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NOTCH3 gene mutations in subjects clinically suspected of CADASIL

Lorena Mosca a, Raffaella Marazzi b, Alfonso Ciccone b, Ignazio Santilli b, Anna Bersano c, Valeria Sansone d, Enrico Grosso e, Giorgia Mandrile f, Daniela Francesca Giachino f, Laura Adobbati g, Elisabetta Corengia h, Elio Agostoni i, Anna Fiumani i, Salvatore Gallone j, Elio Scarpini c, Mario Guidotti h, Roberto Sterzi b, Clara Ajmone k, Alessandro Marocchi a, Silvana Penco a,*

a Department of Laboratory Medicine, Medical Genetics, Niguarda Ca' Granda Hospital, Milan, Italy

b Department of Neurology, Niguarda Ca' Granda Hospital, Milan, Italy

c Dino Ferrari Centre, Department of Neurological Sciences, University of Milan, IRCCS Foundation Ospedale Maggiore Policlinico, Mangiagalli and Regina Elena, Milan, Italy

d Department of Neurology, University of Milan, Istituto Policlinico San Donato, Milan, Italy

e Department of Genetics, Biology and Biochemistry, University of Turin, and SCU Medical Genetics, AOU San Giovanni Battista

f Department of Clinical and Biological Sciences, Medical Genetics Unit, S. Luigi Hospital, University of Turin, Turin, Italy

g Department of Neurology and Lab. Neuroscience, Dino Ferrari Center, University of Milan, IRCCS Istituto Auxologico Italiano, Milan, Italy

h Neurologic Unit, Valduce General Hospital, Como, Italy

i Department of Neurosciences, Division of Neurology, A. Manzoni Hospital, Lecco, Italy

j Department of Neuroscience, Neurogenetics, University of Turin, Turin, Italy

k Department of Mental Health, Psychological and Psicoterapia counseling Service, Niguarda Ca' Granda Hospital, Milan, Italy

* Corresponding author.

Tel.: +39 02 64442830/2803;

fax: +39 02 64442783.

E-mail addresses: silvana.penco@ospedaleniguarda.it, penco.silvana@libero.it

ABSTRACT Background: Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is an inherited cerebrovascular disease due to mutations involving loss or gain of a cysteine residue in the NOTCH3 gene. A cluster of mutations around exons 3 and 4 was originally reported. Identification of pathogenic mutation is important for diagnostic confirmation of the disease, however genetic counselling and testing of relatives at risk is critical in mutation carriers. Methods: Mutation analysis of the NOTCH3 gene was performed through direct sequencing in 140 patients with clinical suspicion of CADASIL. Patients underwent genetic counselling pre and post testing. The 2–23 exons containing all EGF-like domains were screened. Results: 14 familial forms of the disease have been identified with 14 different causative mutations in exons 2, 3, 4, 5, 7, 10, 14, 19, 20 and 22 of the NOTCH3 gene; no pathogenic mutations have been identified in exons 6 and 8; several genetic variations both in coding as well as in intronic regions were identified too. Conclusions: Our data confirm the importance of screening the whole EGF-like domains region of NOTCH3 gene for the molecular diagnosis of CADASIL among the Italian population too. Moreover genetic variants different from loss or gain of a cysteine residue are identified and presented.

1. Introduction

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL; OMIM 125310) is a rare hereditary vascular disease with neurological manifestations [1]. The disease occurs worldwide and in many different ethnic groups; however the greatest number of families has so far been identified among European Caucasians, while in the United States and Canada the number of reported cases has been surprisingly low considering the size of the populations. Exact prevalence numbers have not been reported, but the estimates are that in Finland, with a relatively high frequency and fairly good general awareness of this entity among clinicians, the prevalence is about 4 per 100000 [2]. CADASIL is characterized by four main features like migrainous headache with aura, recurrent ischemic attacks, cognitive decline and psychiatric symptoms [3]. The age of onset varies greatly, also depending on the criteria used for the onset of the disease. There are several infrequent clinical features too, such as intracerebral haemorrhages, encephalopathy, mental retardation and retinal and optic nerve abnormalities [4]. The pathological hallmark of the disease is loss of vascular smooth muscle cells (VSMC) in the tunica media of small arteries, and accumulation of granular osmiophilic material (GOM) that is detected by electron microscopy (EM) [5]. The disorder is inherited in an autosomal dominant fashion with wide expressivity within families [6]. The disease-causing mutations are located in the NOTCH3 gene, mapped on 19p13.12 which encodes a transmembrane receptor involved in cellular differentiation and cell cycle regulation [7]. All mutations causing CADASIL lie in the EGF-like repeats and result in gain or loss of a cysteine residue [8]; to date, more than 95% of cases are due to at least 170 different mutations in 20 different exons [3]. More recently, five small deletions have been described too [9] resulting in a loss of either 1 or 3 cysteine residues and thereby changing an even to uneven number of cysteines in the deleted EGF repeat [2]. A three-nucleotide insertion has been described too as the first pathogenic insertion [10]. Since clinical diagnosis is difficult, skin biopsies identifying GOM deposits can be used for diagnostic purpose; in fact pathomorphological changes in the small vessels are observed not only in the brain, but also in the skin [11]. However, the accuracy of GOM test is quite variable and CADASIL can be diagnosed only if GOM are present [3,4,12]. So far, the most definitive diagnostic test is sequence analysis of the gene [11]. A cluster of mutations around exons 3 and 4 was originally reported [13], nevertheless, the mutation spectrum analysis in a significant number of unrelated Italian families with CADASIL suggested that a different mutational approach is needed from country to country, and perhaps

also from region to region in the same country [14]. In the present study we report the molecular analysis of NOTCH3 gene in 139 Italian subjects and one individual coming from China; all subjects were clinically suspected of CADASIL. We identified 14 familiar forms for which clinical characteristics are reported. Moreover, we identified several genetic variants in NOTCH3.

2. Materials and methods

2.1. Patients

The study population consists of 140 subjects referred to our laboratory from different regions of Italy and from China with suspected CADASIL. All the patients were offered genetic counselling and all signed the informed consent approved by the Ethics Committee of our Hospital. A cultural mediator has been involved for informed consent related to the Chinese patient. Neurological examination, brain MRI, and clinical history were evaluated. The patients were selected on the basis of the following criteria: history of premature ischemic strokes; migraine with aura; vascular dementia of unknown etiology; presence of T2-weighted hyperintensities located mainly in the periventricular region, or the external capsule, or the anterior temporal pole at the cerebral MRI scans; a family history of neurological disorders consistent with an autosomal dominant pattern of inheritance; and a combination of the above criteria. All positive results of the NOTCH3 test were communicated in the presence of a multidisciplinary team made by geneticist, neurologist and psychologist. All family members who made a request for genetic testing were carefully counselled before and after molecular testing.

2.2. Molecular analysis

DNA was extracted from whole blood samples using standard procedures [15]. Blood and DNA samples are conserved and available upon request, according to the informed consent signed, for further studies. DNA from patients was amplified using primers, kindly provided by Prof. Dichgans (Ludwig Maximilians University, Munich, Germany), designed to amplify the 2–23 exons of the NOTCH3 gene, including the intron–exon boundaries. The PCR reactions were performed using AmpliTaq Gold® DNA polymerase (Applied Biosystems). Direct sequence was performed on an automated sequencing system (Applied Biosystems 3730 DNA Analyzer) using the BigDye™

Terminator Cycle Sequencing Kit Version 1.1 (Applied Biosystems). Sequencing of exons 3, 4, 6 and 8 was done first; and screening of the 18 remaining exons encoding the EGF repeats was pursued until a mutation creating or deleting a cysteine residue was identified. The nucleotide position of mutation present in the coding regions refers to the mRNA sequence (NM_000435). Haplotypes and their frequencies were estimated in probands free of pathogenetic mutations by using the HAPSTAT software (<http://www.bios.unc.edu/~lin/hapstat/>) under HardyWeinberg Equilibrium (HWE).

3. Results

3.1. Patients

We identified 14 different causative mutations localized in 10 different exons in 14 probands (Table 1). Analysis of 15 additional family members, both symptomatic as well as asymptomatic was performed. Among the tested family members, 11 resulted positive to genetic test, details are reported in Table 2 and pedigrees are shown in Fig. 1. The remaining 126 probands free of pathogenetic mutations presented the clinical onset as summarized in Table 3; comparison with NOTCH3 positive subjects are reported too.

3.2. Characterization of NOTCH3 genetic variants in 126 subjects with suspected CADASIL

21 different but not pathogenetic variants were detected in the NOTCH3 gene. The most common polymorphism was c.606 A/G (A202A) in exon 4: 21% subjects were A/G, while 60% were G/G. However exon 4 also shows the rare polymorphism c.660 C/T (Y220Y) found in two patients (2%) in the heterozygous state. Polymorphisms in exon 16 (c.2538 C/T; C846C) and in exon 17 (c.2742 A/G; P914P) were quite frequent too: the first one was detected in 82% of subjects (C/T=57%; T/T=43%) whereas the second one was detected in 88% (A/G=25%; G/G=75%). In exon 3, the c.303 C/T (T101T) variant was found in heterozygous state in 26% of individuals, while 5% were in homozygous state. Polymorphism in exon 7 (c.1140 T/C, P380P) was detected in 7% of subjects and polymorphism in exon 9 (c.1487 C/T, P496L) in 5%; in both cases, they were present in the homozygous state in one patient only. In exon 11 we detected two polymorphisms at the same codon (c.1725 G/A and c.1725 G/C) not changing the corresponding aminoacid (T575T): they were

present in 3% of subjects in the heterozygous state. The genetic variant in exon 19 (c.3058 G/C, A1020P) was found only in the heterozygous state in 4% of subjects. Variants c.2155 G/T (V719L) in exon 14 and c.3399 C/A (H1133G) in exon 21 were in the heterozygous state in one person only. Two different polymorphisms were detected in exon 22, both only in the heterozygous state: c.3547 G/A (V1183M) was found in the in 2% of subjects and c.3704 A/T (H1235L) in 1%. In addition, seven polymorphisms located in the intronic regions of the gene were identified (IVS9-22 C/G, IVS12-23 G/A, IVS15+7 C/A, IVS15-4 C/G, IVS16+24 G/T, IVS22-24 A/T, and IVS23+21 T/A); they were found only in the heterozygous state and were present in 1%, 3%, 1%, 1%, 4%, 1% and 7% of subjects, respectively.

3.3. Haplotype

Genotyping results enable us to identify different haplotypes; three of them were more frequent within the group of patients without pathogenetic mutations (Table 4). Any possible correlation with clinical status and haplotypes was not performed because of the reduced number of subjects.

4. Discussion

CADASIL is a rare heritable cause of stroke and vascular dementia in adults [1] and the pathophysiology of the disease is not yet defined as well as there is no useful treatment to cure CADASIL, but only drugs to relieve the symptoms. Due to its clinical heterogeneity, diagnosis of CADASIL through genetic testing plays an important role, and the whole extracellular domain of the NOTCH3 gene should be analyzed. We tested 139 Italian and 1 Chinese subjects with brain MRI and neuroradiological features suggestive of CADASIL: 14 families were identified through the genetic analysis thus confirming the diagnosis of CADASIL. Clinical characteristics of the identified families were subcortical recurrent ischemic strokes, headache, migraine with aura, cognitive impairment and depressive disorder, which are typical of CADASIL [18]; also the mean age of onset of the index patients (47 years) was in agreement with the literature [11,19]. We identified mutations in 14/140 (10%) probands (7 males), with the majority lying in exon 4. Two patients from the Centre of Italy showed mutation in exon 19, according to the mutation regional distribution reported by Dotti et al. [14]; exons 2, 5 and 7 were not described as Italian mutational hot spots, whereas exons 3, 10, 14, and 20 have been reported to be mutated in Italian CADASIL

subjects [9,20,21]. Surprisingly enough, no mutations were localized in exons 6 and 8 as described by Ungaro et al. [22] in the NOTCH3 gene analysis on 684 CADASIL patients and by Dotti et al. [14] in their study on 28 Italian CADASIL families. The IVS15-4 C/G variant detected in our patient, was already described by Ungaro et al. [22] and classified as polymorphism, although detailed functional analyses were not performed in that study. The nucleotidic substitution is very close to the 3' acceptor site of intron 15 and two groups have recently reported NOTCH3 splice site mutations in IVS3 and IVS15, respectively [23,24]. The exon skipping mechanism in NOTCH3 resulted in an elimination of cysteine residues and therefore was believed to be causative of CADASIL. A similar consequence could be caused by the presence of the variant IVS15-4 C/G: we found that variant in a single patient and never in 150 chromosomes from healthy subjects. We are not able to prove the pathological meaning of that variant since the affected patient didn't give consent for mRNA collection. In the remaining 126 probands, only six were free of nucleotidic substitutions in the NOTCH3 gene; indeed 21 different polymorphisms were identified, some of them leading to amino acid substitutions, others present in non-coding portions of the gene. Fifteen of these polymorphisms were found by Ungaro et al. [22] too as well as by Pantoni et al. [21] in their Italian cases and the pathogenic significance of these genetic variants is uncertain. To date it is unknown whether all these polymorphisms affect the signalling or whether they are involved in cerebrovascular disease [22]. Indeed we do not know either whether large gene deletions or duplications, not identifiable through direct sequencing, may play a role in CADASIL: the heterozygous presence of genetic variants in NOTCH3 gene could be informative on the integrity of the whole exon/allele. Recently, a different molecular approach such as MLPA (multiple ligation probe assay) has been developed and applied to CADASIL, with interesting results [25]. We also performed haplotype estimation in the EGF-like repeats of the NOTCH3 gene for the subjects free of mutations. This study allowed the identification of different haplotypes, three of which with particular frequency, however this is a preliminary study and further efforts in larger series of patients are needed to better understand a possible relationship between clinical manifestations and each haplotype.

5. Conclusion

The finding of mutations localized in 10 different exons strengthens the necessity to screen all the EGF-like repeats (exons 2–23) of NOTCH3 gene, in order to improve the diagnosis of the disease.

Moreover, the identification of mutations in splice sites causing exon skipping shows the importance of the analysis of the intron–exon boundaries too. New different approaches for the identification of mutations causing the removal of one or more exons that can't be detected by sequence analysis are also needed. A limitation of this study is that NOTCH3 negative patients did not undergo skin biopsy to search for GOM. Moreover, the molecular characterization of additional genetic variations present within NOTCH3 gene may shed light on phenotypic variations. Indeed, we need to obtain more data, to increase the number of studied subjects in order to correlate the genetic status with the observed clinical status.

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Fig. 1. Pedigrees of the studied families.

The arrow indicates the index case. Squares represent males; circles, females. Symptomatic family members are shown in black. A diagonal line through the symbol represents a deceased person.

