Clinical and molecular analysis of 30 patients with multiple lentigines LEOPARD syndrome

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M ultiple lentigines LEOPARD syndrome (MIM 151100) is an autosomal dominant multiple congenital anomaly syndrome, with high penetrance and markedly variable expression. The acronym LEOPARD was coined by Gorlin et al. in 1971 as a mnemonic of the major features of this disorder: multiple lentigines, ECG conduction abnormalities, ocular hypertelorism, pulmonary stenosis, abnormal genitalia, retardation of growth, and sensorineural deafness. It is also known as cardio-cutaneous syndrome, Moynahan syndrome, lentigiosis profuse, and progressive cardiomyopathic lentigiosis. Voron et al. proposed some diagnostic criteria for multiple lentigines LEOPARD syndrome. More than 100 cases have been described, and one review has been published. Multiple lentigines LEOPARD syndrome shares many features with Noonan syndrome (MIM 163950), in which lentigines and deafness usually are not present. About 40% of patients with Noonan syndrome have missense mutations in the PTPN11 gene, which encodes for the protein tyrosine phosphatase SHP2. Multiple lentigines LEOPARD syndrome has proved to be allelic to Noonan syndrome, with pulmonary valve stenosis prevailing in the former and hypertrophic cardiomyopathy in the latter.

We examined the PTPN11 gene in a consecutive series of 30 patients with multiple lentigines LEOPARD syndrome, including 16 new patients. We report the full spectrum of molecular defects of these patients and describe the clinical features related to PTPN11 gene mutations.

MATERIALS AND METHODS

Patient selection
A clinical geneticist or a paediatric cardiologist chose the patients. Thirty patients of Caucasian origin (24 from central and southern Italy and six from Sydney, Australia), including 12 men and 18 women, were enrolled in this study. The age range was 8 months to 49 years (mean 16.37 (SD) 13.60 years). Clinical assessment for the diagnosis of multiple lentigines LEOPARD syndrome included physical evaluation of dysmorphisms and malformations, according to the clinical features outlined by Voron et al. Additional investigations included anthropometric measurements, renal ultrasonography, and radiological studies. In addition to multiple lentigines, other cutaneous abnormalities were recorded, particularly café au lait spots, axillary freckling, localised hypopigmentation, and other pigmented lesions. Patients with characteristic cephalofacial dysmorphisms, such as triangular face with biparietal bossing, hypertelorism, ptosis, epicanthic folds, pronathism, and large and low set auricles, were classified as mild or full dysmorphic. Cardiac evaluation included electrocardiogram, two dimensional echocardiography, and colour Doppler echocardiography. Pulmonary valve stenosis and pulmonary valve leaflet...
dysplasia were assessed according to classic echocardiographic criteria. Hypertrophic cardiomyopathy was diagnosed when the thickness of the left ventricular wall was more than two standard deviations from that of an age matched control group.\textsuperscript{19} Cardiac catheterisation with angiocardiography was performed in eight patients to attempt pulmonary valve dilatation or show the left ventricular shape and confirm the diagnosis. As a few signs of multiple lentigines LEOPARD syndrome are age dependent, we also included three unrelated children of nonaffected parents (aged eight months to four years), who were considered likely examples of multiple lentigines LEOPARD syndrome on the basis of café au lait spots, distinct facial characteristics, and congenital heart defects (two children) or hypertrophic cardiomyopathy and deafness without cutaneous abnormalities (one child).

The patients’ parents, when available, were examined for major or minor dysmorphisms and cardiac and extracardiac defects. Informed consent was obtained from all patients or their parents. Cytogenetic analysis was normal in all patients.

Molecular analysis

We extracted DNA from the peripheral blood lymphocytes of all patients and available affected relatives. We screened the entire \textit{PTPN11} gene coding region, including the 15 exons and exon–intron boundaries (GenBank accession number NM_002834), for mutations. All fragments were amplified by polymerase chain reaction (PCR) with primers located around 50 bp upstream and downstream from the splicing sites. We analysed the PCR fragments with single strand conformation polymorphism (Amersham Pharmacia Biotech, Uppsala, Sweden). We performed bidirectional direct sequencing on fragments with anomalous mobility shifts by using the Big Dye Terminator Abi Prism Sequencing Kit (Applied Biosystems, Foster City, CA) and the ABI PRISM 3100 Genetic Analyser Automated Sequencer (Applied Biosystems, Foster City, CA). We sequenced the entire \textit{PTPN11} coding region in samples with no anomalous mobility shifting fragments on single strand conformation polymorphism analysis.

Statistical analysis

Statistical analysis was performed with Fisher’s exact test, with a significance threshold of \( p \leq 0.05 \).

RESULTS

The study group comprised 30 participants: 20 sporadic patients and 10 familial cases from four kindreds. We reevaluated 14 previously described participants and added 16 new patients, including the second sibling of a previously reported mother–sibling pair with multiple lentigines LEOPARD syndrome (patient N.15 (family B)) (table 1).\textsuperscript{15 17 18} Table 1 summarises the clinical findings. Twenty-seven patients fulfilled Voron’s diagnostic criteria (patients N.1–24 and N.28–30),\textsuperscript{3} while three young patients were considered to have “partial” phenotypes of multiple lentigines LEOPARD syndrome because of a negative family history and absence of multiple lentigines in the presence of two or more other cardiac features of multiple lentigines LEOPARD syndrome (patients N.25–27). In particular, patient N.25 had severe hypertrophic cardiomyopathy, bilateral sensorineural deafness, hypotonia, and retarded developmental milestones but no cutaneous anomalies or facial dysmorphisms and patient N.26 had full cardiac dysmorphic hypertrophic cardiomyopathy, and three café au lait spots. Patient N.27 had hypertrophic cardiomyopathy with subaortic stenosis, a broad chest, 15 sparse café au lait spots, and facial anomalies, including epicantal folds, bilateral palpebral ptosis, and dysmorphic ears.

The most common features among the 30 patients were multiple lentigines (25 patients), mild to full facial dysmorphisms (29 patients), and structural heart defects (18 patients; most often hypertrophic cardiomyopathy (14 patients)). Sensorineural deafness was diagnosed in six patients.

Analysis with single strand conformation polymorphism showed anomalous mobility shifting fragments in exons 7, 12, and 13 only. Of 24 index patients, 21 (88%) harboured a \textit{PTPN11} gene mutation. The \textit{PTPN11} gene was mutated in 17/20 sporadic patients and in all familial cases (table 1); the mutations cosegregated with the disease. Mutations were not found in the unaffected parents or in 200 control chromosomes. None of the mutations has been described previously in Noonan syndrome, with the notable exception of the Tyr279Cys mutation, which has been reported in a single patient with Noonan syndrome.\textsuperscript{11} We identified seven different missense \textit{PTPN11} mutations, which clustered in the SHP2 protein tyrosine phosphatase domain (fig 1). Sixteen unrelated patients had one of the two common mutations: eight of these had the 836A→G transition, which leads to a Tyr279Cys change and eight had the 1403C→T transition (Thr468Met). Seven patients had five rare mutations. One of these (Tyr279Ser in patient N.25) occurred at nucleotide 836 (836A>C), which is also involved in the more common Tyr279Cys mutation. A G>C transition at position 1391 in exon 12, which led to a Gly464Ala substitution, was found in a mother and her daughter (patients N.19 and N.20). Three other mutations were detected in exon 13. The Gln506Pro (1517A>C) change was reported in one sporadic patient (patient N.24).\textsuperscript{17} The remaining two sequence variations at codon 498 predicted different amino acid substitutions. The first change was a C>T transition at position 1492, which led to an Arg498Leu substitution (patient N.23); the second change was a G>T transversion at position 1493, which segregated from an affected mother to her daughter and predicted an Arg498Trp change (patients N.21 and N.22).

Three patients who satisfied Voron’s criteria for multiple lentigines LEOPARD syndrome had no anomalous mobility pattern on single strand conformation polymorphism analysis (patients N.28–30), and direct sequencing of the whole \textit{PTPN11} coding region failed to show any change in nucleotides. Among patients with a negative molecular analysis, patient N.28 had multiple café au lait spots, axillary and neck freckling, facial choristosis pilaris, valvar pulmonary stenosis, left sensorineural deafness, macrocephaly, and facial anomalies including prominent eyes with bilateral palpebral ptosis and dysmorphic ears. Patient N.29 had multiple lentigines, mitral valve dysplasia, broad chest, moderate mental retardation, macrocephaly, and facial anomalies including bilateral palpebral ptosis, hypertelorism, epicantal folds, and dysmorphic ears. Patient N.30 had multiple lentigines, short stature, hypertrophic cardiomyopathy, broad chest with pectus carinatum, facial anomalies including prominent eyes, bilateral palpebral ptosis, and dysmorphic ears.

DISCUSSION

In this study, we analysed the \textit{PTPN11} gene in 30 patients with multiple lentigines LEOPARD syndrome. In addition to the two already described recurrent missense mutations (Tyr279Cys and Thr468Met) and the Gln506Pro mutation found in a single patient, four additional novel and rare changes were detected in the \textit{PTPN11} gene. Mutations were found in 88% of the index patients, which indicated that \textit{PTPN11} is the major gene behind multiple lentigines LEOPARD syndrome. Patients with multiple lentigines LEOPARD syndrome with \textit{PTPN11} mutations had marked
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* Novel patients
† Familial cases marked with letters (families A, B, C, and D): N.5, N.13, N.19, and N.21 were the index patients for families A, B, C, and D, respectively.
of the SHP2-PTP domain. The numbers indicate amino acid positions. Variations in the clinical expression, which did not fulfill Voron’s diagnostic criteria in about 14% of the cases.

All of the mutations occurred in exons that code for the protein tyrosine phosphatase domain (fig 1). The most common changes, in exons 7 (Thr468Met) and 12 (Thr468Met), accounted for 8/21 (67%) of mutations. One rare mutation involved the same nucleotide as the common Thr468Met change, but it produced a different amino acid substitution (Thr468Met). Another rare mutation in exon 12 affected amino acid Gly464, which, like the more commonly mutated Thr468, is located in the consensus sequence of the active site that is necessary for activity of protein tyrosine phosphatase (amino acids 457–469, VHCSAGIGRTGTF). Two novel mutations were identified in exon 13. They both involved Arg498, which is located between the Thr468 mutation hot spot (Thr468Met) and Gln506, which was mutated in a sporadic patient with full blown multiple lentigines LEOPARD syndrome.

Figure 1 Nucleotide and amino acid sequence of the SHP2-PTP domain.

The rate of detection of PTPN11 mutations in patients with multiple lentigines LEOPARD syndrome consistently is higher than in patients with Noonan syndrome.10–14 Although genetic heterogeneity has been established in patients with Noonan syndrome,10–14,20 it has not been proven in those with multiple lentigines LEOPARD syndrome.21 Although intrinsic and regulatory regions of the PTPN11 gene were not analysed in our study, our inability to detect mutations in the three unrelated children of unaffected parents could point to the supposed genetic heterogeneity of multiple lentigines LEOPARD syndrome.

This study investigated one of the largest cohorts of patients with multiple lentigines LEOPARD syndrome. In addition to 18 patients with mutations and a full blown phenotype of multiple lentigines LEOPARD syndrome, three patients with a partial phenotype of multiple lentigines LEOPARD syndrome showed PTPN11 mutations in the two common mutation hot spots.

Distinct facial dysmorphism, including hypertelorism, ptosis, and large low set auricles, occurred in 90% of patients with PTPN11 mutations independently of age. As expected, multiple lentigines were present in most patients (86%). They presented as flat, black-brown macules anywhere on the skin, but mostly on the face, neck, and upper part of the trunk. The number of lentigines increased into the thousands until puberty. Café au lait spots also were observed, alone or in association with multiple lentigines (61%). Two children (patient N.15 (family B) and patient N.22 (family D)) with PTPN11 mutations, who were evaluated at the ages of four and two years, had no pigmented macules. Both children fulfilled Voron’s criteria, however, because their mothers were affected (patients N.13 (family B) and N.21 (family D)) and onset of lentigines in the mothers occurred only in late childhood. Structural cardiac defects occurred in 71% of the index patients with PTPN11 mutations. Hypertrophic cardiomyopathy was the most common defect (12/15 (80%) patients) and was not related to any specific mutation site. Pulmonary valve stenosis with valve leaflet dysplasia was less common than previously thought, affecting only 2/21 patients with PTPN11 mutations.5,6,9,15–17 Partial atrioventricular canal defect was diagnosed in one patient (patient N.11), who had developed hypertrophic cardiomyopathy during the past year, soon after the onset of multiple lentigines. In agreement with a previous report,22 we found that the first diagnosis or worsening of hypertrophic cardiomyopathy, or both, was concomitant with the appearance of multiple lentigines. This observation leads to the recommendation that any patient with isolated multiple lentigines or multiple lentigines LEOPARD syndrome should undergo cardiac assessment during infancy and adolescence. Hearing loss was documented at any age in about one quarter of patients.5,6,9 Interestingly, the four patients with bilateral sensorineural deafness who we evaluated harboured mutations at position 279, while a patient with the rare Gln506Pro mutation presented unilateral deafness. Coeliac disease was diagnosed in two (9.5%) patients with mutations, a value significantly higher than that seen in the general population (0.54%) (p = 0.02) and comparable with that seen in patients with Down syndrome, Turner syndrome, and Williams syndrome.22–25 This observation deserves further evaluation in other patients to assess if coeliac disease must be included among the features of multiple lentigines LEOPARD syndrome, and screening for antigliadin and antiendomysium antibodies is indicated in these people. Mental retardation, if present, usually was mild, at times manifesting only with mild learning difficulties and a frequency comparable with that in previous
studies, having been found in three sporadic patients and two families (5/21, 24%), in association with different PTPN11 mutations.

Patients who presented with a “partial” phenotype of multiple lentigines LEOPARD syndrome and harboured PTPN11 mutations had an incomplete clinical spectrum, almost certainly because of their young age. Two patients (N.26 and N.27) showed the characteristic facial appearance, hypertrophic cardiomyopathy, and café au lait spots. The third patient (patient N.25) was aged eight months and had hypertrophic cardiomyopathy and congenital deafness, with no obvious facial dysmorphism or skin macules. The occurrence of hypertrophic cardiomyopathy with deafness is rarely seen in patients. In the absence of lentigines, Voron’s criteria for the diagnosis of multiple lentigines LEOPARD syndrome include at least three major features and an immediate affected relative. Personal experience suggests that any young patient without multiple lentigines and a positive family history should be evaluated for the presence of de novo PTPN11 gene mutations when other features of multiple lentigines LEOPARD syndrome are found, particularly hypertrophic cardiomyopathy or pulmonary valve stenosis (with leaflet dysplasia) in association with deafness. The identification in two of these patients of the recurrent Thr468Met mutation may anticipate a later onset of multiple lentigines. On the other hand, in the presence of the newly recognised Tyr279Ser mutation, we could only guess that the full blown phenotype of multiple lentigines LEOPARD syndrome would develop in the forthcoming years. The clinical follow up of this patient and of the unique patient with Noonan syndrome who also harboured the common Tyr279Cys mutation of multiple lentigines LEOPARD syndrome should prove the assumption that codon 279 mutations are pathognomonic of the phenotype of multiple lentigines LEOPARD syndrome.

Skin features, congenital heart disease, and mental status mostly were concordant in our four families with multiple lentigines LEOPARD syndrome. In particular, after the diagnosis of hypertrophic cardiomyopathy in a patient aged two years, the reevaluation of the affected mother in family D, who was aged 34 years, disclosed the same congenital heart disease. Familial concordance could be an important clue for the assessment of the reproductive risk of affected parents. In this respect, the 23% occurrence rate of congenital heart disease in familial cases is lower than expected, possibly because of the reduced fitness of severe congenital heart disease. A number of families without congenital heart disease have been reported, which suggests that multiple lentigines might be an autosomal dominant disorder distinct from LEOPARD syndrome. Others have considered both disorders as unique clinical conditions, although the term LEOPARD has been restricted at times to patients who present with visceral abnormalities. Detection of the same PTPN11 mutation in patients with multiple lentigines, those with full blown multiple lentigines LEOPARD syndrome, and those with the “partial” phenotype of multiple lentigines LEOPARD syndrome suggests that these different phenotypes mark the variable expression of a unique genetic condition.

Clinical overlap between multiple lentigines LEOPARD syndrome and neurofibromatosis 1 is well known. In fact, café au lait spots and multiple lentigines are skin manifestations common to both disorders. Café au lait spots increase in number with age in both conditions, whereas the lentigines characteristic of multiple lentigines LEOPARD syndrome are not present at younger ages and develop during childhood, so that differential diagnosis in children may be difficult. On the contrary, the lentigines of multiple lentigines LEOPARD syndrome in adolescence or adult age are distinguishable from those of neurofibromatosis 1, being generalised and dark in colour. In addition, in children with café au lait spots, the presence of facial anomalies or congenital heart disease may be more suggestive for multiple lentigines LEOPARD syndrome, although intermediate phenotypes such as neurofibromatosis 1–Noonan syndrome or Watson syndrome have been described. In practical genetic evaluation, we believe that PTPN11 screening should be carried out at first in children with café au lait spots associated with facial anomalies or congenital heart disease (hypertrophic cardiomyopathy or pulmonary valve stenosis), or both, although in the absence of multiple lentigines. In addition, PTPN11 analysis should be considered in patients with café au lait spots or multiple lentigines when neurofibromas or Lisch nodules are not detectable and molecular analysis for mutations of NF1 is negative. Conversely, screening of the NF1 gene should be performed in patients with skin manifestations of multiple lentigines LEOPARD syndrome without a detectable PTPN11 mutation. Nevertheless, the existence of a third locus for multiple lentigines LEOPARD syndrome also should be considered.

In our series, no obvious clinical difference was seen between patients with allelic mutations, except for bilateral sensorineural deafness, which we found always in association with mutations of the amino acid Tyr279 (table 1) (p = 0.021), although one patient with monolateral deafness carried a mutation in exon 13. Screening of additional patients with multiple lentigines LEOPARD syndrome with bilateral and monolateral deafness is needed to confirm or exclude this observation. Clinical variability seen in patients with the same mutation likely reflects the effect of modifier genes or environmental factors, or both, which influences the phenotypes associated with PTPN11 mutations.

The high prevalence of hypertrophic cardiomyopathy in patients with multiple lentigines LEOPARD syndrome and PTPN11 mutations (57%) indicates that mutations related to multiple lentigines LEOPARD syndrome confer an increased risk for this congenital heart disease. This conclusion also seems to be strengthened by the relatively rare occurrence of hypertrophic cardiomyopathy in Noonan syndrome (15%), where, in general, PTPN11 mutations cluster to the N-SH2 domain. Detection of hypertrophic cardiomyopathy, therefore, should be considered an important hint in the search for PTPN11 mutations in the absence of multiple lentigines.

Conclusion

PTPN11 is the major gene for multiple lentigines LEOPARD syndrome, being mutated in about 90% of patients with the condition. Seven different mutations have been identified so far. Gene analysis in these patients should first include screening by direct sequencing for mutations in exons 7 and 12, which are present in 86% of the cases, and then analysis of exon 13. The phenotypic spectrum of patients with mutations is associated with a marked clinical variation in the absence of any pathognomonic feature. Hypertrophic cardiomyopathy should be suspected and screened for in patients with multiple lentigines LEOPARD syndrome who do not harbour PTPN11 mutations, and careful cardiac evaluation and follow up also should be performed in asymptomatic people. Identification of PTPN11 mutations also in patients with the “partial” phenotype of multiple lentigines LEOPARD syndrome suggests the inclusion in the analysis of people who do not fulfil Voron’s diagnostic criteria. Consequently, the diagnosis of multiple lentigines LEOPARD syndrome should not be ruled out in any young patients with no multiple lentigines and a negative family history who present with hypertrophic cardiomyopathy or
pulmonary valve stenosis in association with an extracardiac feature, such as hearing loss.

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