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Exome sequencing reveals an unexpected genetic cause of disease: *NOTCH3* mutation in a Turkish family with Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is a genetically complex disorder for which the definite diagnosis is only accomplished post mortem. Mutations in three genes (*APP*, *PSEN1* and *PSEN2*) are known to cause AD, but a large number of familial cases do not harbor mutations in these genes and several unidentified genes that contain disease-causing mutations are thought to exist. We performed whole exome sequencing in a Turkish patient clinically diagnosed with Alzheimer's disease from a consanguineous family with a complex history of neurological and immunological disorders and identified a mutation in *NOTCH3* (p.R1231C), previously described as causing cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). Complete screening of *NOTCH3* in a cohort of 95 early onset AD cases and 95 controls did not reveal any additional pathogenic mutations.

Although the complex history of disease in this family precluded us to establish segregation of the mutation found with disease, our results show that exome sequencing is a rapid, cost-effective and comprehensive tool to detect genetic mutations, allowing for the identification of unexpected genetic causes of clinical phenotypes. As etiological based therapeutics become more common, this method will be key in diagnosing and treating disease.

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Keywords

Alzheimer's disease; NOTCH3; CADASIL; Exome sequencing

1. Introduction

Several recent studies have succeeded in identifying the genetic basis of different disorders by using exome and genome sequencing. In an early study, a homozygous missense mutation in *SLC26A3* was identified as the cause of congenital chloride diarrhea in a patient who was referred with a preliminary diagnosis of Bartter syndrome, (a renal salt-wasting disease) (Choi et al., 2009). This work serves as an example of the power of exome sequencing in the diagnosis of Mendelian diseases, and also demonstrates that discordance between clinical findings and molecular diagnosis may exist. More recently, this premise was demonstrated in a neurological disorder: mutations in *VCP* were identified as the cause of amyotrophic lateral sclerosis by exome sequencing. Mutations in *VCP* were known to be the cause of Inclusion Body Myopathy, Paget disease and Frontotemporal Dementia (IBMPFD) in several families and this study expanded the phenotype of IBMPFD to include motor neuron degeneration (Johnson et al., 2010).

Taken together, these studies clearly demonstrate that using even a very small number of samples, it is now possible to uncover unexpected genetic alterations in Mendelian diseases. This has great clinical utility with implications for disease gene discovery, diagnosis and, ultimately, therapeutic approaches.

We sequenced the whole exome of a clinically diagnosed Turkish AD patient from a consanguineous family presenting with complex neurological and immunological disorders. In this patient we identified a previously described pathogenic mutation in *NOTCH3*, a gene that has been previously molecularly associated with CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy).

2. Methods

2.1. Human subjects

The Turkish Alzheimer disease patients are followed in the Outpatient Clinic of the Department of Neurology, Istanbul Faculty of Medicine, Turkey. Samples were collected by the same service and consent for participation was obtained in accordance with institutional review board standards. Genomic DNA was prepared from venous blood samples by standard procedures.

Alzheimer disease and healthy control samples were used to screen for mutations in *NOTCH3*. Exon 22, where the mutation was found, was analyzed in a cohort of 124 Turkish dementia patients. It was not possible to sequence the whole gene in these samples due to lack of DNA availability. The whole gene was sequenced in 95 early onset AD cases and 95 healthy controls from different geographical origins. The AD cases were obtained from the Coriell Cell Repositories, the National Cell Repository for Alzheimer's Disease (NCRAD) and the Neurology Service of Coimbra University Hospitals, Portugal. Healthy controls were obtained from Coriell Cell Repositories. Details for these samples are provided in Table 1. The study protocol was approved by the NIH IRB Committee. Written informed consent was obtained from all patients (or guardians of patients) participating in the study.

2.2. Exome sequencing

Sequences corresponding to all annotated human exons were enriched by hybridization using the SureSelect Human All Exon Kit for Illumina GA (Agilent Technologies), as per manufacturers' protocols. The DNA sample was sequenced in three flow cell lanes, on a paired-end 55bp GAIx runs (Illumina), yielding about 6 billion high quality bases. Image analysis and base calling was performed using Illumina's pipeline (version 1.5.1) with default parameters. Sequence reads were mapped to the reference genome (hg18) using BWA (Li and Durbin, 2009). SAMtools was used to generate bam files (Li et al., 2009). SNPs and Indels were called using GATK (Depristo et al., 2011). Visual inspection of variants was performed, when necessary, using IGV (Robinson et al., 2011).

2.3. Sanger sequencing

In order to exclude *PSEN1*, *PSEN2* or *APP* mutations as the cause of disease, exons 3-12 of *PSEN1* and *PSEN2* and exons 16 and 17 of *APP* were PCR amplified and sequenced as previously described (Goate et al., 1991; Cruts et al., 1998).

In order to confirm the mutation found in the proband, analyze other family members and screen a cohort of Turkish dementia cases, ExonPrimer (<http://bit.ly/9W4KkC>) was used to generate primers for amplification of *NOTCH3* exon 22. This exon was PCR amplified using Qiagen Taq DNA polymerase (Qiagen) and 7-deaza-dGTP (Sigma-Aldrich). PCR products were sequenced using the same forward and reverse primers with Applied Biosystems BigDye terminator v3.1 sequencing chemistry and run on an ABI3730xl genetic analyzer as per manufacturer's instructions (Applied Biosystems). The sequences were analyzed with Sequencher software, version 4.2 (Genecodes, VA).

Screening of mutations in the whole *NOTCH3* gene (NM_000435.2) was performed as described above, either by using Roche FastStart PCR Master Mix polymerase (Roche Diagnostics Corp., IN) or Qiagen Taq DNA polymerase and 7-deaza-dGTP, as appropriate. Primers and conditions are available upon request.

3. Results

3.1. Genetic analysis

In order to exclude mutations in the genes known to be associated with AD, *APP*, *PSEN1* and *PSEN2* were sequenced in the proband (patient IV.4 in Figure 1). No mutations were found in any of these genes and we proceeded to sequence the patient's whole exome. This analysis resulted in the identification of 211,029 single nucleotide variants, 166,379 of which were present in dbSNP(v130), 153,005 in 1K Genomes and 1,453 were new transcribed variants (including variants located in exon/intron boundaries +/- 4bp).

SIFT was used as a first-pass filter to predict how the amino acid substitutions found would affect protein function taking into account sequence homology and the physical properties of amino acids. 178 novel missense variants were predicted to be damaging. Inspection of these 178 variants revealed a previously described pathogenic mutation in *NOTCH3* gene (p.R1231C in exon 22). This mutation results in the inclusion of a cysteine residue in the epidermal growth factor-like 31 (EGF-like 31) domain, in the extracellular domain of the protein. Sanger sequencing confirmed this result.

Additional screening of other family members (IV.5; V.1; V.2 and V.3 in Figure 1) revealed that individual V.1 also harbors p.R1231C.

In order to assess if the mutation found was a common cause of AD or other dementias in the Turkish population, exon 22 of *NOTCH3* was sequenced in a cohort of 124 Turkish

dementia cases. This mutation was found to be absent from these cases. Only the common SNP p.V1183M was found in seven samples (four AD, two classified as mild cognitive impairment (MCI) and one as frontotemporal lobar degeneration (FTLD)).

In order to evaluate the frequency of mutations in the *NOTCH3* gene in early onset AD and in healthy individuals, we sequenced the whole gene in 95 AD samples originating from different regions as well as in 95 healthy elderly controls. Several genetic variants were found in the gene (Table 2) both in cases and controls, but none of these were found to alter cysteine residues of the protein.

3.2. Clinical details of the family

The family here described has at least two loops of consanguinity. DNA samples from individuals IV.4, IV.5, V.1, V.2 and V.3 were available for analysis (Figure 1).

Proband (IV.4)—This 74 year old man is reported to have had onset of memory loss at around 62 years of age. His parents, originating from Giresun, a Turkish town on the Black sea were first cousins. The mother (III.5) died at a young age and the father (III.4) died at 65 years following cancer. The patient's uncle (III.1) was also reported to have suffered from memory problems and died of a cerebral vascular accident around 65 years of age, though, unfortunately, no more details are known. The patient was examined in the Department of Neurology, Istanbul Faculty of Medicine, Turkey for the first time at age of 69 years. At this time he had problems in remembering recent events and in abstract reasoning. No particular medical history was known except that he was treated for hypothyroidism with levothyroxin since the age of 60 and he also took vitamin B12 substitution because of a low level found within a general check up at age of 62 years. The clinical examination showed normal results; only deep tendon reflexes of the lower limbs were decreased. Neuropsychological examination showed deficits in visual and verbal memory, and attention. Administration of the Short Blessed Orientation Memory Concentrating test was revealed a score of 16/28 and the patient scored 18/30 on the Mini Mental State Examination (MMSE). Brain magnetic resonance imaging (MRI) was reported to show right hippocampal as well as cerebello-cortical atrophy (Figure 2). Treatment by donepezil 5mg/day was introduced. Over the next three years the memory problems of the patient continued to worsen, and he saw physicians in different hospitals. At age of 72 years his MMSE score was 10/30 and his Addenbrooke' cognitive examination score was 21/100 (attention and orientation: 7/18, memory: 2/26, fluency: 0/14, language: 6/26, visuospatial examination: 6/16). There was no change in the clinical examination, but a mild bradykinesic-rigid syndrome was noted and the patient's family reported that the patient very rarely initiated communication and experienced periods of insomnia. A new cerebral MRI showed marked bilateral hippocampal atrophy, global atrophy and mild frontal white matter changes. Plasma homocysteine level was at 30.4 µg/ml. One year later the patient started to suffer from incontinence, hallucinations and agitation. His general physical condition deteriorated rapidly. Now, at age of 74 years, he is bedridden.

Individual IV.5—The proband's wife was examined at age of 73 years. Her father, who also suffered from memory problems, and the proband's father are known to be cousins (not shown in Figure 1). Her clinical and neurological examination was normal. She receives treatment for arterial hypertension and a chronic peptic ulcer. Her homocysteine level was 14.2µg/ml.

Individual V.1—The youngest son was recently seen at age of 46 years. Neurological examination was normal. He had previously been diagnosed with Systemic Lupus Erythematosus (SLE).

Individual V.2—The oldest son of the proband was reported to be diagnosed with Morbus Behçet. Aged 51 years he said not to suffer from memory problems but he was not available for a more detailed clinical examination.

Individual V.3—This 50-year-old lady, born at term without any complications, from consanguineous parents did not have any particular medical history until the age of 40 years except for an appendectomy at the age at 29 years. She started to complain about painful dysesthesia in her feet and also in her legs at age of 41 years and developed in the following two years difficulties in walking and standing up. In 2004, at age of 45 years, the diagnosis of a bilateral tarsal tunnel syndrome was established and the patient underwent surgical treatment. Nevertheless symptoms worsened over the next years. A cervical-spinal MRI from 2006 is reported to show generalized degeneration of the intervertebral discs, but no focal lesion. The cerebral MRI showed a marked cortico and mild cerebellar atrophy and a mild atrophy of the corpus callosum (CC). Electromyographic (EMG) examination revealed a chronic affection of the L5 radix-segment, but no polyneuropathy was found. One year later, in 2007, EMG showed a very mild asymmetric axonal sensorimotor polyneuropathy. Research of several antibodies (ENA panel, anti-DNA, c-ANCA, ANA, RF, ACA) was negative. In 2008 the diagnosis of dystonic syndrome was suggested and levodopa therapy was introduced under which the patient started to have hallucinations and psychotic symptoms. Both disappeared when the daily levodopa doses were reduced. In 2009 the patient was seen in our outpatient clinic for the first time. The clinical examination showed the typical picture of spastic paraplegia with very brisk reflexes, extensor plantar reflexes, reported bladder disturbances, abnormal gait and difficulty in walking and decreased vibratory sense at the ankles. Upper extremity muscle tone and strength were normal, but bilateral thenar atrophy was found. In the lower extremities, muscle tone was increased at the hamstrings, quadriceps and ankles. Weakness was most notable at the iliopsoas and tibialis anterior. Furthermore problems with speech and also memory were noted. A new MRI again showed a progressed cortical atrophy, mild periventricular white matter changes and thin CC. Detailed neuropsychological testing detected significantly reduced memory and frontal functioning as well as moderately reduced attention and processing speed. At first examination, patient's skin was flushed and she had a red rash in her face. Against the background of Lupus and Behçet disease in the family, a skin biopsy was performed but did not reveal any abnormalities. Skin symptoms disappeared when the levodopa therapy was stopped and replaced by Tizanidin and physical therapy.

4. Discussion

Our results demonstrate for the first time the value of exome sequencing for identification of disease causing mutations in Alzheimer's disease. In this study, a mutation in *NOTCH3* is associated with AD.

NOTCH3 (chromosome 19p13; MIM 600276) consists of 33 exons spanning 7 kb and encodes a transmembrane receptor of 2,321 amino acids involved in cellular signaling and fate during embryonic development (Joutel et al., 1996). More than 130 mutations in this gene are known to cause cerebral arteriopathy autosomal dominant with subcortical infarcts and leukoencephalopathy (CADASIL; MIM 125310) (Ungaro et al., 2009).

CADASIL is an inherited cerebrovascular disease mainly characterized by recurrent transient subcortical ischemic attacks, strokes, migraine with aura, vascular dementia, and diffuse white matter abnormalities detectable through neuroimaging, being stroke the most common sign (occurring in up to 80% of individuals) (Chabriat et al., 1995). All symptomatic patients present typical MRI findings, including clear signal abnormalities with hyperintense lesions on the T2-weighted images in the subcortical white matter and basal

ganglia (Tournier-Lasserre et al., 1991; Tournier-Lasserre et al., 1993). With rare exceptions, all CADASIL-associated mutations result in a gain or loss of cysteine residues, thus the pathogenic role of this protein is thought to arise from the aberrant dimerization of Notch3 caused by an abnormal disulfide bridging with another Notch3 molecule or with another protein (Dichgans et al., 2001; Federico et al., 2005). The highly stereotyped nature of the mutations has allowed the identification of a cysteine *NOTCH3* mutation to be considered the gold standard in the confirmation of CADASIL as a diagnosis.

The family described in the present study has more than one loop of consanguinity and presents a constellation of diverse phenotypes. The concurrent presentation of different autoimmune diseases (SLE and Behçet's syndrome) in some family members suggests additional genetic loci involved in these phenotypes. Furthermore, the proband's daughter (individual V.3) shows a very distinct clinical course with a diagnosis of spastic paraparesia. One of the proband's sons (individual V.1) was diagnosed with SLE and is reported to be cognitively normal at 46 years of age. The onset of dementia in his father (IV. 4) occurred at 65 years of age, in this way, he is still expected to develop a clinical picture of dementia since he is positive for the *NOTCH3* mutation p.R1231.

These disparities in phenotypes preclude us from demonstrating segregation of the mutation with AD. Nonetheless, p.R1231C has been repeatedly described before as a cause of CADASIL (Joutel et al., 1997; Markus et al., 2002; Singhal et al., 2004; Ungaro et al., 2009; Adib-Samii et al., 2010) and since it is predicted to result in a gain of a cysteine residue, it is highly likely to be pathogenic (Ungaro et al., 2009). The clinical phenotype associated with this mutation is not very well documented, however one female patient was reported to be hypertensive and suffer from multiple depressive episodes (Valenti et al., 2008), while a male patient had an age at onset of 52 years, MMSE of 27.3 and presented no recurrent strokes (Formichi et al., 2009).

Although rare cases of co-occurrence of CADASIL and AD have been reported (to our knowledge, only two cases are described in the literature (Gray et al., 1994; Thijs et al., 2003)), knowing the proteins involved in the pathologic process of both diseases, one could expect a larger superposition. In fact, presenilin proteins are essential in regulating the proteolytic cleavage of APP into A β -amyloid and have a very similar role in the control of the Notch signaling pathway. Presenilins (or an associated cofactor) appears to cleave Notch into an extracellular domain responsible for ligand binding and an intracellular domain that carries the signal transducing capacity (De Strooper et al., 1999). Previously proposed hypotheses highlight that in CADASIL the cleaved ectodomain of the protein accumulates in the cell and this excess protein may lead to a toxic cascade that induces amyloid deposition (Joutel et al., 2000). Dysfunctional Notch signaling may also induce or inhibit genes that are important in the pathogenesis of AD or in presenilin function.

We have sequenced *NOTCH3* in a cohort of AD patients. The absence of *NOTCH3* mutations in this cohort indicates these are not a common cause of Alzheimer's disease. In the same way, we didn't find the p.R1231C mutation or any other cysteine altering variants in neurological normal controls, which indicates these are not common polymorphisms. The data presented in this report raises, for the first time, several important and interesting hypotheses:

1. p.R1231C in *NOTCH3* was identified in an individual without CADASIL. This may lead us to question the pathogenicity of this variant. More likely, it broadens the phenotype spectrum associated with this mutation (related to the next point).
2. p.R1231C in *NOTCH3* is found in a patient clinically diagnosed with AD. Due to the fact that both CADASIL and AD are dementias, the first tendency is to question

the clinical diagnosis. However, after the molecular finding was established, two independent neurologists (blinded to the molecular results) went back and re-analyzed all the clinical and diagnostic data. The result was two additional independent diagnoses of probable AD for the proband (individual IV.4), indicating that even if neuropathologically this does not meet criteria for AD, clinically it was consistently diagnosed as such. With this in mind, the results demonstrate the utility of comprehensive exome sequencing in the differential diagnosis of clinically characterized disease.

3. p.R1231C was found in an unaffected subject (individual V.1). Although this subject is currently under the age of onset of the disease reported in his father, it is possible that the mutation has an incomplete penetrance in this family.

In summary, here we demonstrate that exome sequencing is a valid, rapid and cost-effective tool to identify genetic mutations in complex diseases. More specifically, this technology led us to a finding that was unexpected, given the clinical diagnosis, and reveals the power of this method in differential diagnosis.

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Figure 1.

Pedigree of the family where the *NOTCH3* p.R1231C mutation was found.

The arrow indicates the proband. (+) indicates positivity for p.R1231C and (-) indicates the individual does not carry the mutation. Black filled squares represent affected family members with AD. Grey filled circle represents atypical cognitive deficits together with other neurological symptoms (see text).

Figure 2.

Brain magnetic resonance imaging for proband (patient IV.4).

A. Coronal T1 weighted image demonstrates a right hippocampal as well as cerebello-cortical atrophy. B. Axial fluid-attenuated inversion recovery (FLAIR) MR reveals confluent white-matter hyperintensities adjacent to the anterior and posterior horns of the lateral ventricle and single lesions in the right frontal white-matter. C. In sagittal T2 weighted images corpus callosum seems normal.

Table 1

Characteristics of the different cohorts of samples used to screen for mutations in the *NOTCH3* gene.

	95 AD cases (whole <i>NOTCH3</i> gene sequenced)	124 Turkish dementia cases (exon 22 of <i>NOTCH3</i> sequenced)	95 healthy controls (whole <i>NOTCH3</i> gene sequenced)
Origin	10 from Coriell (American, Italian, English) 52 from Indiana University (American) 33 Portuguese	Turkey (included 1 patient originally from Iran and 6 from Yugoslavia)	USA, Israel, Uruguay, Poland, Greece, Ireland, Australia, United Kingdom, Germany, Wales, Netherlands, Canada, Colombia, Cuba
Diagnosis	Alzheimer disease	Dementia (including 62 AD, 30 MCI, 13 FTLD and 19 atypical)	Absence of neurological conditions
Ages Average (range)	55 (36 - 78)	69 (29 - 88)	78 (70 - 95)
Ages at onset Average (range)	50 years (33 - 60)	66 (29 - 84)	n.a.
Family history	15 positive from 28 known	Positive for all 124 cases	n.a.
Gender	58 females 37 males	66 females 58 males	46 females 49 males

Table 2

Genetic variants found in the *NOTCH3* gene in 95 AD cases and 95 healthy elderly controls.

Exon	rs number	cDNA	Protein	95 AD cases	95 controls
Exon 3	rs3815188	nt.303C>T	p.Thr101Thr	24 het; 1 hom	25 het; 3 hom
Exon 4	rs1043994	nt.606A>G	p.Ala202Ala	14 het; 2 hom	20 het; 2 hom
		nt.509A>G	p.His170Arg	0	1 het
	rs114457076	nt.660C>T	p.Tyr220Tyr	1 het	0
Exon 6	rs116239440	nt.945C>T	p.Ile315Ile	0	1 het
Exon 7	rs61749020	nt.1140T>C	p.Pro380Pro	9 het	6 het
Exon 9	rs11670799	nt.1487C>T	p.Pro496Leu	0	2 het
Exon 11	rs79926127	nt.1725G>A	p.Thr575Thr	4 het	4 het
	rs35793356	nt.1782C>T	p.Gly594Gly	0	1 het
Exon 14		nt.2202C>T	p.Ala734Ala	1 het	2 het
		nt.2177G>A	p.G726D	1 het	
Exon 16	rs1043996	nt.2538C>T	p.Cys846Cys	38 het; 13 hom	41 het; 9 hom
Exon 17	rs1043997	nt.2742A>G	p.Pro914Pro	15 het; 3 hom	24 het; 2 hom
Exon 19	rs35769976	nt.3058G>C	p.Ala1020Pro	2 het	3 het
Exon 21	rs112197217	nt.3399C>A	p.His1133Gln	1 het	2 het
		nt.3421G>A	p.Val1107Met	1 het	
Exon 22	rs10408676	nt.3547G>A	p.Val1183Met	1 het	3 het
Exon 24	rs78926093	nt.4044C>T	p.Gly1348Gly	2 het	1 het
Exon 25	rs1044006	nt.4563A>G	p.Pro1521Pro	19 het; 1 hom	16 het; 2 hom
		nt.4455C>T	p.Asp1485Asp	1 het	0
Exon 26		nt.4793A>T	p.Asp1598Val	0	1 het
Exon 30	rs16980398	nt.5526T>C	p.Ala1842Ala	1 het	3 het
Exon 32	rs115582213	nt.5854G>A	p.Val1952Met	2 het	1 het
Exon 33	rs1044008	nt.6438G>A	p.Ala2146Ala	2 het; 1 homo	8 het
	rs1044009	nt.6668C>T	p.Ala2223Val	32 het; 4 homo	36 het; 5 hom
	rs61731974	nt.6813 T>C	p.Pro2271Pro	0	1 het
		nt.6838A>G	Met2280Val	1 het	0

Nucleotide numbering ("c.") reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence NM_000435.2. The initiation codon is codon 1. Protein numbering ("p.") refers to sequence NP_000426.2. "Het" refers to heterozygous changes and "hom" to homozygous variants.