Identification of eight novel VEGFR-3 mutations in families with primary congenital lymphoedema

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rimary lymphoedema is oedema that occurs as a consequ-ence of a failure of lymph drainage and arises from an intrinsic abnormality of the lymphatic system.1 Familial lymphoedema usually segregates as an autosomal dominant trait with reported variable expression and reduced pene-trance.2 Primary lymphoedema can be classified according to age of onset, at birth as primary congenital lymphoedema (PCL) or Milroy disease (MIM 136352); more commonly, after puberty as Meige disease (MIM 153200).

Lymphoedema may occur as part of a well recognised syndrome, where the genetic defect may or may not be known, for example in Turner or Noonan syndrome (MIM 163950).3 Lymphoedema can also occur in association with other clinical features—for example, pubertal onset auto-somal dominant lymphoedema with distichiasis (LD; MIM 135400), which has been linked to 16q24.3.56 The gene for LD has recently been identified as FOXC2 (MFH-1) (MIM 602402), a member of the forkhead/winged-helix family of transcription factors, and mutations within this gene have been identified in families with LD.6,7

We have previously reported linkage to 5q35.3 in one large American family and four British families with PCL.8 This has also been shown independently by two other groups.9,10 Subsequent mutations were reported in the gene encoding the vascular endothelial growth factor receptor 3 (VEGFR-3, also known as FLT4; MIM 136352; GenBank X68203 and S66407), which resulted in defective VEGF-3 tyrosine kinase activity and signalling, suggesting this was the cause of primary lymphoedema.11,12 The VEGFR-3 gene is expressed in the lymphatic endothelium of adult tissues.14 Targeted disruption of Vagf-3 in mice leads to embryonic death at day 9.5 owing to defective development of large blood vessels and cardiovascular failure.15 Moreover, cutaneous overexpression of its ligand, vascular endothelial growth factor C (VEGF-C), induces selective hyperplasia of the lymphatic vasculature.16 This prompted the suggestion that lymphatic growth may be induced by VEGF-C and mediated through VEGFR-3.

In this report we describe 12 PCL families consistent with linkage to the 5q35.3 region and report eight novel mutations (G854S, A915P, C916W, G933R, R1041W, R1041Q, del F 1108, and P1137L), two of which each occur in two separate families. Seven of the mutations are missense mutations, as is the type of mutation found previously, and one is a deletion mutation, which has not so far been reported.12,13 We have also found eight polymorphic variations (N149D, 507 G>T, T494A, Q890H, P954S, P1008L, R1146H, and R1324L). Because, as with all previously reported mutations, the eight mutations identified in this study lie in the region of VEGFR-3 encoding the tyrosine kinase domain, they are predicted to interfere with VEGFR-3 signalling.

METHODS

Ascertainment and examination of patients

Twelve white PCL families (10 from the United Kingdom and two American) who had been referred to AHC/PM were clinically examined. A complete family history was obtained and the clinical status of all the examined subjects documented. Diagnostic criteria included evidence of idiopathic painless oedema with characteristic skin thickening and associated hyperkeratosis and papillomatosis. Skin thickening was indicated by a positive Kaposi-Stemmer sign, which is the inability to pick up or pinch a fold of skin at the base of the second toe.17 The 12 families were examined by one of us (GR, AHC, or PM) specifically to exclude the presence of other syndromes associated with lymphoedema such as Turner, Noonan, and LD syndromes. In contrast with findings in patients with LD syndrome,18 there were no occurrences of distichiasis, ptosis, cleft palate, or congenital heart disease, and true early onset varicose veins were rare. Blood samples (20 ml in EDTA) or buccal rubbings were obtained from each person for DNA extraction, as described elsewhere.19 All samples were taken with informed written consent of the individual concerned, or in the case of a child under 18, of the parent. The study protocol and consent forms were approved by the local research ethics committee.

Clinical analysis of patients

For the largest American family (PCL-900), a detailed clinical description has been published previously.9

The mode of inheritance for all families was consistent with autosomal dominance, with a history of up to five generations affected. In addition to family PCL-900, in whom 84% penetrance was indicated by haplotype analysis,19 one additional family, PCL-101, showed reduced penetrance clinically, with one adult female having an affected parent and child, although she herself was clinically unaffected. Pedigree analysis of the remaining 10 families showed no evidence of skipped generations; however, in two families (PCL-66 and PCL-72) males were less severely affected than females.

The 11 additional families had clinical features consistent with those found in PCL-900—that is, variable degrees of painless pitting lower limb oedema of congenital onset. Other associated features observed were recurrent cellulitis, papil-
Restriction enzyme analysis
To confirm the variations found, digests were made using restriction endonucleases identified on DNA Strider (version 1.2). Restriction enzymes used were as follows: Kas I (G8545S); Sau96 I (A913P, C916W); Dde I (R1041Q); Hpa II (P1137L); Fnu4HI 1 (Q900H, R1146H); Bbs I (P954S); MpoAI 1 (P1008L); A1a I (R1324L). The reactions were carried out in a 20 μl volume containing 10 μl of the PCR product, 1 × buffer (New England Biolabs, Hitchin, Hertfordshire, UK), 100 μg/ml BSA where required, and 2 units of enzyme (New England Biolabs). The reactions were incubated overnight at 37°C, after which 8 μl of the digest were run on a 3% agarose gel and visualised under ultraviolet light.

RESULTS
Seven families with PCL were genotyped with six STRP and SNP markers which were previously found to be most informative for linkage to the 5q35.3 region. Haplotypes were constructed for the families in order to determine whether the disease was segregating with these markers in the seven families (figs 2 and 3). Genotypes were referenced against those obtained for the original five families (PCL-66, 72, 100, 102, and 902) in order to determine whether there was a common ancestral haplotype, particularly with respect to the 10 British families (PCL-001, 66, 72, 100, 101, 102, 104, 107, 144, and 163). However, inspection of the “affected” haplotype between all 12 families which link to 5q35.3 does not indicate a common haplotype segregating in these different families. The same allele is shared between PCL-163 and PCL-902 (sharing the mutation G933R) and between PCL-101 and PCL-102 (sharing the mutation G933R) for the two SNPs and the most telomeric marker, D5S2006. However, we do not think this is strong evidence for a shared haplotype as they do not share the haplotype for marker D5S408, which is in closest proximity to VEGFR-3 and occurs in the same BAC. Furthermore, SNPs are bi-allelic and so are not particularly informative.

For PCL-001, 101, 107, 163, and 902 there are no affected recombinants for any of the six markers (figs 2 and 3). However, in PCL-104, affected individual V:1 has recombined for the top three centromerically positioned markers (D5S2030, WIAF-616, and WIAF-2213) but is not a recombinant for the three telomeric markers (D5S1354, D5S408, and D5S2006) (fig 2). This narrows the PCL locus in this family to below the SNP marker WIAF-2213, assuming that there is linkage to 5q35.3 in the family. In PCL-144, affected individual V:1 has recombined for marker D5S2030 but not for the other five telomeric markers, placing the region below this marker in this family, again assuming that there is linkage to 5q35.3 in this family (fig 3).

Individual III:2 of PCL-101 was found to have inherited the affected haplotype but is herself clinically unaffected (fig 2). Individuals II:1, II:5, and III:6 of PCL-101 also appear to have inherited the affected haplotype, although they are clinically unaffected. As the clinical status of the parents (I:1 and I:2) of II:1 and II:5 is not known, and the markers are not entirely informative, it is not possible to say for certain that this is the haplotype segregating with the affected status. In the case of III:6, although the haplotype of the father (II:10) is not known, she is an obligate carrier, as her mother (II:9) and daughter (IV:2) are both clinically affected. These findings are consistent with the reported incomplete penetrance of this disorder.\(^2\)\(^,\)\(^3\)\(^,\)\(^9\)\(^,\)\(^10\)

Individuals III:4 and III:5 of PCL-144 have inherited the affected haplotype despite being apparently clinically unaffected, although their clinical status has not been confirmed (fig 3). However, both individuals are presumed to be obligate carriers as they have affected offspring (individuals...
IV:4, IV:5, and IV:7) and their mother (individual II:2) was believed to be affected. Individual IV:2 of PCL-144 has inherited the affected haplotype despite being clinically unaffected, although his clinical status has again not been confirmed. However, as markers D5S408 and D5S2006 are not informative for his father, individual III:1, it is not possible to determine whether he has inherited the affected chromosome or if there has been a recombination with these two markers and he has inherited the father’s ‘normal’ chromosome. This is important because the VEGFR-3 gene lies very close to marker D5S408 (within the same BAC).

Individual II:6 of PCL-144 also appears to have inherited the affected haplotype. As the haplotype of her parents cannot be fully inferred, one cannot determine this for certain (fig 3).

One affected member from each of the families consistent with linkage to the PCL locus was selected, giving a total of 12 subjects. Eight different mutations and eight DNA variants were found, as detailed in tables 2 and 3 (note that the G854S and G933R mutations were found both in PCL-163 and PCL-902 and in PCL-101 and PCL-102, respectively).

**DISCUSSION**

In this study, we analysed 12 families (10 British and two American) who were consistent with linkage to the PCL locus on 5q35.3. Eight novel VEGFR-3 mutations (G854S, A915P, C916W, G933R, R1041W, R1041Q, del F 1108, and P1137L) and eight polymorphic variations, four of which have been previously reported, were identified among the 12 families (tables 2 and 3).

Seven of the eight novel mutations, identified in nine separate families (PCL-001, 100, 107, 144, 163, 900, and 902; PCL-163 and PCL-902 sharing the same mutation and PCL-101 and PCL-102 sharing another mutation), were missense mutations. The remaining mutation found was a deletion of three nucleotides resulting in the deletion of phenylalanine. This mutation is interesting as to date only missense
Mutations have been reported. All eight mutations were found to co-segregate with the PCL phenotype, and, where a clinically unaffected patient carried the mutation, they were also found to carry the affected haplotype. The only exception was clinically unaffected individual II:6 from PCL-144 who appears to carry the affected haplotype but not the VEGFR-3 mutation. However, as both her parents are deceased and their haplotype cannot be reconstructed, it was not possible to determine for certain whether she has the affected haplotype. Furthermore, the clinical status of this individual has not been confirmed.

Mutations R1041Q and R1041W involve the same codon in the region of VEGFR-3 encoding the tyrosine kinase domain II. The catalytic sites of many receptor tyrosine kinases contain a conserved HRDLAARN sequence, of which R1041 in VEGFR-3 corresponds to the second arginine. Previous studies have shown that mutations affecting this residue resulted in proteins with an inactive tyrosine kinase which prevented downstream gene activation. Hence R1041Q and R1041W could be predicted to have the same effect, suggesting that mutations interfering with VEGFR-3 signal transduction are the cause of primary lymphoedema in these two families.

The G854S, A915P, C916W, and G933R mutations all occur in the tyrosine kinase domain I, which is also known to be important for the catalytic activity of protein kinases and so could again be predicted to result in a defective receptor. The G854S mutation affects the second glycine residue in the conserved GXGXXG kinase motif, which forms a critical portion of the ATP-binding site. The GXGXXG sequence forms a turn between β strands 1 and 2 of the five stranded β sheet. Interaction between this motif—conserved residues on β strand 3—and the phosphates of ATP is believed to be important for the productive binding of ATP and catalysis.
Interestingly, this mutation was found to occur in two separate families, which may suggest that this region will prove to be a hot spot for mutations in PCL. Furthermore, another mutation in this GXGXXG kinase motif has been found independently in a family with PCL (G857R). The P1137L mutation occurs in subdomain X, which corresponds to the larger COOH-terminal lobe, mainly responsible for binding the peptide substrate and initiating phosphotransfer. It is predominantly helical in content. The P1137L mutation occurs within the tyrosine kinase domain II.

The deletion (del F 1108) also occurs within the tyrosine kinase domain II. This mutation is of particular interest as it is, to our knowledge, the first disease-causing mutation involving the deletion of an amino acid. All polymorphisms found in this study, with the exception of one (P1008L), were either ones which had previously been reported (N149D, S954G, T494A, and R1324L) or were present in normal controls at a frequency greater than 1% (Q890H (16%), P954S (3%), and R1324L (28%)). The P1008L substitution, in PCL-902, was found in only two (II:1 and II:4) of six affected family members tested, and so is not seen to be causative. Furthermore, upon closer inspection of the haplotype data, it became apparent that the two individuals with the P1008L substitution had both inherited the same normal haplotype from their parent (presumably individual I:2), which was not inherited by any other family members. This suggests that the P1008L mutation is being transmitted along with the “normal” allele and hence it is likely to be a rare polymorphism, although it has not been found in 50 normal controls tested (fig 3).

None of the eight mutations described above were found in the unaffected family members or in 50 normal controls. Thus these alterations could contribute to causing the observed lymphoedema in the corresponding families. Although no phenotype–genotype correlation can be
extracted from these results, it is interesting to note that all eight causative mutations occurred in the kinase domains of the VEGFR-3 protein. These findings support previously published mutations.12 13

As, to date, probable causative mutations have only been found in 10 of the families, the underlying genetic cause of lymphoedema in the remaining two PCL families which appear to map to 5q35.3 remains to be determined (PCL-72 had a maximum LOD score of 0.938 (θ = 0.1) for the closest marker, D5S408, and although PCL-104 is too small to produce a significant LOD score, the haplotype data are consistent with linkage to this region). It is unlikely that there has been a major deletion resulting in the lymphoedema phenotype as we have found polymorphic biallelic variations in the VEGFR-3 promoter. As such, it is possible that mutations will be found here, or there could be other, as yet unidentified, genes responsible for the lymphoedema phenotype observed in these families.

Clinically there is little to separate those families in which mutations were found and those without a VEGFR-3 mutation. Cellulitis and tense dilated leg veins occurred in both groups, and the severity of leg swelling varied both within and between families. Although this a small group it is noteworthy that a hydropoecele occurred in at least one male member of all seven families with a VEGFR-3 mutation and in none of the families without a mutation.

Conclusions

In summary, we report here the identification of eight novel mutations in the VEGFR-3 gene in 12 white families with primary congenital lymphoedema linking to 5q35.3. Our results confirm the causative role of the VEGFR-3 gene in the pathogenesis of this disease.

ACKNOWLEDGEMENTS

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**Table 3 Summary of variants found in the VEGFR-3 gene**

<table>
<thead>
<tr>
<th>Family</th>
<th>Position</th>
<th>Nucleotide position</th>
<th>Amino acid substitution</th>
<th>Domain</th>
<th>Type of change</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL-001</td>
<td>Ex 4</td>
<td>445 A&gt;G</td>
<td>N149D</td>
<td>Between Ig-like domains 1 and 2</td>
<td>Polymorphic substitution*</td>
</tr>
<tr>
<td>PCL-72</td>
<td>Ex 4</td>
<td>507 G&gt;T</td>
<td>—</td>
<td>Ig-like domain 2</td>
<td>synonymous substitution*</td>
</tr>
<tr>
<td>PCL-72</td>
<td>Ex 11</td>
<td>1480 A&gt;G</td>
<td>T494A</td>
<td>Ig-like domain 5</td>
<td>Polymorphic substitution*</td>
</tr>
<tr>
<td>PCL-101</td>
<td>Ex 19</td>
<td>2670 G&gt;C</td>
<td>Q890H</td>
<td>TK I domain</td>
<td>Polymorphic substitution†</td>
</tr>
<tr>
<td>PCL-101</td>
<td>Ex 21</td>
<td>2860 C&gt;T</td>
<td>P954S</td>
<td>Kinase insert</td>
<td>Polymorphic substitution†</td>
</tr>
<tr>
<td>PCL-902</td>
<td>Ex 22</td>
<td>3023 C&gt;T</td>
<td>P1008L</td>
<td>Kinase insert</td>
<td>Polymorphic substitution†</td>
</tr>
<tr>
<td>PCL-001</td>
<td>Ex 26</td>
<td>3437 G&gt;A</td>
<td>R1146H</td>
<td>TK II domain</td>
<td>Polymorphic substitution*</td>
</tr>
<tr>
<td>PCL-72</td>
<td>Ex 4</td>
<td>507 G&gt;T</td>
<td>—</td>
<td>Ig-like domain 2</td>
<td>synonymous substitution*</td>
</tr>
<tr>
<td>PCL-104</td>
<td>Ex 21</td>
<td>2860 C&gt;T</td>
<td>P954S</td>
<td>Kinase insert</td>
<td>Polymorphic substitution†</td>
</tr>
<tr>
<td>PCL-902</td>
<td>Ex 22</td>
<td>3023 C&gt;T</td>
<td>P1008L</td>
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<tr>
<td>PCL-001</td>
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<td>R1146H</td>
<td>TK II domain</td>
<td>Polymorphic substitution*</td>
</tr>
</tbody>
</table>

*Previously reported. †Novel polymorphism found in 50 unrelated control individuals. ‡Novel polymorphism not found in 50 unrelated control individuals.

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