Prolidase deficiency (PD) is a rare, pan-ethnic, autosomal recessive disease with a broad phenotypic spectrum. Seventeen causative mutations in the *PEPD* gene have been reported worldwide. The purpose of this study is to characterize, clinically and molecularly, 20 prolidase deficient patients of Arab Moslem and Druze origin from 10 kindreds residing in northern Israel. All PD patients manifested developmental delay and facial dysmorphism. Typical PD dermatological symptoms, splenomegaly, and recurrent respiratory infections presented in varying degrees. Two patients had systemic lupus erythematosus (SLE), and one a novel cystic fibrosis phenotype. Direct DNA sequencing revealed two novel missense mutations, A212P and L368R. In addition, a previously reported S202F mutation was detected in 17 patients from seven Druze and three Arab Moslem kindreds. Patients homozygous for the S202F mutation manifest considerable interfamilial and intrafamilial phenotypic variability. The high prevalence of this mutation among Arab Moslems and Druze residing in northern Israel, and the presence of an identical haplotype along 500,000 bp in patients and their parents, suggests a founder event tracing back to before the breakaway of the Druze from mainstream Moslem society.

How to Cite this Article:

Key words: prolidase deficiency; *PEPD* gene; novel mutation; founder mutation; developmental delay

INTRODUCTION

A myriad of genetic defects result in developmental delay. Prolidase deficiency (PD) (OMIM 170100) is a rare and most probably under-diagnosed one. This autosomal recessive, pan-ethnic disorder is characterized by a highly variable phenotype. Fewer than 60 PD patients have been documented in the literature (www.ncbi.nlm.nih.gov/Omim). One study in Quebec assessed the incidence of PD, as 1–2 per million live births [Lemieux et al., 1984]. Testing of 1,000 Japanese individuals in another study failed to detect a single PD patient [Endo and Matsuda, 1981]. However, population screening of 1,425 residents in a Druze village in northern Israel revealed a very high carrier frequency of 1:21 [Falik-Zaccai et al., 2008].
The variable phenotype of PD has not been well delineated. The most common clinical manifestations of PD include developmental delay, facial dysmorphism, splenomegaly, recurrent pulmonary infections [Luder et al., 2007], and skin lesions. The latter include erythematous papular eruptions, impetigo-like eruptions, pruritic eczematous lesions, pseudo-psoriasis skin lesions, and intractable skin ulcerations [Mandel et al., 2000; Hechtman, 2001; Lupi et al., 2006; Wang et al., 2006; Luder et al., 2007]. Frank vasculitis and hepatitis-like symptoms present in some patients. Typical features of systemic lupus erythematosus (SLE) and hyper-IgE syndrome have also been documented [Bissonnette et al., 1993; Shrinath et al., 1997; Hershkovitz et al., 2006; Di Rocco et al., 2007]. PD is generally diagnosed in early childhood, with some patients apparently remaining asymptomatic [Royce and Steinmann, 2002]. Hyperiminodipeptiduria is the characteristic biochemical abnormality. Reduced prolidase activity in leukocytes, erythrocytes, cultured fibroblasts, or amniocytes confirms diagnosis [Mandel et al., 2000; Hechtman et al., 2001; Lupi et al., 2006; Viglio et al., 2006].

Prolidase enzyme hydrolyzes dipeptides with carboxy-terminal proline or hydroxyproline. This enzyme has a major role in the recycling of proline released during the degradation of collagen and dietary proteins [Lupi et al., 2004]. Although there is considerable knowledge concerning the putative roles of the prolidase enzyme [Lupi et al., 2008], the pathophysiology of PD remains an enigma.

The prolidase gene (PEPD) has been localized to chromosome 19q12-q13.2 [Garca et al., 1995]. Currently, 17 causative mutations have been characterized [Lupi et al., 2008]. Marked phenotypic variability, including intrafamilial, has been documented [Yasuda et al., 1999; Mandel et al., 2000; Lupi et al., 2006; Luder et al., 2007]. Thus, the number and nature of molecular defects do not fully explain the clinical variability of PD [Hechtman, 2001].

In the present study, we characterized the phenotype and genotype of 20 PD patients residing in a small geographic area in northern Israel, the largest cohort described. We identified two novel mutations and identified 17 patients with the S202F mutation. The high prevalence of the latter, and its association with a shared haplotype in seemingly unrelated Druze and Arab Moslem kindreds, supports the possibility of a founder effect for this mutation, probably antedating the establishment of the Druze religion in 1017 AD.

**PATIENTS AND METHODS**

**Patients**

We have diagnosed 20 patients with PD originating from 10 inbred kindreds, 7 Druze, and 3 Arab Moslem, all residents of northern Israel (Fig. 1). The institutional Ethics Review Committee and the National Committee for Genetic Studies of the Israeli Ministry of Health approved the study protocol. The participants or their legal guardians gave their informed consent.

**Phenotype Analysis: Clinical and Laboratory Evaluation**

All affected individuals were clinically and biochemically evaluated by pediatric, genetic, and metabolic specialists. Hyperiminopeptiduria was identified by bi-dimensional thin-layer chromatography on cellulose and/or amino acid analysis using a Beckman 7300 amino acid analyzer, which measures peptides and free amino acids in urine. The method is based on cation-exchange chromatography with lithium citrate buffers and post-column ninhydrin detection, as previously described [Moore et al., 1958]. Prolidase activity was measured in leukocytes, fibroblasts, and amniocytes as previously described [Butterworth and Priestman, 1984; Mandel et al., 2000].

**Mutation Analysis**

Total RNA was isolated from fibroblasts of patient V_4 Family 1 (Fig. 1), as previously described [Chirgwin et al., 1979]. Synthesis of cDNA was performed using 5 µg of total RNA, as previously described [Ledoux et al., 1994]. The cDNA containing the 15 exons of PEPD was used to amplify four PCR products [Ledoux et al., 1994]. Direct sequencing of the four fragments was performed using standard methods.

Using a salting-out standard method [Sambrook et al., 1989], genomic DNA was isolated from members of the 10 families under our care. Mutation analysis was applied to the 15 exons and to the flanking splice junction consensus sequences of the PEPD gene, using genomic DNA from patients VI_1 Family 5, V_3 Family 9, and IV_2 Family 10 (Fig. 1). Sequence analysis was performed using the Big Dye Terminator Kit (Applied Biosystems, Foster City, CA), and analyzed using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), according to the manufacturers' instructions. Primers and annealing temperature used for sequence analysis are described in Table I.

**Molecular Assay for Mutation Detection**

We analyzed the PEPD gene in the 20 affected individuals, their parents, and other high-risk family members. Mutation analysis of the mutation c.605C>T in exon 8 was performed as described previously [Falk-Zaccai et al., 2008].

Mutation analysis of c.834G>C in exon 9 was performed by amplification of a 133-bp fragment containing the mutation from genomic DNA. The sense primer 5'-ATCCGTCTCTCCTCAGGTAAATCGAC-3' and the antisense primer 5'-TTGACCTCTCTCAGGTAAATCGAC-3' were used with the following PCR conditions: denaturation at 94°C for 5 min; 35 subsequent amplification cycles at 94°C for 15 sec, at 55°C for 30 sec, and at 72°C for 10 sec; and a final step at 72°C for 5 min. The PCR products were subsequently digested with Fnu4HI (New England Biolabs, Beverly, MA) and analyzed on 8% acrylamide gels (Biological Industries, Beit Haemek, Israel). Normal alleles do not digest. Mutant alleles digest to two fragments, 113 and 20 bp.

Mutation analysis of c.1103T>G in exon 13 was performed by amplification of a 377-bp fragment containing the mutation from genomic DNA. The sense primer 5'-TCCACAGGACCTGTAAGG-3' and the antisense primer 5'-TGCTGCTCTAGTGTGGTAC-3' were used to amplify the PCR products according to the conditions described above. The PCR products were subsequently digested with MspI (New England Biolabs) and analyzed on 8% acrylamide gels (Biological Industries). Normal alleles digest to...
FIG. 1. Ten family pedigrees including 20 PD patients. Eight unrelated and two related families of Druze (Families 3–8 and 10) and Arab Moslem (Families 1, 2, and 9) origin. Mutation S202F: detected in Families 1–8. Mutation A212P: detected in Family 9. Mutation L368R: detected in Family 10. The inheritance of the disease is compatible with an autosomal-recessive trait. Roman capitals indicate generations. Arabic numerals indicate serial numbers of study participants. Blackened symbols indicate affected individuals. Black dots indicate carriers. Diagonal lines across symbols indicate deceased individuals. E+ indicates individuals who underwent mutational analysis, with positive evaluation. E− indicates individuals who underwent mutational analysis, with negative evaluation.
two fragments, 258 and 119 bp. Mutant alleles digest to three fragments, 235, 119, and 23 bp.

Haplotype Analysis

We studied five polymorphic markers around the PEPD locus spanning about 500,000 bp on genomic DNA of eight PD patients from five families, three of Druze origin (Families 4, 7, and 8) and two of Arab Moslem origin (Families 1 and 2). Two of the markers are proximal to PEPD (D19S868; D19S555), one is an intragenic marker (D19S719), and two are distal to PEPD (D19S416; D19S874). These markers were selected from the UCSC genome bioinformatics database site. The sense set of the PCR primers were fluorescently labeled. We analyzed the PCR products of these reactions using the GeneScan system of the ABI PRISIM 3100.

Prenatal Diagnosis (PND)

Prenatal diagnosis (PND) based on molecular analysis was performed twice in Family 1. PND was performed twice in Family 7: once consisting of combined biochemical assay [Mandel et al., 2000] confirmed by molecular analysis; and a second time based on molecular analysis alone. DNA was isolated directly from 10 mg of CVS using standard methods. A molecular assay to detect the causative mutation was performed using 50–100 ng of DNA. Maternal contamination was excluded using 10 highly polymorphic markers.

RESULTS

Clinical Manifestations

Table II presents the clinical features of the 20 PD patients. All presented with some degree of developmental delay, most of them with moderate cognitive or speech delay. Facial dysmorphism (Fig. 2), with great variance in the severity, was also noted in all 20 patients. The dysmorphic facial features consisted of ocular hypertelorism, exophthalmus, upward or downward slanting palpebral fissures, small-beaked nose, low posterior hairline, facial hirsutism, and a slender upper lip. Dermatological manifestations included erythematous papular eruptions, impetigo-like eruptions, pseudo-psoriasis skin lesions, and pruritic eczematous lesions. Splenomegaly, hepatitis-like symptoms, osteomyelitis, recurrent lung infections, asthma, and chronic lung disease resembling cystic fibrosis (CF) [Mandel et al., 2000; Luder et al., 2007] appeared in some. Two PD patients fulfilled the diagnostic criteria for SLE.

Significant interfamilial and intrafamilial variability is evident in this cohort. Clinical symptoms do not discriminate between patients carrying different causative mutations. Phenotypic variance is high among the 17 patients in families 1–8 with mutation S202F. While the phenotype of the two patients in Family 10 with mutation L368R is identical, it is not distinguishable from the phenotype of the other two mutations.

Systemic Lupus Erythematosus (SLE) Phenotype in PD

SLE was diagnosed prior to PD in a Druze patient (V 28 Family 6), and subsequent to it in a Moslem patient (V 1 Family 9). The former carries mutation S202F, and the latter mutation L368R. V 28 Family 6: This girl suffered recurrent episodes of bacterial pneumonia from the age of 10 years. She developed bronchiectasis, clubbing, maculopapular rash, splenomegaly, and pancytopenia. The diagnosis of SLE was based on typical hematological findings, including Coombs-positive hemolytic anemia, severe thrombocytopenia, high levels of anti-cardiolipin antibodies, anti-nuclear antibodies, and strongly positive anti-double-stranded DNA antibodies. Severe thrombocytopenia did not respond to high-dose steroid therapy, cyclophosphamide, or intravenous immunoglobulin therapy (IVIG), thus necessitating splenectomy. Hematological and immunological signs of SLE resolved 9 months

<table>
<thead>
<tr>
<th>Exon</th>
<th>5’ primer</th>
<th>3’ primer</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)</th>
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<tr>
<td>1</td>
<td>GCTGACGCCGCACTTCACGT</td>
<td>GAGGGTGAGAGGAGCTGGGC</td>
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<td>265</td>
</tr>
<tr>
<td>2</td>
<td>ACGTACAGGTGGGCTGATC</td>
<td>CTCACACCTCTGCTCAAGGA</td>
<td>55</td>
<td>326</td>
</tr>
<tr>
<td>3</td>
<td>GTGGCTGCTAGGTGGCTCTTCA</td>
<td>ACCAGCCTCTCCTACCCA</td>
<td>55</td>
<td>312</td>
</tr>
<tr>
<td>4</td>
<td>CAGGAAAGTTGCGCATGCTGA</td>
<td>CAGCAAGGTTGAGCAAT</td>
<td>62</td>
<td>225</td>
</tr>
<tr>
<td>5</td>
<td>ATGGCAACTCTGATTAAGAGGA</td>
<td>CCCTTCCATTATCCTCCCC</td>
<td>55</td>
<td>249</td>
</tr>
<tr>
<td>6</td>
<td>TGCTCTACCTCCGCTGACG</td>
<td>CTAGTAGGTGGGAGTGGG</td>
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<td>245</td>
</tr>
<tr>
<td>7</td>
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<td>TCTGCTCCTAGGAGGGTCT</td>
<td>55</td>
<td>233</td>
</tr>
<tr>
<td>8</td>
<td>GTGGTGAACGGCTGGACCTCT</td>
<td>TACAGCAGTTTTCCTCAGC</td>
<td>55</td>
<td>218</td>
</tr>
<tr>
<td>9</td>
<td>AGGAAGAGTGTCTTTGGAGAAG</td>
<td>TGTATCAGTGCCAGGACATAAT</td>
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<td>203</td>
</tr>
<tr>
<td>10</td>
<td>GCTTCCCTTGGGCTTCAT</td>
<td>GCTCACTTACTGCTACCT</td>
<td>55</td>
<td>213</td>
</tr>
<tr>
<td>11</td>
<td>TCAAGGCAGGAGGATCAGAGCAT</td>
<td>GGAAGTGTGGCAGATTCAAGCTA</td>
<td>55</td>
<td>220</td>
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<tr>
<td>12</td>
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<td>AGGTGAGGCAGATCTAC</td>
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<td>268</td>
</tr>
<tr>
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<td>TCCACACAAGCAGCTGAGTA</td>
<td>TCTGTCTCCTAGAATGCTCCA</td>
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<tr>
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<td>CGAAGCTTGGGTACCTGATTCTG</td>
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<td>320</td>
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TABLE I. PCR Primers and Annealing Temperatures Used for Sequence Analysis of PEPD Gene
<table>
<thead>
<tr>
<th>Family/patient</th>
<th>Sex</th>
<th>Age (y)</th>
<th>AO</th>
<th>Origin</th>
<th>Initial symptoms</th>
<th>Eyes: proptosis/ hypertelorism</th>
<th>Skin: crusting dermatitis face and extremities</th>
<th>Pulmonary disease</th>
<th>Splenomegaly</th>
<th>SLE phenotype</th>
<th>Psychomotor development</th>
<th>Prolidase activity (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/IV7</td>
<td>F</td>
<td>25</td>
<td>1 m</td>
<td>Moslem</td>
<td>FTT, infections, splenomegaly</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>—</td>
<td>Mild DR</td>
<td>NT</td>
</tr>
<tr>
<td>1/IV8</td>
<td>M</td>
<td>40</td>
<td>30 y</td>
<td>Moslem</td>
<td>Dysmorphism</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Mild delay</td>
<td>NT</td>
</tr>
<tr>
<td>1/IV7</td>
<td>F</td>
<td>10</td>
<td>1 y</td>
<td>Moslem</td>
<td>Developmental delay</td>
<td>++</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Speech delay</td>
<td>NT</td>
</tr>
<tr>
<td>1/IV3</td>
<td>F</td>
<td>8</td>
<td>1 y</td>
<td>Moslem</td>
<td>Developmental delay</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Speech delay</td>
<td>NT</td>
</tr>
<tr>
<td>1/IV4</td>
<td>M</td>
<td>20 m died</td>
<td>1 m</td>
<td>Moslem</td>
<td>FTT, diarrhea, bronchiolitis</td>
<td>++++</td>
<td>—</td>
<td>Respiratory failure</td>
<td>—</td>
<td>—</td>
<td>Severe DR</td>
<td>PND: undetectable postnatal: 8 (controls: 44.2 ± 11.4)</td>
</tr>
<tr>
<td>2/II1</td>
<td>F</td>
<td>8</td>
<td>16 m</td>
<td>Moslem</td>
<td>Dysmorphism, splenomegaly, dermatitis</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>+</td>
<td>—</td>
<td>Mild delay</td>
<td>NT</td>
</tr>
<tr>
<td>2/II3</td>
<td>M</td>
<td>5</td>
<td>8 m</td>
<td>Moslem</td>
<td>Dysmorphism, splenomegaly, dermatitis</td>
<td>+++</td>
<td>++</td>
<td>—</td>
<td>+</td>
<td>—</td>
<td>Mild delay</td>
<td>NT</td>
</tr>
<tr>
<td>3/II1</td>
<td>F</td>
<td>3 m</td>
<td>20 d</td>
<td>Druze</td>
<td>Diarrhea, jaundice, splenomegaly</td>
<td>+</td>
<td>+</td>
<td>Slight facial dysmorphism</td>
<td>—</td>
<td>+++</td>
<td>Hypotonia</td>
<td>NT</td>
</tr>
<tr>
<td>4/IV2</td>
<td>M</td>
<td>2</td>
<td>1 m</td>
<td>Druze</td>
<td>Purulent otitis, pneumonia</td>
<td>+</td>
<td>+</td>
<td>Asthma</td>
<td>—</td>
<td>—</td>
<td>Mild delay</td>
<td>NT</td>
</tr>
<tr>
<td>5/IV1</td>
<td>M</td>
<td>13</td>
<td>6 y</td>
<td>Druze</td>
<td>Dermatitis, recurrent wheezing, developmental delay</td>
<td>+</td>
<td>+</td>
<td>Asthma</td>
<td>+</td>
<td>—</td>
<td>Moderate DR</td>
<td>NT</td>
</tr>
<tr>
<td>6/IV28</td>
<td>F</td>
<td>18</td>
<td>3 m</td>
<td>Druze</td>
<td>Diarrhea, pneumonia, dermatitis</td>
<td>+ Down slanted</td>
<td>++ Skin ulcers</td>
<td>+++</td>
<td>+++</td>
<td>Splenectomy</td>
<td>—</td>
<td>Moderate delay</td>
</tr>
<tr>
<td>7/IV2</td>
<td>F</td>
<td>4 died</td>
<td>1 m</td>
<td>Druze</td>
<td>Diarrhea, pneumonia, jaundice</td>
<td>++ Down slanted</td>
<td>+</td>
<td>+++</td>
<td>Asthma and bronchiolitis</td>
<td>+++</td>
<td>—</td>
<td>Severe DR</td>
</tr>
<tr>
<td>7/IV5</td>
<td>M</td>
<td>6</td>
<td>PND</td>
<td>Druze</td>
<td>Asthma</td>
<td>+</td>
<td>+</td>
<td>Asthma</td>
<td>—</td>
<td>—</td>
<td>Mild delay</td>
<td>Undetectable</td>
</tr>
<tr>
<td>8/IV3</td>
<td>F</td>
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<td>10 m</td>
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<td>Dermatitis, splenomegaly</td>
<td>++</td>
<td>++</td>
<td>—</td>
<td>+++</td>
<td>—</td>
<td>Mild delay</td>
<td>NT</td>
</tr>
<tr>
<td>8/IV4</td>
<td>F</td>
<td>15</td>
<td>Birth</td>
<td>Druze</td>
<td>SFD, FTT, DR severe</td>
<td>++</td>
<td>Low hairline</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Severe DR since birth</td>
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</tr>
<tr>
<td>8/IV7</td>
<td>M</td>
<td>13</td>
<td>7 m</td>
<td>Druze</td>
<td>FTT, anemia hypotonia</td>
<td>+</td>
<td>+ Pseudo-psoriasis</td>
<td>Cystic fibrosis</td>
<td>+</td>
<td>—</td>
<td>Moderate delay</td>
<td>NT</td>
</tr>
<tr>
<td>9/IV3</td>
<td>F</td>
<td>8</td>
<td>2 m</td>
<td>Moslem</td>
<td>FTT, diarrhea, dermatitis, dysmorphism</td>
<td>+++</td>
<td>+++ Hirsutism</td>
<td>Pneumonia recurrent</td>
<td>+++</td>
<td>SLE</td>
<td>Mild delay</td>
<td>9.5 (controls: 44.2 ± 11.4)</td>
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<tr>
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<td>M</td>
<td></td>
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<td>Druze</td>
<td>FTT, dysmorphism</td>
<td>+++</td>
<td>—</td>
<td>—</td>
<td>+++</td>
<td>—</td>
<td>Moderate delay</td>
<td>NT</td>
</tr>
<tr>
<td>10/IV7</td>
<td>M</td>
<td></td>
<td></td>
<td>Druze</td>
<td>FTT, dysmorphism</td>
<td>+++</td>
<td>—</td>
<td>—</td>
<td>+++</td>
<td>—</td>
<td>Moderate delay</td>
<td>NT</td>
</tr>
</tbody>
</table>

AO, age at onset; DR, developmental retardation; NT, not tested; SFD, small for date; SLE, systemic lupus erythematosus; m, months; y, years; d, days.
after splenectomy. At age 13 years, the possibility of PD was raised following the diagnosis of SLE in patient V3 Family 9, who presented with similar dysmorphic facial features, splenomegaly, and skin lesions.

V3 Family 9: At age 6 years, dysmorphism, splenomegaly, and developmental delay raised suspicion of PD, which was subsequently confirmed by the detection of massive dipeptiduria (Table II). At age 8, she presented with fever lymphadenopathy, aphthous stomatitis, and cellulitis of her left foot. Four months later, she presented with pleuro-pneumonia, macroscopic hematuria, proteinuria, and pancytopenia, Coombs-positive hemolytic anemia, hypocomplementemia (low C3 and C4), and elevated levels of anti-nuclear and anti-DNA antibodies. A month later, despite treatment with high-dose methylprednisone, she developed acute renal failure with severe fluid overload and hypertension, necessitating temporary hemodialysis. Renal biopsy revealed diffuse proliferative glomerulonephritis, compatible with lupus nephritis. Seizures began 2 months later. Brain MRI findings were compatible with vasculitis. Treatment included prednisone, cyclophosphamide, azathioprine, and several anti-hypertensive drugs. Within 5 months renal function started to improve, and she was gradually weaned off hemodialysis. Brain MRI normalized. She remained, however, severely proteinuric. Despite ongoing therapy with low-dose steroids, azathioprine, anti-proteinuric, and anti-hypertensive drugs, she gradually developed end-stage renal failure. Two years ago she resumed hemodialysis. At age 13, she awaits renal transplantation.

Cystic Fibrosis (CF) Phenotype in PD
We previously described patient VI1 Family 8 (Fig. 1) [Luder et al., 2007], who was diagnosed with CF at the age of 11 months. Despite positive response of the lung disease to standard management, and careful attention to nutrition and pancreatic enzyme replacement, growth remained poor. At age 4 years developmental delay, dysmorphism and skin disease (pseudo-psoriasis) led to PD diagnosis. Extensive molecular studies have failed to reveal any abnormality in the CFTR gene. At age 16 years his lung function is preserved, his growth improved, and his skin disease in remission. However, he is unable to read or write, and is socially incompetent.

Metabolic Analysis
Hyperimidopeptiduria was present in all 20 patients. Low prolidase activity was detected in leukocytes, fibroblasts, and/or amniocytes of four studied patients (Table II).

Mutation Analysis
A C > T transition at nucleotide 605 in exon 8 (c.605C > T), resulting in an S202F missense mutation, was identified in proband
V4 Family 1 (Fig. 1, Table II). Amplification and sequencing of genomic DNA spanning this region showed that this patient and 16 additional patients in Families 1–8 were all homozygous for the S202F mutation. This mutation was previously described in one PD patient from a Druze family [Hershkovitz et al., 2006]. Parents of our patients tested heterozygous for the same mutation, except IV8, the father of V4, who tested homozygous (Fig. 3A). Seven of 12 siblings from Families 1, 2, 6, 7, and 8, were heterozygous, and 5 were homozygous, for the wild-type genotype. Genomic sequencing of the PEPD gene of V1 Family 9 (Fig. 1, Table II) showed a G > C homozygous transition at nucleotide 634 (c.634G > C) in exon 9, resulting in an A212P missense mutation (Fig. 3B). Genomic sequencing of the PEPD gene of VI2 Family 10 (Fig. 1, Table II) showed a T > G homozygous transition at nucleotide 1103 (c.1103T > C) in exon 13, resulting in an L368R missense mutation (Fig. 3C). The parents of each patient were heterozygous for their offspring’s mutation. VI2 Family 10 (Fig. 1, Table II) was also homozygous for the L368R mutation (Fig. 3C). The nucleotide changes detected in all families are not among known SNPs in the NCBI and Ensemble human single nucleotide polymorphism (SNP) databases. The mutations A212P and L368R were not present in 100 chromosomes of healthy ethnically matched control samples, indicating that the possibility of polymorphism is lower than 2% (binomial test, \( P > 0.05 \)).

**Alignment Analysis**

The S202F missense mutation causes the transition of serine, a hydrophilic amino acid, to phenylalanine, an aromatic-hydrophobic amino acid. The A212P missense mutation causes the transition of alanine, an aliphatic hydrophobic amino acid, to proline, an aromatic hydrophobic amino acid. The L368R missense mutation causes the transition of leucine, an aliphatic hydrophobic amino acid, to arginine, a polar hydrophilic amino acid. A comparison of the 27 amino acids containing the mutated serine at position 202, the mutated alanine at position 212, and the 12 amino acids containing the mutated leucine at position 368, of chimpanzee, mouse, chicken, fish, xenopus, and sponge to human PEPD is presented in Figure 4. These alignments reveal conservation of the mutated serine and leucine in all compared species, and the mutated alanine in all compared species except fish and sponge.

**FIG. 3.** Mutation analysis of PD patients and their families. The PCR products were amplified from genomic DNA. A: Representative digestion of exon 8 PCR products, containing the c.605C > T mutation with MboII restriction enzyme. B: Representative digestion of exon 9 PCR products, containing the c.634G > C mutation with Fnu4HI restriction enzyme. C: Representative digestion of exon 13 PCR products, containing the c.1103T > G mutation with MslI restriction enzyme. MW, molecular weight; UC, un-cut PCR product; N, normal control; O, no DNA in this lane.
Haplotype Analysis

Three out of the five markers (D19S555, D19S719, and D19S874) were informative for mutation S202F. Haplotype analysis of eight affected individuals from three Druze and two Arab Moslem families showed that all affected individuals present with the same homozygous haplotype along approximately 500,000 bp, except for the proximal marker D19S555 in V2 from Family 4 (Table III).

Prenatal Diagnosis

Fetus V 4 Family 1 tested homozygous. His parents decided to continue the pregnancy. An affected child was born (Table II). PND from CVS identified fetus V5 heterozygous for mutation S202F mutation (Fig. 3A). A healthy child, carrier of PD, was born, as predicted.

Fetus VI5 Family 7 tested homozygous for mutation S202F (Fig. 3A). The parents decided to continue the pregnancy. An affected child (Table II) was born, as predicted. The second PND identified fetus VI6 heterozygous for the mutation S202F (Fig. 3A). Physical examination and absence of iminopeptiduria confirmed that the child does not suffer from PD. Unfortunately, he is developmentally delayed, but no diagnosis has been made.

DISCUSSION

In this study, we describe the largest cohort of PD patients: 20 individuals from 10 large highly inbred families, 7 Druze and 3 Arab Moslem.

Clinical Phenotype

The phenotype of PD is highly pleomorphic, with great variability in the age it is first recognized. The present cohort of patients showed recognized features of PD, with different degrees of developmental delay and marked facial dysmorphism. Of note, the classic PD facial dysmorphism was the first feature to raise suspicion of PD in 14 of 20 patients. Dermatological manifestations, splenomegaly, recurrent lung infections, and CF-like disease presented in some.

The rarity and the wide phenotypic variability of PD result in low awareness, delayed diagnosis, and likely an underestimation of its prevalence. Physicians from any medical discipline could be the first to confront a PD patient. Familiarity with PD features led to cancellation by a general pediatrician of bone marrow sampling in patient V3, Family 9, and to the diagnosis by a rheumatologist of PD in a patient suffering from SLE (V28, Family 6). Of particular interest is the CF syndrome [Mandel et al., 2000; Luder et al., 2007] and the two patients with SLE. Four cases of SLE phenotype associated with PD have previously been described [Bissonnette et al., 1993; Shrinath et al., 1997; Di Rocco et al., 2007]. SLE and PD are multi-system disorders associated with immune system dysfunction. Their shared clinical features raise the possibility that PD may be a genetic risk factor for the more common disorder SLE. Both disorders should be considered in patients presenting with either one.

Prolidase Deficiency and Developmental Delay

All 20 of our patients presented with some degree of developmental delay, most with moderate cognitive or speech delay. The underlying pathophysiology of developmental delay in PD is unknown, though a number of hypotheses have been proposed. High amounts of proline residues accumulating in neuro-peptides have been suggested as a possible explanation [Hui and Lajtha, 1980]. Alternatively, neutrophil superoxide, primed by iminodipeptides, may cause thickening of cerebral blood vessels and consequent mental retardation [Yasuda et al., 1999]. The proponents of the latter claim that the clinical heterogeneity they found between two sisters with PD could be due to the differences detected in their neutrophil responses to iminodipeptides [Yasuda et al., 1999].

Phenotypic Heterogeneity

Distinctive genotype–phenotype correlations for the three mutations were not discerned.
Documentation of intrafamilial phenotypic variability of PD includes two sisters with the same molecular defect [Lupi et al., 2006]. The present study presents the largest reported sample of phenotypic pleomorphism related to a single mutation. A striking example is the marked phenotypic variability within Family 1 with mutation S202F, including six affected members, a father, his four affected children, and his sister. This father, who was asymptomatic and unaware of his having PD, married his first-degree cousin, an unaware carrier of the S202F mutation. Their first child died at age 2 weeks from a sepsis-like disease including hepatosplenomegaly and jaundice. Two subsequent pregnancies yielded two girls with psychomotor retardation and dysmorphism (not known at that time to be characteristic of PD). They were diagnosed with PD only following detection of iminodipeptiduria in their aunt, IV7, during metabolic evaluation of her growth failure and developmental delay. We previously reported patient V4 [Luder et al., 2007], who was diagnosed prenatally with PD. At age 1 month, he was hospitalized with severe RSV bronchiolitis and growth failure. He had remarkable facial dysmorphism, including hypertelorism and markedly protruding eyes. He continued to suffer from recurrent pneumonia, leading to oxygen dependency and severe malnutrition. At the age of 22 months he died of respiratory failure. His father, IV8, seemed healthy, although mildly retarded, when we first met him with his son. It was the father's facial dysmorphism that led to suspicion of PD, which was confirmed by the finding of dipeptiduria and homozygosity for the S202F mutation. At age 45, he developed severe progressive restrictive lung disease. This family raises the question of whether true asymptomatic PD patients do in fact exist. Once a proband is detected, all family members should be tested. Moreover, the large multi-ethnic cohort described here suggests that population-wide genetic screening amongst Druze and Arab Moslems in northern Israel might be indicated.

Genetic Counseling

Prevention of PD is possible via PND using enzymatic and/or molecular evaluation of amniocytes or chorionic villi [Mandel et al., 2000]. However, prenatal genetic counseling for this condition is complicated due to the marked heterogeneity of the phenotype [Mandel et al., 2000]. Although most of our patients had only mild mental retardation, and might be only mildly affected, a couple at risk should be informed of the possibility of severe developmental retardation, as well as of other medical problems, including life-threatening infections, leg ulcers, and SLE, which could lead to renal failure, cerebral involvement, and chronic lung disease. In our population, the religious and cultural constraints regarding genetic counseling and PND among Arab Moslems and Druze further hinder prevention via PND [Mandel et al., 2000; Luder et al., 2007; Falik-Zaccai et al., 2008]. However, once the familial mutation is identified, preimplantation genetic diagnosis (PGD) might be a good option in high-risk populations.

The Causative Mutations

Direct DNA sequencing revealed two novel missense mutations: A212P in one Arab Moslem patient, and L368R in two Druze patients originating from the same kindred. The other 17 patients
were all homozygous for S202F, a missense mutation that was recently documented in one Druze patient from northern Israel [Hershkovitz et al., 2006]. A number of findings support the causative role of mutations A1212P, L368R, and S202F in the pathogenesis of PD. These include (1) segregation of each mutation with the disease-associated phenotype in all investigated family members; (2) the high degree of conservation of A1212P, L368R, and S202F residues across the PEPD homolog of various nonhuman species; (3) the location of all three mutations in one of the two functional domains of PEPD, substantiating the likelihood that they disrupt the enzymatic activity responsible for release of C-terminal proline and hydroxyproline from di- and tripeptides [Hershkovitz et al., 2006].

The researchers who identified mutation S202F did not detect this mutation among 223 healthy Druze individuals, who they presumed to represent all known Druze families residing in northern Israel [Hershkovitz et al., 2006]. Such findings suggested that the mutation is both rare and recent. We, in contrast, detected the same mutation in 17 patients who originated from six Druze kindreds residing in four different villages in northern Israel, and in two Arab Moslem kindreds residing in two different villages.

Although geographic proximity of Druze and Arab Moslems would allow for genetic interchange, their separate practice of consanguinity and endogamy reduces its likelihood. Thus, the presence of an identical PEPD mutation (S202F) in Druze and Arab Moslems is indeed surprising. Two possible explanations are the presence of a mutational hot spot in the PEPD gene and a founder effect. The fact that this mutation was not described in patients elsewhere militates against the former. The homozygous haplotype, identical along approximately 500,000 bp in all five families tested, three of Druze origin and two of Arab Moslem origin, is compatible with the second option, namely that of a founder mutation. Founder effects have been suggested for other monogenic recessive diseases [Stoll et al., 1999; Zlotogora et al., 2003]. We determined the carrier frequency for PD in a Druze village as 1:21 [Falik-Zaccai et al., 2008]. The relationship to other disease phenotypes such as SLE and CF suggests that PD should be considered in atypical cases of these disorders, and perhaps others, possibly as a modifier gene.

Greater awareness of the PD phenotype, especially the developmental delay and facial dysmorphism, together with understanding of its molecular basis, will contribute to the recognition and treatment of this multi-system disorder.

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