LETTER TO JMG

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

A L Evans, R Bell, G Brice, P Comeglio, C Lipede, S Jeffery, P Mortimer, M Sarfarazi, A H Child

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Primary lymphoedema is oedema that occurs as a consequence 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 penetrance.[2][3] Primary lymphoedema can be classified according to age of onset, at birth as primary congenital lymphoedema (PCL) or Milroy disease (MIM 153100) or, 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).[4] Lymphoedema can also occur in association with other clinical features—for example, pubertal onset autosomal dominant lymphoedema with distichiasis (LD; MIM 153400), which has been linked to 16q24.3.[5][6] 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.[7–10]

We have previously reported linkage to 5q35.3 in one large American family and four British families with PCL.[3] This has also been shown independently by two other groups.[11][12] 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 VEGFR-3 tyrosine kinase activity and signalling, suggesting this was the cause of primary lymphoedema.[12][13] The *VEGFR-3* gene is expressed in the lymphatic endothelium of adult tissues.[14] Targeted disruption of *Vegfr-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

Key points

- Twelve families with primary congenital lymphoedema (PCL) are described in which there is linkage to 5q35.3.
- Eight novel mutations were identified in *VEGFR-3*, all of which occur in the tyrosine kinase domain.
- We conclude that mutations occurring in the region of *VEGFR-3* encoding the tyrosine kinase domain interfere with *VEGFR-3* signalling, resulting in the PCL phenotype.

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 (GB, 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,[10] 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.[18] 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.[3]

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,[3] 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-

Figure 1 Evidence of lymphoedema in a 45 year old man. This individual from PCL-900[3] has had bilateral swelling of the lower limbs since birth, with papillomatosis and nail changes.

lomatosis, hydroceles, and prominent veins in the lower legs (fig 1).

Controls

One hundred chromosomes from 50 unrelated control individuals were tested for the newly identified DNA variants.

Genotyping

Before mutation analysis we undertook manual genotyping in the additional seven unpublished families (PCL-001, 101, 104, 107, 144, 163, and 902) using six chromosome 5 specific STRP and SNP markers from the 5q35.3 region as described by Evans et al.[3] Genotypes were then used to construct the inherited haplotype in all seven families.

Polymerase chain reaction

Primers used to amplify all 30 exons of the *VEGFR-3* gene in the polymerase chain reaction (PCR) were designed around intronic sequences obtained from Iljin et al,[19] and the Homo sapiens chromosome 5 clone CTB-36B8 (GenBank accession No AC022095). Primers to screen the part of the *VEGFR-3* gene encoding the intracellular kinase domain, encoded in part by exons 18, 23, and 25, were as published by Karkkainen et al.[13] The complete list of primers is reported in table 1. Detailed protocols are available on request.

Direct sequencing

Mutation analysis was done by direct sequencing of the amplified exons using the same oligonucleotides used for the PCR. Sequence analysis was carried out using the Big Dye terminator kit (Applied Biosystems, Warrington, Cheshire, UK), and the samples run on an ABI 310 genetic analyser (Applied Biosystems), following the manufacturer's directions. Sequencing data were analysed on ABI 310 sequencing analysis software to check for the presence of any base changes.

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 (G854S); *Sau*96 I (A915P, C916W); *Dde* I (R1041Q); *Hpa* II (P1137L); *Fnu*4H I (Q890H, R1146H); *Bbs* I (P954S); *Msp*A1 I (P1008L); *Alu* 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℃, 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.[3] 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 900)[3] 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 13 20]

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

Table 1 Details of primers used to sequence *VEGFR-3* and conditions for amplification

Exon	Forward primer	Reverse primer	Annealing temperature (°C)	Magnesium concentration (mmol/l)
1	ccgcgcggacactttcagcc	gtacccggcggagcggtctc	64	1.5
2	cgatgcagaatgcccagccatg	cacagccaccacctcctgcc	66	1.5
3	ctggtgaggctcggcctgag	agcccagggtccacaggctg	65	1
4	tggctggttcaggaccagcac	ccttgctcctggccagacag	64	1.5
5	ctgtctggccaggagcaagg	caggacgacacccactgagg	64	1.5
6	gggctgtcactgcctcactgg	cagtgtgtgctgtacagtcacag	65	1.5
7	gacgcggggcttccctg	cacaaggaccctggtttcc	61	1
8	cctggtcagtccacgacggg	tctccgtgtgcaggtcctgac	65	1.5
9	gggccctgacctccttc	cccgtgcgctctcccgtc	59	1
10	cagctgaccggccatctg	ggggacctgaggctggag	61	1
11	ctgcccttgcctcttctg	gggcactcagcagcgcg	61	1
12	gcacagcgacctccttgcag	ctcaatgagcccaaacctgcc	65	1.5
13	tgccacagggcttcttcc	gtccccgccctccccg	61	1
14	gtcctcctgctaagtgcagctc	gcctcgcgggcctccggac	65	1.5
15	gtccggaggcccgcgaggc	cttctggtggccacgacttgc	65	1
16	gcaagtcgtggccaccagaag	gaactagggcgggcaccttattc	65	1.5
17	ctgtcctgggcaaagttctgcc	atggagggattcaggcactccg	65	1.5
18	tgtctccacgctcaccc	ccgctgaccccacacctt	55	1
19	tgccttagctaagcggccagtg	gctccgcgtttgcacccgcg	65	1
20	cgcgggtgcaaacgcggagc	cgcagaggcgcctccattcc	65	1
21	gccctcgagccagcttcg	aggaaaagggaagaggccag	61	1
22	acatgcttgttagctgttccctg	caagcacttcttggcggactggt	63	1.5
23	gagttgacctcccaaggt	tctcctggacaggcagtc	56	1.5
24	ttcaacccaggcagaggaccc	cgtaacctgccgccagtgacc	64	1.5
25	caggacggggtgacttga	gcccaggcctgtctactg	56	1
26	gggcactgaaggtcctgggac	gcagctcgggcccagggacc	67	1
27	gctgagggcagtggctggtg	aaatgcaccttgtccacatggc	65	1
28	cctcagcaccttctgatttc	cccaccccttcatgtgaagt	55	1.5
29	ggtggctgccctgcaggagg	ggagggcaacatcgatacctgc	65	1.5
30	tactaacaccaccttccctg	gtgctgggggtcttgtcc	60	1.5

Table 2 Summary of mutations found in the *VEGFR-3* gene

Family	Position	Nucleotide position	Amino acid substitution	Domain	Type of change
PCL-163	Ex 18	2560 G>A	G854S	TK I domain	Missense mutation
PCL-902	Ex 18	2560 G>A	G854S	TK I domain	Missense mutation
PCL-100	Ex 19	2743 G>C	A915P	TK I domain	Missense mutation
PCL-900	Ex 19	2748 C>G	C916W	TK I domain	Missense mutation
PCL-101	Ex 20	2797 G>C	G933R	TK I domain	Missense mutation
PCL-102	Ex 20	2797 G>C	G933R	TK I domain	Missense mutation
PCL-001	Ex 23	3121 C>T	R1041W	TK II domain	Missense mutation
PCL-107	Ex 23	3122 G>A	R1041Q	TK II domain	Missense mutation
PCL-66	Ex 24	nt 3323-3325 del TCT	del F 1108	TK II domain	Deletion
PCL-144	Ex 25	3410 C>T	P1137L	TK II domain	Missense mutation

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 in the region of these two markers. 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,[19] 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
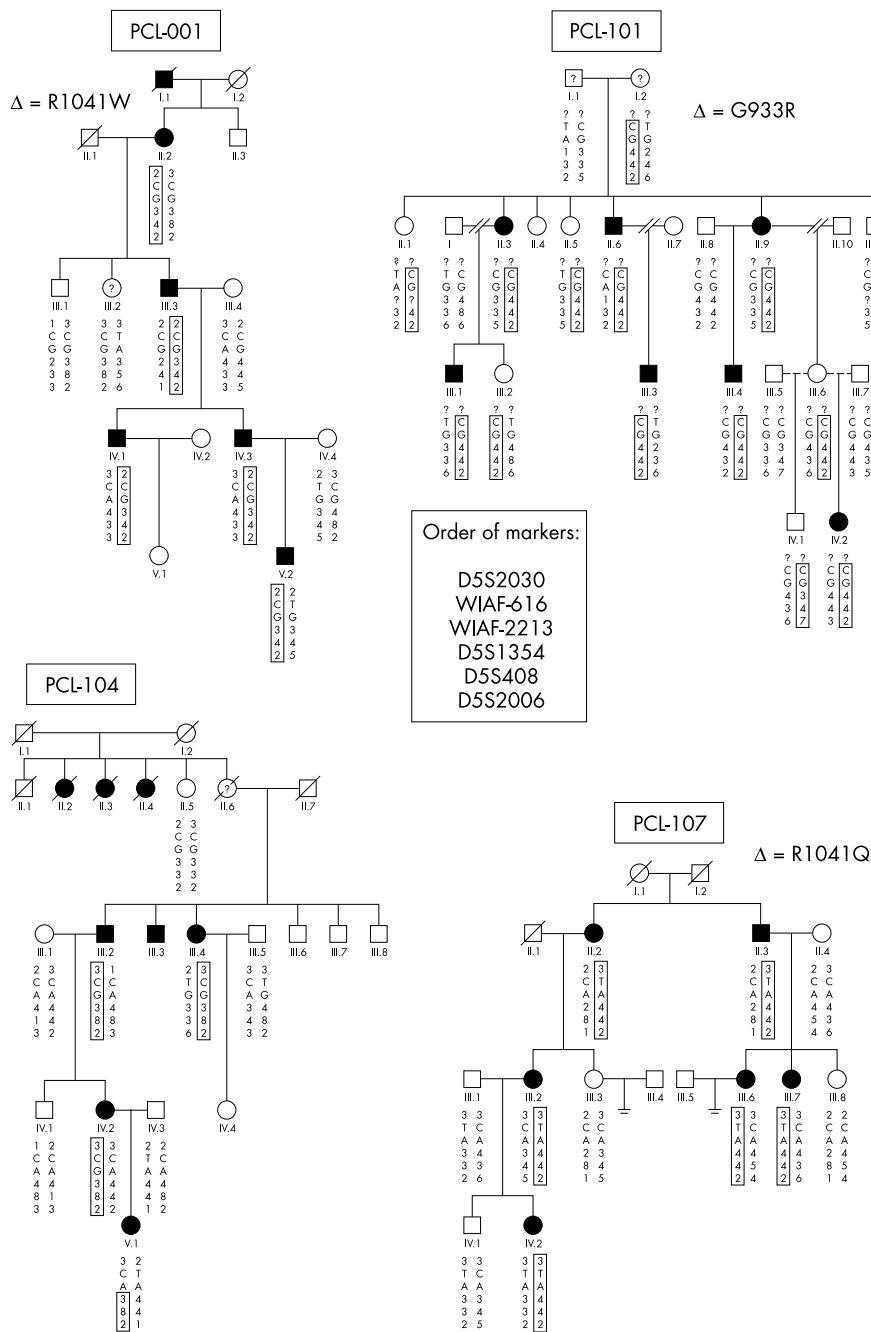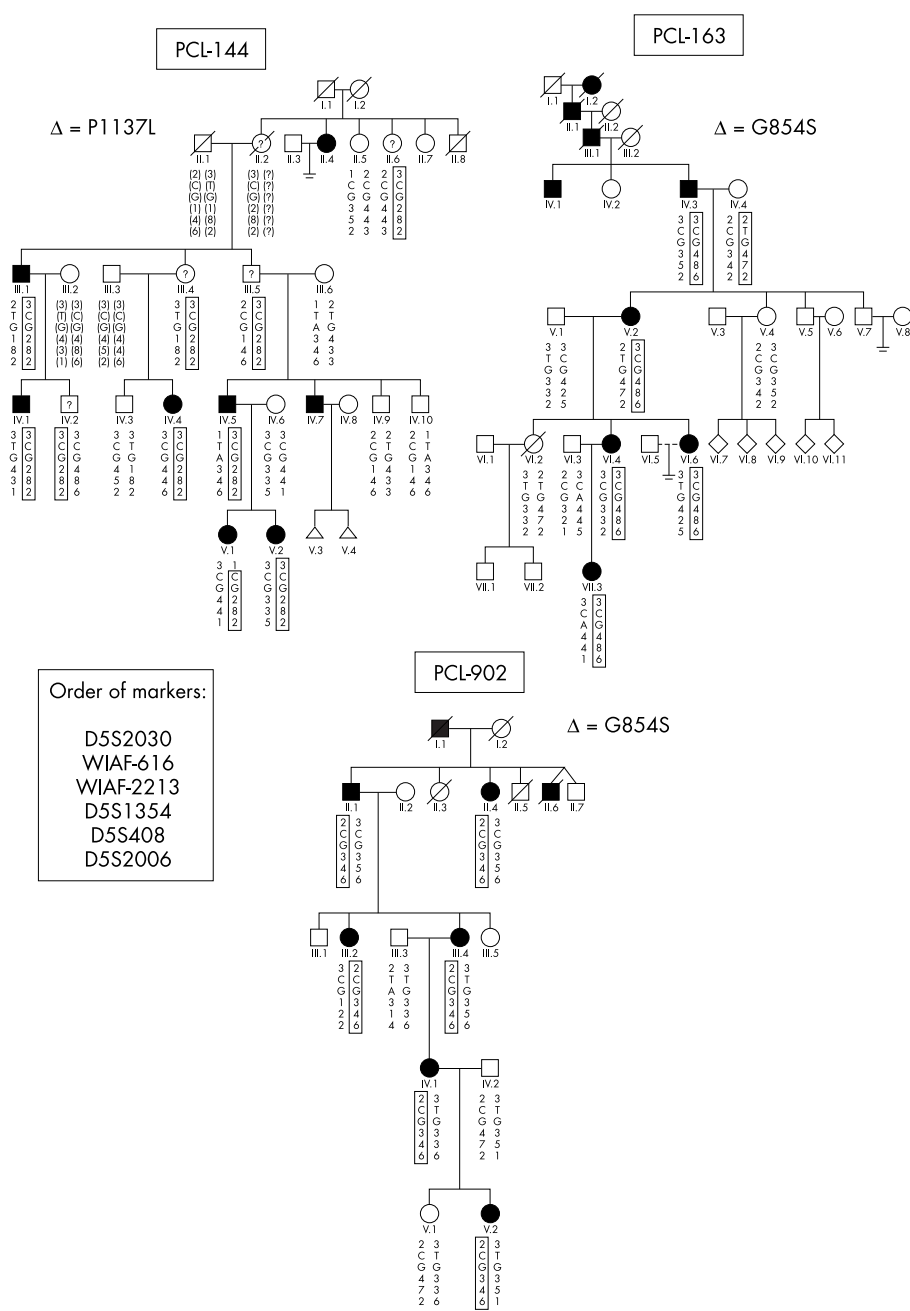
Figure 2 Pedigree structure and genotypic data for PCL-001, 101, 104, and 107. The "affected" haplotype is boxed. Note that in PCL-101, four unaffected family members have also inherited the affected haplotype (II:1, II:5, III:2, and III:6). The clinical status has not been confirmed in individuals III:2 from PCL-001, I:1 and I:2 from PCL-101, and II:6 from PCL-104, so they have been marked with a ? in their symbol. Δ, mutation.

mutations have been reported.[12][13] 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.[12][21] Previous studies have shown that mutations affecting this residue

resulted in proteins with an inactive tyrosine kinase which prevented downstream gene activation.[13] 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.[13][21] 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.[13][22]

Figure 3 Pedigree structure and genotypic data for PCL-144, 163, and 902. The ''affected'' haplotype is boxed. Note that in PCL-144, four unaffected family members have also inherited the affected haplotype (II:6, III:4, III:5, and IV:2) and have been marked with a ? in their symbol, as their clinical status has not been confirmed. Δ, mutation.

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).[13]

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 α helical in content.[21] 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, 507 G>T, T494A, and R1146H)[19] 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

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

Family	Position	Nucleotide position	Amino acid substitution	Domain	Type of change
PCL-001 PCL-72 PCL-104 PCL-900	Ex 4	445 A>G	N149D	Between Ig-like domains 1 and 2	Polymorphic substitution*
PCL-66	Ex 4	507 G>T	–	Ig-like domain 2	Synonymous substitution*
PCL-902	Ex 11	1480 A>G	T494A	Ig-like domain 5	Polymorphic substitution*
PCL-101	Ex 19	2670 G>C	Q890H	TK I domain	Polymorphic substitution†
PCL-101	Ex 21	2860 C>T	P954S	Kinase insert	Polymorphic substitution†
PCL-902	Ex 22	3023 C>T	P1008L	Kinase insert	Polymorphic substitution‡
PCL-001 PCL-72 PCL-104 PCL-107 PCL-900	Ex 26	3437 G>A	R1146H	TK II domain	Polymorphic substitution*
PCL-66 PCL-902	Ex 30b	3971 G>T	R1324L	Carboxy terminus	Polymorphic substitution†

*Previously reported.[19]
†Novel polymorphism found in 50 unrelated control individuals.
‡Novel polymorphism not found in 50 unrelated control individuals.

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 *VEGFR-3* in both families (N149D and R1146H; table 3). The *VEGFR-3* promoter has recently been isolated[19] and 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 whom 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 hydrocele 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.

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Authors' affiliations
A L Evans, G Brice, P Comeglio, A H Child, Department of Cardiological Sciences, St George's Hospital Medical School, London SW17, UK
R Bell, C Lipede, S Jeffery, Medical Genetics Unit, St George's Hospital Medical School
P Mortimer, Department of Medicine (Dermatology), St George's Hospital Medical School
M Sarfarazi, Department of Surgery, University of Connecticut Health Center, Farmington, Connecticut, USA

Correspondence to: Dr Alison Evans, Department of Cardiological Sciences, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, UK; a.evans@sghms.ac.uk

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