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ORIGINAL ARTICLE

Normal immunofluorescence pattern of skin basement membranes in a family with porencephaly due to *COL4A1* G749S mutation

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Abstract COL4A1 mutations have been associated with cerebral small-vessel disease, including perinatal intracerebral hemorrhage with consequent porencephaly, microbleeds, and lacunar strokes. Moreover, involvement of multiple organs and tissues like kidney, muscle, and large vessels have been reported. Three related patients with porencephaly bearing the G749S mutation in the *COL4A1* gene and one healthy control belonging to the same family underwent skin biopsy. Tissue was examined by means of immunofluorescence microscopy and immunoreactivity for collagen type IV in skin basement membranes was tested. In subjects with *COL4A1* mutation, we did not detect significant alterations of immunofluorescence patterns in basal membranes of different skin structures. Heterozygous *COL4A1* G749S mutation is associated with a normal immunofluorescence pattern of skin basement membranes. Further studies are needed to clarify the role of possible functional abnormalities of the basement membranes in patients with this mutation.

Keywords COL4a1 · Immunofluorescence · Skin · Basement membrane · G749S mutation

Introduction

Collagen IV is the major protein found in basement membranes (BMs). This protein, which consists of three heterotrimers (α1α1α2, α3α4α5, and α5α5α6) forming distinct networks, is responsible for the strength and integrity of the membrane [1–3]. The α1 (IV) and α2 (IV) chains are widely expressed and form the α1α1α2 (IV) heterotrimers [1–3]. After secretion into the extracellular matrix, the molecules of collagen IV self-associate to form a supramolecular network that provides the biomechanical stability of BMs. Mutations in the *COL4A1* gene, resulting in alteration of α1 chain, have been associated with different autosomal dominant phenotypes including cerebral small-vessel disease with ischaemic or haemorragic stroke with or without Axenfeld–Rieger anomaly [4, 5], type I Porencephaly [6–9], and the hereditary angiopathy, nephropathy, aneurysms and muscle cramps (HANAC) syndrome [10]. It is now well known that these clinical phenotypes may overlap: a genotype–phenotype correlation is only partially established [11, 12]. Abnormalities of skin BM have also been found in subjects with porencephaly due to *COL4A1* mutations [8]. Data about the structure of BM of skin in patients carrying the G749S mutation are lacking. In this work, we executed biopsies from normal-appearing skin in four family members including three patients carrying the *COL4A1* G749S mutation and one healthy control and we performed immunofluorescence microscopy to assess the integrity and morphology of the BM in epidermal–dermal junction and in other skin structures.

S. Gasparini and A. Qualtieri contributed equally to this work.

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Materials and methods

Patients

Three patients and one healthy control belonging to the same family were examined (Fig. 1). All these subjects were described before [6, 7] and the affected members carried a heterozygous *COL4A1* G749S mutation. The patients were a father and two children, aged 60, 29 and 20, respectively. The father (patient I:1) had a mild clinical picture with modest left hemiparesis and buccofacial apraxia. Intellectual level was normal and he did not suffer from epileptic seizures. He also presented a mitral valve prolapse and severe cramps in lower limbs. No skin lesions were observed. His older daughter (patient II:1) had a spastic tetraparesis with generalized dystonia, moderate mental retardation and epileptic seizures with secondary generalization. Furthermore, she presented bilateral congenital hip dislocation and muscle cramps in left lower limb. His youngest son (patient II:4) had a spastic tetraparesis, left homonymous hemianopia, severe mental retardation, epileptic seizures, mitral valve prolapse and congenital left hip dislocation. The healthy control (II:3) was patient I:1's third child.

Punch biopsy

All experiments were conducted in agreement with the Declaration of Helskinki. The experiment was approved by local Ethics Committee at Bianchi-Melacrino-Morelli Hospital. Written informed consent was obtained from patients. Skin biopsies were performed with a 3-mm punch in patients and control. In each subject, skin samples were obtained from two different sites: mid-thigh and distal portion of one leg. After excision, tissue samples were immediately immersed in Zamboni' s fixative and left overnight at 4 °C. The specimens were transferred to a cryoprotecting solution containing 30 % sucrose in 0.1 m phosphate buffer (PB), pH 7.4 overnight and included in OCT.

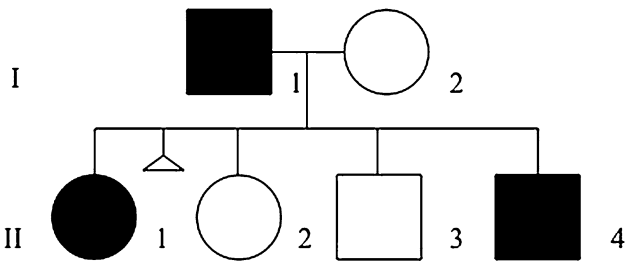

Fig. 1 Pedegree of investigated family

Immunofluorescence microscopy

Fifty-μm-thick frozen sections were processed for immunofluorescence stain using the free-floating staining procedure. A primary antibody directed toward the COL IV alpha 2 chain (mouse 1:200; Chemicon, Temecula, CA, USA) was utilised to stain the basal membranes. A primary antibody directed toward protein gene product (PGP) 9.5 (rabbit 1:10000 Ultraclone, UK), an ubiquitin hydrolase utilized as a pan-neuronal marker, was employed to visualize all nerves in sections (including full thickness of the epidermidis and superficial derma). The secondary antibodies Alexa-Fluor 488 goat anti-rabbit IgG (Molecular probes, Oregon USA) and Alexa-Fluor 546 goat anti-mouse IgG (Molecular probes) were used at 1:200 dilution. Fluorescence-labeled sections were mounted in Santa-Cruz mounting medium (Santa-Cruz Biotechnology Inc., Dallas, TX, USA) to prevent fluorescent quenching and to stain nuclei by 6-Diamidino-2-phenylindole (DAPI). Fluorescent samples were viewed using a conventional fluorescence microscope (Microphot FX, Nikon Instrument Inc.) equipped with a Nikon D100 digital camera. Acquired fluorescent images were processed using ImageJ software.

Results

At low magnification, immunoreactivity to colIV revealed main skin structures in three affected subjects (subject II:1, Fig. 2a; subject I:1, Fig. 2b; subject II:4, Fig. 2c) and in the healthy control (subject II:3, Fig. 2d).

Epidermal basement membrane

In all examined subjects, the epidermal–dermal basement membrane showed intense immunoreactivity to colIVα2 antibody and homogeneous thickness. The intensity of fluorescence was apparently comparable in patients and in the control subject (Fig. 3a).

Blood vessels

In arterioles and in capillaries, colIVα2 immunoreactivity was clearly evident in the BM close to endothelium and lining the pericytes. The pericytes and vascular smooth muscle cells could be easily identified for their nuclei projecting on the wall of the vessels. We were unable to detect differences in thickness and continuity of these structures between patients and control, even at a high magnification (Fig. 3b).

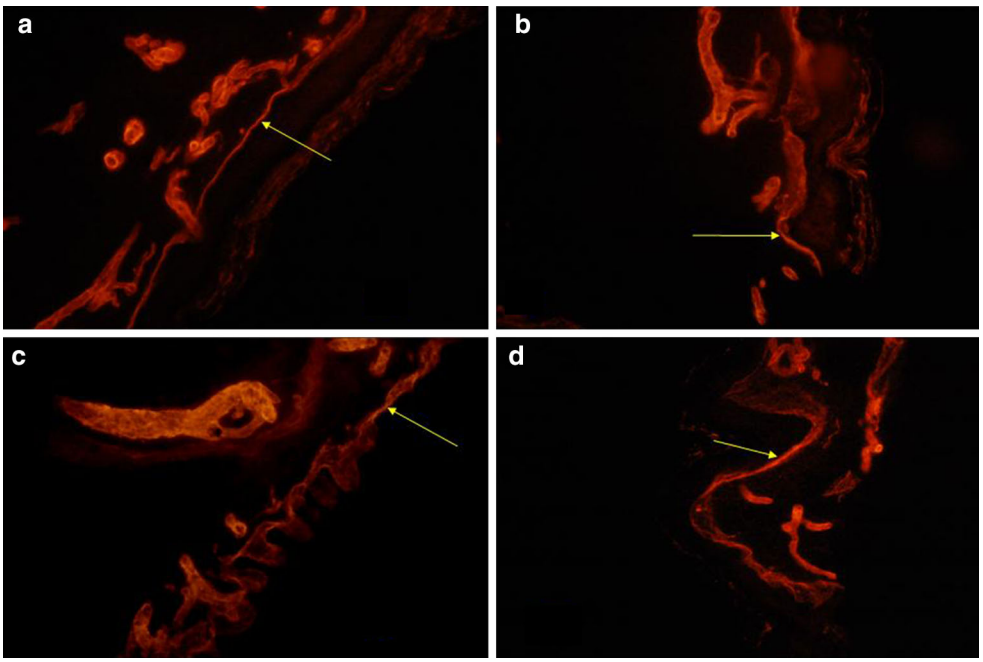

Fig. 2 Low magnification (10×) fluorescent 50 μm skin section of the normal control (II:3; letter **d**) and patients (I:1; letter **b**, II:1; letter **a**, and II:4; letter **c**) with *COLIV* mutation. The immunoreactivity for basal membrane (Col IV a2) is well evident in different structures such as blood vessels and epidermal basal membrane (*arrows*)

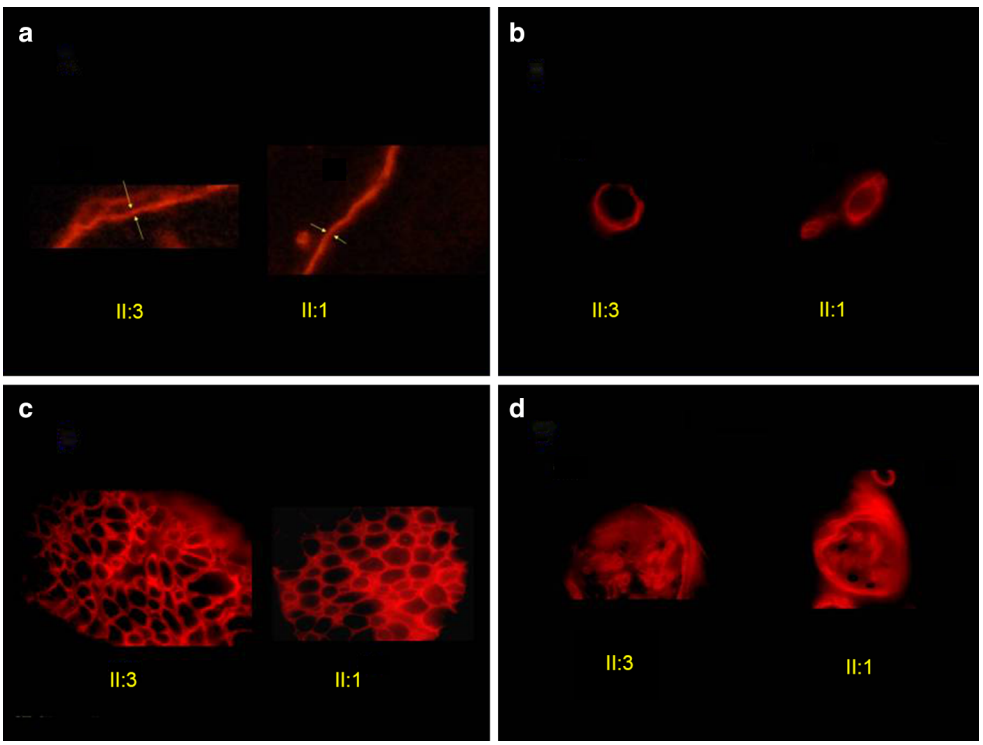

Fig. 3 COLIVα2 immunoreactivity in 50 μm section from normal control (*II:3*) and patient (*II:1*) of the epidermal basal membrane (*arrows*) (panel **a**); blood vessels (panel **b**); sebaceous gland (panel **c**) and nervous trunk (panel **d**)

Sebaceous glands

The sebaceous glands were also observed in skin biopsies (Fig. 3c). These structures showed an intense colIVα2 immunoreactivity. No substantial differences were visible between patients and the control subject.

Nerves

In nervous trunk, the epineurium showed colIV immunoreactivity with multilamellar appearance (Fig. 3d); in myelinated nervous fibers, immunoreactivity was localized externally to the myelin sheath, as shown in Fig. 4. We also observed immunoreactivity in unmyelinated peripheral fibers at medium and high magnification by using double labeling for peripheral axons (PGP 9.5) and basement membrane (colIVα2). Double labeling analysis revealed no noteworthy differences between patients and control (Fig. 5a, b).

Discussion

We demonstrated the normality and micro-structure of the BM as detected by immunofluorescence in different skin structures of three related subjects with G749S mutation in *COL4A1* gene.

Patients with *COL4A1* mutations show a prominent clinical heterogeneity, which has also been observed within the same family [5, 6, 8–11]. The first observations [4–7, 10] described three main clinical pictures (type I porencephaly, small-vessel disease with haemorrhagic and ischaemic strokes, HANAC syndrome) that seemed distinct from each other. More recently, other authors [9, 11–13] have observed overlapping phenotypes, so that the term "*COL4A1*-related disease" has been proposed to include all possible clinical manifestations. Most *COL4A1* mutations described so far affect highly conserved glycine residues within the collagenous domain of the colIVα1 protein: these changes may disrupt the formation of collagen α1α1α2 (IV) triple helix and undermine its stability [12]. Given the widespread localization of collagen α1α1α2 (IV), the involvement of multiple organs and systems seems likely. In particular, the involvement of skin BMs has been documented in patients with *COL4A1* mutations: in HANAC syndrome [10], electron microscopy of the epidermal-dermal junction revealed an irregular and thickened BM with numerous focal interruptions in some patients. Focal thickening of skin BM was also appreciable in biopsied tissues from patients with prominent cerebrovascular disease and porencephaly [8]. Pathogenic mechanisms causing disease expression in different tissues are far from being understood. The absence of a clinical phenotype in mice with one null *Col4a1* allele suggests a detrimental effect of mutated protein [12]. Intracellular accumulation of the mutated protein may lead to activation

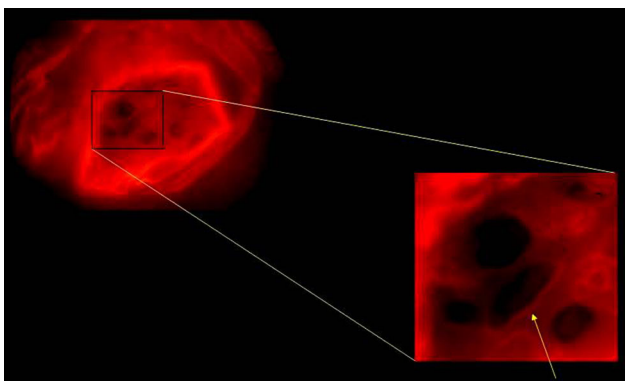

Fig. 4 COLIVα2 immunoreactivity in nervous trunk showing the appearence of the basal membrane around a single myelinated fiber external to the myelin sheath (*arrow* in the enlarged frame)

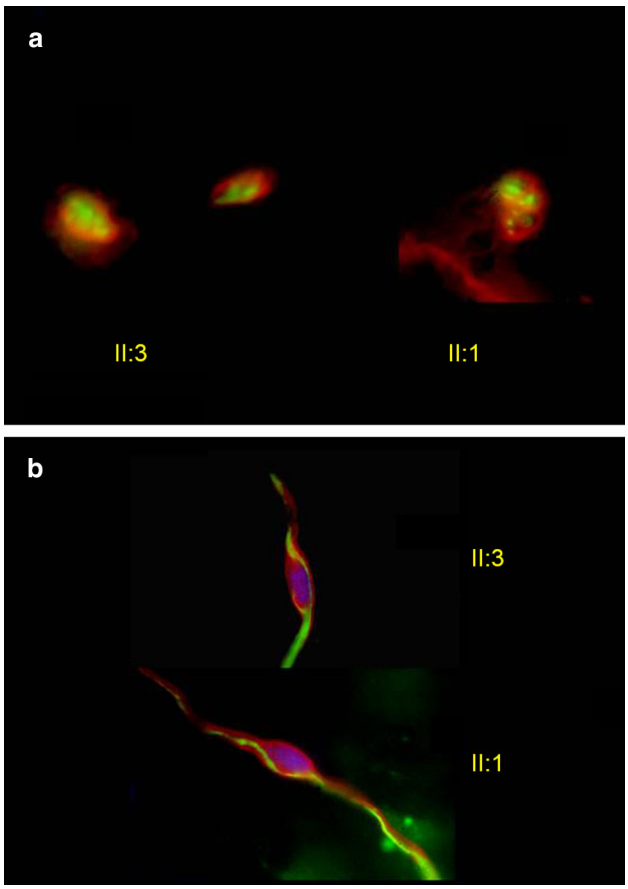

Fig. 5 Double labeling of PGP 9.5 (*green*) and COLIVα2 (*red*) of unmyelinated fibers. Nuclei are stained with DAPI (*blue*). Panel **a** shows trasversal sections of small dermal nerves; panel **b** shows a longitudinally sectioned single unmyelinated fiber in contact with its Schwann cell. *II:3* normal control, *II:1* patient

of stress response. A dominant negative effect of the same mutated protein upon normal heterotrimers has also been postulated [12]. However, these hypotheses do not account for the clinical manifestations (porencephaly and cerebrovascular disease) described by Lemmens et al. [8] in two families with *COL4A1* mutations causing haploinsufficiency. Finally, more complex factors like feedback mechanisms and transcriptional control may be involved [12]. In the patients described here, the normality of immunofluorescence pattern in skin basement membranes suggests that none of the hypothesized mechanisms is able to determine pathology. However, electron microscopy was not performed, and this constitutes a limit of our study. Further studies are needed to clarify the role of ultrastructural alterations and possible functional abnormalities of the basement membranes in patients with *COL4A1* G749S mutation.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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