BRAIN
A JOURNAL OF NEUROLOGY

Congruence between *NOTCH3* mutations and GOM in 131 CADASIL patients

Saara Tikka,[1,2,]* Kati Mykkänen,[2,3,]* Marie-Magdeleine Ruchoux,[4] Robert Bergholm,[5,6] Maija Junna,[3] Minna Pöyhönen,[7,8] Hannele Yki-Järvinen,[5,6] Anne Joutel,[9,10] Matti Viitanen,[11,12] Marc Baumann[1] and Hannu Kalimo[2,13,14]

1 Protein Chemistry Unit, Institute of Biomedicine/Anatomy, University of Helsinki, Finland
2 Department of Pathology, University of Helsinki, Helsinki, Finland
3 Department of Medical Biochemistry and Genetics, University of Turku, Turku, Finland
4 Institute of Emerging Diseases and Innovative Therapies, CEA, Fontenay-aux-Roses, France
5 Department of Medicine, University of Helsinki, Helsinki, Finland
6 Minerva Medical Research Institute, Helsinki, Finland
7 Department of Medical Genetics, University of Helsinki, Finland
8 Department of Clinical Genetics, Helsinki University Central Hospital, Helsinki, Finland
9 INSERM, U740, Paris, F-75010, France
10 7-Denis Diderot, Faculté de Médecine, Site Lariboisière, Université Paris, Paris F-75010, France
11 Department of Geriatrics, Turku City Hospital and University of Turku, Turku, Finland
12 Division of Clinical Geriatrics, Karolinska Institutet, Stockholm, Sweden
13 Departments of Pathology and Forensic Medicine, University of Turku, Turku, Finland
14 Department of Genetics and Pathology, University of Uppsala, Uppsala, Sweden

*These authors contributed equally to this work.

Correspondence to: Hannu Kalimo,
Department of Pathology, University of Helsinki,
PO Box 21,
FI-00014 Helsingin yliopisto, Finland
E-mail: hannu.kalimo@helsinki.fi

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is the most common hereditary subcortical vascular dementia. It is caused by mutations in *NOTCH3* gene, which encodes a large transmembrane receptor Notch3. The key pathological finding is the accumulation of granular osmiophilic material (GOM), which contains extracellular domains of Notch3, on degenerating vascular smooth muscle cells (VSMCs). GOM has been considered specifically diagnostic for CADASIL, but the reports on the sensitivity of detecting GOM in patients' skin biopsy have been contradictory. To solve this contradiction, we performed a retrospective investigation of 131 Finnish, Swedish and French CADASIL patients, who had been adequately examined for both *NOTCH3* mutation and presence of GOM. The patients were examined according to the diagnostic practice in each country. *NOTCH3* mutations were assessed by restriction enzyme analysis of specific mutations or by sequence analysis. Presence of GOM was examined by electron microscopy (EM) in skin biopsies. Biopsies of 26 mutation-negative relatives from CADASIL families served as the controls. GOM was detected in all 131 mutation positive patients. Altogether our patients had 34 different pathogenic mutations which included three novel point mutations (p.Cys67Ser, p.Cys251Tyr and p.Tyr1069Cys) and a novel duplication (p.Glu434_Leu436dup). The detection of GOM by EM in skin biopsies was a highly reliable diagnostic method: in this cohort the congruence

between *NOTCH3* mutations and presence of GOM was 100%. However, due to the retrospective nature of this study, exact figure for sensitivity cannot be determined, but it would require a prospective study to exclude possible selection bias. The identification of a pathogenic *NOTCH3* mutation is an indisputable evidence for CADASIL, but demonstration of GOM provides a cost-effective guide for estimating how far one should proceed with the extensive search for a new or an uncommon mutations among the presently known over 170 different *NOTCH3* gene defects. The diagnostic skin biopsy should include the border zone between deep dermis and upper subcutis, where small arterial vessels of correct size are located. Detection of GOM requires technically adequate biopsies and distinction of true GOM from fallacious deposits. If GOM is not found in the first vessel or biopsy, other vessels or additional biopsies should be examined.

Keywords: CADASIL; GOM; skin biopsy; *NOTCH3*; genetic testing

Abbreviations: CADASIL = cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; EGF = epidermal growth factor; EM = electron microscopy; GOM = granular osmiophilic material; VSMC = vascular smooth muscle cells

Introduction

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), the most common hereditary vascular dementia, is characterized by migraineous headache with aura, recurrent ischaemic attacks, cognitive decline and psychiatric symptoms as the four main features. It is caused by mutations in *NOTCH3* gene encoding a transmembrane receptor Notch3 (Joutel *et al.*, 1996). Virtually all pathogenic mutations lead to an odd number of cysteine residues in one of the 34 epidermal growth factor (EGF) like repeats in the extracellular domain of Notch3 (N3ECD). The mutations result in a degeneration of vascular smooth muscle cells (VSMC), in which *NOTCH3* is predominantly expressed in adult humans (Joutel *et al.*, 2000a). The main pathological findings are accumulation of N3ECD on degenerating VSMCs as well as fibrosis and thickening of arterial walls, (Ruchoux *et al.*, 1995; Miao *et al.*, 2004). In electron microscopy (EM), the pathognomonic feature of CADASIL is accumulation of granular osmiophilic material (GOM) in indentations of the VSMCs or in the extracellular space in close vicinity to VSMCs (Baudrimont *et al.*, 1993; Ruchoux *et al.*, 1995). The exact composition of GOM has not been elaborated, but a recent immunogold EM study suggested N3ECD to be a component of GOM (Ishiko *et al.*, 2006).

CADASIL is suspected in patients with the typical clinical features and white matter alterations in brain T_2-weighted MRI (O'Sullivan *et al.*, 2001). The definite verification of the diagnosis can be done by identifying a pathogenic mutation in the *NOTCH3* gene. In CADASIL, at least 170 different mutations in 20 different exons have been reported (Supplementary Table 1). Comprehensive analysis of all these exons is time consuming and costly. Thus, most diagnostic laboratories screen only the exons that according to the previous reports harbour majority of the mutations (Joutel *et al.*, 1997; Escary *et al.*, 2000; Kalimo *et al.*, 2002; Opherk *et al.*, 2004; Dotti *et al.*, 2005).

Already before the underlying gene defect was discovered, GOM was detected by EM in skin biopsies from CADASIL patients (Ruchoux *et al.*, 1994). So far GOM has not been described in any other disease entity. However, the reports on the sensitivity of detecting GOM in skin biopsy of patients with genetically verified CADASIL have been contradictory.

Two earlier studies on a smaller number of patients (Ebke *et al.*, 1997; one family with eight patients, mutation not specified; Mayer *et al.*, 1999; three families, 14 patients, mutations not specified) suggested 100% sensitivity and specificity, whereas two more recent papers have reported a low sensitivity: Markus *et al.* (2002) reported a sensitivity of only 44.4% (eight out of 18), while Razvi *et al.* (2003) suspected that the sensitivity might be even lower, although they did not give an exact number. To solve this contradiction, we performed a retrospective investigation of a combined patient material from Finland, Sweden and France comprising 131 patients, from whom both the genetic analysis and EM examination of skin biopsy were available. Skin biopsies from 26 mutation negative members of genetically proven CADASIL families served as control. We showed that electron microscopic demonstration of GOM in skin biopsy is a highly reliable and practical method to screen for or even specifically diagnose CADASIL. Furthermore, the intensive search for mutations based on confidence in the diagnostic specificity of GOM resulted in discovery of four novel, previously unreported mutations, among them the first duplication of three codons.

Materials and Methods

Subjects

The subjects comprise of 131 patients in whom both the analysis of *NOTCH3* gene and EM examination of skin biopsy had been adequately performed (38 from Finland, 13 from Sweden and 80 from France) as well as 26 control subjects (mutation negative members of the patients' families: four from Finland, two from Sweden and 20 from France). Genetic analyses and biopsy examinations were, in general, performed without knowledge of the other test result. However, these analyses were part of the true clinical practice with the aim to establish the patient's diagnosis with all possible diagnostic methods, thus in several cases the detection of GOM in skin biopsy led to extended genetic analyses. Only a single subject, in whose biopsy no representative vessels of correct size were identified, was excluded from the study. Subjects have been clinically examined at different hospitals in Finland, Sweden or France as well as in different international hospitals from which blood and biopsy samples have been sent to France for diagnostic CADASIL analyses.

Molecular genetics

In Finland and Sweden, the diagnostic genetic analyses were originally limited to restriction enzyme analysis of two previously found mutations in Finland (p.Arg133Cys and p.Arg182Cys) and in negative cases complemented by sequence analysis of exons 3 and 4. Because the skin biopsy had shown presence of GOM in nine Finnish and Swedish suspected CADASIL patients without mutation being detected, an extended genetic analysis for *NOTCH3* was established. For the detection of CADASIL type *NOTCH3*, mutation exons 2–24 of gene were amplified with specific primers and subsequently amplicons were sequenced using an automated sequencing system (Applied Biosystems, Foster City, CA, USA).

Blood samples from hospitals in France and other countries were sent to Laboratoire de Cytogénétique, Hôpital Lariboisière, Paris, France and analysed as a commercial diagnostic service.

Electron microscopy

In Finland and Sweden, skin biopsies were requested from as many CADASIL suspected patients as possible, especially from those with a strong suspicion of CADASIL and negative result in limited genetic tests mentioned above. The skin biopsies were taken at the hospitals, where the CADASIL patients or their relatives were examined. Either full thickness punch biopsies or small incision biopsies were taken (usually from the upper arm) and fixed in phosphate buffered 3–4% glutaraldehyde, in which solution the biopsies were sent in Finland to the Laboratory of Electron Microscopy of the University of Turku or Helsinki, in Sweden to the Laboratory of Electron Microscopy at the Department of Pathology, University of Uppsala. In France the biopsies fixed in either Carson or Trump liquids or as above were sent to the Laboratories of Electron Microscopy at Hôpital Roger Salengro, University of Lille or Hôpital Bretonneau, University of Tours, France. The samples were postfixed in buffered osmium tetroxide, dehydrated in ascending grades of ethanol and embedded in Epon. Semithin sections were cut and stained with toluidine blue for selecting arteries of appropriate size (usually in the borderzone between dermis and subcutis) for thin sectioning. Thin sections were double stained with uranyl acetate and lead citrate and then examined in transmission electron microscopes at the EM laboratories mentioned above. The Finnish and Swedish skin biopsies were analysed by two Finnish (S.T. and H.K.) electron microscopists and in France by a French (MMR) electron microscopist.

Results

Altogether, GOM was detected in skin biopsies from all 131 patients, who in the genetic analyses were determined to carry a pathogenic *NOTCH3* mutation for CADASIL (Table 1). GOM was present above all in arterial vessels, whereas the veins and capillaries were either GOM negative or seldom GOM positive (Figs 1 and 2). In most GOM positive cases, GOM was detected in the first biopsy but in a few cases a repeated biopsy was needed. During the diagnostic activity, we examined many suspected cases in whose biopsies we detected, instead of GOM, cellular debris between the VSMCs with structure that might lead unfamiliar electron microscopists astray. These cases were also negative in genetic tests (limited to the

Table 1 The spectrum of *NOTCH3* mutations in combined CADASIL patient cohort from Finland, Sweden and France

	Amino acid change	Nucleotide change in coding sequence	Exon	Finnish (F) French (Fr) Swedish (S)
1	p.Cys49Tyr	c.146G>A	2	Fr
2	p.Cys67Ser[a]	c.199T>A	3	S
3	p.Thr71Cys	c.213G>T	3	Fr
4	p.Arg90Cys	c.268C>T	3	Fr/S
5	p.Arg110Cys	c.328C>T	3	Fr
6	p.Arg133Cys	c.397C>T	4	F/Fr/S
7	p.Cys134Trp	c.402C>G	4	Fr
8	p.Arg141Cys	c.421C>T	4	Fr
9	p.Arg169Cys	c.505C>T	4	Fr
10	p.Gly171Cys	c.511G>T	4	Fr
11	p.Cys174Arg	c.520T>C	4	S
12	p.Arg182Cys	c.544C>T	4	Fr/S
13	p.Cys185Arg	c.553T>C	4	Fr
14	p.Cys185Gly	c.553T>G	4	Fr
15	p.Cys206Tyr	c.617G>A	4	S
16	p.Cys212Ser	c.634T>A	4	Fr
17	p.Cys222Gly	c.664T>G	4	Fr
18	p.Cys224Tyr	c.671G>A	4	Fr
19	p.Cys251Tyr[a]	c.752G>A	5	S
20	p.Tyr258Cys	c.773A>G	5	Fr
21	p.Arg332Cys	c.994C>T	6	S
22	p.Cys428Ser	c.1283G>C	8	Fr
23	p.Glu434_Leu436dup[a]	c.1300_1308dup	8	F
24	p.Gly528Cys	c.1660G>T	10	F
25	p.Cys542Tyr	c.1625G>A	11	Fr
26	p.Arg558Cys	c.1672C>T	11	Fr/S
27	p.Arg578Cys	c.1732C>T	11	Fr
28	p.Arg728Cys	c.2182C>T	14	Fr
29	p.Arg985Cys	c.2953C>T	18	Fr
30	p.Arg1006Cys	c.3016C>T	19	Fr
31	p.Arg1031Cys	c.3091C>T	19	Fr
32	p.Tyr1069Cys[a]	c.3206A>G	20	F
33	p.Arg1231Cys	c.3691C>T	22	Fr
34	p.Cys1261Arg	c.3782T>C	23	Fr

a Mutation previously unpublished.

common mutations). Some common deposits of fallacious appearance are shown in Fig. 3.

Representative skin biopsies were negative for GOM in the 26 subjects, who were also negative for the *NOTCH3* mutation that was detected in their relatives. Therefore, these subjects were identified as definite negative cases for CADASIL.

In Finland and Sweden, GOM was detected in 51 patients, from 28 families with verified *NOTCH3* mutations. In these families 12 different *NOTCH3* mutations were identified, among these three point mutations (p.Cys67Ser, p.Cys251Tyr and p.Tyr1069Cys) and one duplication (p.Glu434_Leu436dup, producing an unpaired cysteine in the affected EGF repeat) were novel, previously unpublished (Table 1). In one Finnish patient, repeated skin biopsies were positive for GOM, although no mutation was found in screening of the *NOTCH3* exons 2–24. Repeated genetic analysis with another set of primers revealed a heterozygous c.1582GT (p.Gly528Cys) mutation.
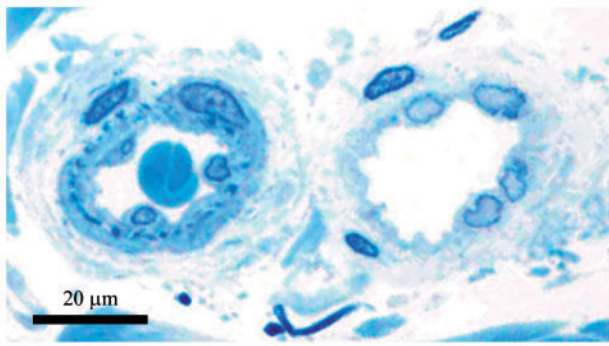
Figure 1 A small arteriole (left) and venule (right) in the lower dermis of the skin biopsy from a Finnish CADASIL patient with p.Arg133Cys mutation. Note the somewhat thicker wall and lamina elastica interna (dark blue spots beneath the endothelium) in the arteriole. Toluidine blue-stained semithin epon section.
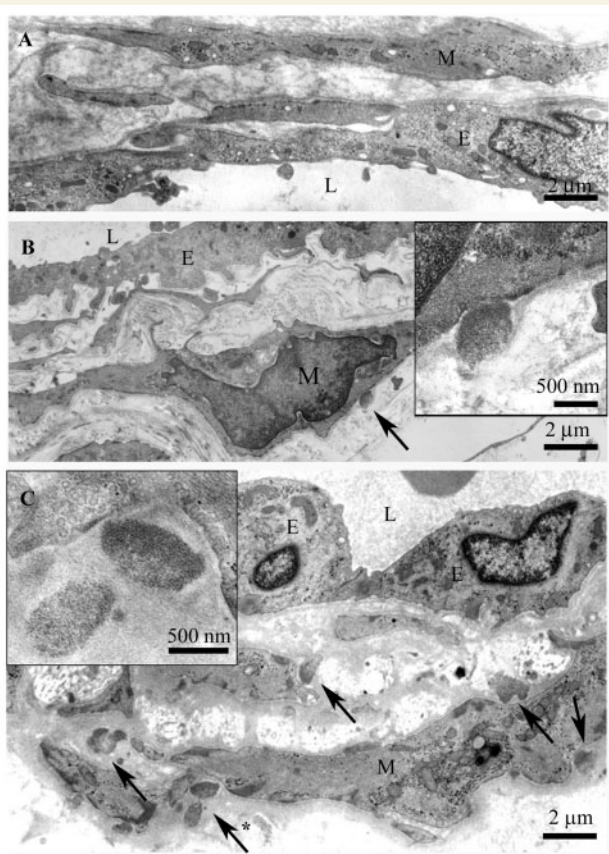

Figure 2 (**A**) The same vein as in Fig. 1 with no GOM. (**B**) A vein from deep dermis with one definite GOM deposit (arrow) shown at a higher magnification in the inset. (**C**) The arteriole in Fig. 1 with several GOM deposits (five shown with arrows), one marked with asterisk is shown at a higher magnification in the inset. Note the characteristic pinocytotic vesicles at the VSMC plasma membrane beneath the GOM. L = lumen, E = endothelium, M = vascular smooth muscle cell.

In France GOM was detected (by MMR) in all 80 *NOTCH3* mutation positive patients—not only from France but also from different parts of the world. Among the patients whose DNA samples were analysed in France 26 different *NOTCH3* mutations were identified (Table 1).

Discussion

In our patients' detection of GOM from skin biopsy was a highly reliable method for the diagnostic workup of suspected CADASIL patients. GOM was detected in the skin biopsy of all 131 patients, in whom the *NOTCH3* mutation had been identified and a representative skin biopsy was available. Thus the congruence was 100%. This study was performed retrospectively and therefore to exclude possible selection bias a prospective study would be required to get an exact figure for sensitivity, which we estimate to be well over 90%. The genetic analysis and EM detection of GOM were primarily made simultaneously and blinded to each other. Since the analysis has been made as a part of the diagnostic work, the EM analysis has been used to support the restricted genetic testing. In some cases, the GOM positive EM analysis prompted us to screen the *NOTCH3* exons 2–24. During the examinations of suspected patients in some cases, no representative vessels were detected. A repeated biopsy was requested and if the biopsy was still non-representative the case was excluded from this study. Most often the parallel genetic analysis (limited as mentioned above) of these patients proved negative. Since our resources did not allow full sequencing of exons 2–24 in all suspected patients, CADASIL diagnosis could not be excluded in these patients with absolute certainty. In only one *NOTCH3* mutation positive patient, we did not find representative vessels in the only available biopsy, and thus, the presence of GOM could not be determined. Based on the inclusion criteria this patient had to be excluded from this cohort.

When using EM analysis of skin biopsy as a diagnostic method in suspected CADASIL cases, special attention should be paid to the quality and analysis of the skin biopsy. The vessels in which the GOM is best detectable are generally medium sized or small arterioles (usually outer diameter 20–40 µm) in deep dermis or upper subcutis, but in a few cases GOM has been detected also in veins. In toluidine blue semi-thin sections the detection of lamina elastica interna as dark blue dots (Fig. 1) is a good marker of representative arterioles. Technical factors in the processing of the samples may also influence the result. Since GOM is osmiophilic, each laboratory should adjust the osmium tetroxide treatment such that GOM becomes sufficiently well contrasted. Furthermore, if GOM is not found in the first vessel investigated, other vessels or even repeat biopsies should be examined. Just like in any laboratory work, first negative result might be due to an unrepresentative sample or improper sample handling. Besides, one should examine rather an artery (with multiple layers of VSMCs and inner elastic lamina) than a vein or a capillary, since according to our experience veins and capillaries are not always GOM positive (Fig. 2). Of course, a prerequisite is that one recognizes GOM correctly and distinguishes it from fallacious deposits (Fig. 3).
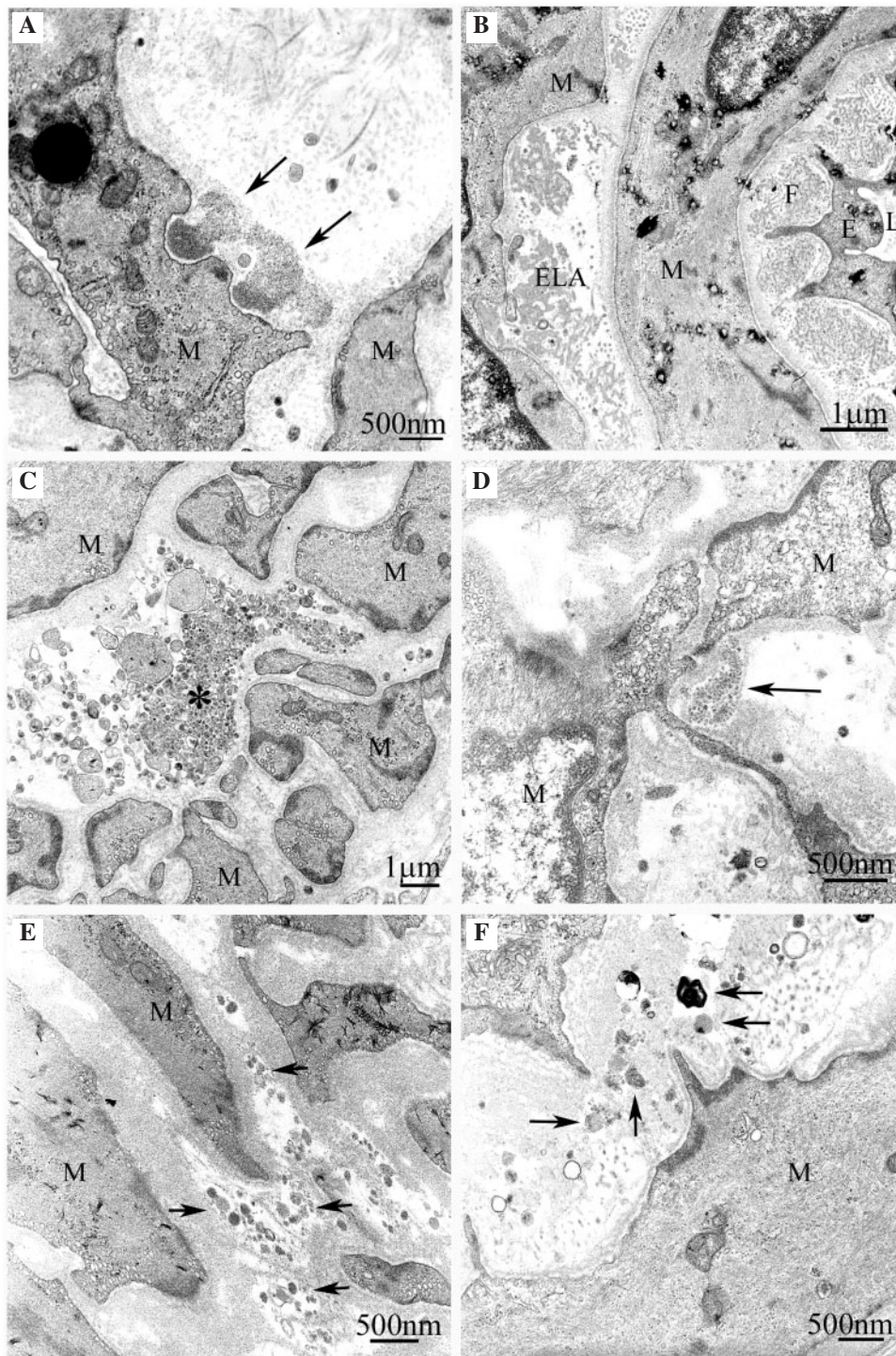
Figure 3 Fallacious deposits which may lead the electron microscopist astray. (**A**) True GOM with a somewhat exceptional mushroom-like form (arrows). Note the characteristic finely granular structure of GOM. (**B**) Fragments of elastica interna (ELA) and granular fibrillin network (**F**) in the widened subendothelial space. (**C**) Granular debris of unknown origin (asterisk). (**D**) Similar granular material as in C (arrow) with a misleading localization in a cove of VSMCs. (**E** and **F**) Small clumps of cell debris of different composition (arrows), possibly from degenerated cells. L = lumen, E = endothelium, M = vascular smooth muscle cell.

Our confidence in the EM analysis prompted us to extend our search for mutations to sequencing the exons 2–24 in the nine patients in whom the limited mutation analyses had been negative. The detection of four novel mutations demonstrated that even though over 170 different *NOTCH3* mutations are known, the presence of six cysteines in each of the translated 34 EGF repeats which may be replaced by another amino acid in addition to the replacement of other amino acids by cysteine,

makes detection of hundreds of novel mutations still possible. This multitude is further widened by possibility of deletions (Dichgans *et al.*, 2000, 2002; Opherk *et al.*, 2004) and splice site mutations (Joutel *et al.*, 2000*b*).

In one Finnish patient with GOM positive skin biopsy the *NOTCH3* mutation was not detected until the analysis was performed with another set of primers (at Hôpital Lariboisière, the most experienced diagnostic CADASIL laboratory). This experience further emphasizes the value of skin biopsy examination as a guide for more comprehensive genetic analyses.

What would be the most efficient strategy to confirm the clinical suspicion of CADASIL? This strongly depends on the patient's family history and the mutational background in the population to which the suspected patient belongs. In families with a known mutation, the method of choice is, of course, to test directly for that mutation. If there are known founder or major mutations in the population, the diagnostic workup is best to begin by first searching for those mutations. In populations with no known founder or other determined mutations, screening of mutational hot spot region of the *NOTCH3* gene should be the first genetic method to search for CADASIL. Of the all reported pathogenic *NOTCH3* mutations 62% locate in exons 3, 4, 5 and 8. Furthermore, to obtain 80% coverage, additional investigation of exons 2, 6, 11 and 18 is required (Supplementary Table 1). At the latest after these analyses electron microscopy for GOM is highly recommended. Similar approach has been suggested by Peters *et al.* (2005). Mutation screening covering the whole region coding for EGF repeats (exons 2–24) is not realistic for all patients and for most laboratories.

In cases with at least a fair amount of accumulated N3ECD the immunostaining has been found reliable (Joutel *et al.*, 2001). However, if only a small amount of the N3ECD has accumulated, e.g. at the early stage of the disease, ultrastructural resolution and characteristic appearance of GOM most likely make the EM analysis more reliable: we have detected GOM even in patients below the age of 20 years (Fig. 4). Besides, non-specific staining is an inherent caveat of immunohistochemistry (Lesnik Oberstein *et al.*, 2003), which may be problematic just in cases with small amounts of N3ECD. Moreover, EM examination provides also information about other pathological changes in the arterial wall, such as those due to hypertension, ageing and possibly even other hereditary arteriopathies (Ruchoux *et al.*, 2000, 2002; Brulin *et al.*, 2002). The absence of GOM in members of the Swedish family with multi-infarct dementia, previously thought to be the first published pedigree with CADASIL (Sourander and Walinder, 1977) was an important piece of evidence in addition to the negative genetic analyses in the demonstration that the family suffers from another hereditary vascular dementia (Low *et al.*, 2007). On the other hand, the team of one of the authors (A.J.) has just identified another cerebral small vessels disease caused by a novel type of pathogenic mutation in the exon 25 of *NOTCH3* outside the domain with EGF like repeats (Fouillade *et al.*, 2008). This mutation, p.Leu1515Pro, causes increased canonical *NOTCH3* signaling in a ligand-independent fashion, possibly due to destabilization of the Notch3 heterodimer. Remarkably, in this single patient reported there is no deposition of N3ECD and GOM on VSMCs.
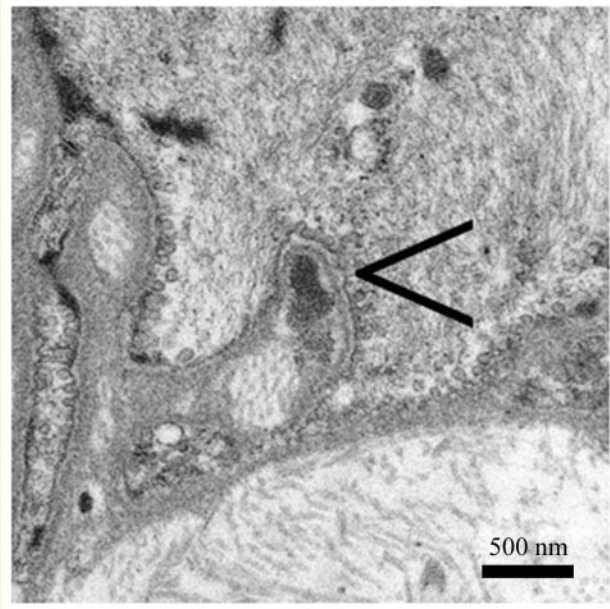

Figure 4 A small GOM deposit is in a deep indentation on a VSMC in a dermal arteriole from a 19-year-old CADASIL patient with p.Arg133Cys mutation. Note the pinocytotic vesicles.

In conclusion, the strategy of the CADASIL workup should be based on logical evaluation of clinical findings, family history as well as on both genetic and morphological methods available. Demonstration of a known pathogenic mutation provides indisputable evidence for the disease and gives a practical tool to clarify genetic counselling in the family. In those cases, in which the mutation is not easy to identify or genetic analysis is not available, skin biopsy is easy to perform and in experienced hands EM is a highly reliable method. Neither is it time consuming nor excessively expensive. Importantly, it is invaluable in guiding, how far one should proceed with the genetic analyses.

Supplementary material

Supplementary material is available at *Brain* online.

Acknowledgements

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