Identification of a PTPN11 hot spot mutation in a child with atypical LEOPARD syndrome

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Received May 20, 2015; Accepted February 15, 2016

DOI: 10.3892/mmr.2016.5547

Abstract. LEOPARD syndrome (LS) is an autosomal dominant inherited disorder primarily caused by mutations in the *PTPN11*, *RAF1* and *BRAF* genes. Characteristic features include lentigines, craniofacial dysmorphism, myocardium or valve abnormalities, eletrocardiographic conduction defects and deafness. LS, neurofibromatosis type 1, Noonan syndrome and Legius syndrome are a group of highly overlapped disorders termed 'RASopathies'. Therefore, clinical discrimination between these syndromes represents a huge challenge. The present study reports a young child diagnosed with LS via identification of a common p.Thr468Met mutation in *PTPN11*. Taking into account two Taiwanese LS cases with an identical mutation, Thr468Met is likely to be the most prevalent mutation in the Chinese population. Furthermore, this study suggests that a clinical diagnosis of LS should be considered for individuals with congenital cardiac defects and atypical lentigines (i.e., light brown freckles) scattered particularly on the face.

Introduction

LEOPARD syndrome (LS, OMIM 151100), an autosomal dominant inherited disorder, presents with phenotypes that strongly overlap with Noonan syndrome (NS, OMIM 163950). These features include ocular hypertelorism, pulmonary stenosis, growth retardation, sensorineural deafness, genitourinary abnormalities and in particular multiple lentigines (1). The majority of the clinical features of LS appear to be age-dependent (2), similar to that observed in neurofibromatosis type 1 (NF1, OMIM 162200). The cutaneous appearance is also similar to Legius syndrome, formerly termed neurofibromatosis type 1-like syndrome (NFLS, OMIM 611431), characterized by multiple café-au-lait spots (CALS) and skin-fold freckling. Thus, NF1, NFLS, NS and LS, which belong to a new class of genetic disorders called the 'RASopathies', may be clinically indistinguishable at early stages.

LS harbors certain genetic heterogeneity. *PTPN11* (proportion, ~85%), *RAF1* and *BRAF* (~0%) are the major pathogenic genes known to cause LS. Two recurrent mutations, Tyr279Cys and Thr468Met in *PTPN11*, were found in ~65% of the cases examined in a previous large study (3). Moreover, recently, a novel heterozygous *MAP2K1* mutation (c.305A>G) was reported to be associated with LS (4).

To date there have been few cases of LS described in the Chinese population, except for a recent study that demonstrated cardiovascular complications in a patient with sporadic LS caused by a Tyr279Cys mutation in the *PTPN11* gene (5) and four previous Taiwanese cases with Thr468Met and Gly464Ala mutations (6). The current study presented another case of LS with atypical symptoms diagnosed via identification of a common Thr468Met mutation in the *PTPN11* gene and reviewed the literature for cases of LS associated with this mutation.

Materials and methods

Case history. A 5 year-old male patient was admitted to the Department of Dermatology, Xinhua Hospital (Shanghai, China) in December 2013. His parents were concerned about several CALS and freckles presenting over the trunk and face of the child from birth. Physical examination revealed 4 CALS with a diameter >0.5 cm and several freckle-like lesions scattered over the body. The patient also presented with short stature (height, 102 cm) and ocular hypertelorism (Fig. 1). Results of neuropsychological examination and hearing tests were normal. No Lisch nodules were found through slit-lamp examination. Following examination seven months later, it was observed that the freckle-like lesions had increased. Moreover, the patient had a medical history of pulmonary stenosis at 5 months and a follow-up surgical history of percutaneous balloon pulmonary valvuloplasty. Therefore, RASopathies, such as NS and LS, were investigated as potential diagnoses using genetic testing. This study was approved by the Institutional Review Board of Xinhua Hospital, Shanghai JiaoTong University School of Medicine and written informed consent was obtained from the parents of the patient. Peripheral blood was collected for

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Key words: LEOPARD syndrome, mutation analysis, Chinese

Table I. Primers of the *PTPN11* gene.

Primer name	Forward primer sequence	Reverse primer sequence	Product size (bp)	Annealing (˚C)
PTPN11-E01	GCCAGCCCGATGTGACCGAG	CTGGAGGGCGAGGGGACGAG	245	64.0
PTPN11-E02	ACTCTGCTCATAATGCGTCT	ACTTCTATGACCTGCTCCAA	452	55.0
PTPN11-E03	TCCTTGGGTTTCTTTCAACA	AGTCATACACAGACCGTCAT	392	53.0
PTPN11-E04	CCCTTGGAGGAATGTGTCTA	GTGTTTGTCCTCTTCCAGCA	552	57.0
PTPN11-E05	TCCCAGGCTGAAGCACAGTTG	GAAGCTGCAATGGGTACATGGAG	677	62.4
PTPN11-E06	CCTCTGTCCGTGCCTTTATG	ACTCACTGCCAACTCCCTTC	441	59.0
PTPN11-E07	TTCTGTGACTCTTTGACACGT	GATTATTTTGGAAACTGCTTG	291	53.0
PTPN11-E08 + 9	TGAATGAACAAAACTTGGAC	CACCAAGGAATAACATAATCA	625	51.0
PTPN11-E10	AACCTAACAGATGCGAAACAG	GATGAGGGCAGGAACACTAC	478	57.0
PTPN11-E11	GCCCAAAAGGAGACGAGTTC	TGGGTAGGTAAAAGCAAGCC	397	57.0
PTPN11-E12	AATGGCTTGGTTTTGAGTCT	TGTAAACAAGGTCAGGTGGC	414	55.0
PTPN11-E13	GAATCCTGACTTCTGCCACT	CAAGAGAATGAGAATCCGCA	405	57.0
PTPN11-E14	TTGGTTCGGTACAGTAAGTT	AGTCACAGATACACTAACAG	526	53.0
PTPN11-E15	GCGTTATTTCACTTCTGCCT	TTAACCAATAGAGCACTTGCA	337	55.0

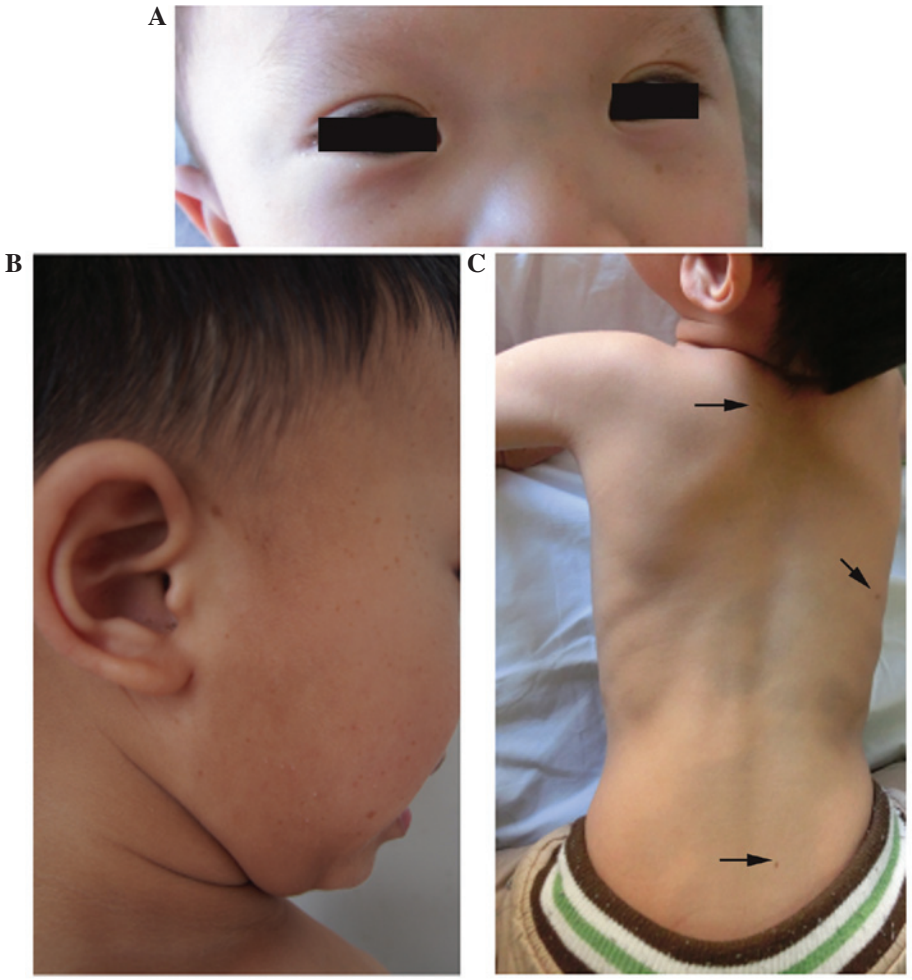

Figure 1. Clinical appearance of the patient in this study. The patient presented with (A) ocular hypertelorism, (B) a few freckle-like lesions mostly on the face from birth, and (C) developed gradually at 4 years old, as well as scattered café-au-lait spots (Arrows indicate the position) over the trunk.

DNA extraction using a TIANamp Blood DNA kit (Beijing Tiangen Biochemical Co., Ltd., Tiangen, China).

DNA sequencing. Primers flanking all coding exons and intron-exon boundaries of *NF1*, *SPRED1* and *PTPN11* were designed by software Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA) (Table I). The extracted genomic DNA (gDNA) samples were amplified by polymerase chain reaction (PCR). Thermal cycling conditions were as follows: Denaturation at 94˚C for 5 min; 31 cycles

Table II. Review of patients with LEOPARD syndrome with Thr468Met mutation in literature.

			Major clinical features								
Reference	Gender/age (years)	Population	Cutaneous features	Dysmorphic face	Skeletal anomalies	Hearing loss	Tumors	Pulmonary stenosis	HCM	Other cardio-vascular anomalies	Ref no.
Present study	M/5	Chinese	CLS AL	+	-	-	-	+	-	-	
Lin *et al* 2009	M/8	Chinese	ML	+	+	-	-	-	+	-	6
	M/8	Chinese	ML	+	+	-	-	-	+	-	
	F/7	Chinese	ML	+	+	-	-	+	-	-	
Digilio *et al* 2002	F/12.8 y	Italian	CLS ML	+	-	-	-	-	-	-	18
	M/15	Italian	CLS ML	+	+	-	-	-	-	+	
	F/15.1	Italian	CLS ML	+	+	-	-	-	-	+	
	F/39	Italian	CLS ML	+	+	-	-	-	-	-	
	F/8.9	Italian	CLS ML	+	-	-	-	-	-	+	
	F/3	Italian	CLS	+	+	-	-	+	+	-	
	M/4.9	Italian	CLS	+	+	-	-	-	+	+	
Sarkozy *et al* 2004	?/4	Italian	-	+	?	-	-	-	-	-	14
	?/34	Italian	ML	+	?	-	-	-	+	-	
Limongelli *et al* 2008	?/13	Italian	CLS ML	+	?	?	-	+	-	+	10
	?/1	Italian	ML	+	?	?	-	-	-	+	
	?/9	Italian	CLS ML	+	?	?	-	-	-	+	
	?/4	Italian	ML	+	?	?	-	-	-	+	
Santoro *et al* 2014	M/8	Italian	CLS AL	+	+	-	-	-	-	+	9
	M/12		CLS ML								
	M/50	Italian	CLS ML	+	+	+	-	-	+	+	
Carcavilla *et al* 2011	M/4	Spanish	CLS AL	+	-	-	-	-	+	+	11
	M/9	Spanish	CLS AL	+	-	-	-	-	+	-	
	M/16	Spanish	CLS AL	+	-	-	-	-	+	+	
Carcavilla *et al* 2013	M/49	Spanish	ML	+	-	-	-	-	+	-	13
	F/3	Spanish	CLS ML	+	+	-	-	+	-	-	
	M/11	Spanish	CLS ML	+	-	-	-	-	+	-	
	M/4	Spanish	ML	+	+	-	-	-	+	-	
	M/1.11	Spanish	-	+	-	-	-	+	-	-	
	F/0.8	Spanish	CLS	+	-	-	-	+	-	-	
	F/2	Spanish	-	+	-	-	-	-	-	-	
	M/14	Spanish	-	+	+	-	-	-	+	-	
Rankin *et al* 2013	M/23	British	CLS ML	+	+	-	+	-	-	-	15
Keren *et al* 2004	Mean: 19	French	ML (7/7) CLS (3/7)	(4/7)	(5/7)	(0/6)	?	(2/7)	(4/7)	(4/7)	12
Total (38)			CLS (23/38)	(35/38)	(19/32)	(1/34)	(1/31)	(9/38)	(17/38)	(16/38)	

+, positive result; -, negative result; ?, unclear result; M, male; F, female; CLS, café-au-lait spots; ML, multiple lentigines; AL, atypical lentigines; HCM, hypertrophic cardiomyopathy.

of denaturation at 94˚C for 30 sec, annealing for 30 sec at a temperature determined by the primers of each fragments and extension at 72˚C for 1 min; followed by extension at 72˚C for 1 min and an extension of 4˚C for 5 min. The experiment was repeated 10-20 times After PCR, products were purified with AxyPrep DNA Gel Extraction Kit (Axygen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Sanger sequencing was conducted using an ABI PRISM 3730 automated sequencer (Applied Biosystems; Thermo Fisher Scientific Inc.) to identify the mutation in the proband and verify it in his unaffected family members. When no pathogenic mutations were found in *NF1* by Sanger sequencing, gDNA samples of the patient were further analyzed using multiplex ligation-dependent probe amplification (MLPA) kits P122, P081 P082 and P295 (MRC-Holland,

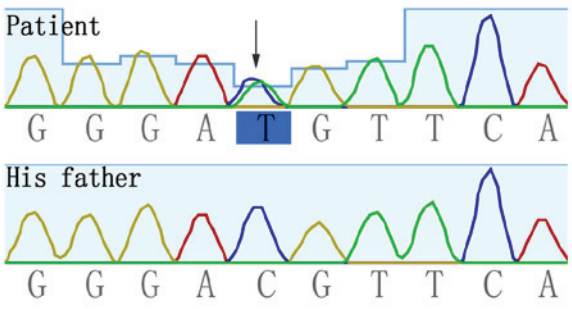

Figure 2. Sequencing results of *PTPN11* by Geneious, version 5.6.7. A heterozygous missense mutation c.1403C>T (p.Thr468Met) in *PTPN11* was revealed in the proband and was absent in his unaffected father. Arrows indicate site of mutation. cDNA reference sequence: NM_002834.2.

Amsterdam, The Netherlands) as previously described (7,8). These kits were able to detect deletions/duplications involving single or multiple exons or the entire *NF1* and *SPRED1* genes. Sequencing results were analyzed using Geneious (version 5.6.7; Biomatters Ltd., Auckland, New Zealand). Full details on the use of Geneious are available on the website (https://support.geneious.com/home). Identified mutations were determined by comparing with the reported cDNA reference sequences (NM_000267.3 for *NF1*, NM_152594.2 for *SPRED1* and NM_002834.2 for *PTPN11*).

Moreover, the literature was reviewed for the reported LS cases with p.Thr468Met mutation to determine the phenotypes of different LS patients with the same mutation (6,9-15). The Pubmed database was searched using the term 'PTPN11 mutation', and all studies investigating LS cases were downloaded prior to the selection of LS cases with a p.Thr468Met mutation.

Results

Sanger sequencing. Sanger sequencing and MLPA analyses for *NF1* and *SPRED1* did not identify any pathogenic mutations. However, a common heterozygous missense mutation c.1403C>T (p.Thr468Met) in *PTPN11* was identified in the proband and was absent in his unaffected parents (Fig. 2).

Discussion

PTPN11 mutations can cause LS and NS. Currently, there are twelve missense mutations in *PTPN11* that are known to be responsible for LS: Tyr279Cys/Ser, Ala461Thr/Ser, Gly464Ala, Thr468Met/Pro, Arg498Leu/Trp, Gln506Pro and Gln510Glu/Pro (3,5,16,17). Mutation loci are important in the pathogenic mechanisms of NS and LS. The genetic discrimination between these two syndromes is primarily dependent on LS-related mutants all located at a PTP domain (amino acid residues 221-524) (18), exerting a dominant negative effect on PTPN11 (19). Moreover, LS-associated loss-of-function mutations in *PTPN11* result in sustained extracellular signal-regulated kinases 1/2 activation by enhanced specific substrate dephosphorylation and cause hypertrophic cardiomyopathy by dysregulating mechanistic target of rapamycin signaling (20-24). Conversely, NS was associated with excessive PTPN11 activity by gain-of-function changes predominantly located at the N-SH2 domain (amino acid residues 3-104) (25).

Typical lentigines were flat, black-brown hyperpigmented macules that appeared all over the body (primarily on the face, neck and upper part of the trunk) during late childhood (Table II), and increased in number and darkened in color with age (2,3). However, the present case presented with light-brown freckle-like lesions (probably atypical lentigines) predominantly on the face since birth, which developed gradually to the current state at 4 years old. In consideration of the presence of several CALS, the most common and relevant disorders NF1 and NF1-like syndrome (i.e., Legius syndrome) were initially suspected. Following exclusion by molecular genetic testing, although there was no diffuse pattern of lentigines, the DNA was sequenced for mutations in the second most common pathogenic gene of RASopathies, *PTPN11*. Finally, a mutation in *PTPN11* that accounts for NS and LS was identified. While patients with NF1 usually have >6 CALS (>0.5 cm in childhood, >1.5 cm in puberty), this study indicated that children with light brown scattered freckling on the face, several CALS and congenital cardiac defects such as pulmonary stenosis or hypertrophic cardiomyopathy should be considered for a diagnosis of LS or NS. Moreover, *PTPN11* should analyzed in these patients.

A previous genotype-phenotype study suggested that hypertrophic cardiomyopathy is characteristic of *PTPN11* mutation-positive LS patients (13), and was particularly associated with mutations in exon 7 and exon 12 (26), and cardiovascular anomalies were also prevalent in previous cases (Table II). Therefore, a long-term cardiac evaluation regarding cardiomyopathy should be concerned in our case. Moreover, overactive RAS signals can result in cell growth and division, ultimately leading to tumors. Therefore, we still should pay attention to the tumor risk of 'RASopathies' (27), and the potential tumor spectrum of LS, such as hematologic malignancies (28), medulloblastoma (15), which have been demonstrated in numerous LS cases.

In conclusion, the present study presents a young Chinese patient with LS with CALS and atypical lentigines who was successfully diagnosed by a series of molecular genetic testing. Considering that two Taiwanese LS cases harbored an identical mutation, Thr468Met is likely to be a mutation hotspot in the Chinese population. Generally, children with NS or NF1 are prone to developing malignancies, while the prognosis of LS is relatively favorable only if no severe cardiac defects and adverse cardiac events, which underline the necessity of definite diagnosis by genetic methods along with subsequent early prevention of congenital cardiac defects. A more recent follow up of the patient showed nearly normal pulmonary artery pressure and no obvious electrocardiogram abnormalities; however, regular monitoring concerning potential complications are required.

Acknowledgements

This study was supported by a grant from the Ph.D. Programs Foundation of Ministry of Education of China (grant no. 20130073120014) and a grant from the Natural Science Foundation of Shanghai Jiaotong University School of Medicine (grant no. 13XJ10023).

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