Original Article

New Evidence for the Role of Calpain 10 in Autosomal Recessive Intellectual Disability: Identification of Two Novel Nonsense Variants by Exome Sequencing in Iranian Families

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Abstract

Background: Knowledge of the genes responsible for intellectual disability, particularly autosomal recessive forms, is rapidly expanding. Increasing numbers of the gene show great heterogeneity and supports the hypothesis that human genome may contain over 2000 causative genes with a critical role in brain development.

Methods: Since 2004, we have applied genome-wide SNP genotyping and next-generation sequencing in large consanguineous Iranian families with intellectual disability, to identify the genes harboring disease-causing mutations. The current study paved the way for identification of responsible genes in two unrelated Iranian families.

Results: We found two novel nonsense mutations, p.C77* and p.Q115*, in the calpain catalytic domain of CAPN10, which is a cysteine protease known to be involved in pathogenesis of noninsulin-dependent diabetes mellitus. Another different mutation in this gene (p.S138_R139ins5) has previously been reported in an Iranian family. All of these patients have common clinical features in spite of specific brain structural abnormalities on MRI.

Conclusions: Different mutations in *CAPN10* have already been found in three independent Iranian families. These results have strongly supported the possible role of CAPN10 in human brain development. Altogether, we proposed *CAPN10* as a promising candidate gene for intellectual disability, which should be considered in diagnostic gene panels.

Keywords: Calpain 10, intellectual disability, Iran, next generation sequencing

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Introduction

Intellectual disability (ID) is a neurodevelopmental disorder that can have a great destructive impact on affected individuals and their families. It is defined by significant limitations in intellectual functioning and adaptive behavior, which start before age 18. As a result of its broad clinical and genetic heterogeneity, the etiologies of ID are still incompletely known. Genetic factors compromise a major group of reasons which might result in syndromic (ID with additional clinical or dysmorphological features) or nonsyndromic (ID with a few subtle clinical features).[1] In the Middle East and Iran, more than one-third of population have parental consanguinity, which highlights the role of autosomal recessive ID in these communities.[2] A definite genetic diagnosis cannot be made for most of the cases of ID. In practice, observation of a specific clinical phenotype, imaging abnormalities or assessment of blood metabolites, could be helpful. Karyotyping, array comparative genomic hybridization (CGH) and scanning the candidate genes are some approaches to discover genetic reasons.[3,4] The contribution of massively parallel next-generation sequencing (NGS), while it focuses on the results of the homozygous linkage intervals, has proven to be a powerful tool in discovering the genes of monogenic forms of ID.[5]

This study presents two novel distinct pathogenic variants in *CAPN10* (MIM number: 605286) responsible for autosomal recessive intellectual disability (ARID). The *CAPN10* gene was previously reported in an Iranian family as a candidate gene for syndromic autosomal recessive intellectual disability (SARID).[2] Here; we present two other different homozygous nonsense mutations in two unrelated Iranian families. Clinical features of the patients were significantly overlapped with previous reports. Thus, this study provides a robust evidence for the critical role of *CAPN10* in neurodevelopmental disorders.

Subjects and Methods

Subjects

Two investigated families were recruited to the Genetics Research Center, in the context of ID project. After obtaining written informed consent from the live parent, physical and mental status of the patients was assessed by an experienced clinical geneticist (Figure 1A, 2A, and Table 1). The Wechsler Intelligence Scales for Children (WISC) were used to analyze the IQ (intelligence quotient) of the patients. Brain MRI was performed to detect any

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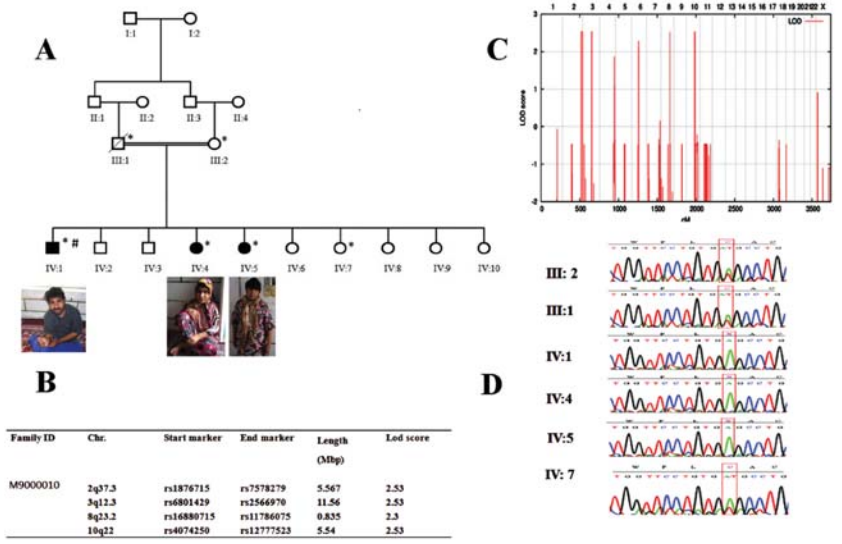
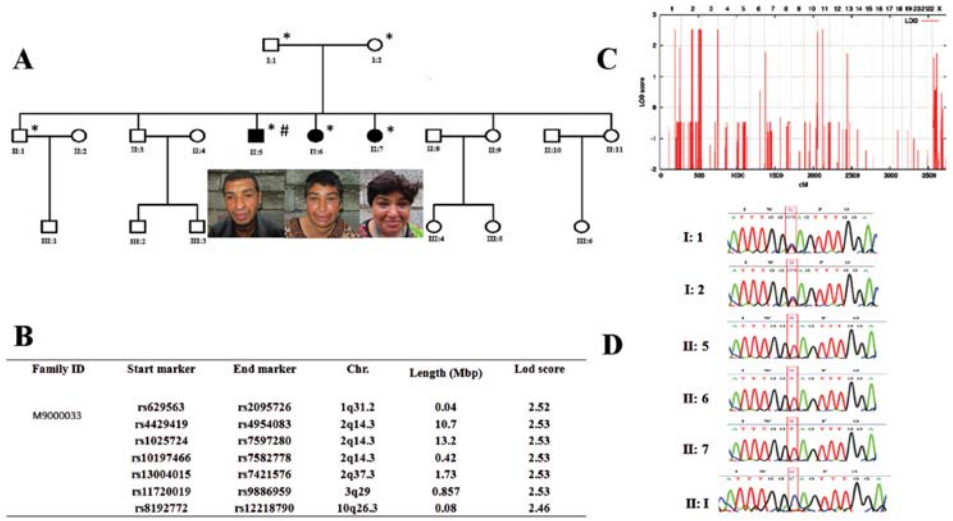
Table 1. Clinical characteristics of the patients representing *CAPN10* mutations

Family ID	**M8600057**			**M9000010**			**M9000033**		
Individual ID	**IV:1**	**IV:8**	**IV:9**	**IV:1**	**IV:4**	**IV:5**	**II:5**	**II:6**	**II:7**
Sex	**Female**	**Male**	**Female**	**Male**	**Female**	**Female**	**Male**	**Female**	**Female**
Age at the time of examination (yr)	26	19	17	35	24	30	32	39	30
Height (cm)	150	170	140	155	145	141	165	148	151
Head circumference (cm)	51 (-3.1SD)	51.5 (-2.4SD)	45.5 (-8.3SD)	55.5 (+0.27 SD)	50.5 (-3.6SD)	50 (-4.1SD)	52 (-2.1SD)	52 (-2.2SD)	52(-2.2SD)
Intelligence quotient score	33	46	30	20 – 34	20 – 34	20 – 34	20 – 34	> 25	30 – 40
Speech delay	yes	yes	yes	yes	yes	yes	yes	yes	yes
Aggression behavior	no	no	no	no	no	no	yes	no	yes



Family ID	Chr.	Start marker	End marker	Length (Mbp)	Lod score
M9000010	2q37.3	rs1876715	rs7578279	5.567	2.53
	3q12.3	rs6801429	rs2566970	11.56	2.53
	8q23.2	rs16880715	rs11786075	0.835	2.3
	10q22	rs4074250	rs12777523	5.54	2.53

Figure 1. Family M9000010. **A:** Pedigree of this family. DNA samples for SNP genotyping and whole exome sequencing have been marked by * and #, respectively. **B, C:** Results of linkage analysis and the position of the homozygous intervals. **D:** The detected variant (c.231T>A) has been clearly co-segregated.



Family ID	Start marker	End marker	Chr.	Length (Mbp)	Lod score
M9000033	rs629563	rs2095726	1q31.2	0.04	2.52
	rs4429419	rs4954083	2q14.3	10.7	2.53
	rs1025724	rs7597280	2q14.3	13.2	2.53
	rs10197466	rs7582778	2q14.3	0.42	2.53
	rs13004015	rs7421576	2q37.3	1.73	2.53
	rs11720019	rs9886959	3q29	0.857	2.53
	rs8192772	rs12218790	10q26.3	0.08	2.46

Figure 2. Family M9000033. **A:** Pedigree of this family. DNA samples for SNP genotyping and whole exome sequencing have been marked by * and #, respectively. **B, C:** Results of linkage analysis and the position of the homozygous intervals. **D:** The detected variant (c.343C>T) has been clearly co-segregated.

structural abnormalities. About 5 – 10 mL of blood samples were collected from parents and each available affected and unaffected individual in the families. DNA extraction was performed from whole blood following the standard salting out protocol. To rule out chromosomal aberrations and Fragile X-syndrome, karyotype analysis by G-banding as well as Fragile X testing by Southern blot analysis and PCR were carried out.

SNP genotyping and whole exome sequencing

Genome-wide linkage analysis was performed by whole genome single nucleotide polymorphism genotyping using the Affymetrix arrays, including Human Axiom® Genome-Wide CEU 1 Array (M9000033), and Genome-Wide Human SNP Array 6.0 (M9000010). The ALOHOMORA software was used for standard data quality control and converting the genotyping data into suitable formats for linkage programs.[6] Parametric linkage analysis was carried out by the Merlin program with complete penetrance, disease allele frequency of 10^{-3}, and based on autosomal recessive mode of inheritance.[7] Whole exome sequencing has been done for one affected individual in each family, after enrichment with Agilent SureSelect DNA enrichment kit. Sequencing has been done by Illumina HiSeq 2000 machine. The coverage of the coding exons with at least 20X was 97%, which provided sufficient depth to call variants at 98% of the coding regions. The reads were aligned to human reference genome (GRCh37/hg19 assembly). All variants including single nucleotide variants (SNVs), and copy number variants (CNVs) were called by MERAP software package (Medical Re-sequencing Analysis Pipeline) and developed at the Max Planck Institute for Molecular Genetics, Berlin, Germany.[8] All changes were filtered against variants reported in dbSNP138, 1000 Genome Project, Exome Variant Server (ESP6500), and 200 Danish individuals, and were absent in Iranian controls. Sanger sequencing was then used to validate the candidate variants.

Results

In the frame of collaboration with Max Planck Institute for Molecular Genetics a cohort of 1000 families with two or more affected individuals, have been recruited to Genetics Research Center since 2004. Until recently, with the help of linkage analysis followed by next generation sequencing, this group has reported several novel genes for ARID.[9–15]

The reason of severe ID in an Iranian family (M8600057) with three affected members (pedigree in supplementary Figure 4) has previously been reported.[2] Clinical examinations revealed thick lower lip without any deformities of the upper or lower extremities (Table 1). Brain MRI of the patient (IV: 1) showed right axial pachygyria with a small right carotid artery (unpublished data). The corpus callosum also appeared slightly small in size. The right cerebellar peduncle was smaller than the left and the insula was also thicker in the right segment (Figure 3A).

These findings raised the possibility that impaired CAPN10 protein can lead to distinct clinical features (microcephaly, brain structural abnormalities) and prompted us to look for similar clinical features in two other families with candidate variants in *CAPN10* gene.

Family M9000010, was from Ahvaz (Khoozestan province), and had three affected patients (one male, and two females). All patients had normal measurements at birth. They developed seizures during infancy that were managed by phenobarbital. Patient IV: 1 could not walk normally and had a right-sided waddling gait with right wrist contracture and inability to use her hand since 7 years of age. Patients IV: 4 and IV: 5, were able to walk but with an unbalanced gait. All of them were unable of self-caring, and toilet training, and showed no behavioral aggression or self-mutilation, (Table 1). The brain MRI of IV: 5 showed thick insula in axial FLAIR MRI, mild cerebellar atrophy, left cerebral hemisphere atrophy and left dilated sulci probably because of a smaller right carotid artery (Figure 3B).

Family 9000033, was recruited from a village near the Caspian Sea and had three affected patients (one male, two females). They had seizures which were controlled by carbamazepine. These patients are able to walk but did not have any interaction with their environment, no verbal communication or toilet training. All had

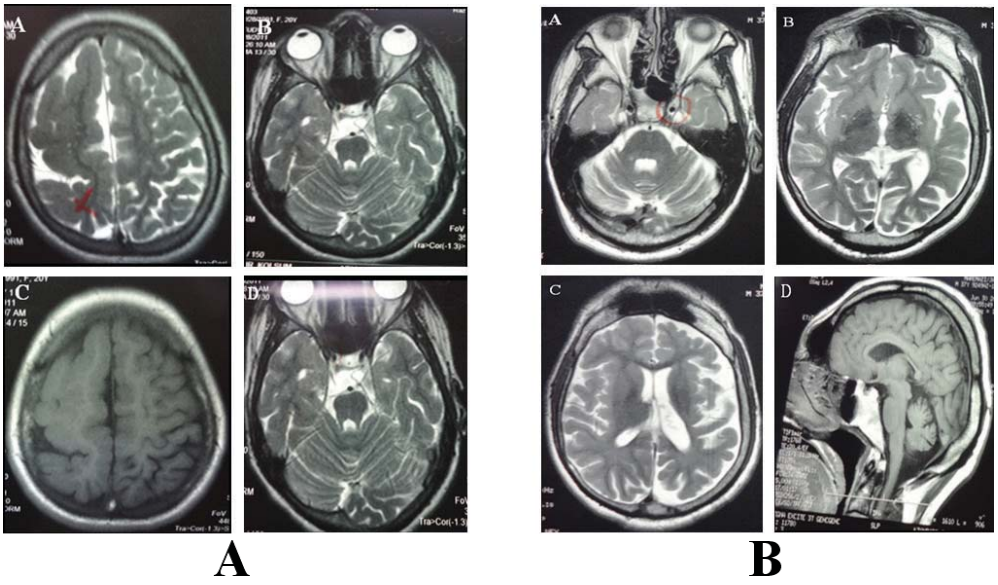


Figure 3. Magnetic resonance brain imaging in patients with *CAPN10* mutations. **A)** M8600057; **B)** M9000010. Cerebral hemisphere atrophy due to smaller right carotid artery can be seen in both figures.

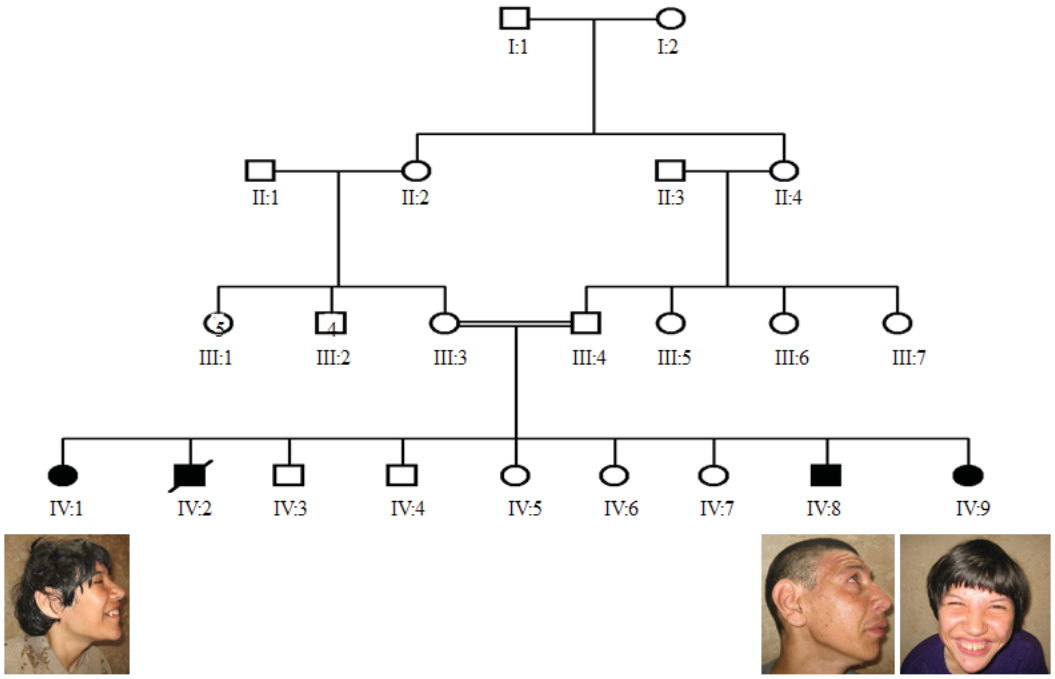


Figure 4. Family M8600057. Previously reported family with three affected ID members. Clinical investigation revealed thick lower lip without any deformities of the upper or lower extremities.[2] We detected *CAPN10* mutations as the reason of severe ID for this family.

thick lower lips, (Table 1). Brain MRI showed cortical thinning in the right cerebral hemisphere. The right cerebral carotid artery was also smaller than the left one (Figure 3B).

Genome-wide SNP genotyping for all affected individuals, parents and one normal healthy sibling in families M9000010, and M9000030, revealed several homozygous regions (Figures 1B, and 1C, Figures 2B, and 2C). There was also one overlapping interval in these two families, flanked by heterozygous SNP markers rs13004015, and rs7578279.

Whole exome sequencing (WES) for one affected individual from each family, showed two novel nonsense variants in *CAPN10* gene (located in the overlapping homozygous region). These variants were clearly co-segregated with the disease. In family M9000010, WES detected a nonsense variant, g.2:241528849T>A, NM_023083: c.231T>A (GRCH37/ hg 19) on chromosome 2. In family M9000033, WES, also detected a nonsense variant, g.2:241530301C>T, NM_023083: c.343C>T (GRCH37/ hg 19) on chromosome 2, (Figures 1D, 2D).

Discussion

CAPN10 was first reported as a candidate gene ID in an Iranian family from the northern Khorasan province (M8600057). In a cohort of 1000 Iranian families with ID, we have so far identified three homozygous mutations in this gene. Sever developmental delay, minor facial deformities (thick lower lip), and structural brain abnormalities were the main manifestations.[2]

Calpains represent a well-conserved family of calcium-dependent cysteine proteases. Their substrates include cytoskeletal proteins, kinases, phosphatases, membrane and steroid receptors.[16] Calpain 10, an intracellular non-lysosomal calcium-dependent cysteine protease, is a ubiquitous calpain. The expression and localization of CAPN10 correlate mainly with cellular calcium level.[17] CAPN10 can be found in the cytosol, nucleus and mitochondria.[18]

Calpain 10 is implicated in a wide range of cellular activities including remodeling of cytoskeleton, signal transduction, long-term potentiation, apoptosis, ischemia and inflammation.[2,19] Several Calpain 10 transcripts have been detected in both fetal and adult tissues. A homology comparison study has demonstrated that the *CAPN10* gene has been extremely well conserved among mammals.[20]

It has already been proven that there is a close relationship between *CAPN10* gene and noninsulin-dependent diabetes mellitus (MIM number 601283).[21,22] Several genome wide association studies in different populations have identified variations in this gene regarding association with noninsulin-dependent diabetes mellitus.[23] Calpains have been involved in regulation of insulin receptors and can impact the regulation of adipocyte differentiation.[24–26]

Its role in insulin secretion and trafficking may help in regulation of cytoskeletal changes. It has also been demonstrated that Calpain 10 is the predominant mitochondrial Calpain. Epithelial cell death may happen due to the loss of Calpain 10, while *CAPN10* over-expression may lead to mitochondrial dysfunction.[27]

Inhibition of Calpain 10 by antisense experiments showed the impairment of actin organization and translocation of glucose transporter type 4 by insulin that provides evidence for the role of Calpain 10 in the development of type 2 diabetes.[28]

Single nucleotide variations in the introns of *CAPN10* have been reported in association of polycystic ovary syndrome (MIM number 184700) and microvascular functions.[29,30] Although it has not functionally proven, variations in *CAPN10* have also been associated with carotid artery intima-media thickness, which propose CAPN10 as an atherogenic factor both in atherosclerosis and diabetes. Calpains have a role in the production of nitric oxide, expression of adhesion molecules, and proliferation of vascular smooth muscle cells.[31]

Protein expression of Calpain 10 has been shown in astrocytes of temporal cortex by immunohistochemistry. Up-regulation of *CAPN10* expression which impaired calcium signaling in astrocytes, showed its role in Alzheimer-type diseases.[32]

The first reported homozygous mutation in *CAPN10* was an in frame insertion of five amino acids in Calpain catalytic domain, which may disturb CAPN10 activity and protein interactions.[2]

Here, we provide the second report of *CAPN10* mutations in two other Iranian families. Interestingly, none of these families were related to each other or even have the same ethnicities.

These variants made premature termination codons in the N-terminus of CAPN10 protein. They may result in truncated proteins, being interrupted in the middle of Calpain catalytic domain, and lacking the C-terminus. Due to the generation of premature termination codons, nonsense-mediated decay and mRNA degradation might also happen, which may result in the absence of the CAPN10 protein.[33,34] Further investigations by reverse transcription PCR and Western blotting should be done, to elucidate the effect of these mutations on CAPN10 protein.

Calpain-mediated proteolysis is a regulatory process in synapses, which is important in pathophysiology of nervous system diseases.[35] In spite of the role of CAPN10 in calcium signaling, it may also have a role in the neuronal mechanisms of breaking down.

Brain MRI scans of the patients showed a slightly smaller right carotid artery, which may explain all abnormalities mentioned earlier. It seems that the influence of CAPN10 on proliferation and migration of vascular smooth muscle cells and its related polymorphisms on vascular defects,[30,36] may increase the carotid intima-media thickness and also lead to subclinical atherosclerosis.[31]

Functional investigations are necessary to elucidate the exact mechanism regarding the role of CAPN10 in impaired cognitive disorders.

In conclusion, this study strongly supports the role of CAPN10 in cognitive function. It could be proposed that individuals with *CAPN10* gene mutations usually display a distinct syndromic pattern of ID. Although no functional assessment has been done, the overlapping phenotypes of the patients can be sufficiently persuasive. We suggest that in similar clinical phenotypes *CAPN10* mutation isconsidered, especially in Iranian population. It is reasonable to include *CAPN10* in NGS diagnostic panels for intellectual disability.

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