Application of Next-Generation Sequencing in Noonan Spectrum Disorders

Hui Mei, Hongzheng Dai, Jinglan Zhang, Lee-Jun Wong, and Pilar L. Magoulas

Abstract Noonan spectrum disorders (NSDs) are a group of disorders with aberrant signal transduction in the RAS/mitogen-activated protein kinase (MAPK) pathway, and therefore they are also known as RASopathies. All NSDs known to date are caused by germline dominant mutations in genes encoding proteins participating in the RAS-MAPK pathway. The molecular characterization thus far explains approximately 80% of individuals affected with a NSD. Pathogenic variants in the *PTPN11* gene cause about 50% of all NSD cases. The other 17 genes account for an additional 20–30% NDS cases. High genetic heterogeneity in NSDs and their considerable overlap in clinical presentations had made the diagnosis of these disorders expensive and time consuming in a gene by gene approach. In this chapter, we provide a brief overview of clinical features of Noonan syndrome and closely related conditions, the molecular mechanisms underlying pathogenesis, and the advantages and challenges in implementing next generation sequencing (NGS) in clinical laboratories for the molecular diagnosis of NSDs.

Keywords RAS/MAPK • Signal transduction pathway • RASopathies • Noonan syndrome • Cardiofaciocutaneous syndrome • Costello syndrome • Noonan syndrome with multiple lentigines (LEOPARD syndrome) • NGS • Prenatal testing • Whole genome amplification

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1 Introduction

1.1 RAS/MAPK Pathway Biology

The RAS/mitogen-activated protein kinase (MAPK) pathway is a key signal transduction pathway that plays an essential role in cell determination, proliferation, differentiation, and survival. RAS proteins are members of the small guanosine-binding protein family. The RAS subfamily includes HRAS, NRAS, and KRAS. They act as signal switch molecules that transmit extracellular signals to activate downstream effectors (Fig. 1). Activation of the RAS/MAPK pathway is initiated by growth factors binding to the transmembrane tyrosine kinase receptors, which undergo dimerization, autophosphorylation, and subsequent activation. The activated receptors interact with a set of specific adaptor proteins, including growth factor receptor-bound protein (GRB2), CBL, and SHP2. GRB2 recruits son of sevenless (SOS), a guanine nucleotide exchange factor (GEF). SOS1 is the major GEF that activates the RAS proteins by facilitating a conformational switch that is dependent on the exchange of GDP for GTP. Activated RAS propagates the signaling cascade by activating the effector MAPKKK (RAF). There are three RAF serine/threonine kinases (ARAF, BRAF, and RAF1). Phosphorylated RAF then activates the MAPKKs -MAP2K1 and/or MAP2K2, which in turn activates the MAPK proteins, ERK1 and ERK2. The substrates of ERK1 and ERK2 include nuclear components, transcription factors, membrane proteins, and protein kinases that in turn control vital cellular functions.

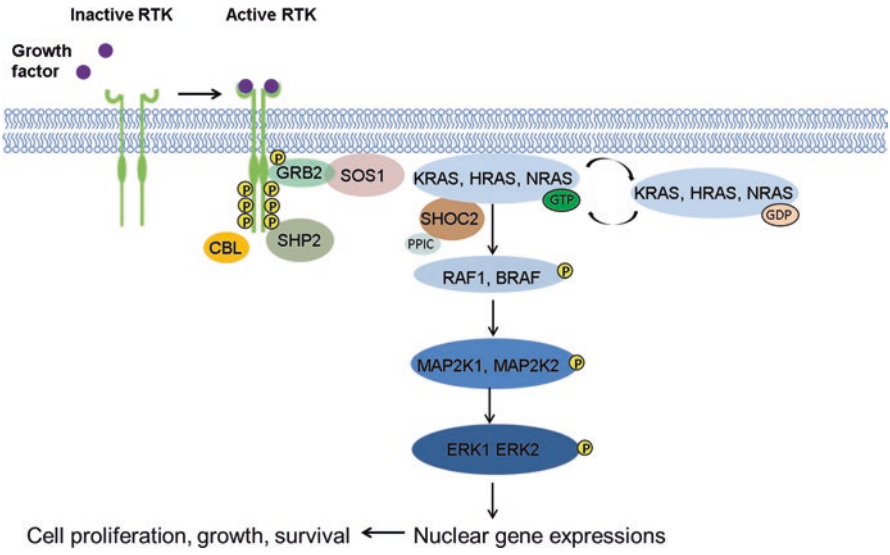

Fig. 1 The RAS/ MAPK signal transduction pathway

1.2 The RASopathies

The RASopathies are a group of genetically heterogeneous developmental disorders caused by defects in genes involved in the RAS/MAPK signaling pathway [43]. These disorders include Noonan syndrome (NS), Costello syndrome, Noonan syndrome with multiple lentigines (NSML; formerly called LEOPARD syndrome), cardio-facio-cutaneous syndrome (CFC), capillary malformation-arteriovenous malformation syndrome, Legius syndrome, and neurofibromatosis type 1 (NF1). While these individual disorders are rare, as a group, the prevalence of these disorders is between 1 in 1000 and 1 in 2500 live births. Although each RASopathy exhibits distinctive phenotypic features, the common dysregulation of RAS/MAPK signaling likely underlies the overlapping clinical manifestations, including dysmorphic craniofacial features, cardiac malformations, skin abnormalities, musculoskeletal and ocular abnormalities, varying degrees of intellectual disability, and increased cancer risk (Fig. 2; Table 1).

NF1 was the first disease gene identified in the RAS/MAPK pathway [8, 65, 67]. Recently, a number of additional genes causative for the RASopathies have been identified, including *PTPN11, SOS1, RAF1, KRAS, NRAS, HRAS, SHOC2, RIT1, BRAF, MAP2K1,MAP2K2, CBL, SOS2, LZTR1, RRAS, RASA2, and A2ML1* (Table 2a) [3–5, 9, 10, 12, 13, 15, 19, 30, 35, 36, 41, 44, 46–48, 53, 58, 61, 66, 68]. Table 2b summarizes several RASopathy genes, their respective phenotypes, and percentage of that gene accounts for all NSD cases. Discovery of specific RASopathy genes made it possible to develop genetic tests that facilitate the postnatal diagnosis of symptomatic individuals with characteristic features of a RASopathy as well as prenatal diagnosis of fetuses with normal karyotypes that present with specific ultrasound findings [4, 27, 37].

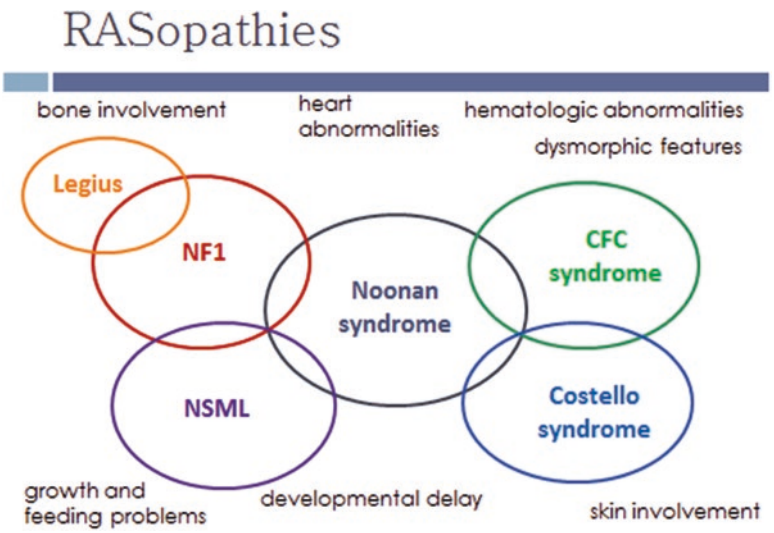

Fig. 2 Overlapping clinical features of RASopathies

Table 1 Summary of RASopathies

Syndromes	RAS/MA PK gene	Characteristic clinical features
Neurofibromatosis I	*NF1*	Multiple café-au-lait spots; axillary and inguinal freckling; multiple
Noonan syndrome	*PTPN11, SOS1, RAF1, KRAS, NRAS, SHOC2, RIT1, CBL*	Short stature; broad or webbed neck; characterisitic facies, including a borad forehead, hypertelorism, down-slanting palpebral fissures, ptosis, and low-set posteriorly rotated ears; congenital heart defect; coagulation defects; ocular abnormalities; normal intelligence to mild intellectual disability; predispostion to cancer
Noonan syndrome with multiple lentigines	*PTPN11, RAF1*	Similar to Noonan syndrome; but with multiple skin lentigines present as dispersed flat, black-brown macules
Costello syndrome	*HRAS*	Failure to thrive; short stature; Noonan facies but more coarse, including
Cardiofaciocutaneous syndrome	*BRAF1,*	Noonan facies; congenital heart defects; cutaneous abnormalities, including xerosis, hyperkeratosis, ichthyosis, keratosis pilaris, eczema; developmental delay or intellectual disability;
	MAP2K1,	
	MAP2k2,KR AS	
Leguis syndrome	*SPRED1*	Multiple café-au-lait spots; intertriginous freckling; lipoma; macrocephaly;
Capillary malformation-arteriovenous malformation	*RASA1*	Multiple, small capillary malformations; arteriovenous malformations and/or arteriovenous fistulas

2 Noonan Spectrum Disorders

2.1 Noonan Syndrome

Noonan syndrome (NS) is an autosomal dominant disorder characterized by short stature, congenital heart defect, and developmental delay of variable degree. Short stature is present in over 70% of the NS patients; the heart defects are one of the key features presented in approximately 50–80% of the NS patients, including primarily pulmonary valve stenosis and hypertrophic cardiomyopathy (Allanson and Roberts GeneReviews; http://www-ncbi-nlm-nih-gov.ezproxyhost.library.tmc.edu/books/NBK1124/). Other important findings include broad or webbed neck, unusual chest shape with superior pectus carinatus and inferior pectus excavatum, cryptorchidism, and bleeding disorders. Patients with NS exhibit distinctive craniofacial features, including a broad forehead, hypertelorism, down-slanting palpebral fissures, and low-set, posteriorly rotated ears. In addition, individuals with NS have an increased risk of hematological malignancy, including juvenile myelomonocytic leukemia (JMML) [60]. While the postnatal presentation of NS is well known, the recurrent prenatal findings of NS have also been noted, including polyhydramnios, increased nuchal translucency (NT), cystic hygroma, pleural effusions, hydrops, and cardiac defects [27, 37].

Table 2a Selected genes involved in RASopathies

RAS/MAPK pathway gene	Chromosome location	OMIM	NM#	Number of exons	Protein	Protein function	Inheritance
NF1	17q11.2	162,200	NM_000267.3	57	Neurofibromin	RASGAP	AD
PTPN11	12q24.1	176,876	NM_002834.3	16	Tyrosine-protein phosphatase non-receptor type 11	Phosphatase	AD
SOS1	2p22.1	182,530	NM_005633.3	23	Son of sevenless homolog1	RAS GEF	AD
RAF1	3p25.1	164,760	NM_002880.3	17	RAF proto-oncogene serine/threonine-protein kinase	Kinase	AD
BRAF	7q34	164,757	NM_004333.4	18	V-RAF murine sarcoma viral oncogene homolog B1	Kinase	AD
KRAS	12p12.1	190,070	NM_004985.3	5	GTPase KRas	GTPase	AD
NRAS	1p13.2	164,790	NM_002524.3	7	GTPase NRas	GTPase	AD
HRAS	11p15.5	190,020	NM_005343.2	6	GTPase HRas	GTPase	AD
CBL	11q23.3	165,360	NM_005188.3	16	CBL	E3 ubiquitin ligase	AD
SHOC2	10q25.2	602,775	NM_007373.3	9	SHOC2	Scaffolding	AD
RIT1	1q22	609,591	NM_006912.5	6	RIC-like protein without CAAX motif 1	GTPase	AD
MAP2K1	15q22.31	176,872	NM_002755.3	11	Dual specificity mitogen-activated protein kinase kinase 1	Kinase	AD
MAP2K2	19p13.3	601,263	NM_030662.3	11	Dual specificity mitogen-activated protein kinase kinase 2	kinase	AD
SPRED1	15q14	609,291	NM_152594.2	7	Sprouty-related EVH1 domian-containing protein 1	SPROUTY-related EVH1 domain-containing protein 1	AD

NS is caused by activating pathogenic variants in genes in the RAS/MAPK signaling pathway (Table 1). The heterozygous missense pathogenic variants in these genes lead to the constitutive activation or enhanced activity of proteins encoded by these genes, resulting in increased signaling through the RAS/MAPK pathway.

2.1.1 PTPN11

Gain-of-function missense pathogenic variants in the *PTPN11* are the most common cause of NS. Approximately 50% of the patients with a clinical diagnosis of NS harbor pathogenic variants in *PTPN11* (Allanson and Roberts GeneReviews; http://www-ncbi-nlm-nih-gov.ezproxyhost.library.tmc.edu/books/NBK1124/; [62]). The *PTPN11* gene is located at 12q24.1 and consists of 16 exons (Table 2a and 2b, Fig. 3). The protein product of *PTPN11*, SHP2, is a non-receptor protein tyrosine phosphatase (PTP) [62, 69]. It is composed of N- and C-terminal SH2 domains and a single catalytic PTP domain (Fig. 3). Interactions between the N-terminal SH2 domain and the PTP domain are involved in switching the protein between its inactive and active conformation. In the inactive state, the N-terminal SH2 domain directly binds the PTP domain and inhibits its catalytic activity by blocking the access of the substrate to the catalytic site. Once the N-terminal SH2 domain binds the phosphotyrosine peptide, conformational change results in the active state. The majority of NS-causing pathogenic variants in *PTPN11* cluster in and around the interacting residues of the N-terminal SH2 domain and the PTP domain. Pathogenic variants in this region disrupt the stability of the catalytically inactive form of SHP2, causing constitutive or prolonged activation of the protein (gain of function). Most pathogenic variants indentified in Noonan syndrome were missense gain of function pathogenic variants (Table 3). Four pathogenic variants affecting residues involved in the N-terminal SH2/PTP interaction, Y63C, Q79R, N308D, and N308S, were identified in approximately 40% of Noonan patients with

Table 2b Summary of RASopathy genes by phenotype and diagnostic yield

Gene	Phenotype(s)	Percentage of cases
PTPN11	NS	50%
SOS1	NS	10–13%
RIT1	NS	9%
RAF1	NS	3–7%
KRAS	NS, CFC	<5%, <5%
NRAS	NS	Unknown (<1%)
LZTR1	NS	Unknown
SOS2	NS	Unknown
SHOC2	Noonan-like	Unknown
CBL	Noonan-like	Unknown (<1%)
BRAF	CFC	75–80%
MEK1	CFC	10–15%
MEK2	CFC	
HRAS	CS	80–90%

PTPN11 pathogenic variants (Table 3) [2]. Pathogenic variants affecting residues not only involved in N-terminal SH2/PTP interaction but also involved in controlling the catalytic activity, substrate specificity, or the flexibility of the linker stretch between N-terminal SH2 and C-terminal SH2, have all been identified in NS patients. Two pathogenic variants affecting residues binding the phosphopeptide in SH2 domain, Thr42 (N-terminal SH2) and Glu139 (C-terminal SH2), were identified in approximately 6% of Noonan patients with *PTPN11* pathogenic variants [2]. Some germ line *PTPN11* pathogenic variants overlap with somatically acquired *PTPN11* pathogenic variants associated with JMML. Some *PTPN11* pathogenic variants are distinctively associated with Noonan syndrome or cancer [2]. Although most of the *PTPN11* pathogenic variants arise as *de novo* events, PTPN11 pathogenic variants have been detected in 30–75% of patients with familial NS [59, 69].

2.1.2 SOS1

The second most common cause of NS is missense pathogenic variant in the *SOS1* gene, accounting for approximately 15% of the NS cases [28, 46, 47, 61, 70]. The *SOS1* gene is located at 2p22.1 and consistent of 23 exons. *SOS1* encodes the guanine nucleotide exchange factors for RAS and acts as a positive regulator of RAS by stimulating the guanine nucleotide exchange. N-terminal of SOS1 is an autoinhibition regulatory domain including tandem histone-like folds (HF), a Dbl-homology domain (DH) and a pleckstrin-homology domain (PH). C-terminal of SOS1 is the catalytic domain including the RAS exchanger motif (REM), CDC25 domain, and a tail providing docking sites for adaptor proteins required for receptor anchoring. The N-terminal inhibits the SOS1's GEF activity by blocking the GDP-RAS allosteric binding site resided in the C-terminal. Once SOS1 is recruited to the membrane, the N-terminal autoinhibition is relieved, allowing the RAS binding to the allosteric site, which in turn, promotes the RAS binding to the catalytic site through the conformational change of the CDC25 domain.

The most common pathogenic variant, c.2536G > A (p.Q846K), is located in the CDC25 domain, which accounts for approximately 12% of the *SOS1* pathogenic variants (Table 4) [28, 46, 47, 61, 70]. Other SOS1 pathogenic variants also tend to cluster in specific regions. Pathogenic variants at three residues located in the PH-REM linker (Ser 548, Leu550, and Arg552) account for approximately 40% of SOS1 pathogenic variants (Table 4). The second pathogenic variant cluster is located in the PH domain, which accounts for approximately 20% of *SOS1* pathogenic variant. The last pathogenic variant cluster is resided in the interaction region of DH domain and REM domain (16% of all pathogenic variants). Most SOS1 pathogenic variants arise as *de novo* events. Rarely, familial cases have been reported. In these cases, the parents who transmitted pathogenic variants had similar clinical features as the affected children [46, 47, 70].

Individuals with *SOS1* pathogenic variants have typical features of Noonan syndrome, however some are reported to have more ectodermal manifestations, including sparse eyebrows and skin abnormalities, similar to what is more typically seen

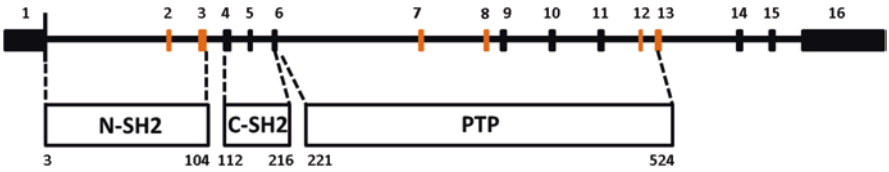

Fig. 3 Genomic organization and function domains of PTPN11 gene. Coding exons are shown as filled boxes. The peptide structure shows functional domains with their amino acid boundaries. *The hot spots for the pathogenic variants* are shown as orange filled boxes. (Adapted from [62, 69])

Table 3 Selected recurrent PTPN11 pathogenic variants in Noonan Syndrome. The four most common PTPN11 pathogenic variants are highlighted in red

DNA Nucleotide change	Protein amino acid change	Exon	Function domain
c.124A>G	p.T42A	2	N-SH2
c.179G>C	p.G60A	3	N-SH2
c.181G>A	p.D61N	3	N-SH2
c.182A>G	p.D61G	3	N-SH2
c.184T>G	p.Y62D	3	N-SH2
c.188A>G	**p.Y63C**	**3**	**N-SH2**
c.214G>A	p.A72S	3	N-SH2
C.215C>G	p.A72G	3	N-SH2
c.218C>T	p.T73I	3	N-SH2
c.228G>C	p.E76D	3	N-SH2
c.236A>G	**p.Q79R**	**3**	**N-SH2**
c.317A>C	p.D106A	3	Linker
c.417G>C,T	p.E139D	4	C-SH2
c.836A>G	p.Y279C	7	PTP
c.922A>G	**p.N308D**	**8**	**PTP**
C.923A>G	**p.N308S**	**8**	**PTP**
c.1403C>T	p.T468M	12	PTP
c.1510A>G	p.M504V	13	PTP

in CFC syndrome, when compared to other individuals with NS. Growth may also be less affected in *SOS1*-related NS when compared to other genetic causes of NS (such as *PTPN11*-related NS) [28].

2.1.3 KRAS and NRAS

Pathogenic variants in RAS genes have been reported in a small portion of NS patients, including *KRAS* and *NRAS*. RAS contains a G domain and a C terminal membrane targeting region. The G domain directly binds to GDP or GTP, and two

switch motifs within the G domain, G2 (switch I) or G3 (switch II), are the main parts that facilitate the activation of RAF by GTP [64]. The Thr35 that binds directly to GTP is located in G2 (switch I) domain. The conformation change of these two switch motifs mediates the switch between ON-state of GTP bound RAS and OFF-state of GDP bound RAS. In the active state, RAS has high affinity of downstream effectors, which in turn, stimulates downstream signaling pathways. RAS also has an intrinsic GTPase activity that needs the binding of GTPase activating proteins (GAPs) to hydrolyze a bound GTP molecule into GDP. The G3 play a crucial role in hydrolysis of GTP to GDP. The balance between SOS1 and GAP activity determines the guanine nucleotide status of RAS, thus regulating RAS activity.

The *KRAS* gene is located at 12p12.1 and encodes two isoforms; the KRASA expressed in a tissue-specific and developmentally restricted manner, and a ubiquitously expressed KRASB. The *KRAS* pathogenic variants have been identified in approximately 2% NSD patients [7, 53, 70]. The KRAS pathogenic variants increase the signaling of the RAS/MAPK pathway through three distinct mechanisms: reducing the RAS GTPase activity; interfering with the guanine nucleotide binding of KRAS; or disrupting the KRAS autoinhibition by membrane sequestration of its effector-binding site [31]. In fact, the most common pathogenic variant in *KRAS*, D153V in exon 6 of *KARSB*, activates signaling by perturbing membrane orientation to unleash autoinhibition. Somatic KRAS pathogenic variants are frequently detected in lung, colon, and pancreatic cancers. However, the most common somatic pathogenic variants at codons 12, 13, and 61 have not been indentified as germline pathogenic variants. Pathogenic variants in *KRAS* indentified in other RASopathy disorders are discussed later.

A few pathogenic variants in *NRAS* have been found in patients with NS [10, 16, 49]. The *NRAS* gene is located at 11p13.2 and consistent of seven exons. *NRAS* pathogenic variants are located within or near the switch I and switch II regions and thought to activate the MAPK pathway by accumulating in the GTP-bound conformation or to reducing the GTPase function.

2.1.4 RIT1

Recently, whole exome sequencing studies have identified pathogenic variants in a new RAS like gene, *RIT1*, in RASopathies patients with no detectable pathogenic variants in known Noonan-related genes [3]. The RIT1 gene is located at 1q22 and consists of six exons. RIT1 shares approximately 50% sequence identity with RAS at amino acid level, and it has an additional N-terminal extension. Similar to *NRAS* pathogenic variants, *RIT1* pathogenic variants are clustered in the G3 (switch II) GTPase activity domain. A few *RIT1* pathogenic variants also have been identified in G1 and G2 domains. Overall, *RIT1* pathogenic variants are identified in 4–9% NS patients.

Table 4 Selected recurrent SOS1 pathogenic variants in Noonan Syndrome

DNA nucleotide change	Protein amino acid change	Exon	Function domain
c.322G>A	P.E108K	4	HF
c.806T>C	P.M269 T	6	DH
c.806T>G	p.M269R	6	DH
c.1642A>C	p.S548R	10	PH-REM linker
c.1649T>C	p.L550R	10	PH-REM linker
c.1654A>G	p.R552G	10	PH-REM linker
c.1655G>A	p.R552K	10	PH-REM linker
c.1655G>T	p.R552M	10	PH-REM linker
C.1656G>C	p.R552S	10	PH-REM linker
c.210T>C	p.Y702H	14	REM
c.2536G>A	p.E846K	16	CDC25

2.1.5 RAF1 and BRAF

Pathogenic variants in *RAF1* have been identified in 3–17% NS patients. The *RAF1* gene is located at 3p25.2 and consistent of 17 exons. *RAF1* encodes a serine/threonine kinase that is one of the direct downstream RAS effectors. RAF1 share three conserved cysteine-rich regions, CR1, CR2, and CR3 with the other two RAFs, ARAF and BRAF [32]. CR1 domain (CRD) and most of the RAS binding domain (RBD) bind to RAS-GTP. CR2 is rich in serine and threonine residues. CR3 is the kinase domain containing the highly conserved glycine-rich G-loop GXGXXG motif. Pathogenic variants in RAF1 identified in NS patients are clustered in CR2 and CR3 domains. CR2 domain contains an inhibitory phosphorylation site (Ser 259). The dephosphorylation of Ser259, which is required for RAF1 translocation to the cell membrane and its catalytic activity, has been shown to be the primary pathogenic mechanism in the activation of RAF1. This pathogenic variant accounts for approximately 70% of all the identified *RAF1* pathogenic variants in NS patients. The other four pathogenic variants that reside within the C-terminal kinase domain, Asp 486, Thr491, Ser612, and Leu613, account for almost all the remaining 30% of *RAF1* pathogenic variants. Individuals with *RAF1*-related NS have a significant risk of hypertrophic cardiomyopathy, which often presents in the newborn period as severe cardiac involvement.

Pathogenic variants in *BRAF* have been reported in a few NSD patients, the majority pathogenic variants of *BRAF* have been identified in patients with CFC syndrome. The detailed discussion of BRAF is under the CFC syndrome section.

2.1.6 SHOC2

A recurrent pathogenic variant, p.S2G in the *SHOC2* gene, has been identified in NS patient with a unique phenotypic feature of loose anagen hair [12]. The *SHOC2* gene is located at 10q25.2 and consists of nine exons. The *SHOC2* gene encodes a protein composed almost entirely of leucine-rich repeats (LRR) with a lysine-rich sequence at the N-terminus. The LRR of SHOC2 functions as a scaffold linking

RAS to the downstream effector, RAF1. SHOC2 binds to RAS-GTP and promotes the catalytic subunit of protein phosphatase 1 (PP1C) translocation to the cell membrane. This facilitates PP1C dephosphorylation at residue Ser259 of RAF1. As mentioned previously, the Ser259 is the major hot spot for NS-causing pathogenic variant in RAF1. The recurrent pathogenic variant p.S2G in SHOC2 is proposed to promote an aberrant protein N-myristoylation that results in constitutive membrane targeting of SHOC2, leading to prolonged PP1C-mediated RAF1 dephosphorylation at Ser259 and consequently increased MAPK pathway activation [12, 24].

2.1.7 CBL

Pathogenic variants in a tumor-suppressor gene, *CBL*, have been reported as a rare cause of NS [30]. The *CBL* gene is located at 11q23.3, consisting of 16 exons. The CBL gene encodes an E3 ubiquitin ligase that negatively regulates the downstream signaling of receptor tyrosine kinases (RTKS). CBL contains an N-terminal tyrosine kinase-binding (TKB) domain, a short linker and a C-terminal zinc-binding RING-finger domain mediating the E3 ubiquitin ligase activity. CBL catalyzes the ubiquitination of activated RTKs to switch off signaling via receptor degradation or recycling [52]. Pathogenic variants in CBL identified in NS patients are clustered within the RING finger domain or the adjacent linker region.

2.2 Costello Syndrome

Costello syndrome (CS) is one of the rare RASopathies characterized by failure to thrive in infancy, short stature, developmental delay, coarse facial features, ectodermal abnormalities, hypotonia, and cardiac abnormalities. Clinical features of CS overlap with other RASopathy disorders. Relative or absolute macrocephaly is typical in CS patient and the characteristic facial appearances include coarse face (full cheeks, wide mouth with full lips and broad nasal base and full nasal tip). Ectodermic abnormalities in CS patient include soft skin, curly or spare, fine hair, deep palmar and plantar creases. The majority of CS individuals have cardiac abnormalities including hypertrophic cardiomyopathy, valve abnormalities (usually valvar pulmonic stenosis), and arrhythmia. Individuals with CS have an approximately 15% lifetime risk to develop malignant tumors, including rhabdomyosarcoma, ganglioneuroblastoma, and bladder carcinoma.

Only a single gene, *HRAS*, has been reported to cause CS. The *HRAS* gene is located at 11p15.5 and consists of six exons. The most common *HRAS* pathogenic variants affect residues Gly12 and Gly13 that are found in approximately 80–90% CS individuals. Especially, pathogenic variant p.G12S is found in more than 80% CS individuals and also found in cancers. The second most common pathogenic variant causes CS is p.G12A, unlike p.G12S, this pathogenic variant is not commonly found in cancers. These pathogenic variants disrupt guanine nucleotide binding and cause

a reduction of GTP hydrolysis, resulting in HRAS remaining in the active state. Interestingly, the two most common residues mutated in CS are also the most frequently mutated positions in cancers. Other HRAS pathogenic variants are also observed in CS individuals with very low frequency. Up to date, there is only one documented case of germline mosaicism reported in CS [56]. The p.G12S pathogenic variant identified in the affected son was present in 7–8% cells of the father.

2.3 Cardio-Facio-Cutaneous (CFC) Syndrome

CFC is another rare RASopathy syndrome characterized by cardiac abnormalities, distinctive craniofacial appearance, and cutaneous abnormalities. CFCS has considerable clinical features overlap with NS and CS. Individuals with CFC have distinct facial features similar to NS, including macrocephaly, a high forehead, bitemporal narrowing, and facial dysmorphia that is coarser compared to NS. The ectodermal findings include dry and hyperkeratotic skin, ichthyosis, eczema, sparse, curly hair, and sparse eyebrows and eyelashes. Cardiac abnormalities also occur in the majority of individuals with CFC; the most common being pulmonic stenosis, septal defects and hypertrophic cardiomyopathy. Neurologic and/or cognitive deficits are present in nearly all individuals with CFC, ranging from mild to severe. Neoplasia, mostly acute lymphoblastic leukemia (ALL), has been reported in some individuals.

Four genes have been identified to be associated with CFC syndrome, *BRAF, MAP2K1, MAP2K2*, and *KRAS*. Pathogenic variants in *BRAF* are found in approximately 75% of individuals with CFC. The *BRAF* gene is located at 7q34 and consists of 18 exons. The *BRAF* gene is also known as a proto-oncogene. Somatic pathogenic variants in this gene are frequently found in various cancers, including malignant melanoma, thyroid, colorectal, ovary and lung cancers. However, the CFC-associated pathogenic variants only partially overlap with the cancer-associated pathogenic variants in *BRAF*. Unlike the cancer-associated pathogenic variants, which are clustered in the glycine-rich loop and activation segment, the majority of CFC-associated pathogenic variants are clustered in the cysteine-rich domain (CR1) in exon 6 and in the protein kinase domain (CR3). The pathogenic variant Q257R in the CR1 domain is the most common *BRAF* pathogenic variant identified in CFC individuals, followed by E501G, G469E and N581D. The functional analyses of *BRAF* pathogenic variant proteins have demonstrated that some *BRAF* pathogenic variants have increased kinase activity and some BRAF pathogenic variants have exhibited impaired kinase activity [36, 48]. However, further *in vivo* studies in Zebrafish have demonstrated that both kinase-active and kinase-impaired *BRAF* pathogenic variants result in similar phenotypic dysregulation of MAPK signaling [1]. The functional difference between the kinase-active and kinase-impaired *BRAF* pathogenic variants remains unknown.

Pathogenic variants in *MAP2K1* and *MAP2K2* are identified in the approximately 25% of the pathogenic variant positive CFC individuals [17, 33, 34, 40, 48, 54]. *MAP2K1* and *MAP2K2* are threonine/tyrosine kinases (MEK1 and MEK2) and both

isoforms have the equally ability to phosphorylate and activate ERK substrates (ERK1 and ERK2) [71]. Functional studies of MEK CFC mutant proteins by examining the ERK phosphorylation have showed that all pathogenic variants are more active than wild-type MEK [48].

In addition, pathogenic variants in *KRAS* have been identified in 2–3% CFC individuals (Rauen [42], gene reviews).

2.4 Noonan Syndrome with Multiple Lentigines (NSML)

NSML (formerly referred to as LEOPARD syndrome) is a rare autosomal dominant disorder that is an allelic NS. NSML is characterized by the craniofacial features of NS as well as multiple lentigines, electrocardiogram (ECG) conduction abnormalities, ocular hypertelorism, pulmonary valve stenosis, abnormal genitalia, growth retardation, and sensorineural deafness [21, 50]. Multiple lentigines present as flat, black-brown macules, mostly on face, neck and upper part of the trunk with sparing of mucosa (Gelb and Tartagila [20], gene reviews). In general lentigines appear at the age of 4–5 years and increase to the thousands by puberty. Heart defects are observed in approximately 85% of NSML individuals, including ECG anomalies, HCM and pulmonary valve stenosis. Growth retardation is observed in approximately 50% of NSML individuals with a final height in most individuals less the 25th percentile for age. Pathogenic variants in the PTPN11 gene have been identified in approximately 90% NSML individuals. However, unlike the PTPN11 pathogenic variants associated with NS, the most common PTPN11 pathogenic variants associated with NSML are clustered in the catalytic PTP domain, causing a loss of function of SHP2 catalytic activity [18, 25]. In addition to PTPN11, pathogenic variants in RAF1 have been reported in about 5% of NSML individuals. Rarely NSML individuals have pathogenic variants in the BRAF and MAP2K1 genes [26, 38, 50].

3 Molecular Diagnosis of NSDs by NGS

As of 2016, 18 genes have been discovered to play a role in the pathogenesis of RASopathies, including *A2ML1, BRAF, CBL, HRAS, KRAS, LZTR1, MAP2K1, MAP2K2, NF1, NRAS, PTPN11, RAF1, RASA2, RIT1, SHOC2, SPRED1, SOS1,* and *SOS2* [3, 5, 8–10, 12, 30, 35, 36, 41, 44, 46–48, 53, 58, 61, 65–68]. The genes associated with NSDs over 30 kb coding sequences in total in terms of coding region and 20 bp adjacent intronic sequences which make standard stepwise molecule testing of NSDs expensive by Sanger sequencing. Therefore, thorough clinical evaluation and preliminary differential diagnosis based on presenting symptoms for suspected NSDs are often the prerequisite to warrant a high diagnostic yield. However, the clinical delineation of NSDs can be difficult, as these disorders present with wide variabilities in the affected organs as discussed above. Prioritization of genes to be tested is

primarily dependent on the distinct phenotypes seen in the disorders and the disease prevalence. The molecular diagnosis can be achieved for the most of NSD cases by taking this gene-by-gene approach. Some s cannot be identified in primary screening for prevalent mutations in genes such as PTPN11, RAF1 or SOS1. A follow-up sequencing test should be considered for other less common NSDs.

All of known NSD pathogenic variants are single nucleotide changes and no deletions or duplications involving *PTPN11, KRAS, SOS1, RAF1, BRAF*, or *MAP2K1* have been reported to cause NSDs. Therefore, sequencing for these genes is considered to be sufficient the molecular testing for NSDs. However, unlike other NSDs, NF1 is caused by loss of function pathogenic variants in NF1 and exonic copy number variation in this gene represents an important fraction of NF1 pathogenic variants. A different and more comprehensive test strategy should be considered if NF1 is included in the NSD panel. Recent report on *MAP2K2* suggests deletion could also be a novel mechanism for the etiology of RASopathy [39].

When designing an NGS panel for NSD genes by a capture approach, the paralogues sequences in three genes, *PTPN11, MAP2K and MAP2K2*, and the high GC contents in *BRAF* and *HRAS* genes should be considered in order to achieve 100% coverage of the coding regions. For example, higher probe density in the high-GC content exon 1 of the PTPN11 gene might improve the capture efficiency and reduce gap-filling by Sanger sequencing. Any probe that may capture the paralogous sequences such as exons in the NF1 gene should be avoided, so the enriched DNA fragments by hybridization will be less prone to ambiguous mapping after sequencing Amplicon-based NGS for 12 NSD genes has been clinically validated and demonstrated satisfactory detection rate [29]. As any other amplicon based NGS tests, redundant primer pairs should be included to avoid allele drop-out caused by rare SNPs. In addition, the amplicon size has to be optimized for sequencers and to generate overlapping reads in long exons to ensure full coverage. Recent studies have reported the pathogenic variant detection rates from 19% to 68% in patients who had sequencing test for RASopathies [6, 11, 29]. We have designed a capture based 12 gene NSD NGS panel, including the *BRAF, CBL, HRAS, KRAS, MAP2K1, MAP2K2, NRAS, PTPN11, RAF1, RIT1, SHOC2,* and *SOS1* genes. For the first 73 patients evaluated by our laboratory, 38 pathogenic or likely pathogenic variants (52% of the cases) were detected (data not published). It is worth noting that the recently discovered RIT1 gene accounts for about 10% of the diagnosed patients in our cohort.

3.1 NGS Based Prenatal Diagnosis

It has been estimated that Noonan spectrum syndrome has high prevalence as 1 in 1000–2500 live birth previously [3]. Most of those typical presentations of postnatal Noonan spectrum disorders are not identified in the first or second trimester of pregnancy. Although indications of Noonan spectrum disorders related prenatal abnormalities have been observed as mention above, such associations are only suggestive. Precise molecular diagnosis then can play a critical role to provide early medical intervention, especially for patients with structural cardiac defects identified usually after delivery.

3.1.1 Whole Genome Amplification for Prenatal DNA

The quantity of DNA isolated from chorionic villi sampling (CVS) or amniotic fluid is usually not sufficient for NGS based targeted gene enrichment. For example, it has been estimatated that about 100 ng DNA per ml of direct amniotic fluid at early gestational age. Thus, it is often a challenge to obtain sufficient amount of DNA for NGS analysis of multiple target genes, which usually requires at least one microgram of DNA for target gene enrichment. Thus, unbiased whole genome amplification (WGA) is necessary to generate sufficient DNA. Efforts have been reported in various samples with low DNA input, such as cancer tissues from micro dissect [47] and cells derived from *in vitro* fertilization for preimplementation examination [14].

WGA can be achieved by different mechanisms. In principle they can be categorized into PCR-based and Non-PCR based. PCR based WGA utilizes PCR amplification with modifications of primers to incorporate randomized nucleotides for PCR reaction working on whole genome as uniformly as possible. Some of those PCR based WGA can be regarded as modified library preparation protocols for NGS. Non-PCR based WGA methods are isothermal amplification using Phi29 DNA polymerase (MDA) [57], T4 replisome [51] or T7 RNA polymerase(LinDA or LADS) [23, 55]. Performance of each WGA methods has been evaluated under different platforms: MDA or GenomePlex in aCGH [63], in amplicon-NGS [45], MDA in whole exome sequencing [22].

3.1.2 Validation of Capture/NGS Based Prenatal Diagnosis

A custom-designed capture library targeted to 12 genes *(BRAF, CBL, HRAS, KRAS, MAP2K1, MAP2K2, NRAS, PTPN11, RAT1, RIT1, SHOC2, SOS1*), was designed to capture a total of 135 CDS of these genes with a total target region of 24.7 kb. DNA extracted from varies kind of validation samples, such as blood, cultured chorionic villus sampling (CVS), cultured amniocentesis (AMNIO) and tissues were amplified using MDA based method Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Waukesha, Wisconsin) with 100 ng of DNA input for WGA. Amplified DNA products were used for library construction and then for NimbleGen based capture NGS. WGA performance was accessed first on representativeness. As shown in Table 5 an average 95.3% of total captured CDS (ranged from 84.4% to 100%) have relative fold change within 0.5 ~ 1.5 compared to non-WGA samples, which is acceptable for evenness of amplification efficiency. So the amplification performance in terms of representativeness is consistently good for most regions of genes in Noonan Spectrum test.

Accuracy of whole genome amplification was accessed by the pathogenic variant calling. All negative samples and positive pathogenic variants were correctly identified through double blinded test and listed in Table 5. Sample 4, i.e. DNA from Sample 1 mixed with 5% of DNA from Sample 2, was used to mimic the maternal contamination in fetus sample, which is not a rare event during clinical sample collection for direct AMNIO or CVS. NGS results of variants calling indicated that WGA process we used here would not exaggerate the contamination so maternal

Table 5 Summary of Noonan NGS panel validation

Validation ssample	Sample type	Percentage of CDS with relative fold change within 0.5~1.5	Variant	Concordant with Sanger sequencing
1	Prenatal(tissue)/trio	88.10%	neg	Yes
2	Postnatal(blood)/trio	99.30%	neg	Yes
3	Postnatal(blood)/trio	99.30%	neg	Yes
4	Mixed of sample 1 and sample 2	94.80%	neg	Yes
5	Prenatal(CCVS)	97.10%	neg	Yes
6	Prenatal(CCVS)	91.10%	neg	Yes
7	Prenatal(CCVS)	84.40%	PTPN11:c.215C>G	Yes
8	Prenatal(CAMNIO)	100%	neg	Yes
9	Prenatal(CCVS)	99.20%	neg	Yes
10	Postnatal(blood)	93.30%	SOS1:c.1656G>C	Yes
11	Postnatal(blood)	98.50%	RAF1:c.781C>A	Yes
12	Postnatal(blood)	99.30%	HRAS:c.34G>A	Yes

alleles could be distinguishable for the low (~5%) heterozygosity, which would be expected from the validation results on evenness of amplification efficiency as mentioned above. To further rule distinguish possible maternal contamination and possible chimerical pathogenic variants in prenatal samples, we always perform Trio-analysis (simultaneously run prenatal sample and parental samples) by which maternal alleles can be identified from variants calls in prenatal samples.

3.1.3 Insufficiently Covered Regions and Pseudogene

At the average coverage depth of 500X, there are 3 recurrent insufficiently (<20X) covered exons (*PTPN11*-exon1, *BRAF*-exon1, and *MAP2K2*-exon1) that require specific amplification followed by sequencing. These insufficiently covered exons have been consistently observed in both WGA and nonWGA analyses, and are similar to what have been previously reported ([29]).

Pseudogene interference has been observed in *PTPN11*-exon 6, *MAP2K2*-exon 6. *PTPN11* exon6 has several highly homologous regions across the genome (with identities ranging from 91.0% to 97.5%). Capture based enrichment methods would easily bring these pseudogene sequences into the final sequencing libraries. Routine bioinformatic analytical pipeline is usually not designed to distinguish homologous or pseudogene sequences from active genes. Thus, sequences in these regions with problematic alignment or without effective specific capture probes should be carefully reviewed, and re-aligned if necessary. The pseudogene sequences can be distinguished by their recurrent appearance and *in cis* alignment pattern. Nevertheless, it is necessary to use active gene specific primers for Sanger sequencing to rule out ambiguous NGS calls.

3.2 Trio Analysis for the Detection of De Novo Findings

As mentioned above, trio analysis can be used to distinguish between possible maternal allele from contamination and possible chimerical low heterozygocity allele in fetus DNA. Moreover, observance of parental alleles could provide valuable information on pathogenicity of a variant detected in prenatal samples because of autosomal dominant pattern caused by pathogenic alleles in RASopathies. From 100 trio prenatal cased analyzed we identified four *de novo* novel pathogenic/likely pathogenic variants and one probably *de novo* pathogenic variant (paternal DNA unavailable), eight inherited novel variants with unknown significance and one inherited reported variant with unknown significance (Table 6). For novel VUS and reported VUS, it is interesting to notice that these VUS were observed paternally or maternally, which could provide additional information for variant classification if clinical presentations of parents are available. On the other hand, some cases appeared to be *de novo* at the time of test request. However, the molecular results indicated that one of the parents carried the same pathogenic variant. Thus, due to variable penetrance and expressivity, careful clinical evaluation of the parents should be a general practice before and after prenatal diagnosis. The success of trio analysis with WGA suggests possible application of non-invasive prenatal genetic testing (NIPT) for RASopathies in the near future.

Table 6 Variants identified in 100 prenatal cases

	Gene	Variant	Inherited or De novo	Variant classification	Sample type
1	PTPN11	c.215C>T (p.A72V)	De novo	Likely pathogenic	CCVS
2	PTPN11	c.1505OT(p.S502L)	Not inherited from mother	Pathogenic	CCVS
3	RIT1	c.268A>G (p.M90V)	De novo	Likely pathogenic	CAMNIO
4	RIT1	c.170OG (p.A57G)	De novo	Likely pathogenic	CAMNIO
5	SOS1	c.508A>G (p.K170E)	De novo	Pathogenic	CAMNIO
6	SOS1	c.1051OG (p.L351V	Inherited from mother	VUS	CCVS
7	SOS1	c.911G>C (p.R304P)	Inherited from father	VUS	CCVS
8	MAP2K2	c.1112G>A (p.R371Q)	Inherited from mother	VUS	CAMNIO
9	RAF1	c.124_125GOAT,p.A42l	Inherited from father	VUS	CAMNIO
10	CBL	c.1324C>A(p.L442M)	Inherited from father	VUS	CCVS
11	CBL	c.2635G>A (p.V879l)	Inherited from father	VUS	CCVS
12	RIT1	c.634C>T (p.R212W)	Inherited from mother	VUS	CCVS
13	SHOC2	c.1594A>G (p.S532G)	Inherited from father	VUS	CCVS

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