

CADASIL: A Critical Look at a Notch Disease

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Key Words

CADASIL · Central nervous system degenerative disorder · Notch 3 mutations

Abstract

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a catastrophic late-onset syndrome which manifests itself mainly as a central nervous system degenerative disorder. CADASIL has been associated with mutations in the Notch 3 receptor which appear to cause, mainly, vascular abnormalities. Although more than a decade has passed since Notch 3 mutations were linked with this disease, we still do not have a good grasp on the molecular mechanisms underlying the CADASIL-associated Notch 3 receptor malfunction, nor do we understand many aspects of the CADASIL pathobiology. In this review, we discuss the CADASIL-related literature and attempt to evaluate the various experimental systems and approaches used to address what seems to be a paradigm for studying the pathobiology and genetics of vascular cognitive impairment.

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Introduction

Notch signaling plays a fundamental and evolutionarily conserved role in metazoan development. Extensive molecular and genetic analyses, mostly based on invertebrate systems, revealed the Notch pathway as one of the fundamental signaling pathways that define the backbone of multicellular development. Notch signals are highly pleiotropic, dictating cellular fates in a way that depends on cellular context. Thus, depending on how the Notch signals integrate their action with other signaling pathways and other cellular elements in a specific tissue, they can influence differentiation, proliferation or apoptotic events in a very broad spectrum of tissues [1–3].

The Notch locus encodes a transmembrane surface protein that represents the central element of a cell signaling mechanism, extensively studied in *Drosophila* and other invertebrate organisms [4]. The Notch receptor is composed of a single-pass 2,700-amino-acid-long protein with a 1,700-amino-acid-long extracellular domain which harbors a characteristic tandem array of approximately 36 epidermal growth factor (EGF)-like repeats. The basic architecture of the Notch signaling circuitry has the extracellular domain of either of the two single-pass transmembrane ligands, Delta and Serrate/Jagged, on the surface of one cell, interacting with the extracellular domain of the single-pass transmembrane, heterodimeric Notch

receptor on the adjacent cell. Ligand-receptor binding is regulated by posttranslational events such as glycosylation as well as other modifications involving the extracellular domains of both the receptor and the ligand [5]. Normal folding of the Notch protein is facilitated by the chaperone activity of protein O-fucosyltransferase 1 in the endoplasmic reticulum [6]. Ligand binding appears to trigger a series of proteolytic events resulting in cleavage of the intracellular domain of the receptor, which carries nuclear localization signals and thus can translocate into the nucleus. The translocation events are very poorly understood and indeed none of the cellular parameters that can influence it are known. The crucial signal-producing cleavage that releases the intracellular domain from the membrane depends on the Presenilin/ γ -secretase complex. Once in the nucleus, the intracellular sequences act as an activation/recruitment element of a complex that contains the Notch signaling effectors, Suppressor of Hairless (a DNA-binding protein with both enhancer and suppressor activities [7, 8]) and Mastermind (a nuclear protein), which in turn directs the assembly of transcriptional complexes that drive target gene expression. These molecular elements define a basic and evolutionarily conserved framework of the Notch signaling mechanism.

In humans, as in other mammals, while the core and as far as we know, the biochemistry of Notch signaling is completely conserved, each Notch pathway element has several paralogues [9]. The large volume of the existing experimental evidence supports the notion that while the biochemical core of each of the four mammalian Notch receptors and the downstream targets of the signals may be to a large degree the same, the expression pattern of the receptors and possibly quantitative aspects of the signal are specific to each receptor. The available evidence does not in our view support the notion that each receptor may target qualitatively distinct gene activities. It is noteworthy that Notch 3 has been reported to suppress Notch 1 function [10, 11]. While it is possible that such differences may reflect context-dependent aspects of Notch 1 versus Notch 3 signaling [11, 12], such behaviour could be in principle explained by differential quantitative aspects of the two Notch receptors.

Extensive studies established that the general developmental role of Notch signals, which can influence differentiation, proliferation and apoptotic cellular decisions, is to link cell fate choices of one cell to those of a cellular neighbour. Given the fundamental role that Notch signaling plays in metazoans, it is not surprising that mutations in Notch pathway elements invariably lead to develop-

mental abnormalities in all animal models tested, affecting a very broad spectrum of cells. Notch malfunction in humans has been associated with pathologies, including acute T-cell lymphoblastic leukaemias (Notch 1 [13, 14]), Alagille, an autosomal dominant developmental syndrome (Jagged 1 [15, 16]), spondylocostal dysostosis, an autosomal recessive developmental disorder (Delta-like 3 [17]) and last, but not least, CADASIL (Notch 3 [18]), which is the focus of this review.

CADASIL: Clinical Findings and Cellular Phenotype

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL; MIM 125310) is an autosomal dominant disease that was first described in 1977 independently by two groups [19, 20]. Tournier-Lasserre and her colleagues [21] assigned the disease locus to chromosome 19 by genetic linkage analysis, coined the acronym CADASIL and established that mutations exclusively affecting the extracellular EGF-like repeat-containing domain of the Notch 3 receptor underlie this syndrome [18].

Clinical studies in large cohorts of patients demonstrated that CADASIL manifests itself mainly as a central nervous system disorder of progressive nature [22, 23]. Migraine with aura is a common initial symptom in young patients (range of age at onset: 5–40 years), while transient ischaemic attacks and ischaemic strokes appear at a later age (range of age at onset: 19–66 years) [22–25]. Older patients develop progressive vascular cognitive impairment and usually depend on others to perform daily activities. Although less common, psychiatric disturbances, hearing loss, and epileptic seizures have also been reported [23, 24, 26]. Magnetic resonance imaging (MRI) studies of the brain in CADASIL patients show white matter pathology (leukoencephalopathy) in subcortical, periventricular and temporal lobe areas [27, 28]. In addition, MRI abnormalities often compromise the basal ganglia and brain stem [29].

Post-mortem pathological examination of the brain and other tissues from CADASIL patients revealed cerebral infarcts and arteriole wall degeneration with deposits of granular osmiophilic material (GOM; evident under electronic microscopy analysis) in the vicinity of vascular smooth muscle cells [30–33], indicating that CADASIL is due to a unique type of nonarteriosclerotic, amyloid-negative arteriopathy involving small arteries and capillaries primarily in the brain but also in other organs in-

cluding skin, peripheral nerves and heart [31, 33–36]. Perivascular deposition of GOM in the brain, muscle, skin and other organs precedes the destruction of vascular smooth muscle cells. Endothelial cell changes in muscle and skin biopsies from CADASIL patients have also been described [37]. Although the arteriopathy is widespread, it is now believed that strokes caused by the pathology of deep perforating vessels in the brain account for most of the clinical manifestations of the disease [38].

The clinical features of CADASIL can vary dramatically among individuals from different families but also among carriers of the same mutation [25, 39]. In general, clinical studies have failed to indicate strong correlations between mutation site and particular clinical manifestations [40]. However, a splice site mutation leading to a small in-frame deletion has been associated with rarity of stroke events, high prevalence of migraine with aura and confusion or coma episodes [41], while a mutation in the ligand-binding domain of Notch 3 (C455R) caused very early onset of migraine and stroke and severe MRI abnormalities in a Colombian family [25]. Moreover, lower age at death (C117F) or at onset of stroke (C174Y) has been described for two mutations [42]. However, the mechanism(s) by which the different mutations result in particular phenotypes remain elusive. It is conceivable that polymorphisms in other genes known to regulate expression, trafficking, and activity of the Notch receptor (ligands, Fringe, Deltex and others) could contribute to phenotypic variation in CADASIL.

GOMs: A CADASIL Hallmark?

GOM deposits in the vicinity of smooth muscle cells are peculiar to CADASIL and were in fact first described in biopsies from non-elderly demented patients even before the syndrome acquired a name [43]. In proximity of the GOM deposits, but distinct from them, and within the cell membrane of vascular smooth muscle cells, the Notch 3 ectodomain appears to accumulate in brain and skin biopsies of CADASIL patients [33, 44]. Despite efforts directed at its characterization, the nature of the GOM remains obscure. Moreover, how these deposits arise as well as their relationship to the mutant Notch 3 receptors remains a key question in the field of CADASIL pathobiology.

Since their initial identification, numerous groups have reported GOM deposits in skin biopsies as well as post-mortem brains from CADASIL patients and GOMs are considered to be pathognomonic of CADASIL [45]. However, some reports question whether widespread

GOM deposition is indeed a *sine qua non* hallmark of the disease, at least as far as skin biopsies are concerned [46–48]. Interestingly, GOM deposits have also been observed in vessels devoid of smooth muscle cells [49].

The CADASIL-Linked Notch 3 Mutations Are Highly Stereotypical

The general structure of the mammalian Notch 3 gene, first characterized by Lendahl and colleagues is very similar to the other Notch receptors, except that it has 34 (instead of the canonical 36) EGF-like repeats (repeat 21 is absent, as are parts of repeats 2 and 3) and a shorter cytoplasmic domain [50]. In adults, Notch 3 is primarily expressed in vascular smooth muscle cells [33, 51–55]. Based on immunocytochemical analysis, it has been suggested that maybe not all smooth muscle cells express Notch 3; while this observation could have interesting implications, it should be pointed out that this particular study did not include double staining with a general smooth muscle cell marker together with Notch 3 [54].

More than 70 mutations in the extracellular domain of Notch 3 have been identified in CADASIL-affected families, originally in Europe, where the first families were described [21, 23, 56], later in the USA [57], and more recently in Colombia [24, 25], Japan [58, 59] and Singapore (ethnic Chinese) [60]. CADASIL patients are heterozygous, with the exception of one homozygous individual who presents with the classic, albeit severe, clinical symptoms of the disease [61]. The prevalence of CADASIL remains largely unknown and likely underestimated. However, CADASIL made it to the movies, as one of the characters in Alejandro Amenábar's 2004 film 'Mar adentro' (The Sea Inside) is afflicted with this disorder.

The CADASIL-linked mutations identified to date are almost exclusively single-base substitutions/missense mutations (only three are short in-frame deletions). The striking observation regarding these mutations is that they result in an extracellular Notch 3 domain that contains an odd number of cysteine residues implying the existence of free, presumably reactive sulfhydryl groups. Rare Notch 3 mutations not affecting cysteines have been reported in some CADASIL patients; however, as these same individuals also carried a mutation affecting a cysteine, it is quite possible that the atypical mutations reflected in fact genome heterogeneities [62]. Three other atypical mutations recently reported may also still represent polymorphisms [36, 59, 63]. In view of the over-

Table 1. Effects of CADASIL mutations on Notch 3 function

Mutation	EGF repeat	System	Trafficking	Ligand binding	Reference
R90C C212S	2 5	293T transient transfection	normal	not assayed	Joutel et al. [33]
R171C H184C C544Y	4 4 13	HEK 293T transient transfection	normal	normal (Delta1Fc)	Haritunians et al. [79]
R142C	3	HEK 293 stable transfection	S1 cleavage affected impaired cell surface expression	normal (Delta1 and Jagged1)	Karlström et al. [78]
R133C C185R	3 4	SHSY5Y (neuroblastoma) HEK 293	normal	not reported	mentioned by Kalaria et al. [90]
R90C C212S C428S C542Y	2 5 10 13	293T NIH3T3 transient transfection	normal normal normal impaired cell surface expression	normal (Jagged1Fc) normal (Jagged1Fc) no Jagged1 binding normal (Jagged1Fc)	Joutel et al. [80]
R133C C183R C455R	3 4 11	NIH3T3 A7r5 (aortic smooth muscle) transient transfection	normal cell surface expression but delayed S1 cleavage	normal (Jagged1Fc; Delta1Fc) no ligand binding	Peters et al. [81]

whelming evidence that CADASIL-linked mutations in Notch 3 affect cysteine residues, rigorous documentation is essential prior to suggesting that atypical mutations are indeed pathognomonic and causative.

What Is the Nature of the Mutations?

While the association between mutations in the extracellular domain of Notch 3 and the CADASIL syndrome is clear, the functional consequences of these mutations on the Notch receptor remain unknown. Yet, if one is to contemplate therapeutic approaches it is essential to resolve such functional questions. It is unclear if the CADASIL mutations reflect receptor gain or loss of function. Normal Notch function is exquisitely sensitive to gene dosage and in fact analyses in *Drosophila*, where such questions can be addressed systematically, demonstrated that either less (haplo-insufficiency) or more (duplications of the gene) copies of the gene result in aberrant development. In addition, the ligands also behave in a haplo-insufficient manner [64] (<http://flybase.bio.indiana.edu/>). These quantitative aspects are not peculiar to *Drosophila*, but are also encountered in mammals, where haplo-insufficient and dosage-sensitive behaviour of Notch signaling components has also been document-

ed, most notably in the case of Alagille patients [65, 66], but also in several Notch pathway mouse mutants [67–70, 91]. Thus, development seems to be sensitive to the quantity of signal-competent Notch receptors in a cell.

The difficulty in evaluating the functional consequences of the CADASIL mutations is reflected by controversial contentions, which are based, however, on what we see as non-conclusive analyses. For instance, CADASIL mutations have been compared to the *Abruptex* class of Notch alleles in *Drosophila* [71], as this class of mutations is associated with extracellular point mutations. The *Abruptex* mutations are, according to classic genetic as well as molecular criteria, gain of function, ligand-dependent mutations that seem to result in overactive receptors [72, 73]. Irrespective of the initial merits of such theoretical considerations, evidence that CADASIL mutations produce analogous overactive receptors is lacking.

In spite of numerous hypotheses on the nature of the CADASIL-linked Notch 3 mutations [74–77], their impact on Notch 3 function, as well as their molecular link to the pathophysiology of the syndrome, remain poorly understood. The observation that the mutant receptors harbor an odd number of cysteine residues has provoked proposals on the effect of the mutations on folding or increased reactivity of the Notch 3 protein. Another pro-

posal is that the mutant receptors cannot be efficiently removed from the membrane, where they could be sequestering ligand, perhaps dominantly inhibiting the normal pathway, or alternatively, having toxic effects. Finally, because CADASIL mutations cluster in regions of sequence diversity not only between the Notch receptors but also between Notch 3 proteins from different species, they were proposed to cause gain of function. Although these possibilities may be attractive, they remain to be tested.

Sporadic attempts to analyse the molecular nature of the CADASIL mutations have been made but failed to reveal an underlying common mechanism. These studies (summarized in table 1) are hampered by limited availability of Notch-3-specific reagents and, more importantly, are difficult to compare, as they are based on heterologous experimental systems. Thus, CADASIL mutations have been associated with impaired intracellular trafficking and processing of the receptor, ligand-binding aberrations, hypomorphic signaling as judged by reporter assays, and more recently, impaired fringe-mediated glycosylation and aberrant dimerization of mutant Notch 3 fragments. Finally, several studies failed to detect any molecular abnormalities [78–82].

Given the complexity of the effects CADASIL mutations appear to have on receptor function as reflected by all these studies, this begs the question of how best to approach experimentally this very important problem. Obviously, coordinated genotype-phenotype correlations, paralleled by systematic *in vitro* analysis and animal models would help elucidate the molecular underpinning of CADASIL-linked mutations. It should also be pointed out that any mechanistic studies on Notch signaling are inevitably complicated by the well-documented and unusual susceptibility of normal development to gene dosage, and therefore, the expression levels of the Notch receptor [1].

Animal Models

The Notch 3 Knockout

The first characterization of a Notch 3 loss of function mouse model that lacks EGF-like repeats 8–12 yielded little information, as at a gross level, the homozygous mutants, likely to be functional nulls, appeared normal [83]. Upon detailed analysis, however, the mutants were found to have arterial defects as adults due to impaired differentiation and maturation of vascular smooth muscle cells [84]. A second knockout model showed some defects in the thymus [55], while yet another, similarly viable and fertile, has not been characterized [85].

Animal Models of CADASIL

In the field of disease-oriented research it is becoming increasingly recognized that animal models may indeed provide a powerful tool in the quest of rational therapeutic approaches. However, a coordinated approach that includes animal models, molecular biological analysis and clinical data is optimal in addressing a specific disease.

To date, two different attempts have been undertaken to generate animal models of CADASIL. In the first approach, a mouse in which the endogenous Notch 3 gene was replaced by a transgene harbouring the R142C mutation (the mouse equivalent of the common human mutation R141C) using a ‘knock-in’ approach, failed to produce a discernible phenotype, despite an exhaustive analysis [86]. It should be noted, however, that these mice have not yet been analysed in terms of vascular mechanotransduction or cerebral vasoreactivity. This was a careful design, which did not perturb gene dosage and of course permitted direct comparison of knock-in animals with their wild-type counterparts. Among the several hypotheses proposed to explain the lack of phenotype, the use of mouse sequence which, despite the very high amino acid identity to the human protein and the fact that the overwhelming majority of residues known to be CADASIL targets are conserved between the two species, is nevertheless not formally known to be disease-prone when mutated, represents an interesting possibility. One may also need to consider whether the lifespan of the mouse might not be long enough to allow for the manifestation of the disease [86].

In the second approach, a transgenic mouse in which the smooth muscle cell-specific SM22 α promoter drives full-length human Notch 3 harbouring the R90C mutation has been generated [87]. Transgenic mice aged 17–20 months show vascular changes (including GOM deposits and accumulation of the Notch 3 extracellular domain) and evidence of smooth muscle cell degeneration. Vessel changes, interestingly preceding GOM deposit formation, were observed in cerebral and peripheral vessels, but were most prominent in tail arteries. Further analyses on these transgenic mice indicated mechanotransduction defects in isolated tail arteries [88] as well as impaired cerebral vasoreactivity [89]. Although these findings appear to parallel the vascular smooth muscle cell alterations, GOM deposits and Notch 3 extracellular domain accumulation detected in patients carrying CADASIL mutations, conclusions based on this model are hampered by the lack of appropriate controls. In all studies using this mouse model, the expression of the mutant transgene is not compared with the expression of the wild-

type counterpart, but rather with non-transgenic, wild-type animals. It is however important to consider that development is, as already mentioned, exceptionally sensitive to quantitative aspects of Notch signaling and to recognize that Notch signaling depends on (and thus can be affected by) feedback loops that may further complicate the analysis. With this in mind, even a transgenic animal carrying a wild-type Notch 3 allele is not in fact the optimal control, given that both the random insertion site and copy number can affect the expression levels of the transgene.

Conclusions

Attaining a molecular understanding of CADASIL pathophysiology is of paramount importance if we are to contemplate a rational therapeutic approach to this devastating disease. In particular, determining the nature of the CADASIL-linked mutations is essential as gain or loss of function abnormalities would be treated radically dif-

ferently. Moreover, if the cause of the disease is associated with an abnormal clearing of the extracellular domain of Notch 3, then a therapeutic approach that aims at modulating the Notch pathway may not even be appropriate. In genetic terms, such behaviour would be classified as neomorphic and may be counteracted by the modulation of cellular functions that have never been associated with Notch signaling.

We consider that elucidating CADASIL goes beyond the disease itself. The monogenic nature of CADASIL and the fact that it affects one of the best-studied signaling pathways in development renders it a paradigm for studying the pathobiology and genetics of vascular cognitive impairment.

Acknowledgements

We are grateful to Dr. Urban Lendahl for his critical reading of the manuscript and his insightful comments. S. A.-T. is supported by the NIH.

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