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ARTICLE

Locus for susceptibility for familial capillary malformation ('port-wine stain') maps to 5q

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Capillary malformation (CM; 'port-wine stain'), is a common vascular malformation affecting cutaneous capillary vessels in 0.3% of newborns. Increased incidence of lesions in first-degree relatives of these patients and several reported familial cases suggest that genetic factors may play a role in the pathogenesis of CM. We report the first genome-wide linkage analysis of familial CM. In the non-parametric linkage analysis, strong evidence of linkage (peak Z-score 6.72, *P*-value 0.000136) was obtained in an interval of 69 cM between markers *D5S407* and *D5S2098*, corresponding to *5q11–5q23*. Parametric linkage analysis gave a maximum combined HLOD score of 4.84 (α-value 0.67) at marker D5S2044 on *5q15*, and analysis using only the linked families, defined a smaller, statistically significant locus *CMC1* of 23 cM (peak LOD score 7.22) between markers *D5S1962* and *D5S652* corresponding *to 5q13–5q15*. Interesting candidate genes implicated in vascular and neural development, such as *MEF2C*, *RASA1*, and *THBS4*, are in this locus.

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Introduction

Vascular anomalies are a heterogenous group of disorders manifesting abnormal development of blood vessels. They are divided into tumours and malformations, and the latter are sub-categorised according to the defective type of vessel.[1] Capillary malformation (CM, 'port-wine stain'), (OMIM 163000) is a common vascular malformation, occurring in 0.3% of neonates.[2] CMs are flat cutaneous lesions (Figure 1) that are typically located in the head and neck region, and change in colour from pink-to-purple with age.[3] These lesions are usually obvious at birth and grow proportionally with the child. Similar macular stains of the face and neck, called 'salmon patch', 'angel's kiss', or '*nevus flammeus neonatorum*' occur in up to 40% of newborns,[2,4] however, they predictably fade during infancy. In contrast, CMs do not disappear; they darken, and often thicken with age, and cause psychological distress.[5]

Although CM is usually sporadic, families in which these lesions segregate in a dominant manner, with incomplete penetrance, have been reported.[6–9] Further evidence of genetic predisposition has been obtained in demographic studies showing that 7–22% of CM patients have relatives with a cutaneous vascular stain.[8,10,11] Thus, susceptibility to CM can be familial, but low penetrance and high frequency of sporadic cases complicate determination of an inheritance pattern.

CM also occurs in the combined vascular syndromes. A patch of CM is frequently found in the skin overlying a pure (classic) lymphatic malformation.[3] In Klippel–Trenaunay syndrome (OMIM 149000), capillary malformation is associated with vascular anomalies of lymphatics and veins, and accompanied by hypertrophy of bone and soft tissues.[3]

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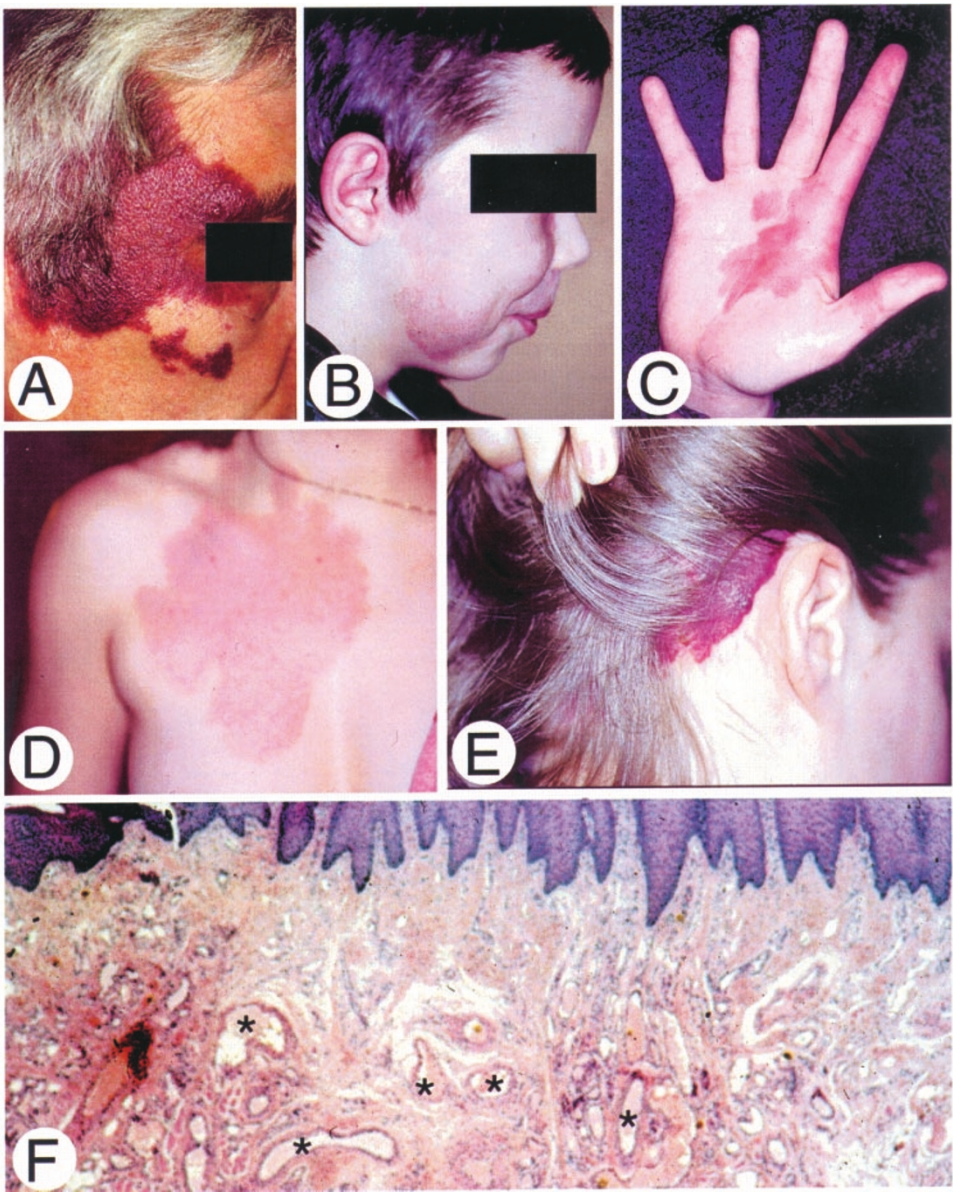

Figure 1 Clinical and histological characteristics of capillary malformation (CM). (**A** and **B)** CM on face, subject I-2 (family M) and subject III-11 (family C), respectively. Subject III-11 (family C) had also an intramaxillar arteriovenous malformation. (**C)** CM on hand, subject III-1 (family A). (**D**) CM on thorax, subject III-4 (family C). (**E**), retroauricular CM, subject III-1 (family F). (**F**) hematoxylin eosin staining of CM. Asterisks (*) indicate dilated capillary-like channels within papillary dermis.

Interestingly, the prevalence of CM in the first-degree relatives of patients with Klippel–Trenaunay syndrome is 1.8%, significantly higher than in the general population.[12] This suggests that descendants of patients with CM have an increased risk for Klippel–Trenaunay syndrome. Parkes–Weber syndrome is another combined vascular disorder composed of CM, multiple arteriovenous fistulas, and tissue hypertrophy.[3] Sturge–Weber syndrome (OMIM 185300) is defined as a cutaneous CM located in V_1, or V_1–V_2 trigeminal neurotomal distribution associated with choroidal and leptomeningeal anomalies and causing facial hypertrophy, glaucoma, and epilepsy.[13]

Etiopathogenesis of CM is poorly understood. Histologically, these lesions have an increased number of ectatic capillary-like channels within the papillary dermis, suggesting defective angiogenesis (Figure 1F).[14] Walls of the dilated vessels have normal morphology, based on immunohistochemical analysis of major structural components, such as type IV collagen and fibronectin.[15] Similarly, endothelial-specific antigens such as factor VIII, PAL-E (pathologische

anatomie Leiden-endothelium), ICAM-1 (intercellular adhesion molecule-1) and ELAM-1 (endothelial leukocyte adhesion molecule-1) exhibit normal staining patterns in the ectatic capillaries.[16] In contrast, studies using antibodies against neuronal cytoplasmic protein (PGP 9.5), neuron-specific enolase (NSE), and S100 protein have demonstrated that the density of cutaneous nerves is significantly decreased in CM.[17,18] These findings support the hypothesis that the gradual dilatation of the dermal vessels is the result of abnormal neural regulation of blood flow.[17] However, the decreased density of cutaneous nerves can be a secondary effect due to reduced blood flow and chronic ischemia in the lesions.[19]

In an effort to understand the genetic mechanisms that underlie CM, and are likely to control cutaneous vascular morphogenesis, we performed genome-wide linkage analysis on six families with inherited CM (families A–F, Figure 2). Using non-parametric and parametric linkage analysis, we identified cosegregation of CM with a large locus on chromosome *5q*. This region of susceptibility was reinforced by analysis of seven additional families with inherited CM, and a minimal locus of 23 cM could be identified with a subset of the families.

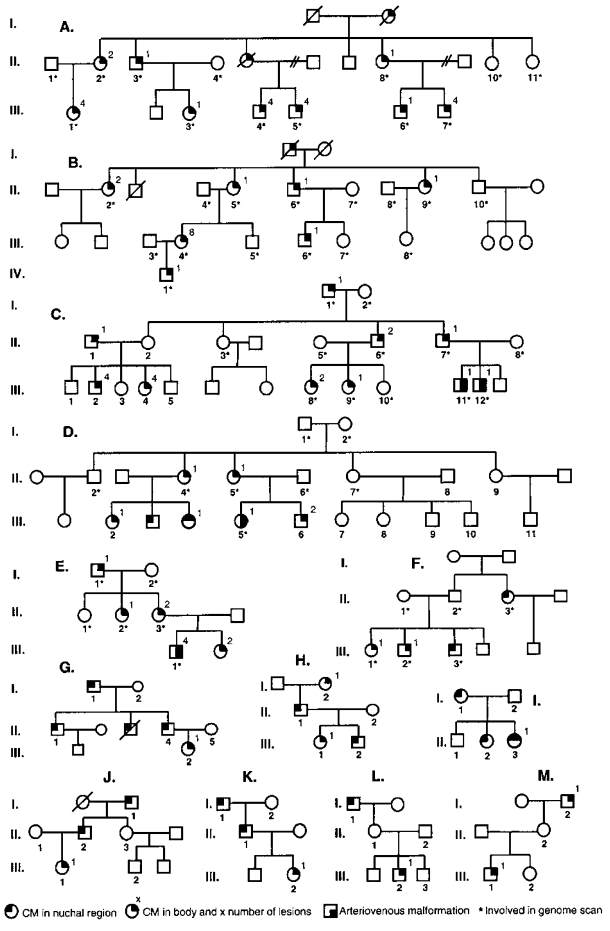

Figure 2 Pedigrees of 13 families with inherited capillary malformation. Incomplete penetrance in families D, F, L and M. Numbered individuals participated in study.

Materials and methods

Informed consent was obtained from all subjects participating in the study, in accordance with the ethics committees of the Faculty of Medecine of Université catholique de Louvain, Saitama Children's Medical Center and Boston Children's Hospital. Blood or buccal brush samples were collected from 60 affected and 51 unaffected individuals (Figure 2). Patients were clinically examined by a plastic surgeon (LM Boon, JB Mulliken and S Watanabe), or general practitioner (H Grynberg). In the 13 families involved in this study, most CMs were pink-to-purple macular lesions, measuring a few centimeters in diameter (Figure 1). All subjects with a CM of at least 1 cm in diameter were considered affected. Individuals with only one lesion, smaller than 1 cm, or with faint nuchal stain, reminiscent of a fading birthmark, were considered to be unaffected. Out of 60 affected subjects, 19 had a lesion on the face, 15 in the nuchal region and 26 in other parts of the body. Fifteen subjects had multiple lesions (Figure 2). In subject III-12, in family C, and subject III-1 in family E, an arteriovenous malformation underlay the cutaneous vascular stain (Figure 2). Subject III-5, in family D, had an arteriovenous fistula between the left carotid artery and jugular vein, a cutaneous vascular stain and soft tissue hypertrophy of the homolateral face. Subject III-11, in family C, had a hemifacial CM associated with left intramaxillar arteriovenous malformation (Figure 1B).

Genomic DNA was extracted from blood samples using the Qiagen DNA purification kit (Westburg, the Netherlands) or from buccal cells using a lysis method, as described.[20] The six most informative families (A–F) were selected for a genome scan. Due to space constraints on acrylamide gels, some unaffected individuals were left out and the screening was performed on 34 affected and 26 unaffected subjects (Figure 2). Since none of the six families showed evidence of sex-linked inheritance, the genome scan was restricted to the autosomes. Fluorescently labelled polymorphic markers from Human MapPairs genomewide screening set (n=356, 10 cM average resolution) were amplified by PCR using the conditions recommended by the supplier (LI-COR, Westburg, the Netherlands). Amplified markers were electrophoresed on 6.5% acrylamide gels on Gene Readir 4200 DNA analyser, and genotyped with SAGA GT 2.0 software (LI-COR, Westburg, the Netherlands). Altogether, 168 additional markers, synthesized by Gibco Lifetechnologies (UK) or Isogen (the Netherlands), were used to cover genomic regions where Human MapPairs markers were uninformative. These markers were radioactively end-labelled with γ-[^{32}P] using polynucleotide kinase (TAKARA/BioWhittaker, Belgium) before amplification by PCR, and electrophoresed on 5%

acrylamide gels, and scored manually after autoradiography overnight. Multipoint linkage analyses were performed with Genehunter 2.0.[21] The unaffected grandparents in family D (I-1 and I-2) and unaffected subject II-2 in family F were considered unknown for CM phenotype in all linkage calculations (Figure 2).

Results

CM segregated as a dominant trait in the 13 studied families. Evidence for incomplete penetrance was noted in families D, F, L and M (Figure 2). In addition, phenotypic variation from single small CM in extremities to large facial lesion with arteriovenous involvement, was observed (Figures 1 and 2).

A non-parametric multipoint linkage analysis was performed first. This identified strong evidence of linkage between CMs and chromosome *5q*. A maximum Z-score of 4.50 with a *P*-value of 0.0025 was found between markers *D5S401* and *D5S2044*, and a 28 cM region with a *P*-value <0.01 was observed between markers *D5S357* and *D5S652*. Suggestive evidence of linkage (*P*-value <0.05) was also found on chromosomes *2p* (*P*=0.031), *4q* (*P*=0.049), *6q* (*P*=0.015), *7q* (*P*=0.045), *8p* (*P*=0.045), *10q* (*P*=0.045) and *12p* (*P*=0.028).

Genomewide multipoint linkage analysis was then performed under the assumption of autosomal dominant mode of inheritance with an allelic frequency of 0.0001 for the disease. The analysis was carried out with 90 and 80% penetrances, and the phenocopy rate was set at 0.3%, corresponding to the incidence of CM in the general population. With 90% penetrance, a statistically significant multipoint HLOD of 4.58 (α-value 0.92) was obtained on *5q* between markers *D5S357* and *D5S2003*, confirming the results of non-parametric analysis. There was also suggestive evidence of linkage (HLOD >1.0) on *6q*, with a multipoint HLOD score of 1.06 (α-value 0.25). No other chromosomes exhibited evidence of linkage. *5q* and *6q* also gave the highest multipoint HLOD scores under 80% penetrance: 4.41 and 0.98, respectively.

In order to further define the linked region on chromosome *5q*, 27 additional markers were genotyped for the six families, including the family members, mostly unaffected, who were excluded in the initial screening (Figure 2). Furthermore, seven additional small CM families (Figure 2) were genotyped with eight markers on chromosome *5q*. Non-parametric linkage analysis using these 13 families yielded a maximum Z-score of 6.72 (*P*-value=0.000136) at marker *AFM205WG7* on *5q15* (Figure 3). The Z-score remained significant ($P<0.01$) over a 69 cM region between *D5S407* and *D5S2098*, with the exemption of an interval of 1 cM (proximal to marker *D5S2084*) (*P*=0.011).

Parametric multipoint linkage analyses of chromosome *5q*, under various penetrances (50–90%) with all the 13 families, gave the highest multipoint HLOD scores with 90% penetrance. A maximum HLOD of 4.84 (α-value 0.67) was obtained at marker *D5S2044*, which is 1 cM centromeric of marker *AFM205WG7* that yielded the peak in the NPL analysis (Figure 3). Another peak of HLOD >3.0 was 4.09 (α-value 0.51), between markers *D5S2084* and *D5S1453*. In the studied *5q* region, the estimated fraction of families linked (α-values) varied between 0.51 and 0.67, suggesting genetic heterogeneity. When the families B, E, G and M, which yielded negative multipoint LOD scores at marker *D5S2044,* were excluded from linkage analysis, a maximum multipoint LOD score of 7.22 (α-value 1.00) was obtained at marker *D5S2044* using 90% penetrance (Figure 4). The most likely linked region, defined by borders of multipoint LOD score <-2.00, was between markers *D5S1962* and *D5S652*, covering 23 cM (Figure 4).

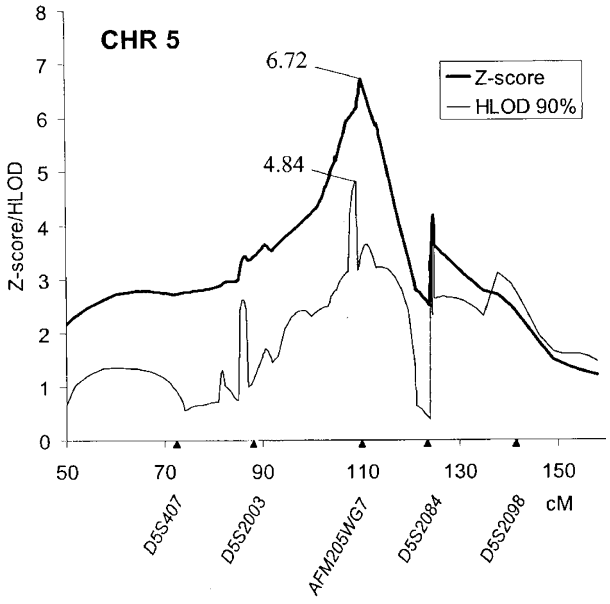

Figure 3 Multipoint linkage analysis of chromosome 5q on 13 families. Thick line, multipoint Z-score; thin line, multipoint HLOD score with 90% penetrance and 0.3% phenocopy rate. Maximum multipoint Z-score of 6.72 obtained at marker *AFM205WG7*, and maximum multipoint HLOD score of 4.84 1 cM centromeric of *AFM205WG7*. Genetic distance, in cM, from 5pter shown below.

Discussion

The results of the non-parametric and parametric linkage analyses showed that the *CMC1*-locus for susceptibility for capillary malformations is located on *5q*. However, the high rate of phenocopies (0.3%), the incomplete penetrance, and the obvious genetic heterogeneity complicate determination of the exact boundaries for the locus. In the non-parametric linkage analysis, strong evidence of linkage (peak Z-score 6.72, *P*-value 0.000136) was obtained in an interval of 69 cM between markers *D5S407* and *D5S2098,* corresponding to *5q11–5q23*. Parametric linkage analysis,

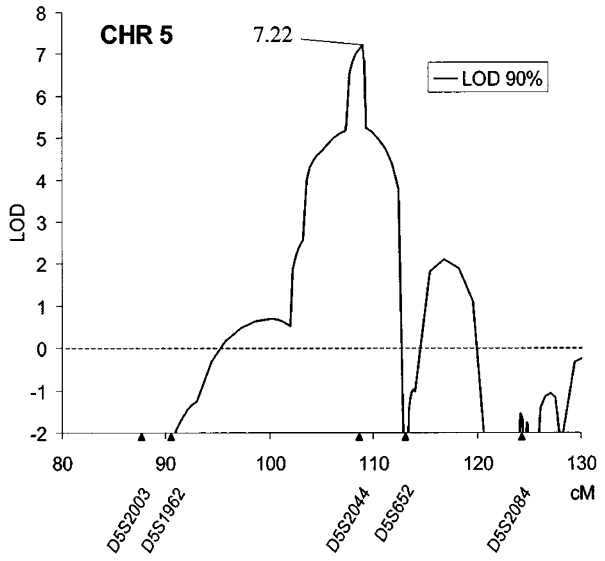

Figure 4 Multipoint linkage analysis on nine families (A, C, D, F, H, I, J, K and L) linked to *CMC1*-locus. Analysis performed under 90% penetrance and 0.3% phenocopy rate. Maximum LOD score of 7.22 obtained at marker *D5S2044* (1 cM centromeric of marker *AFM205WG7*). Most likely linked region located between markers *D5S1962* and *D5S652*. Genetic distance, in cM, from 5pter shown below.

using only the linked families, defined a smaller, statistically significant locus of 23 cM (LOD score 7.22) between markers *D5S1962* and *D5S652* corresponding to *5q13–5q15*.

Genes expressed in embryonal angiogenesis should be prime positional candidates for mutations that cause CM. However, it is difficult to pinpoint the best positional candidate genes, as studies on other vascular malformations have shown that the defective proteins have very different functions.[22,23] For example, mutations in cell surface receptors are known, such as TIE2 (endothelial cell-specific tyrosine kinase) in familial mucocutaneous venous malformation;[24] VEGFR3 (vascular endothelial growth factor receptor-3) in inheritable lymphedema;[25,26] and endoglin and activin in hereditary haemorrhagic telangiectasia.[27,28] Transcription factor FOXC2 (forkhead family transcription factor) is responsible for multiple lymphedema syndromes,[29] and mutations in a gene coding for intracellular putative signalling molecule, KRIT1 (Krev1 interaction trapped-1), cause cerebral capillary malformations, sometimes associated with hyperkeratotic cutaneous capillary–venous malformation.[30–32] Moreover, positional cloning has led to identification of a factor with unknown function as the cause for glomuvenous malformation ('glomangioma').[33]

The 23 cM *CMC1*-locus reported here contains a number of potential genes, such as *MEF2C* (myocyte enhancer factor-2C) and *RASA1* (RAS p21 protein activator-1). Mice deficient in MEF2C manifested lumen size abnormalities of the large vessels close to the heart, as well as diminished peripheral capillary vasculature.[34] This was suggested to be due to defects in remodelling of the primary vascular network to mature vasculature. In the RASA1 null mice, the primary capillary plexus of the yolk sac failed to mature and remained in a honeycomb structure at E10.5.[35] Mosaic mice composed of wild-type and RASA1 null cells survived longer, and at E15, embryos exhibited localized vascular defects resembling CM. The large 69 cM region contains also *FER* (FPS/FES related tyrosine kinase) gene, an activating mutation of which resulted in hypervascularity and multifocal vascular anomalies in transgenic mice.[36] In addition, molecules connected to neurogenesis should be considered. *THBS4 (*thrombospondin-4) gene, involved in neuronal growth and sprouting,[37] is located in the 23 cM interval. Furthermore, *SIAT8D* (Sialyl-transferase-8) and *EFNA5* (ephrin-A5) genes, which have been implicated in the development of the central nervous system, are located in the larger linked region.[38,39]

Interestingly, there is some evidence for chromosome *5q* being involved in the genesis of other vascular anomalies, specifically familial hemangiomas and Klippel–Trenaunay syndrome. Hemangiomas, which are histologically characterized by overgrowth of capillaries, are benign vascular tumours occurring in up to 10% of infants. Recent studies on clonality have suggested that hemangiomas arise from an intrinsic defect, ie a somatic mutation, in vascular endothelial cells.[40] Interestingly, six hemangioma tissues showed evidence of LOH in chromosome *5q* in a large region overlapping with the *CMC1*-locus.[41] There is also suggestive linkage for familial hemangiomas to *5q31-33,* an interval distal to *CMC1*.[42] In addition, a patient with a balanced translocation between chromosomes *5q13.3* and *11p15.1* was found in Klippel–Trenaunay syndrome.[43] Thus, it is possible that inherited CMs, familial hemangiomas and Klippel–Trenaunay syndrome, three conditions with capillary involvement, share some common pathogenic mechanisms.

In summary, we identified a susceptibility locus, *CMC1*, for familial capillary malformation on chromosome *5q*. Identification of the causative gene(s) should give insight into ethiopathogenic mechanisms that cause inherited and sporadic CM, and CM in combination with other vascular defects. It should also shed light into embryonal capillary morphogenesis and thus to angiogenesis in general.

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