A Defect in the *TUSC3* Gene Is Associated with Autosomal Recessive Mental Retardation

Masoud Garshasbi,[1,2] Valeh Hadavi,[3] Haleh Habibi,[4] Kimia Kahrizi,[2] Roxana Kariminejad,[3] Farkhondeh Behjati,[2] Andreas Tzschach,[1] Hossein Najmabadi,[2,3] Hans Hilger Ropers,[1] and Andreas Walter Kuss[1,*]

Recent studies have shown that autosomal recessive mental retardation (ARMR) is extremely heterogeneous, and there is reason to believe that the number of underlying gene defects goes into the thousands. To date, however, only four genes have been implicated in nonsyndromic ARMR (NS-ARMR): *PRSS12* (neurotrypsin), *CRBN* (cereblon), *CC2D1A*, and *GRIK2*. As part of an ongoing systematic study aiming to identify ARMR genes, we investigated a large consanguineous family comprising seven patients with nonsyndromic ARMR in four sibships. Genome-wide SNP typing enabled us to map the relevant genetic defect to a 4.6 Mbp interval on chromosome 8. Haplotype analyses and copy-number studies led to the identification of a homozygous deletion partly removing *TUSC3* (*N33*) in all patients. All obligate carriers of this family were heterozygous, but none of 192 unrelated healthy individuals from the same population carried this deletion. We excluded other disease-causing mutations in the coding regions of all genes within the linkage interval by sequencing; moreover, we verified the complete absence of a functional *TUSC3* transcript in all patients through RT-PCR. *TUSC3* is thought to encode a subunit of the endoplasmic reticulum-bound oligosaccharyltransferase complex that catalyzes a pivotal step in the protein N-glycosylation process. Our data suggest that in contrast to other genetic defects of glycosylation, inactivation of *TUSC3* causes nonsyndromic MR, a conclusion that is supported by a separate report in this issue of *AJHG*. *TUSC3* is only the fifth gene implicated in NS-ARMR and the first for which mutations have been reported in more than one family.

With a prevalence of about 2%, mental retardation (MR) is one of the most important unsolved problems in health care. X-linked MR (XLMR) has been the subject of extensive research during the past decade, and more than 60 XLMR genes have been identified so far (for review, see Ropers 2006[1]). In contrast, very little is known about the role of autosomal genes, even though there is evidence that autosomal-recessive forms of MR (ARMR) are far more common than X-linked ones. To date, only four genes have been implicated in nonsyndromic ARMR (NS-ARMR). These are *PRSS12* (neurotrypsin; MIM 606709),[2] *CRBN* (cereblon; MIM 609262),[3] *CC2D1A* (MIM 610055),[4] and *GRIK2* (glutamate receptor 6; MIM 138244).[5] Neurotrypsin is a trypsin-like serine protease, and cereblon is an ATP-dependent Lon protease, whereas CC2D1A (Freud-1) is a putative signal transducer participating in the positive regulation of the I-κB kinase/NF-κB cascade, which plays a role in neurotrophin-regulated signaling pathways that control many aspects of survival, development, and function of neurons (for review see Reichardt 2006[6]). *GRIK2* encodes a kainate receptor subunit involved in synaptic transmission. The functional diversity of these four genes reflects the extreme genetic heterogeneity of NS-ARMR, which we recently established in a study involving 78 consanguineous Iranian families of varying size.[7]

In the meantime, follow-up studies focusing on large to very large consanguineous families have yielded numerous additional loci for syndromic and nonsyndromic ARMR (unpublished data). Here we describe how in one of these families, autozygosity mapping has led to the identification of a deletion in *TUSC3* (*N33*; MIM 601385), a gene that is assumed to encode a subunit of the ER-bound oligosaccharyltransferase (OST) complex that catalyzes a pivotal step in the protein N-glycosylation process.[8]

The pedigree and facial aspects of the patients are shown in Figure 1. The degree of mental retardation in the affected family members ranged from moderate to severe (Table 1). None of the patients showed any other neurological problems, congenital malformations, or facial dysmorphisms. Head circumferences, body heights, and weights were normal (Table 1). For two of the patients (IV:5 and IV:7), we performed an MRI scan, which revealed no morphological abnormalities. Our study was approved by the appropriate review boards in both Germany and Iran. Sample collection and clinical evaluation were carried out as previously described,[7] with the informed, written consent of the parents. Individuals III:1, III:2, III:9, III:10, IV:1–IV:3, IV:5, IV:7, IV:9, IV:13, and IV:14 were genotyped with the Human Mapping 250K (Nsp) Array (Affymetrix) following the protocol of the manufacturer. To reduce the noise and to simplify the analysis, we ranked the markers according to the quality of the array hybridization results, by using the GeneChip Genotyping Analysis Software package (GTYPE 4.1, Affymetrix). Linkage analysis was performed with ~50,000 selected markers yielding the highest-quality scores per family.

To generate appropriate input files for the linkage-analysis programs Merlin[9] and Allegro,[10] we applied the

[1]Max Planck Institute for Molecular Genetics, D-14195 Berlin, Germany; [2]Genetics Research Center, University of Social Welfare and Rehabilitation Sciences, Tehran 19834, Iran; [3]Kariminejad-Najmabadi Pathology & Genetics Center, Tehran 14667, Iran; [4]Medical Genetics Counselling Centre, Hamedan Welfare Organization, Hamedan 6516815353, Iran
*Correspondence: kuss_a@molgen.mpg.de
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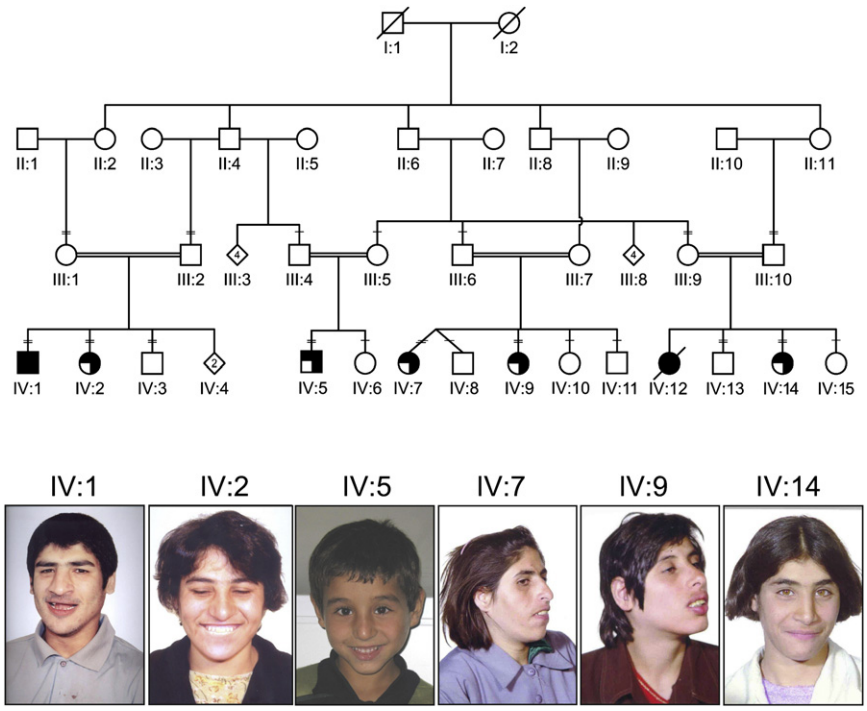

Figure 1. Pedigree and Facial Aspects of Affected Family Members
Filled symbols indicate severe MR, and three-quarter-filled symbols depict moderate MR.

ALOHOMORA software[11] with subsets of 300–700 markers in a sliding-window mode based on mapping information from DeCode. Allele frequencies pertaining to the Caucasian population were used. As a first level of quality control, we checked the gender of sampled individuals by counting the heterozygous single-nucleotide polymorphisms (SNPs) on the X chromosome and comparing the results with the pedigree file information, with the gender-check tool of the ALOHOMORA software. In addition, we employed the Chromosome Copy Number Analysis Tool 4.0 (CNAT 4.0, Affymetrix) for determining the copy number of X chromosomal markers. To verify the relationships between individuals within families, we checked the data with the graphical representation of relationships errors software (GRR).[12] Mendelian inconsistencies and unlikely genotypes were detected by the PedCheck[13] and Merlin[9] programs, respectively, and excluded from genotyping data prior to linkage analysis. Assuming autosomal-recessive inheritance with complete penetrance, we then carried out parametric linkage analysis based on ~50,000 markers with high-quality genotyping scores. This analysis yielded a LOD score of 6.26 for a 4.6 Mbp segment on chromosome 8p22 that contained 14 genes (Figure 2).

Table 1. Clinical Features

Patient	Sex	Age at Examination	Mental Retardation (IQ)	Height	OFC
IV:1	M	22 years	Severe (20–30)	162 cm	54 cm
IV:2	F	21 years	Moderate (35–40)	147 cm	54.5 cm
IV:5	M	8 years	Moderate (40–49)	119 cm	50 cm
IV:7	F	29 years	Moderate (30–40)	151 cm	55 cm
IV:9	F	26 years	Moderate (35–40)	149 cm	51.5 cm
IV:14	F	17 years	Moderate (40–49)	156 cm	54.5 cm

OFC denotes occipitofrontal circumference.

In parallel, we performed DNA copy-number analysis for the complete set of SNP markers by using two different software tools (Copy Number Analyzer for Affymetrix GeneChip [CNAG2.0][14] and the CNAT 4.0 tool, Affymetrix) with 40 Iranian controls as reference panel. In this way, we identified 16 adjacent markers within the linkage interval that were not called in the patients (Figures 3A and 3B), indicative of a homozygous deletion of approximately 120–150 Kbp and including the first exon of the *TUSC3* gene. By PCR amplification (forward primer 5′-TTGGGTAC ACCTCCCAGATG-3′; reverse primer: 5′-ATCCCAACCCAT CATGTCAC-3′) and sequencing of the junction fragment, we could define the exact borders of the deletion (Figure 4A) and show that 121,595 bp (between positions 15347852 and 15469447, NCBI genome build 36.1) were homozygously deleted in all patients. Heterozygous carriers were identified by PCR amplification of the junction fragment and a PCR product specific for the normal allele (forward primer: 5′-TACTTGTGAAAATAACCTGCCATT-3′; reverse primer: 5′-TCTCACCAAAATGGTCCACA-3′). All parents of patients turned out to be heterozygous for this deletion (Figure 4B), but we did not find any homozygous or heterozygous deletion carriers among 192 unrelated healthy Iranian individuals screened as controls.

To exclude other potentially disease-causing mutations, we then sequenced the exons and exon-intron boundaries of all genes in the relevant linkage interval. Apart from two known SNPs and one silent nucleotide exchange, no further sequence alterations were found (data not shown). Thus, the deletion affecting *TUSC3* was the only functionally relevant change observed in this interval.

To check for *TUSC3* expression, we extracted total RNA from Epstein-Barr Virus (EBV)-transformed lymphoblastoid cell lines (LCLs) of a patient and two controls by using the TRIzol reagent (Invitrogen) and generated cDNA by using the SuperScript III Reverse Transcriptase (Invitrogen) together with random hexamers. This cDNA was used to perform PCRs with a series of primer combinations for one or several adjacent exon sequences and together encompassing the entire gene. All predicted PCR products were found to be present in the controls but not in the

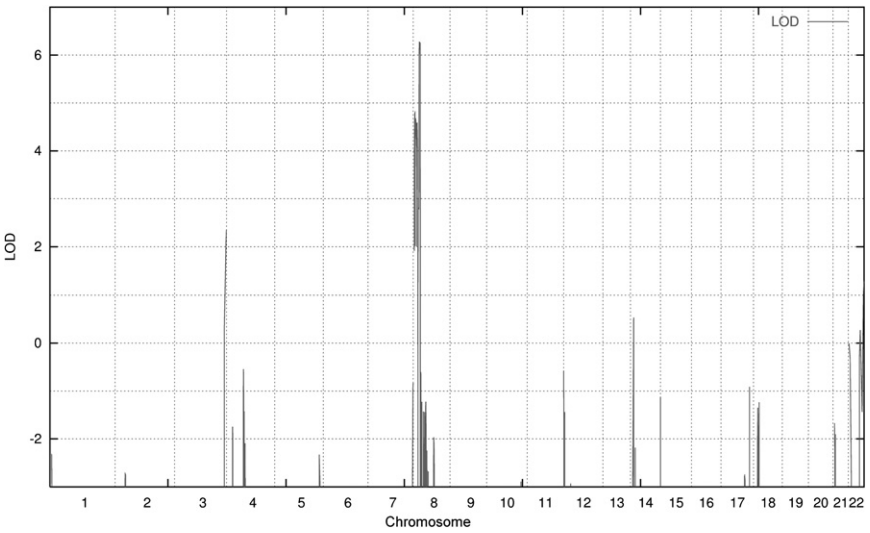

Figure 2. Results of Parametric Linkage Analysis with Merlin Software
A single significant peak (LOD score 6.26) was observed on chromosome 8p22 between rs613566 and rs11203893.

patient, proving the complete absence of a *TUSC3* transcript in homozygous deletion carriers (Figure 4C). This result was substantiated by quantitative PCR, with blood-derived cDNA from four healthy individuals as well as four patients (not shown). We therefore conclude that the deletion causes a complete loss of TUSC3 function, and that this is responsible for the observed MR phenotype.

It is of note that copy-number variation involving *TUSC3* has been described in a parent and child from the HapMap project,[15–18] but this change involved only one of the two alleles. Still, this observation prompted us to re-examine array comparative genomic hybridization (CGH) results from more than 700 unrelated individuals previously analyzed with whole-genome tiling-path BAC arrays in our laboratory. We identified two heterozygous duplications in the vicinity of *TUSC3* that did however not include the gene itself (Reinhard Ullmann, personal communication).

TUSC3 is believed to be the ortholog of the yeast *Ost3* protein that was initially identified as a 34 kD subunit in the yeast oligosaccharyltransferase (OST) complex[8,19] (for a review on eukaryotic OST see, e.g., Kelleher and Gilmore 2006[20]). It is expressed in a wide range of human tissues, including the brain. *TUSC3* has 11 exons spanning ~224 Kbp of the genomic DNA on chromosome 8p22. According to the UniProtKB database, *TUSC3* encodes a predicted 348 amino acid protein with five potential transmembrane domains (Figure 5) and seems to be involved in catalyzing the transfer of a 14-sugar oligosaccharide from dolichol to nascent protein. This reaction is the central step in the N-linked protein glycosylation pathway. Unlike other patients with congenital disorders of glycosylation (CDG), which are characterized by ataxia, seizures, retinopathy, liver fibrosis, coagulopathies, dysmorphic features, and ocular abnormalities,[21] our patients only present with nonsyndromic mental retardation. An explanation for the conspicuous absence of additional symptoms in our patients may be the presence of a closely related gene on Xq21.1, which encodes the implantation-associated protein precursor (IAP or MAGT1). MAGT1 is also assumed to be involved in N-glycosylation through its association with N-oligosaccharyl transferase.[22] It might thus be able to partly compensate for the loss of TUSC3, probably in a tissue-specific manner. Our finding that affected individuals show no aberrant glycosylation of serum transferrin (determined by isoelectric focusing analysis; data not shown) is in keeping with this speculation.

Our observation that loss of function of TUSC3 gives rise to nonsyndromic MR is supported by a separate study, appearing in this issue of *AJHG*, which reports on a small French family with two mentally retarded sibs that carry a homozygous frameshift mutation in the *TUSC3* gene.[23] However, neither this report nor ours has provided conclusive evidence for the assumption that TUSC3 plays a role in protein glycosylation, which is solely based on its 20% sequence similarity with the yeast *Ost3* gene.[8] Indeed, the normal glycosylation patterns seen in serum of TUSC3-deficient patients may argue against a central role of this protein in the glycosylation process. Similarly, the fact that none of these patients has a history of cancer casts doubt on the original assumption that TUSC3 acts as tumor suppressor.[8,24] As to the role of this gene in the brain, it is noteworthy that TUSC3 interacts with the alpha isoform of the catalytic subunit of protein phosphatase 1 (PPPC1A; MIM 176875).[25] Protein phosphatase 1 has been implicated in the modulation of synaptic and structural plasticity (for review see Munton et al. 2004[26]) and was shown to have an impact on learning and memory in mice.[27] It is therefore conceivable that MR in TUSC3-deficient patients is caused by an impairment of PPPC1A function. This opens up interesting perspectives for future studies into the function of TUSC3.

In summary, our study, as well as the report by Molinari et al.,[23] indicate that mutations leading to loss of function of *TUSC3* give rise to NS-MR. Thus, *TUSC3* is the first gene for NS-ARMR for which mutations have been observed in more than a single family. Moreover, it is only the fifth gene that has been implicated in NS-ARMR so far. It remains to be seen whether TUSC3 has a direct role in glycosylation and why in patients with TUSC3 deficiency clinical signs are confined to the brain.

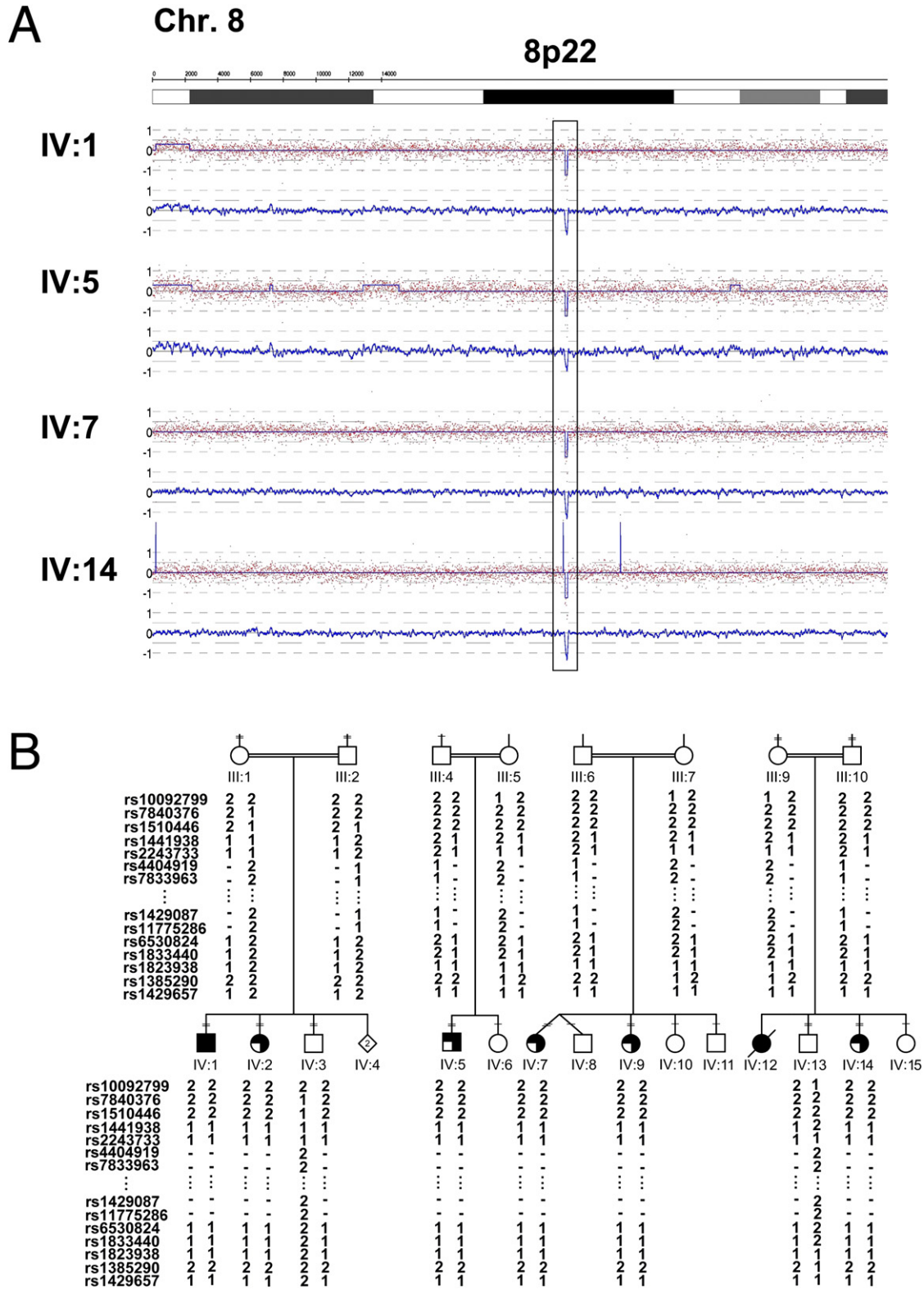

Figure 3. Copy-Number Analysis and Haplotyping of MR Patients

(A) Results of nonpaired DNA copy-number analysis (CNAG2 tool for copy-number variations): Copy-number state (upper) and log2 ratios (lower) for Nsp-array SNP markers located inside the first ~30 Mb of chromosome 8 are displayed for one individual per branch, showing a 120 Kbp homozygous deletion of 8p22 comprising the first exon of *TUSC3*.

(B) The markers bordering the deletion as well as the first and last two deleted markers are shown, revealing that all the affected members are homozygous for the same haplotype, whereas parents and healthy sibs are heterozygous carriers.

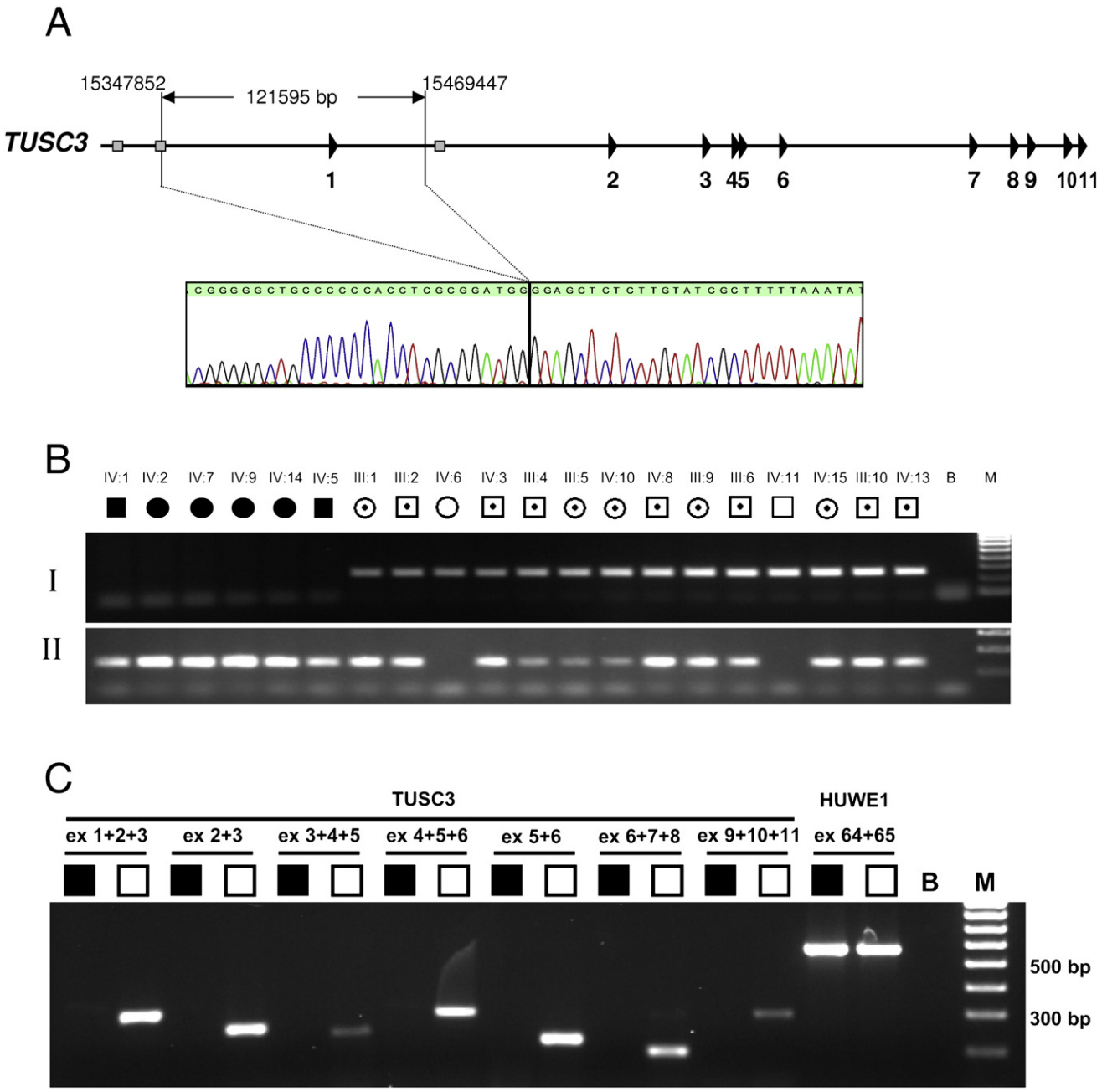

Figure 4. Deletion Encompassing the First Exon of *TUSC3* in MR Patients

(A) Schematic representation of *TUSC3*: Arrowheads represent exons, and gray boxes mark the positions of simple tandem repeats that could be causatively involved in the genesis of the deletion. The positions of the borders of the 121,595 bp deletion (based on NCBI Build 36.1) are indicated. The sequence chromatogram shows part of the PCR amplicon covering the junction of the deletion borders.

(B) Cosegregation analysis by PCR: Results of a deletion-specific PCR (I) and a deletion-spanning PCR (II) are shown for all the available family members. All homozygous carriers show only amplification of the junction fragment (II), heterozygous carriers show both amplicons, and noncarriers show only amplicon I.

(C) RT-PCR results from an experiment with cDNA derived from RNA preparations of one patient (IV:5) and one control lymphoblastoid cell line sample. With a sequence of primer pairs specific for amplicons covering two to three consecutive exons each, the complete *TUSC3* transcript was detected in the control but could not be amplified from patient cDNA. The results of an agarose gel electrophoresis of 5 μl from a 25 μl RT-PCR reaction (primers and conditions are available upon request) are shown. Patient and control products for a specific amplicon (the exons covered by each amplicon are indicated) were loaded on neighboring lanes in ascending order of the amplified exons. As positive control, a PCR specific for the X-chromosomal *HUWE1* gene (MIM 300697) was performed (lane 15 and 16). Filled squares represent the patient and open squares the control, "B" marks the lane loaded with the negative control, and "M" indicates the marker lane (HyperLadder IV, Bioline).

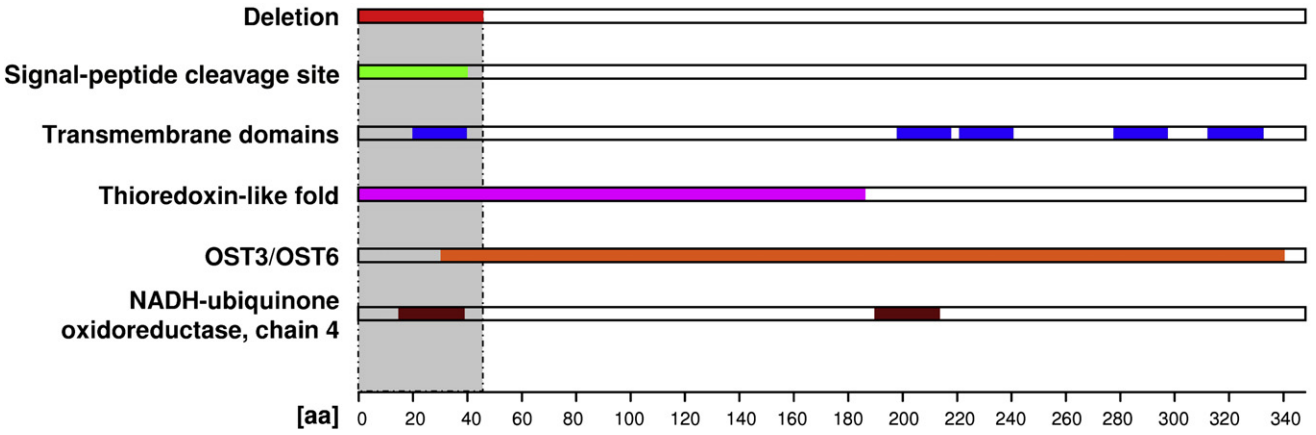

Figure 5. Schematic Representations of Predicted Functional Domains in the *TUSC3* Gene Product
The 348 amino acid TUSC3 protein is shown as an open box. Different functional domains are indicated. Differently colored shading marks their extent and position within the protein. The deletion encompasses the first 46 amino acids and is indicated by gray shading.

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Web Resources

The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

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