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ARTICLE

Linkage disequilibrium narrows locus for venous malformation with glomus cells (*VMGLOM*) to a single 1.48 Mbp YAC

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Venous malformations with glomus cells are localised cutaneous lesions of vascular dysmorphogenesis. They are usually sporadic, but sometimes familial. Using five families, we mapped the locus, *VMGLOM*, to chromosome 1p21–p22. In order to refine this locus, spanning 4–6 Mbp, we then studied seven additional families. They exhibited linkage to *VMGLOM* and the combined lod score for all 12 families was 18.41 at $\theta = 0.0$ for marker *D1S188*. We found a distinct haplotype shared by seven families, comprising seven alleles which are rare in the general population ($P < 0.01$). This indicates that the haplotype is identical by descent in all seven families, and hence the locus can be refined by inferring ancestral crossovers. Using this approach, we position the causative gene between two markers on the same non-chimeric YAC of 1.48 Mbp, a feasible size for positional cloning. As there is no known gene involved in vasculogenesis and/or angiogenesis in this YAC, the identification of the causative gene is likely to reveal a novel regulator or vascular development. *European Journal of Human Genetics* (2001) **9**, 34–38.

Keywords: vascular anomaly; haplotype sharing; glomangioma; multiple glomus tumor; chromosome 1p21–p22

Introduction

Vascular malformations are *localised* errors of morphogenesis.[1] They are classified by the histologic appearance of the anomalous channels as either arterial, capillary, lymphatic, venous or combined malformations.[2] Vascular lesions are usually cutaneous and often disfiguring. Functional and even life-threatening problems can occur if these anomalies involve deeper structures or viscera. Venous malformations (VMs) are the most common in referral centres. They appear as bluish cutaneous lesions that are localised, either small or extensive.[1]

To identify genes involved in venous morphogenesis, we studied families with inherited cutaneous venous malformations (OMIM No 600195), and found the first genetic locus on chromosome 9p21.[3] Subsequently, we identified the causative activating R849W mutation in TIE2/TEK, a receptor tyrosine-kinase specifically expressed in endothelial cells.[4] In addition to this locus, we discovered a second 4–6 cM locus on chromosome 1p21–p22 that is linked to venous malformations in five families (*VMGLOM*, OMIM No 138000).[5] Although the cutaneous lesions in these families clinically looked very similar to the 9p-linked VMs, histologic analysis of the lesions revealed the presence of 'glomus cells' around the lumens of the distorted vessels, hence the traditional histopathologic name 'glomangioma'. The causative gene is

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unknown and we are using positional cloning for its identification.

In order to refine the locus, we here report seven additional families with venous anomalies, show linkage to the *VMGLOM* locus and reveal the existence of a founder effect for some of the families, thereby refining the locus to the size of a single YAC.

Material and methods

The pedigrees of the seven new families with venous malformations with glomus cells are shown in Figure 1. Each participant gave informed consent, as approved by the ethics committee of the medical faculty of the Université catholique de Louvain, Brussels, Belgium. Clinical history was taken and physical examination was performed on all family members participating in the study. Venous blood samples were drawn and DNA was extracted from the buffy coats (Qiagen QIAamp DNA Blood Mini Kit, Westburg, Leusden, The Netherlands). Histological characterisation was done on lesions excised from individuals in all families except family A1.

Genotyping of individuals was performed, as described elsewhere.[3] We used microsatellite markers located in the *VMGLOM* region on the basis of CEPH (Le Centre d'Etude du Polymorphisme Humain: http://www.cephb.fr/), CHLC (The Cooperative Human Linkage Center: http://chlc1.fccc.edu/ChlcMarkerMaps.html) and Whitehead/MIT databases (The Whitehead Institute for Biomedical Research/MIT Center for Genome Research: http://www-genome.wi.mit.edu/). Marker *D1S2849* gave mediocre results that were improved by replacing primer AFM350tg9m by primer 5'-ATTTCCC-TACCCTCCAGAC-3', giving a shorter PCR product. We also used novel CA repeat microsatellites that had been isolated in our laboratory as part of the construction of a physical map for the region (33CA1, 50CA1, 69CA1 and 75CA1).[6] Calculations were performed using the MLINK program of the LINKAGE package,[7] with parameters as described.[5] Phenocopies were not allowed and three penetrance classes were defined on the basis of Iqbal *et al*[8] (Table 1).

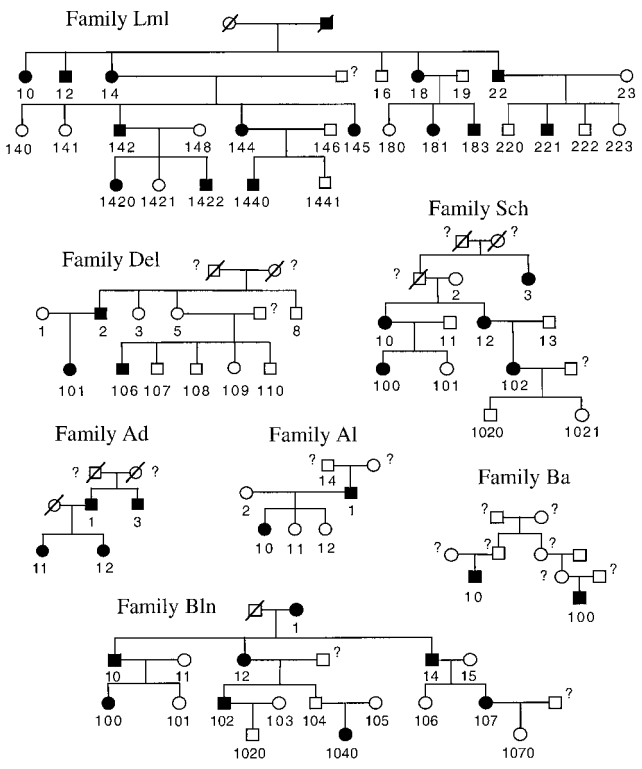

Figure 1 Pedigrees of seven families with venous malformations with glomus cells. Solid symbol: affected person; ?: unknown affection status; /: deceased person. Individuals with a number have been genotyped.

In order to detect putative haplotype sharing, three affected individuals from each family (except for families Ba and A1, in which there are only two affected individuals) were genotyped for every available microsatellite marker in the *VMGLOM* locus (Figure 2). The precise order of the markers was known by construction of the physical map of the locus.[6] The radioactive PCR products for each marker were resolved on a separate polyacrylamide gel to allow consistent scoring of the alleles across the families. Absolute sizes of the alleles were determined by running a pUC18 plasmid sequencing reaction on the same gel. As an additional control for the sizes of the alleles, PCR reactions from CEPH reference individuals (Nos 88415, 134702, 133101 and 133102, http://www.cephb.fr/cephdb/) were also included. To estimate the frequencies of the shared alleles and haplotypes in the general population, 16 triplets (father, mother, child), belonging to the genetically heterogeneous Belgian population, were also genotyped. The frequencies of the shared alleles from the first and second haplotypes were calculated in the affected families and in our control dataset, comprising 64 haplotypes. Statistical significance of the frequency bias was assessed using the uncorrected chi-square test of independence.

In order to retrieve from databases genomic sequences in the *VMGLOM* candidate region, the sequences of the markers

Table 1 Lod scores for marker *D1S188* in the seven families, for disease-allele frequency 0.0001, 10 marker alleles with equal frequencies 0.1, and three liability classes with penetrances 90%, 80% and 70%, respectively, for individuals of age ≥16 years, 12 years ≤age <16 years, and <12 years

Family	*θ=0.0*	*θ=0.01*	*θ=0.05*	*θ=0.1*	*θ=0.2*	*θ=0.3*	*θ=0.4*
Lml	3.78	3.75	3.56	3.27	2.56	1.70	0.70
Sch	1.28	1.26	1.14	1.00	0.70	0.42	0.18
Bln	0.75	0.74	0.70	0.63	0.47	0.29	0.12
Del	0.56	0.54	0.47	0.39	0.23	0.09	0.02
Ba	0.32	0.31	0.26	0.20	0.11	0.05	0.02
Al[a]	–0.18	–0.15	–0.06	0.01	0.08	0.09	0.07
Ad[b]	–0.52	–0.45	–0.27	–0.15	–0.04	–0.00	–0.00

[a]histological diagnosis not known; [b]uninformative marker.

	HAPLOTYPE A										
	BI USA	Bt Bel	Sh USA	F Sco	T Ita	Bln Fra	Sch Ger	n/N fam.	n/N con.	P	
A205XD5	241	239/R	241/245	245/R	241	241	245	-	-	-	
D1S435	175	157	161	175	175	157	157	5/12	25/60	1.000	
B337XE1	263	263	267	281	259	263	267	4/12	24/54	0.481	
D1S188	162	168	166	166	166	168	166	3/12	6/54	0.205	
D1S1170+	118	118	118	118	122	118	118	10/12	30/60	0.034	
33CA1	156	156	156	156	152	156	156	9/12	18/62	0.002	*
D1S2804	179	179	179	179	179	179	179	7/12	10/62	0.001	*
D1S424	223	225	223	223	223	225	223	5/12	9/62	0.033	
D1S406+	200	200	200	204	200	196	200	5/12	6/64	0.003	*
69CA1	171	169/171	171	171	171	171	171	9/12	15/60	0.001	*
D1S2776	206	206	206	206	206	206	206	8/12	8/62	0.000	*
50CA1	127	125/127	129	127	127	127	127	7/12	2/48	0.000	*
D1S2868	146	146	146	146	146	146	146	7/12	14/62	0.012	
75CA1	171/173	171/173	173	173	171	173	173	6/12	5/54	0.001	*
D1S2849	179	179	179	179	179	179	179	8/12	17/64	0.007	*
D1S2779	229	231	233	231	241	231	231	4/12	21/62	0.971	
D1S236	194	210	194	190	212	190/R	190/R	3/12	37/62	0.027	
D1S2775	201/R	199/201	201	201	201	195/R	201/R	-	-	-	

	HAPLOTYPE B							
	AI USA	Ba Yug	Del Bel	Ad Fra	n/N fam.	n/N con.	P	Lml Fra
A205XD5	241	241	241	241	-	-	-	241/R
D1S435	157/161	161	175	157/161	-	-	-	161/R
B337XE1	259	259/267	259	259	5/12	14/54	0.276	263/R
D1S188	154	160	154	152	2/12	6/54	0.594	168
D1S1170+	118	118/122	118/122	122	10/12	30/60	0.034	118
33CA1	156	156	156	152	9/12	18/62	0.002 *	142
D1S2804	185	185	183	185	3/12	13/62	0.756	191
D1S424	209	209	209	209	4/12	28/60	0.396	225
D1S406+	208	204/208	208	208	5/12	17/64	0.290	208
69CA1	169	169	171	169/171	4/12	30/60	0.291	173
D1S2776	198	198	198	198	4/12	11/62	0.219	206
50CA1	125	125	125	127	5/12	27/48	0.365	125
D1S2868	148	148	148	148	4/12	25/62	0.650	144
75CA1	171	171	171	175	7/12	36/54	0.584	171
D1S2849	181	179	181	183	2/12	10/64	0.928	176
D1S2779	235	233	235	241	2/12	3/62	0.135	229
D1S236	194	212	210	212	-	-	-	212
D1S2775	201	201	199/201	199/201	-	-	-	199

Figure 2 Haplotypes A and B sharing in *VMGLOM*. Numbers indicate sizes of alleles that segregate with the disorder in each family. At the top, symbol and geographic origin of family. USA: The United States of America; Bel: Belgium; Sco: Scotland; Ita: Italy; Fra: France; Ger: Germany; Yug: Yugoslavia. +: tetranucleotide repeat microsatellite. R: a recombinant individual in the family for this marker; X/Y: data not informative for linked allele. Marker alleles with a probable ancestral mutation differ from shared haplotype: white background; n/N: number of the shared allele on total number of alleles; fam: families linked to *VMGLOM*; con: control individuals from the Belgian population. P: *P*-value for the uncorrected chi-square test in a 2 × 2 table; *: significant *P*-value ($P < 0.01$).

were compared to the human high-throughput genomic sequences using the blast sequence similarity search tool (http://www.ncbi.nlm.nih.gov/BLAST/). The ordering of the PAC clones containing the markers was visualised using the Entrez Map Viewer tool (http://www.ncbi.nlm.nih.gov:80/cgi-bin/Entrez/hum_srch?chr=hum_chr.inf&query). Known genes and putative genes in the region were identified by comparing the unfinished PAC sequences to the non-

redundant GenBank database using the NCBI blast tool, and to a database of EST contigs at the Swiss EMBnet server (http://www.ch.embnet.org/software/aBLAST.html).

Results

Physical examination of the patients from the families with venous malformations with glomus cells revealed that the locations, number per individual and histological appearance of the lesions were similar to those documented in the five families initially reported.[5]

The number of affected males and females in the 12 families was 35 and 40, respectively, and 59% (26/44) of children of an affected parent had the disorder. Examination of the seven new pedigrees revealed that the condition skipped a generation twice (individuals Del5 and Bln104, Figure 1). The autosomal dominant pattern of inheritance with high penetrance and the absence of sex bias are consistent with our previous data.[5]

None of the seven new families formally excluded linkage to the *VMGLOM* locus. Lod scores for marker *D1S188* are given for different recombination fractions in Table 1. Family Lml showed strong linkage to the *VMGLOM* locus (lod score 4.05 for marker *D1S2804*). Families Sch, Bln, Del, Ad and Ba also showed evidence for linkage in the locus, with highest lod scores (at $\theta = 0.0$) of 1.69 for marker *D1S2776* (family Sch), 0.75 for marker *D1S188* (family Bln), 0.56 for marker *D1S188* (family Del), 0.56 for marker *D1S2776* (family Ad), and 0.52 for marker *D1S2776* (family Ba). Family A1 gave slightly negative lod scores (–0.18 for marker *D1S188*). The maximum combined lod score for the seven new families was 5.99 ($\theta = 0.0$) for marker *D1S188*, and for all 12 families it was 18.41 ($\theta = 0.0$), with no obvious evidence for locus heterogeneity. However, four young unaffected recombinants were observed (Lml223, A112, Sch1020 and Bln1070, aged 14 years, 10 years, 2 years and 1 year, respectively).

Haplotypic analysis of the seven families defined new obligatory recombinations in *VMGLOM* between markers *AFMB337XE1* and *D1S188* (affected individual Lml22) on the telomeric end of *VMGLOM* and between markers *D1S236* and *D1S2779* (affected individuals Sch3 and Bln100, and unaffected individual Bln1020) in the centromeric region. These findings narrow the locus by 2 cM from *AFMA205XD5–D1S236* [Boon, 1999 No 811] to *AFMB337XE1–D1S236*, a region of about 3 cM (on the basis of the Whitehead/MIT physical map). Comparison of the haplotypes linked to the condition in the 12 families revealed two distinct haplotypes, A and B, shared by seven families (Bl, Bt, Sh, F, T, Bln, Sch), and by four families (Al, Ba, Del, Ad), respectively (Figure 2). Family Lml had a unique haplotype.

Statistical significance of the apparent linkage disequilibrium was assessed using the chi-square independence test. The frequency bias is significant ($P < 0.01$) for seven out of the nine markers in the core of the first haplotype (between *D1S2804* and *D1S2849*, Figure 2, top). This supports the hypothesis of a founder effect for this haplotype, and allows us to refine the locus further by inferring ancestral recombinations. In contrast, alleles of the second haplotype do not show statistically significant enrichment from the general population, and thus the second haplotype is probably due to co-occurrence of frequent alleles by chance (Figure 2, bottom). Thus, based on apparent ancestral crossovers in family T for the first haplotype, the *VMGLOM* locus can be delineated between marker *33CA1* and marker *D1S2779*. These two markers, and all intervening markers, have been localised on the same non-chimeric 1.48 Mbp YAC 957D9 (Whitehead/MIT database).[6]

In this 1.48 Mbp *VMGLOM* region between *33CA1* and *D1S2779*, 13 PACs with unfinished sequences were identified as they contained at least one of the markers in the interval. Moreover, two PACs with finished sequences were also identified as belonging to the same PAC contig. On the basis of the Entrez Genome Map Viewer, most of the region appears to be covered by unfinished sequences, although there are two gaps. The ordering of the markers is consistent with our data.[6] In addition to putative genes and EST clusters, nine known or highly similar to known genes were identified in the region using sequence similarity searches. Known genes are *EVI5* (ecotropic viral integration site 5), *FAP48* (FKBP-associated protein), *GFI1* (growth factor-independent 1) and *RPL5* (ribosomal protein L5). The five other genes are highly similar (>90% identity) to *ACTG1* (actin, gamma 1), *AIF1* (allograft inflammatory factor 1), *BTK* (Bruton agammaglobulinemia tyrosine kinase), *HMG4* (high-mobility group protein 4) and *RPL29* (ribosomal protein L29).

Discussion

In this study, we described seven new families with inherited cutaneous venous malformations with glomus cells that showed linkage to the *VMGLOM* locus on chromosome 1p21–p22. By combining the linkage data from these families with our previous data, the maximum two-point lod score was 18.41 (marker *D1S188*, $\theta = 0.0$). The disease showed autosomal dominant inheritance pattern with high penetrance. Four young children were unaffected carriers. This non-penetrance was expected since it has been shown that the onset of the disease can be pubertal.[5] In addition, unaffected individual Del5, with an affected son, had inherited the haplotype associated with the condition in her family and is thus likely to be a carrier. Individual Bln104, on the contrary, had not inherited the haplotype linked with the disorder in his family, and thus his daughter, Bln1040, who has a single small ventral lesion, is probably a phenocopy. This underscores the uncertainty of clinical diagnosis of single small cutaneous lesions in genetic studies of vascular anomalies. Taken together, our data suggest that the *VMGLOM* locus contains the major gene for venous malformations with glomus cells.

Haplotypic analysis revealed recombinative events in affected individuals, narrowing the region from 5–6 cM to 3 cM. Furthermore, in a subset of the families, we discovered a haplotype that is significantly associated with the disease, underlining the existence of a founder effect. This linkage disequilibrium allowed us to further diminish the locus by inferring ancestral crossovers to the region between markers *33CA1* and *D1S2779*. These markers are contained in a single 1.48 Mbp YAC, and thus this finding constitutes an important step towards positional cloning of the *VMGLOM* gene. Naturally, we cannot rule out the possibility that the apparent crossovers in markers *33CA1* and *D1S1170* in family T are actually the consequence of marker mutations, as such mutations were observed inside the core of the first haplotype for markers *D1S424*, *D1S406*, *50CA1* and *75CA1* (Figure 2, top). Taking this possibility into consideration, a very conservative analysis of the data delineates the locus between markers *D1S188* and *D1S2779*.

Delineation of the region for the *VMGLOM* gene between markers *33CA1* and *D1S2779* confirms our previous exclusion of several genes localised to the initial *VMGLOM* locus, either on the basis of mutation screening, for *DR1* (depression of transcription 1), *TFA* (tissue factor) and *TGRBR3* (transforming growth factor-β receptor, type 3), or on the basis of function, for *ABCR* (ATP-binding-cassette transporter) and *GLCLR* (γ-glutamylcysteine synthetase).[5] Thanks to the human genome draft sequence, the *VMGLOM* region now contains nine genes of known or highly similar to known function. However, it is difficult to highlight the best candidate gene or to formally exclude others on the basis of current knowledge.

Identification of the *VMGLOM* gene will probably enhance understanding of the etiopathogenesis of venous malformations, with and without glomus cells. As mutations in tyrosine-kinase receptors and other signalling molecules important for angiogenesis and vasculogenesis are the causes for vascular dysmorphogenesis in man and genetically engineered mice, it will be of great interest to know if the *VMGLOM* gene is related to these pathways, and in particular, to TIE2/TEK signalling.

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