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Supplemental Data

Gain-of-Function Mutations in *RIT1* Cause

Noonan Syndrome, a RAS/MAPK Pathway Syndrome

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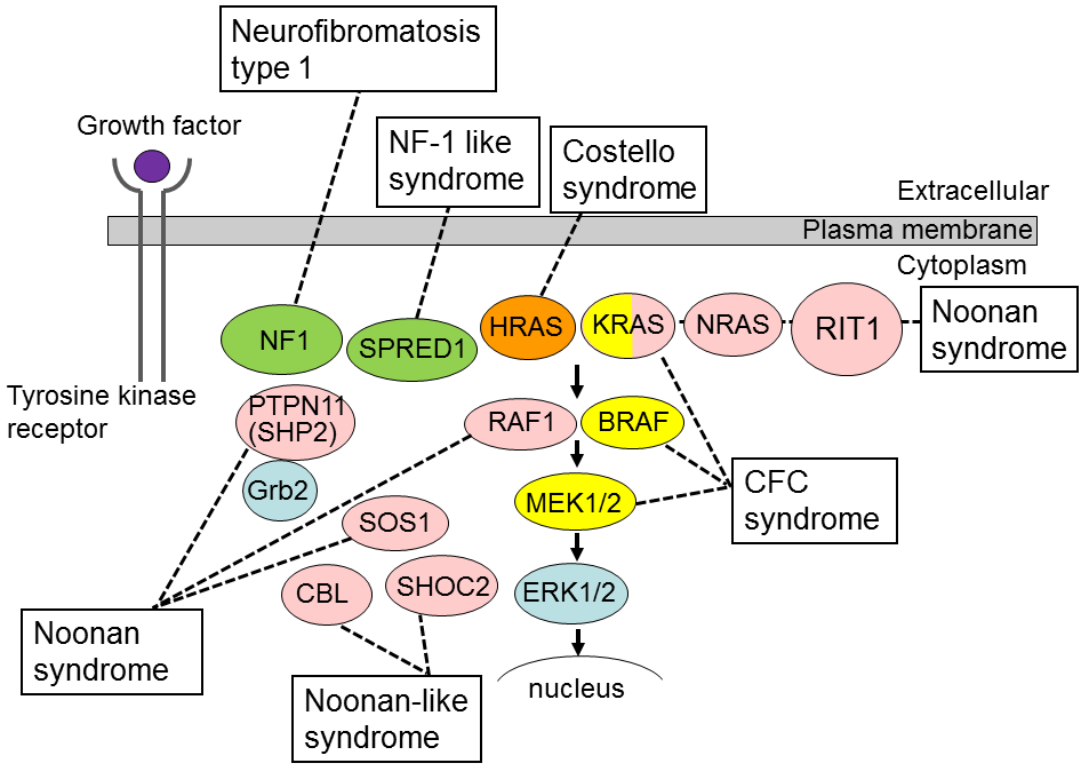


Figure S1. RAS/ERK Pathway and Genetic Disorders (RAS/MAPK Syndromes or RASopathies)

These disorders include: 1) Noonan syndrome caused by mutations in *PTPN11*,*SOS1*, *RAF1*, *KRAS*, and *NRAS*; 2) Noonan syndrome with multiple lentigines caused by mutations in *PTPN11* and *RAF1*; 3) Costello syndrome caused by mutations in *HRAS*; 4) cardio-facio-cutaneous (CFC) syndrome caused by mutations in *BRAF*, *MAP2K1/2* and *KRAS*. 5) Noonan syndrome-like disorder with loose anagen hair caused by mutations in *SHOC2*; 6) Noonan syndrome-like disorder caused by mutations in *CBL*; 7) Neurofibromatosis type I caused by mutations in *NF1*; 8) NF-1 like syndrome (Legius syndrome) caused by mutations in *SPRED1*. *PTPN11* encodes a protein tyrosine phosphatase SHP2. *MAP2K1* and *2* encode MEK1 and MEK2, respectively.

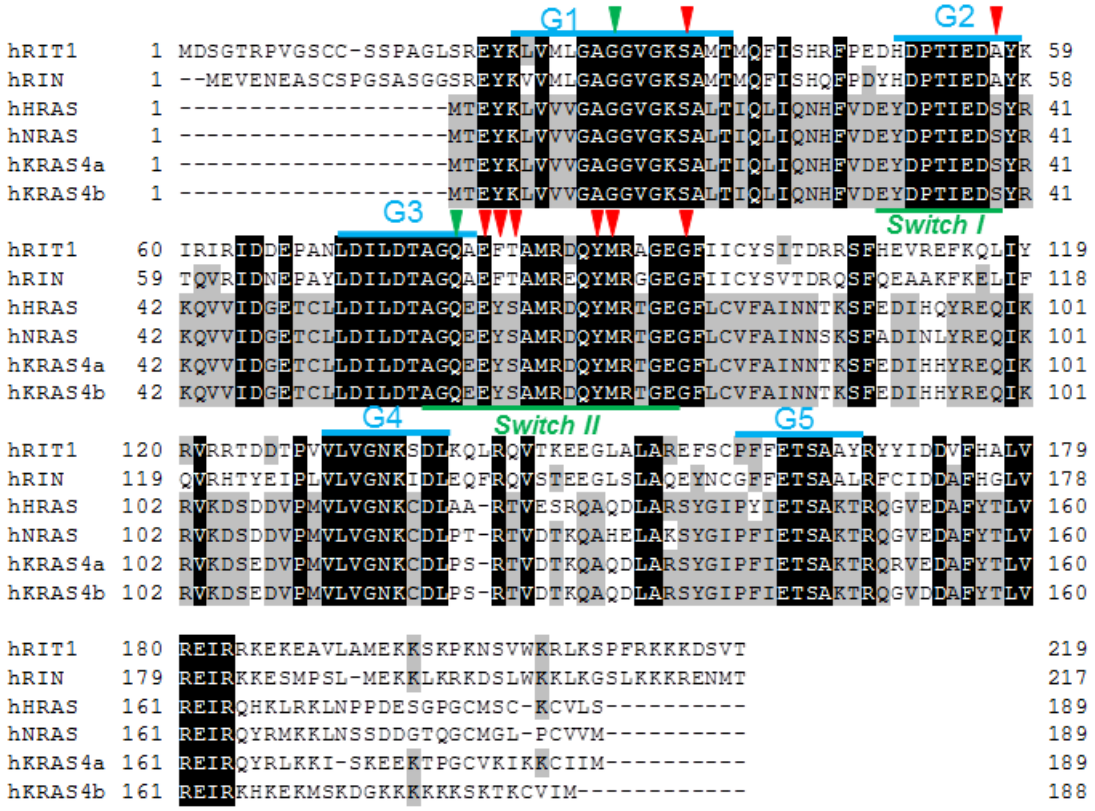


Figure S2. Amino Acid Sequence Alignment of Human RIT1, RIT2/RIN, HRAS, NRAS, and KRAS

The G1-G5 domains are indicated. It has been shown that domains G1 and G3 bind to phosphate, G2 to effectors and G4 and G5 to guanine. Switch I and Switch II regions corresponding to RAS are indicated. Green triangles indicate p.Gly30Val and p.Gln79Leu, which correspond to oncogenic p.Gly12Val and p.Gln61Leu mutations in RAS, respectively. Red triangles indicate amino acids where *RIT1* germline mutations were identified in this study. *RIT1* germline mutations were clustered in Switch I and Switch II regions. Past studies have shown that after GTP binding, the conformational change of RAS occurs in Switch I and Switch II regions, which causes the binding to effectors and Ras GTPase activating protein (GAP). Therefore, it is possible that missense mutations in *RIT1* might have different binding properties to effectors and/or GAP.

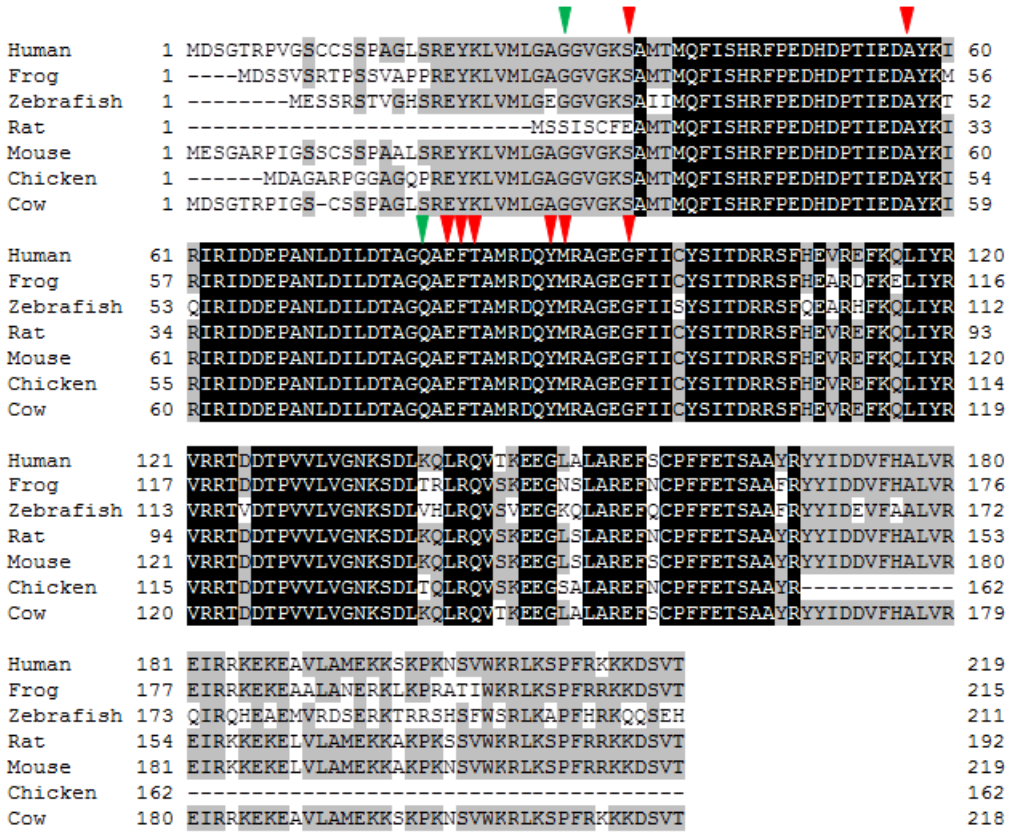
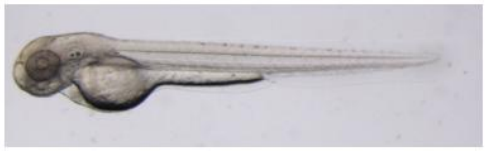
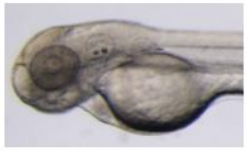


Figure S3. Amino Acid Sequence Alignment of RIT1 among Species

Green triangles indicate p.Gly30Val and p.Gln79Leu, which correspond to oncogenic p.Gly12Val and p.Gln79Leu mutations in RAS, respectively. Red triangles indicate amino acids where *RIT1* germline mutations were identified in this study. Serine at 35 is not conserved in rat. Because of the weakest ELK1 transactivation and familial occurrence, the further analysis will be needed to conclude that p.Ser35Thr is a pathogenic mutation.

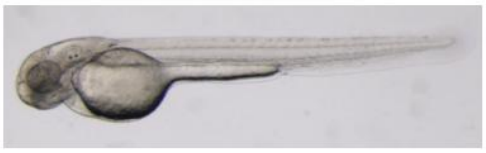
A Uninjected





B

WT Rit1

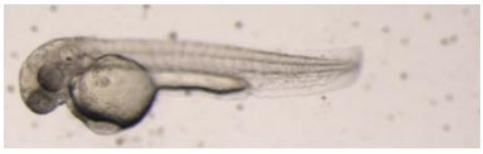


Normal

Craniofacial and heart abnormalities

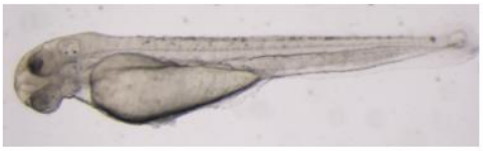
C Gln79Leu Normal n=31

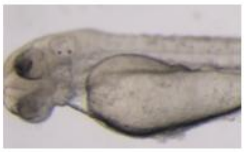
craniofacial and heart abnormalities n=78



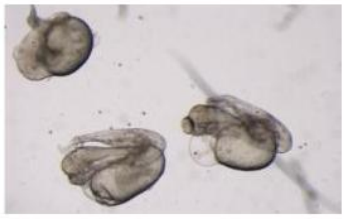
Heavily retarded n=9

D Glu81Gly Normal n=42



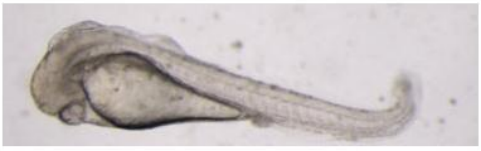


craniofacial and
heart abnormalities
n=55

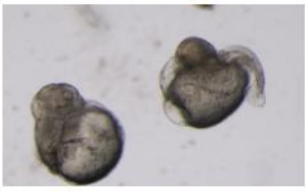


Heavily retarded
n=8

E Gly95Ala Normal n=44



craniofacial and
heart abnormalities
n=34



Heavily retarded
n=6

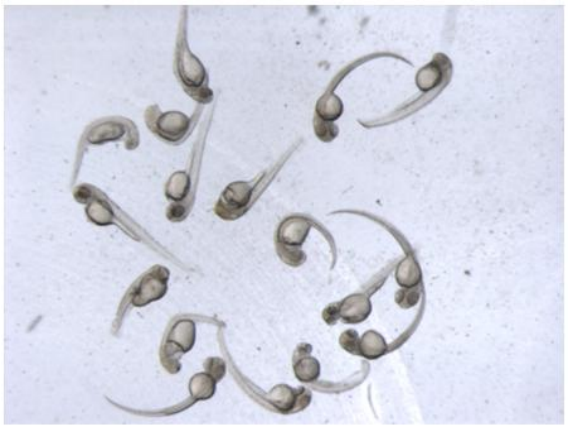


Figure S4. Morphology of Zebrafish Embryos Injected with the Wild-Type (WT) or Mutant mRNA

Some of the photos are also shown in Figure 3.

(A) Uninjected embryos.

(B) Embryos with WT RIT1.

(C) embryos with p.Gln79Leu mutation.

(D) embryos with p.Glu81Gly mutation.

(E) embryos with p.Gly95Ala mutation.

(F) embryos with antisence RNA for WT.

Table S1. Overview of Exome-Sequencing Performance and All Variants Identified by Exome Sequencing in 14 Individuals with Noonan Syndrome and Related Disorders

	NS130	NS265	NS269	NS358	NS336	NS387	NS391	NS392
Exome capture kit	v1 (38 Mb)	v1 (38 Mb)	v1 (38Mb)	v1 (38 Mb)	50 Mb	50 Mb	50 Mb	50 Mb
Mappable reads	28,612,684	25,018,844	27,325,078	35,460,434	88,959,825	105,207,313	65,204,762	86,100,910
Nonduplicated reads	272,65,370	23,498,618	25,535,471	34,353,775	66,451,916	73,303,640	55,591,364	71,416,629
Mean (Median) depth of coverage	39.65(29)	37.74(28)	40.52(30)	42.22(34)	79.88	92.46	68.83	88.78
Total variants								
Exonic	16,645	16,416	16,726	16,194	20,128	20,416	20,228	20,122
Nonsynonymous, nonsense, splicing site, and indel	7,655	7,542	7,785	7,764	9,375	9,589	9,445	9,387
Not in dbSNP135, 1000 genomes	211	177	206	586	332	328	356	309
Not in in-house exomes	188	139	160	282	133	132	155	122

Table S1. Overview of Exome-Sequencing Performance and All Variants Identified by Exome Sequencing in 14 Individuals with Noonan Syndrome and Related Disorders *(continued)*

	KCC7	KCC8	KCC15	KCC19	KCC38	KCC39
Exome capture kit	50 Mb	50 Mb	50 Mb	50 Mb	50 Mb	50 Mb
Mappable reads	88,282,812	77,009,349	75,240,120	78,444,775	65,129,702	82,486,466
Nonduplicated reads	71,923,935	63,964,041	52,981,156	55,068,712	51,911,935	66,498,812
Mean (Median) depth of coverage	88.48	80.22	65.62	70.42	64.47	72.77
Total variants						
Exonic	20,484	20,619	20,440	20,264	20,451	20,609
Nonsynonymous, nonsense, splicing site, and indel	9,676	9,718	9,586	9,537	9,570	9,664
Not in dbSNP135, 1000 genomes	387	390	383	408	377	382
Not in in-house exomes	172	184	172	181	160	165

Table S2. Clinical Manifestations in *RIT1* Mutation-Positive Individuals

	Patient ID					
	NS414	**KCC27**	**NS43**	**NS185**	**NS216**	**NS402**
Nucleotide change	c.104G>C	c.104G>C	c.170C>G	c.170C>G	c.170C>G	c.170C>G
Amino acid change	p.Ser35Thr	p.Ser35Thr	p.Ala57Gly	p.Ala57Gly	p.Ala57Gly	p.Ala57Gly
Sex	F	F	M	F	M	F
Age	3y	4y	8y	9y	5y	15y
Perinatal stage						
Perinatal abnormality	Polyhydr-amnios	–	nd	–	Polyhydr-amnios	Fetal pleural effusion
High birth weight (90 percentile<)	+	–	nd	+	+	–
Craniofacial characteristics						
Relative macrocephaly	+	nd	nd	+	+	+
Hypertelorism	+	+	–	+	+	+
Downslanting palpebral fissures	+	+	–	+	+	–
Ptosis	–	+	–	+	+	–
Epicanthal folds	–	+	–	+	+	+
Low set ears	+		–	+	+	+
Skeletal characteristics						
Short stature (SD)	– (+0.5 SD)	nd	nd	–1.3 SD 9y	–(–1.1 SD)	+ (–3 SD)
Short neck	+	nd	nd	+	–	–
Webbing of neck		nd	+	+	–	–
Pectus abnormalities	+	nd	nd	–	–	–
Cardiac defects						
Hypertrophic cardiomyopathy	+	+	+	+	+	+
Atrial septal defect	–	–	–	+	–	–
Ventricular septal defect	–	–	–		–	–
Pulmonic stenosis	–	+	–	+	–	+
Patent ductus arteriosus	–	–	–	+	–	–
Arrhythmia	–	–	–		–	–
Others	MVP, MR (moderate)		Mild to severe MR, mild TR			
Skin/Hair anomaly						
Curly hair	–	+	nd	+	–	–
Hyperelastic skin	–	nd	nd	+	–	
Eczema	–	nd	nd	–	–	+
Hyperkeratosis	–	nd	nd	–	–	+
Wrinkled palms and soles	–	nd	nd	+	+	
Hyperpigmentation	–	nd	nd	+	+	+
Cryptorchidism	–		+			
Coagulation defects	–	–	–	+	–	nd
Growth and development						
Failure to thrive, feeding difficulty	nd	+	nd	–	+	nd
Intellectual disability	–	nd	–	(IQ=71)	nd	(IQ=74)
Miscellaneous	Hypertrophic cardiomyopathy in mother	Moyamoya disease	Pulmonic stenosis in the mother	Agenesis of corpus callosum		

Table S2. Clinical Manifestations in *RIT1* Mutation-Positive Individuals *(continued)*

	Patient ID					
	NS168	**NS410**	**NS358**	**NS465**	**NS276**	**KCC8**
Nucleotide change	c.242A>G	c.244T>G	c.246T>G	c.246T>G	c.247A>C	c.265T>C
Amino acid change	p.Glu81Gly	p.Phe82Val	p.Phe82Leu	p.Phe82Leu	p.Thr83Pro	p.Tyr89His
Sex	M	F	M	F	M	F
Age	12y	2y	4y	22 mo	4y 7mo	6y
Perinatal stage						
Perinatal abnormality	NT	–	–	Right chylothorax, NT at 12 weeks gestation	TTN	Placental abruption
High birth weight (90 percentile<)	–	+	+	38w, 3390g	–	–
Craniofacial characteristics						
Relative macrocephaly	–	–	+	–	nd	nd
Hypertelorism	–	+	+	–	+	+
Downslanting palpebral fissures	–	+	+	+	+	+
Ptosis	–	+	+	+	+	nd
Epicanthal folds	–	+	+	–	+	nd
Low set ears	–	+ (thick earlobe)	+	–	+	
Skeletal characteristics						
Short stature (SD)	– (–1.4 SD)	(+1.6 SD)	– (–1.0SD)	10th percentile	–2.1 SD at 4y 5m	nd
Short neck	–	+	–	+	+	nd
Webbing of neck	–	–	–	–	–	nd
Pectus abnormalities	–	–	–	–	–	nd
Cardiac defects						
Hypertrophic cardiomyopathy	–	+	–	–	+	+
Atrial septal defect	–	–	+	–	–	nd
Ventricular septal defect	+	–	–	+ (multiple small VSD)	–	nd
Pulmonic stenosis	+	–	+	+ (valvular PS)	+ (valvular PS)	+
Patent ductus arteriosus	–	–	–	–	–	nd
Arrhythmia	–	–	–	–	PVC	nd
Others			PH			
Skin/Hair anomaly						nd
Curly hair	–	–	–	–	–	+
Hyperelastic skin	–	–	+	–	nd	nd
Eczema	–	–	nd	–	nd	nd
Hyperkeratosis	–	–	+	nd	nd	nd
Wrinkled palms and soles	–	+	+	–	nd	nd
Hyperpigmentation	–	–	+	–	nd	nd
Cryptorchidism	+			–	+ (right)	
Coagulation defects	–	nd	nd	–	–	nd
Growth and development						
Failure to thrive, feeding difficulty	–	–	+	–	–	nd
Intellectual disability	(IQ=79)	–	–	nd	nd	nd
Miscellaneous	ALL, intestinal malrotation			Mild motor delay, low muscle tone	Hyponatremia and hyperpotassemia	Head control at 6 months, walk at 2 years

Table S2. Clinical Manifestations in *RIT1* Mutation-Positive Individuals *(continued)*

	Patient ID				
	KCC38	**NS234**	**NS265**	**Og22**	**Og45**
Nucleotide change	c.270G>T	c.284G>C	c.284G>C	c.284G>C	c.284G>C
Amino acid change	p.Met90Ile	p.Gly95Ala	p.Gly95Ala	p.Gly95Ala	p.Gly95Ala
Sex	F	M	M	M	M
Age	3y	5y	8y	11.1 y	0 y
Perinatal stage					
Perinatal abnormality	–	Polyhydr-amnios	–	–	Cystic hygroma in neck, polyhydramnios, pleural effusion in embryo, chylothorax in neonatal period
High birth weight (90 percentile<)	–	+	–	–	+
Craniofacial characteristics					
Relative macrocephaly	–	+	–	–	+
Hypertelorism	+	+	+	+	–
Downslanting palpebral fissures	–	+	+	–	–
Ptosis	+	–	+	+	–
Epicanthal folds	+	+	+	+	–
Low set ears	+	+	+	–	+
Skeletal characteristics					
Short stature (SD)	–2.2 SD at 3y 11m	–	–	(–0.8 SD)	–
Short neck	+	+	–	–	+
Webbing of neck	+	+	–	+	+
Pectus abnormalities	+	–	–	–	nd
Cardiac defects					
Hypertrophic cardiomyopathy	+	–	+	–	+
Atrial septal defect	+	+	–	–	+
Ventricular septal defect	+	–	–	–	–
Pulmonic stenosis	+	–	+	–	+
Patent ductus arteriosus	+	–	–	–	–
Arrhythmia	–	–	–	–	–
Others	Operation for the closure of VSD, ASD, PDA				
Skin/Hair anomaly					
Curly hair	+	–	–	nd	+
Hyperelastic skin	+	–	+	nd	
Eczema	–	–	+	nd	–
Hyperkeratosis	+	–	nd	nd	–
Wrinkled palms and soles	+	–	nd	nd	
Hyperpigmentation	+	+	nd	–	–
Cryptorchidism		+	+	Bilateral migratory testes	+
Coagulation defects	–	–	nd	nd	–
Growth and development					
Failure to thrive, feeding difficulty	+	nd	nd	–	+
Intellectual disability	DQ44 at 1y 11m	–	–	–	nd
Miscellaneous	Hyperuricemia, hyper-triglyceridemia		First word at 2 years of age	Ptosis was operated. His mother had ASD.	G-band: 46XY, hepatomegaly, hyperbilirubinemia, no abnormalities in glycosylation, died at 53 days

MVP, mitral valve prolapse, MR, mitral regurgitation; TR, tricuspid regurgitation; NT, nuchal translucency; TTN, transient tachypnea of the newborn; VSD, ventricular septal defect; PS, pulmonic stenosis; PVC, premature ventricular contraction; IQ, intelligent quotient; ALL, acute lymphoblastic leukemia; ASD, atrial septal defect; PDA, patent ductus arteriosus; nd, no data

Table S3. Primer Pairs Used to Amplify Exons and Their Flanking Introns in *RIT1*

Exon	Forward	Reverse	Product Length (bp)
2	5'-F- gtgactgaactgtctaggagg	5'-R- cacaaaggtgacccacagag	499
3	5'-F- gaccagatgggatacttgc	5'-R- ccaactgctgatacccttgt	331
4	5'-F- cagtggatttagcatgcttcc	5'-R- gcatgtcgattacctgctatc	389
5	5'-F- gtagttgggctagattgcgtc	5'-R- gtaagccaagccaagaatctg	362
6	5'-F- acacctccagaattgagaagc	5'-R- caccatactcagtactgcagg	455

F: 5'- gtaaaacgacggccagt R: 5'- aggaaacagctatgacc