LEOPARD Syndrome: Clinical Features and Gene Mutations

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Key Words

BRAF • Gene • LEOPARD • Mutation • *PTPN11* • *RAF1* • RAR/MAPK pathway

Abstract

The RAS/MAPK pathway proteins with germline mutations in their respective genes are associated with some disorders such as Noonan, LEOPARD (LS), neurofibromatosis type 1, Costello and cardio-facio-cutaneous syndromes. LEOPARD is an acronym, mnemonic for the major manifestations of this disorder, characterized by multiple lentigines, electrocardiographic abnormalities, ocular hypertelorism, pulmonary stenosis, abnormal genitalia, retardation of growth, and sensorineural deafness. Though it is not included in the acronym, hypertrophic cardiomyopathy is the most frequent cardiac anomaly observed, representing a potentially life-threatening problem in these patients. *PTPN11*, *RAF1* and *BRAF* are the genes known to be associated with LS, identifying molecular genetic testing of the 3 gene mutations in about 95% of affected individuals. *PTPN11* mutations are the most frequently found. Eleven different missense *PTPN11* mutations (Tyr279Cys/Ser, Ala461Thr, Gly464Ala, Thr468Met/Pro, Arg498Trp/Leu, Gln506Pro, and Gln510Glu/Pro) have been reported so far in LS, 2 of which (Tyr279Cys and Thr468Met) occur in about 65% of the cases. Here, we pro-

vide an overview of clinical aspects of this disorder, the molecular mechanisms underlying pathogenesis and major genotype-phenotype correlations.

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RAS proteins (HRAS, NRAS and KRAS) are small guanosine-binding proteins, which act as signal switch molecules involved in transmitting extracellular inputs downstream into the cell. The name 'RAS' is an abbreviation of 'Rat sarcoma', reflecting the way the first members of the protein family were discovered. RAS is activated by guanine nucleotide exchange factors that release GDP and allow GTP binding, prompting a conformational change and consequent downstream effects. RAS-GTP activates a cascade of downstream kinases: the MAPK pathway. The RAS/MAPK pathway involves RAF proteins, particularly RAF1 and BRAF, which are MAP kinase kinase kinases (MAP3K) that are activated after binding to RAS-GTP and then phosphorylate and activate MAPK kinases, MEK1 and MEK2, which, in turn, phosphorylate and activate the MAPKs, ERK1 and ERK2. This addition of phosphate groups to a neighboring protein acts as an 'on' or 'off' switch. When one of the proteins in the pathway is mutated, it can be stuck in the 'on' or 'off' position, which is a necessary step in the develop-

Fig. 1. Schematic diagram showing the RAS-MAPK signal transduction pathway. Receptor tyrosine kinases (RTKs) are the high-affinity cell surface receptors for many polypeptide growth factors, cytokines, and hormones. SHP2 is a ubiquitous protein-tyrosine phosphatase (PTP) which plays major biological functions in response to various growth factors, hormones or cytokines. Activation of SHP2 occurs upon interaction of phosphotyrosine residues from substrate or another protein with SH2 domains in these PTPs, promoting GRB2/SOS recruitment through direct binding to GRB2 (growth factor receptor-bound protein 2). GRB2 binds through its SH3 domains to the guanine nucleotide exchange factor (SOS), activating it. Activated SOS then promotes the removal of GDP from a member of the RAS subfamily (most notably HRAS or KRAS). RAS can then bind GTP and become active. Activated RAS activates the protein kinase activity of RAF1 kinase. RAF1 kinase phosphorylates and activates the

MAPK/ERK kinases MEK1 and MEK2. MEK1 and MEK2 then phosphorylate and activate the ERK1 and ERK2 MAPKs. Activated ERKs translocate to the nucleus, where they phosphorylate and regulate various transcription factors leading to changes in gene expression. BRAF is also a member of the RAF kinase family of serine/threonine-specific protein kinases. BRAF is activated following GTP-bound RAS binding, and phosphorylates and activates the mitogen-activated protein kinase kinases (MEK1 and MEK2). Mitogen-activated protein kinase (MAPK) cascades are key signaling pathways involved in the regulation of normal cell proliferation, survival and differentiation. The syndromes and their mutated proteins are as indicated: red asterisk: LEOPARD syndrome; green asterisk: Noonan syndrome; blue asterisk: Cardio-facio-cutaneous syndrome (CFCS); brown asterisk: Costello syndrome (CS); violet asterisk: autoimmune lymphoproliferative syndrome.

ment of many cancers or syndromes. The molecular targets of the MAPK family are usually nuclear transcription factors, cytoplasmic factors responsible for the initiation of translation and apoptosis regulators [Raman et al., 2007]. All of them are critically involved in cell pro-

liferation, differentiation, motility and apoptosis controlling morphology determination, organogenesis and growth (fig. 1).

Recent advances in molecular genetic research have led to the definition of a new group of genetic syndromes

named as the RAS-MAPK pathway disorders or 'RASopathies'. These syndromes, also known as neuro-cardio-facial-cutaneous syndromes (NCFCs), are a result of abnormalities at different steps of this signaling cascade. The RASopathies are caused by germline mutations in genes that encode protein components of the RAS/MAPK pathway. The vast majority of these mutations result in increased signal transduction down the RAS/MAPK pathway, but usually to a lesser extent than somatic mutations associated with oncogenesis. Their common underlying pathogenetic mechanism brings about significant overlap in phenotypic features with characteristic facial anomalies, cardiac defects, cutaneous and ocular abnormalities, growth deficit, varying degrees of neurocognitive impairment and an increased risk of developing cancer. However, in contrast with somatic mutations in RAS/MAPK components that are observed in various malignancies, germline mutations appear to confer only mild, if any increased risks of cancer development.

The RAS/MAPK pathway proteins with germline mutations in their respective genes are associated with Noonan (NS), LEOPARD (LS), gingival fibromatosis type 1, neurofibromatosis type 1 (NF1), capillary malformation-arteriovenous malformation, Costello (CS), autoimmune lymphoproliferative, cardio-facio-cutaneous (CFCS) and Legius syndromes [Gelb and Tartaglia, 2006; Denayer et al., 2008; Tidyman et al., 2009]. Significant locus heterogeneity exists for many of the RASopathies and mutations in the *PTPN11*, *SOS1*, *KRAS*, *NRAS*, *HRAS*, *RAF1*, *BRAF*, *SHOC2*, *MEK1*, *MEK2* and *CBL* genes have been documented to underlie these disorders [Tartaglia et al., 2011; Zenker, 2011; Fahrner et al., 2012] (fig. 1).

Background

Zeisler and Becker [1936] first described LS in a 24-year-old woman with progressive generalized lentiginos increasing in number from birth to puberty, hypertelorism and pectus carinatum. The first familial cases were reported in twins by Rosen [1942]. Subsequent communications added new findings in isolated patients or families. Moynahan first documented the association of the syndrome with cardiac abnormalities and short stature in 1962. A few years later Gorlin et al. [1969] reviewed this disorder and coined, for the first time, the LEOPARD acronym, mnemonic for the major manifestations of this disorder, characterized by multiple lentiginos, electrocardiographic abnormalities, ocular hypertelorism, pul-

monic stenosis, abnormal genitalia, retardation of growth, and sensorineural deafness. After that, Polani and Moynahan [1972] gave a full report of 8 patients and their families. They were impressed with the occurrence of left-sided obstructive cardiomyopathy and that none of their patients was deaf. They proposed the designation 'progressive cardiomyopathic lentiginosis' for this disorder. Digilio et al. [2002] screened for mutations in the *PTPN11* gene in 9 patients with LS and 2 children with NS who had multiple café-au-lait spots. In 10 of the 11 patients, they found 1 of 2 mutations: Tyr279Cys or Thr468Met. Actually, *PTPN11*, *RAF1* and *BRAF* are the genes known to be associated with LS, identifying molecular genetic testing of the 3 gene mutations in about 95% of affected individuals.

Epidemiology

The population prevalence of LS is not known. Although no epidemiologic data are available, the syndrome seems to be rare. Within the group of the so called NCFCs, LS is probably the second most common disorder after NS [van der Burgt, 2007]. However, many cases are under- or misdiagnosed as many of its features are mild and the correct diagnosis may be missed in the absence of lentiginosis.

Clinical Features

The diagnosis of LS (OMIM 151100) is made on clinical grounds by observation of key features. However, to provide a valid overview on symptoms and features of LS we should not overestimate the reports published in the pre-molecular era, because these cohorts may consist of heterogeneous diseases. Instead, anomalies observed in molecularly confirmed cases should be regarded as more reliable.

Facies

Dysmorphic features can occur or be only mildly expressed in the newborns and infants. Hypertelorism is virtually present in all cases, flat nasal bridge and dysmorphic ears in about 87% of the patients. Additionally, less frequently, features include palpebral ptosis, thick lips and low-set ears with overfolded helix (50%), large and everted ears, pterygium colli or redundant neck skin

Fig. 2. **A** Dysmorphic features showing hypertelorism, flat nasal bridge, mild palpebral ptosis, thick lips and low-set ears with diffuse lentiginosis in the upper part of the trunk and face. **B** The patient has hearing aids due to sensorineural deafness.

(37%). In relation to adult patients, they usually manifest hypertelorism, palpebral ptosis, low-set ears, deep nasal-labial folds and premature skin wrinkling (fig. 2) [Somerville and Bonham-Carter, 1972; Sarkozy et al., 2008].

Skin

Lentigines are the most prominent manifestation of LS and are present in more than 90% of the patients; however, an absence of the feature does not exclude the diagnosis of the syndrome. Lentigines are brown macules, usually 2–8 mm in diameter, but sometimes larger. They are usually most heavily concentrated on the upper part of the trunk and neck (fig. 3A), but they are also often present on the face, scalp, limbs, palms, soles and genitalia. Lentigines can also appear in the sclera but spare the mucosa. Lentigines may be present at birth but usually appear during childhood, and they become more numerous and darker with age, independently from sun exposure. They are distinguishable from freckles which appear later than lentigines and are induced by exposure to sunlight.

Biopsy of a lentigo reveals an increased number of melanocytes per skin area unit, accumulation of pigments in the dermis as well as the deeper layers of epidermis and prominent rete ridges (fig. 4) [Voron et al., 1976]. Café-au-lait spots are also observed, alone or in association with lentigines, in up to 70–80% of the patients [Digilio et al.,

Fig. 3. **A** Numerous lentigines on the skin of the chest. **B** Lentigines and a large café-au-lait spot on the neck.

Fig. 4. Histology of a lentigo biopsy (hematoxylin-eosin stain; 4× original magnification) showing hyperpigmentation of the basal membrane, with increased numbers of melanocytes and slight acanthosis, diffuse lymphohistiocytic infiltrate with some scattered melanophages.

2006] and are similar to those found in NF1, although not in a number that fulfils criteria for NF1. They usually precede the appearance of lentigines, being present from the first months of life and appearing much darker in the dark skinned individuals (fig. 3B) [Sarkozy et al., 2008]. Hypopigmented macules may appear in areas were len-

Fig. 5. A Four-chamber apical echocardiographic view showing an asymmetric HCM involving the interventricular septum (arrowhead). LV: left ventricle, RV: right ventricle, LA: left atrium, RA: right atrium. **B** Twelve-lead electrocardiogram showing high R waves in the right precordial leads with ST depression in relation to HCM and systolic ventricular overload.

tigines previously existed, although malignant transformation of the lentigines has not been reported [Kim et al., 2011]. However, Seishima et al. [2007] reported a case of a malignant melanoma in a woman with LS, a germline *PTPN11* mutation and a somatic *BRAF* mutation from an apparently nevi-free region at an old age. Furthermore, onychodystrophy, hyperelastic skin, interdigital webs and patches of hair loss on the scalp can be observed [Shamsadini et al., 1999].

Heart

Though it is not included in the acronym, hypertrophic cardiomyopathy (HCM) is the most frequent anomaly observed, representing a potentially life-threatening problem in these patients. HCM, which is generally asymmetric and progressive and commonly involves the intra-ventricular septum, is detected in up to 80% of the patients with a cardiac defect and may associate with significant left ventricular outflow tract obstruction in up to 40% of the cases [Limongelli et al., 2007; Sarkozy et al., 2008; Martínez-Quintana and Rodríguez-González, 2011a]. Clinical findings of left ventricular hypertrophy are similar to those found in familial HCM (fig. 5A) [Limongelli et al., 2006]. However, despite frequent cardiac involvement, most patients are asymptomatic. In relation to left ventricular diastolic function, the most frequent diastolic patterns in patients with left ventricular hyper-

trophy are abnormal diastolic function, followed closely by abnormal relaxation and more rarely by a restrictive filling pattern [Sarkozy et al., 2008].

Meanwhile, electrocardiographic abnormalities and pulmonary stenosis are included in the acronym. Most patients with electrocardiographic abnormalities have left ventricular hypertrophy (89%) in relation to HCM (fig. 5B) and right ventricular hypertrophy (39%) and right atrial enlargement, followed by the gradual development of right bundle branch block, in relation to pulmonary stenosis illustrating the progressive nature of the underlying heart disease [Limongelli et al., 2007]. Other types of rhythm or conduction abnormalities include paroxysmal atrial tachycardia, atrial fibrillation, prolonged PR interval, left anterior and posterior hemiblocks, bundle branch block or complete atrioventricular block.

Pulmonary stenosis is less frequent than left ventricular hypertrophy in patients with LS. Limongelli et al. [2006] observed pulmonary stenosis in 23% of the patients with LS (valvular, isolated infundibular, valvular and infundibular or with a dysplastic pulmonary valve). In contrast, abnormalities of the aortic valve (23%; e.g. mild aortic regurgitation, discrete subaortic stenosis or aortic valve dysplasia) and mitral valve (38%; e.g. mitral valve prolapse) were more frequent. Furthermore, coronary abnormalities, such as dilatation of the coronary arteries, abnormalities of the left ventricular shape, segmental wall motion, apical aneurysm of the left ventricle,

non-compacted left ventricle, isolated left ventricular enlargement, atrioventricular septal defects and ventricular septal defects were found.

Long-term prognosis seems benign in patients with LS with only mild cardiac abnormalities. Mild pulmonary stenosis has a good prognosis, while valvulotomy or valvuloplasty is indicated for severe stenosis of the pulmonary valve. In contrast, patients with left ventricular hypertrophy may develop significant symptoms and arrhythmias during follow-up. Newborns, infants and children suspected of having LS need a careful cardiac evaluation, including an echocardiogram. Evaluation of the heart in early infancy does not exclude the possibility that an affected child will develop more severe HCM later in childhood. In fact, many patients with LS develop HCM during the toddlerhood. This suggests that serial evaluations during this period are indicated. The onset of HCM usually precedes multiple lentigines, but the hypertrophic process may start or often worsen in parallel with the appearance of lentigines [Limongelli et al., 2008].

Accordingly, treatment in LS patients with ventricular hypertrophy may follow familial HCM algorithms. A beta-blockade or calcium channel blocker therapy is indicated in the presence of a significant left ventricular obstruction. The surgical resection of the left ventricular outflow tract or alcohol septal ablation is indicated in more severe cases or if there is no significant improvement with medical therapy. Previous studies failed to show the benefit of antiarrhythmic drugs, which were used for sudden cardiac death (SCD) prevention. Amiodarone can eliminate complex ventricular arrhythmias, but it does not protect against SCD. It would be used only in patients in whom implantable cardioverter-defibrillator implantation is not feasible. Regarding high-risk patients who have 2 or more risk factors such as family history of SCD (under 45 years old), unexplained syncope, septal thickness ≥ 30 mm, non-sustained ventricular tachycardia on holter (HR > 120 bpm) or abnormal blood pressure response during activity (non-elevation or drop in systolic pressure > 20 mm Hg during activity) [Zipes et al., 2006; Melacini et al., 2007], implantable cardioverter-defibrillator implantation is recommended for primary SCD prevention, with a 5% annual incidence of appropriate shocks [Adriaenssens et al., 2007].

Although treatment algorithms are similar between LS patients with ventricular hypertrophy and patients with familial HCM due to its analogy, without an evidence-based medicine, it is clear that HCM in LS is different in the pathophysiology and dynamics from ventricular hypertrophy of other causes.

Growth

Birth weight is usually normal or above the average in the newborns who develop growth retardation after birth until growth stops. Growth retardation (height below the 3rd centile) is observed in 25% of affected individuals, and final height in 85% of patients is below the 25th centile [Voron et al., 1976; Gorlin et al., 1990]. Growth retardation is not related to endocrine problems or systemic diseases. However, delayed or absent puberty may be contributory in some cases [Voron et al., 1976; Sarkozy et al., 2008].

There are studies indicating that affected NS patients with *PTPN11* gene mutations tend to exhibit mild GH resistance and relatively poor response to GH treatment [Binder et al., 2005; Ferreira et al., 2005]. However, this association has not been confirmed in a recent long-term survey [Noordam et al., 2008] and these studies do not include patients with LS which are functionally different from NS patients.

Skeletal Anomalies

Chest deformity including pectus carinatum or excavatum is the most frequently observed abnormality, been found in up to 75% of newborns [Digilio et al., 2006]. Mandibular prognathism, winging of the scapulae, scoliosis, joint hyperflexibility and other findings are less common [Sarkozy et al., 2008]. Rib anomalies, syndactyly, delayed development, cervical spine fusion and agenesis of permanent teeth or supernumerary teeth are also found. In old age, there is a tendency to develop thoracic kyphosis [Ho et al., 1989; Sarkozy et al., 2004].

Hearing Loss

Sensorineural hearing loss is a form of hearing loss in which sound is conducted normally through the external and middle ear but a defect in the inner ear or auditory nerve results in hearing loss. As the melanocyte participates in the formation of the inner ear, it is reported that this kind of deafness may be due to a primary abnormality in the neural crest [Polani and Moynahan, 1972]. Sensorineural deafness occurs in about 15–25% of patients with LS [Sarkozy et al., 2004], may be unilateral but can be profound. Most cases have deafness diagnosed at birth or during childhood, but some are reported to have developed it in adult life [Sarkozy et al., 2008]. The diagno-

sis of a sensorineural pattern hearing loss is made through audiometry, which shows a significant hearing loss without the 'air-bone gap' that is characteristic of conductive hearing disturbances. Sound discrimination may or may not be affected. Amplification of the sound with a hearing aid will help many people with sensorineural hearing loss. In some cases, limited hearing has been restored by cochlear implants, tiny devices implanted into the inner ear that translate sound waves into electrical impulses that are then transmitted to the auditory nerve.

Genital and Urinary Tract Anomalies

Unilateral or more likely bilateral cryptorchidism occurs in about 50% of males but hypospadias and genital hypoplasia are also frequent. Renal anomalies, including horseshoe kidney, are rare [Digilio et al., 2006]. All these features are distinctly more frequent in males but this predominance can reflect the involvement of visible genitalia and, therefore, be the result of easier diagnosis [Ruggieri et al., 2008].

Neurological Abnormalities

Mild learning difficulties are reported in about 30% of the cases, while mental retardation is rare and usually of mild degree [Sarkozy et al., 2008]. Visual and visuoperceptual abilities are commonly impaired because ocular movements and stereopsis are frequently abnormal [Alfieri et al., 2008]. Other uncommon neurological manifestations include nystagmus, hyposmia, autism [Watanabe et al., 2011], seizures, and abnormalities in electroencephalograms. Hypotonia is common in the newborn and can result in delayed psychomotor development [Digilio et al., 2006]. In patients with LS and neurologic abnormalities such as developmental delay or epilepsy, brain imaging is suggested as part of the diagnostic work-up [Liang et al., 2009].

Tumors

Mutations in *RAS* genes can lead to the production of permanently activated *RAS* proteins. This can cause unintended and overactive signaling inside the cell, even in the absence of incoming signals. As these signals result in cell growth and division, overactive *RAS* signaling can ultimately lead to cancer.

Germline mutations in *PTPN11* cause NS and LS, whereas somatic *PTPN11* mutations occur in several types of hematologic malignancies, most notably juvenile myelomonocytic leukemia [Tartaglia et al., 2003] and more rarely in solid tumors. In fact, *RAS* is the most common oncogene in human cancer and mutations that permanently activate *RAS* are found in 30% of all human tumors and up to 90% in certain types of tumors such as the pancreatic cancer [Saxena et al., 2008].

Although acute lymphoblastic leukemia [Laux et al., 2008], acute myeloid leukemia [Uçar et al., 2006] and multiple granular cell tumor [Schrader et al., 2009] have been reported in patients with LS and *PTPN11* mutation, the association is inconclusive [Kratz et al., 2011]. In fact, single case reports in the literature or translating the knowledge gathered in NS to LS patients do not reliably document an increased risk of malignancy in LS patients. However, and because the predisposition of malignancy in LS remains debatable, LS patients with *PTPN11* mutations should be closely monitored for malignancy, particularly during their childhood.

Mutations

LS is a genetically heterogeneous disorder caused by mutations in 3 loci: the *PTPN11* (protein-tyrosine phosphatase, non-receptor type 11) gene on chromosome 12q24 (OMIM 176876), the *RAF1* (v-Raf-1 murine leukemia viral oncogene homolog 1) gene on chromosome 3p25.2 (OMIM 611554) and the *BRAF* (v-Raf murine sarcoma viral oncogene homolog B1) gene on chromosome 7q34 (OMIM 613707). Molecular genetic testing of these 3 loci identifies mutations in about 95% of affected individuals.

LS with *PTPN11* Mutation

PTPN11 encodes tyrosine-protein phosphatase non-receptor type 11 (also known as protein-tyrosine phosphatase non-receptor type 11, or SHP-2), a widely expressed cytoplasmic tyrosine phosphatase found in embryonic and adult tissues. The protein encoded by this gene is a member of the protein-tyrosine phosphatase (PTP) family. PTPs are a highly polymorphic set of molecules having a role in regulating the responses of eukaryotic cells to extracellular signals. SHP2 is required during development and regulates cell migration, proliferation, survival and differentiation through multiple signaling pathways, including the *RAS*-MAPK cascade,

elicited by growth factors, cytokines, hormones, and extracellular matrix [Feng, 1999; Neel et al., 2003].

SHP2 contains a tandem arrangement of 2 SH2 domains, at the N-terminus (N-SH2 and C-SH2), a single catalytic domain (PTP) and a C-terminal tail containing 2 tyrosyl phosphorylation sites and a proline-rich stretch whose function is not well-characterized [Hof, 1998; Taglia et al., 2006]. SHP2 has inactive and active conformations that are regulated through a molecular switching mechanism. In the inactive state, the backside of the N-SH2 domain forms a loop and is wedged into the PTP domain, blocking the catalytic site. When SHP2 binds phosphotyrosyl-containing proteins at separate sites on the N- and C-SH2 domains, a conformational change releases the N-SH2 domain from the PTP domain, which makes the PTP catalytic cleft available. Kontaridis et al. [2006] examined the enzymatic properties of mutations in *PTPN11* causing LS and found that LS mutants, in contrast to the activating mutations that cause NS and neoplasia, are catalytically defective and act as dominant-negative mutations interfering with growth factor and cytokine-mediated RAS/ERK/MAPK signal flow, perturbing cell proliferation [Hanna et al., 2006; Kontaridis et al., 2006; Whelan et al., 2012]. In fact, a substantial decrease in phosphatase activity has been documented for some recurrent mutations [Hanna et al., 2006; Kontaridis et al., 2006]. Moreover, results from expression studies of mutants in cell culture were consistent with a dominant-negative mechanism.

In approximately 85% of the patients with a definite diagnosis of LS, a missense mutation is found in the *PTPN11* gene. The *PTPN11* gene is divided in 16 exons. All the mutations occur in exons that code for the PTP domain. Exon 1 contains the 5' untranslated region and the translation initiation ATG, and a few additional codons. Exon 15 contains the stop codon and exon 16 contains a major portion of the 3' untranslated region. Eleven different missense *PTPN11* mutations (Tyr279Cys/Ser, Ala461Thr, Gly464Ala, Thr468Met/Pro, Arg498Trp/Leu, Gln506Pro, and Gln510Glu/Pro), have been reported so far in LS, 2 of which (Tyr279Cys and Thr468Met) occur in about 65% of the cases.

In relation to HCM, which represents a potentially life-threatening problem in LS patients, Digilio et al. [2002] found the Tyr279Cys mutation in 3 patients and the Thr468Met mutation in 4 patients from a total of 9 patients. In those 3 patients with Tyr279Cys mutation only 1 of them had obstructive HCM. Legius et al. [2002], on the other hand, reported 3 cases with the Tyr279Cys mutation, all of them having non-obstructive HCM.

Meanwhile, Limongelli et al. [2008] studied 24 LS patients with HCM. Sixteen of them were found to have mutations in the *PTPN11* gene (only 1 of them had the Tyr279Cys mutation), 2 had mutations in the *RAF1* gene, and the mutations in 6 of them could not be found. Patients without *PTPN11* mutations showed a significantly higher frequency of family history of SCD and an increased incidence of bradyarrhythmias, episodes of supraventricular tachycardias, atrial fibrillation, and non-sustained ventricular tachycardia at 24-hour ECG monitoring. During follow-up, 2 patients with *PTPN11* mutations died suddenly.

Corresponding to induced pluripotent stem cells (iPSCs), Carvajal-Vergara et al. [2010] generated iPSCs derived from 2 unrelated LS patients who were heterozygous for the Thr468Met mutation in *PTPN11*. The iPSCs were extensively characterized and produced multiple differentiated cell lineages. These authors showed that in vitro-derived cardiomyocytes from LS iPSCs are larger, have a higher degree of sarcomeric organization, and have a preferential localization of NFATC4 (nuclear factors of activated T cells) in the nucleus when compared with cardiomyocytes derived from human embryonic stem cells or wildtype iPSCs derived from a healthy brother of 1 of the LS patients. These features correlate with a potential hypertrophic state and provide molecular insights into signaling pathways that may promote the disease phenotype. Carvajal-Vergara et al. [2010] also showed that basic fibroblast growth factor treatment increased the phosphorylation of ERK1/2 levels over time in several cell lines but did not have a similar effect in the LS iPSCs despite higher basal phosphorylated ERK levels in the LS iPSCs compared with the other cell lines. Although LS mutations appear to deactivate the gene product (SHP2 protein) [Jopling et al., 2007], these data reinforce the role of increased RAS signaling in cardiomyocyte hypertrophy pathogenesis and suggest that LS pathogenesis should not only be related to a reduced RAS signal transduction [Muslin, 2005]. In fact, it has also been proposed that the residual catalytic activity in the LS mutant SHP2 protein is sufficient to produce a gain-of-function-like phenotype due to dysregulation of the protein causing continuous MAPK pathway activity during development [Oishi et al., 2009].

To date, the exact clinical course of LS patients with HCM is still unclear, especially because many of the affected individuals described in the literature are children and there is not a clear risk figure available based on a follow-up study of a sufficient-size patient cohort. However, anecdotal reports provide enough evidence to state

that long-term prognosis seems benign in LS patients with only mild cardiac abnormalities, whereas HCM in LS is indeed associated with a risk of fatal cardiac events as seen in primary HCM [Martínez-Quintana and Rodríguez-González, 2011b].

LS with *RAF1* Mutation

The *RAF1* gene encodes a MAP3K protein (RAF1 protein), which functions downstream of the RAS family of membrane-associated GTPases to which it binds directly. RAS protein family members belong to a class of proteins called small GTPases, and are involved in transmitting signals within cells [Malumbres and Barbacid, 2003]. Once activated, the cellular RAF1 protein can phosphorylate to activate the dual specificity protein kinases MEK1 and MEK2, which in turn phosphorylate to activate the serine/threonine-specific protein kinases, ERK1 and ERK2. Activated ERKs are pleiotropic effectors of cell physiology and play an important role in the control of gene expression involved in the cell division cycle, apoptosis, cell differentiation and cell migration.

The human *RAF1* gene comprises 17 exons and has 3 conserved regions (CR). CR1, exons 2–5, contains a RAS-binding domain and a cysteine-rich domain, CR2 lies in exon 7 and CR3, which spans exons 10–17, contains the kinase domain and its regulatory element, the activation segment. The gene is highly regulated with numerous serine and threonine residues that can be phosphorylated, resulting in activation or inactivation. RAF1 pathologic allelic variants causing LS are Ser257Leu and Leu613Val.

Pandit et al. [2007] analyzed the *RAF1* gene in 6 individuals with LS who did not have mutations in the *PTPN11* gene, and they identified 2 different missense mutations in 2 unrelated patients, a 35-year-old woman and a 43-year-old woman. These 2 patients carried the Leu613Val and Ser257Leu changes, disclosing a distinct LS phenotype with multiple lentigines, HCM and delayed puberty. These authors investigated the functional effect of different *RAF1* mutants, including the Leu613Val change, and showed that those associated with HCM had increased kinase activity and enhanced ERK activation.

LS with *BRAF* Mutation

The *BRAF* gene is ubiquitously expressed and encodes a protein (BRAF protein) belonging to the RAF/mil family of serine/threonine protein kinases that is activated

following GTP-bound RAS binding and phosphorylates as well as activates the mitogen-activated protein kinase kinases (MEK1 and MEK2). Therefore, this protein plays a role in regulating the MAP kinase/ERK signaling pathway which affects cell division, differentiation and secretion.

Human *BRAF* gene comprises 18 coding exons and pathologic allelic *BRAF* variants causing LS are Thr241Pro and Leu245Phe. Germline mutations in this gene are associated with CFCS and somatic mutations are related with various cancers, including non-Hodgkin lymphoma, colorectal cancer, malignant melanoma, thyroid carcinoma, non-small cell lung carcinoma, and adenocarcinoma of the lung.

Sarkozy et al. [2009] identified a heterozygous de novo Thr241Pro mutation in a patient with LS and *BRAF* mutation. The patient had poor growth, craniofacial anomalies, short and webbed neck, mitral and aortic valve dysplasia, cognitive deficits, neonatal hypotonia, sensorineural deafness, and seizures. Other features included thorax defects, delayed puberty, reduced bone density, and fibrous cystic lesions of the pelvis. The skin showed hyperkeratosis, café-au-lait spots, multiple nevi, and dark colored lentigines that were spread on the whole body including the palms and soles. In vitro functional expression studies showed that the Thr241Pro mutant protein did not show transforming ability to cells in vitro, although there was a slight increase in MEK phosphorylation, suggesting activation of the downstream MAPK pathway.

Furthermore, Koudova et al. [2009] reported a patient with LS and normal intelligence who was found to carry a novel sequence change in *BRAF*. The mutation Leu245Phe was demonstrated to be de novo without evidence of somatic mosaicism.

Diagnosis

Clinical Diagnosis. Voron et al. [1976] proposed diagnostic criteria for LS if the patient had multiple lentigines plus 2 of the other cardinal features or, in the absence of lentigines, 3 of the other cardinal features plus a first-degree relative with LS. Similarly, Digilio et al. [2006] reviewing the clinical characteristics of patients with LS confirmed by molecular study during the first year of life concluded that the diagnosis of LS in the first months of age can be clinically considered in patients presenting with 3 main features, that is, characteristic facial features (100%), HCM (87%) and café-au-lait spots (75%). Common facial features included hypertelorism (100%), malformed ears (87%) and low-set ears with overfolded helix

(50%). Seventy-five percent of patients had skeletal thorax anomalies.

Genetic Diagnosis. LS is caused by autosomal dominant germline mutations with complete penetrance and variable expressivity, accounting de novo mutations for more than half of the LS cases. In about 85% of the cases, a heterozygous missense mutation, a mutation in which a single nucleotide is changed resulting in a codon that codes for a different amino acid, is detected in exons 7, 12 or 13 of the *PTPN11* gene. Furthermore, missense mutations in the *RAF1* gene have been found in 2 out of 6 *PTPN11*-negative LS patients [Pandit et al., 2007; Sarkozy et al., 2008]. However, *RAF1* and *BRAF* mutations appear to be much less frequent than *PTPN11* mutations and, in contrast to *PTPN11* mutations, the *RAF1* and *BRAF* mutations that are described with the LS phenotype have also been observed with other phenotypes such as NS for the *RAF1* mutations and CFCS for the *BRAF* mutations.

To confirm and establish the diagnosis in a proband we should take into account the clinical and genetic findings. Molecular genetic testing can be used to confirm the diagnosis. The following order of testing is recommended: (1) *PTPN11* sequence analysis of coding exons 7, 12 and 13 (these exons encompass all the codons of the *PTPN11* gene identified to be mutated in LS thus far); (2) if no mutation is identified, sequence analysis of coding exons 6, 13 and 16 of *RAF1* and coding exons 6 and 11–17 of *BRAF*; (3) if no mutation is identified, sequence analysis of the remaining coding exons of *PTPN11*, *RAF1*, and *BRAF* [Gelb and Tartaglia, 2010].

Differential Diagnosis

The concept of NCFC syndrome, which includes some overlapping disorders such as NS (OMIM 163950), NF1 (OMIM 16220), CS (OMIM 218040), CFCS (OMIM 11515), and LS itself has been formulated in order to bring together a number of hereditary diseases that include a number of shared phenotypic features to differing degrees: craniofacial dysmorphism, delayed growth, mental retardation or learning difficulties, cardiac malformations (most commonly pulmonary valve stenosis and HCM), cutaneous anomalies, and in some cases predisposition to certain forms of malignant solid tumors and blood diseases associated at the pathophysiological level with deregulation of the RAS-MAP kinase cellular signaling pathways (fig. 1) [Bentires-Alj et al., 2006; Sarkozy et al., 2008]. While the majority of these diseases are readily distinguishable in clinical terms, none of them have

any pathognomonic signs, many different genes causing the same phenotype while identical mutations in the same gene may result in different clinical presentations.

NS consists of short stature, congenital heart defect, pectus deformities, developmental delay of variable degree, cryptorchidism which contributes to the reduced fertility, and characteristic facies including ptosis, downslanting palpebral fissures and pterygium colli. The phenotype of NS shows extreme variability, from mildly short stature with normal intelligence to severe congenital heart disease or HCM, or rarely severe learning disability. The prevalence of NS is estimated at between 1 in 1000 and 1 in 2500 people [van der Burgt, 2007]. It is an autosomal dominant condition, with variable expression, and is genetically heterogeneous, with mutations identified in *PTPN11*, *KRAS*, *NRAS*, *SOS1*, *RAF1*, and *BRAF* [Gelb and Tartaglia, 2010]. Mutations in exons 2–14 of *PTPN11* have been reported in approximately 50% of individuals with NS.

Not surprisingly, many of the phenotypic abnormalities in NS and LS are quite similar: for example, both are characterized by proportionate short stature, facial dysmorphism and skeletal anomalies. However, their cardiac phenotypes are quite distinct. Pulmonary valve stenosis with dysplastic leaflets and atrial/ventricular septal defects are the most prevalent cardiac defects in NS caused by *PTPN11* mutations [Burch et al., 1993]. By contrast, despite the fact that pulmonic stenosis is part of the LEOPARD acronym, the most common cardiac manifestation of this disorder is HCM. These data confirm that LS and NS are allelic conditions but that particular genotype-phenotype correlations exist with certain mutations in *PTPN11* leading to the pigmentary changes and higher prevalence of HCM observed in LS. In fact, the term Noonan-like syndrome with multiple lentigines is frequently used instead of LS.

LS overlaps substantially with NS, which leads to the conclusion of Kontaridis et al. [2006] who advocate that the pathogenesis of LS should be distinguished from NS by mutation analysis rather than clinical presentation. Previous studies have examined the enzymatic properties of mutations in *PTPN11*. Gain-of-function mutations in *PTPN11* have been identified in approximately 50% of individuals with clinically diagnosed NS [Tartaglia et al., 2001; Musante et al., 2003; Niihori et al., 2006]. In contrast, loss-of-function or dominant-negative mutations in *PTPN11* have been reported in patients with LS interfering with growth factor/ERK-MAPK-mediated signaling [Kontaridis et al., 2006; Tartaglia et al., 2006].

NF1, previously known as von Recklinghausen disease, is an autosomal dominant disorder caused by mutation in the *NF1* gene on chromosome 17q11.2 that encodes the tumor suppressor protein neurofibromin. NF1 affects 1 in 2500 people and arises due to a large variety of loss-of-function mutations in the *NF1* gene, which result in increased levels of active, GTP-bound RAS. Nearly half of all cases are the result of de novo mutations [Huson, 2009]. The clinical diagnosis is based on the presence of 2 or more of the following findings: 6 or more café-au-lait macules with diameters >5 mm in prepubertal patients and >15 mm in postpubertal patients, 2 or more neurofibromas of any type or 1 plexiform neurofibroma, axillary or inguinal freckling, optic glioma, 2 or more Lisch nodules of the iris, a distinctive osseous lesion such as sphenoid wing dysplasia or pseudarthrosis and a first-degree relative diagnosed as having NF1. Additional features include short stature, scoliosis, headache, speech disorders, attention deficit disorder, attention-deficit/hyperactivity disorder and learning disabilities [Ferner et al., 2007; Jouhilahti et al., 2011].

CFCS is principally caused by mutations in 4 genes in the MAPK pathway (*BRAF*, *KRAS*, *MEK1*, and *MEK2*), *BRAF* mutation being the most frequent ones (50–75% of cases) [Niihori et al., 2006; Rodriguez-Viciana et al., 2006]. CFCS is characterized by cardiac abnormalities (pulmonic stenosis and other valve dysplasias, septal defects, HCM, rhythm disturbances), distinctive craniofacial appearance, and cutaneous abnormalities. CFCS and LS have similar cardiac findings, pulmonary stenosis being the only anomaly that demonstrates a statistically significant genotype-phenotype correlation in CFCS, being more common in individuals with a *BRAF* mutation [Allanson et al., 2011]. However, in CFCS, intellectual disability is usually more severe than in LS and is associated with short stature and relative macrocephaly. CFCS has also a higher likelihood of structural central nervous system anomalies such as ventriculomegaly/hydrocephalus or epilepsy, more pronounced skin pathology such as absent eyebrows and keratosis pilaris which are characteristic of CFCS and long-lasting gastrointestinal complications including failure to thrive and dysphagia [Wright and Kerr, 2010]. No *PTPN11* mutations have been found in individuals with CFCS [Aoki et al., 2008].

CS shares features with LS, NS and CFCS. Features suggestive of CS include neonatal atrial arrhythmias, ulnar deviation, excess skin which darkens with age, papillomata, usually after the age of 2 years, and childhood cancers, particularly embryonic rhabdomyosarcoma and bladder carcinoma, the latter typically from teenage years

onwards [Kerr et al., 2006]. Two series of individuals with CS have been studied molecularly and no *PTPN11* mutation has been identified [Tartaglia et al., 2003; Tröger et al., 2003]. Although the diagnosis can be suspected clinically, confirmation requires the identification of a heterozygous germline mutation occurring in the first and third coding exons in the proto-oncogene *HRAS* [Aoki et al., 2005; Gelb and Tartaglia, 2010].

Genetic Counseling

LS may be sporadic (de novo mutation) or inherited as an autosomal dominant trait. This means that the patient can be the first affected person in his/her family or that the patient has a 50% chance to inherit the mutated gene, respectively. However, as expressivity is indeed variable, not all individuals who inherit that mutation go on to develop the disease. LS should be suspected in fetuses with a normal karyotype associated with HCM. In these patients, with suspected LS, a DNA test for mutation analysis can be carried out on blood, chorionic villi or amniotic fluid samples.

Genetic counseling helps families and patients to understand and cope with the diagnosis of a genetic condition, its implications, and to make informed decisions. This allows estimating the risks whether other blood relatives, or future children, will be affected by the condition, reviewing the natural history and explaining the manifestations of the disease and its variability. Patients and relatives can also determine if further studies should be done, particularly those in the newborn period and discuss, if appropriate, prenatal testing and other reproductive options to ensure that any decision is made on an informed basis.

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Erratum

In the article ‘Shared Copy Number Variation in Simultaneous Nephroblastoma and Neuroblastoma due to Fanconi Anemia’ by Serra et al. (*Mol Syndromol* 2012;3:120–130) the name of the ninth author is wrong. The correct name is B. Royer-Pokora and not B. Pokora.