beyhan Tüysüz: conceptualization, methodology, formal analysis, investigation, resources, writing—review and editing, supervision.

DATA AVAILABILITY STATEMENT

Authors confirm that all relevant data are included in the article and/or its Supporting information files.

ETHICS COMMITTEE APPROVAL

The data presented in this study were retrieved from the routine clinical care facilities of Cerrahpasa School of Medicine, Istanbul, Turkey.

INFORMED CONSENT

Written informed consent was obtained from the patients or the parents.

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SUPPORTING INFORMATION

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