UBE2A, Which Encodes a Ubiquitin-Conjugating Enzyme, Is Mutated in a Novel X-Linked Mental Retardation Syndrome

Rafaella M. P. Nascimento, Paulo A. Otto, Arjan P. M. de Brouwer, and Angela M. Vianna-Morgante

We report a mutation of *UBE2A/HR6A,* which encodes a ubiquitin-conjugating enzyme (E2), a member of the ubiquitin proteasome pathway, as the cause of a novel X-linked mental retardation (XLMR) syndrome that affects three males in a two-generation family. A single-nucleotide substitution, c.382C→T in *UBE2A,* led to a premature UAG stop codon (Q128X). As a consequence, the predicted polypeptide lacks the 25 C-terminal amino acid residues. The importance of this terminal sequence for UBE2 function is inferred by its conservation in vertebrates and in *Drosophila*. *UBE2A* mutations do not appear to significantly contribute to XLMR, since no *UBE2A* mutations were identified in 15 families with nonsyndromic and 4 families with syndromic idiopathic XLMR previously mapped to intervals encompassing this gene. This is the first description of a mutation in a ubiquitin-conjugating enzyme gene as the cause of a human disease.

Monogenic X-linked mental retardation (XLMR) has been estimated to affect ~10% of mentally retarded males.[1,2] Mutations in 59 genes on the X chromosome have been implicated in familial mental retardation (Greenwood Genetic Center), and they represent about one-third of the X-linked genes demonstrated to be mutated in human monogenic diseases.[3] With the identification of genes involved in XLMR, a picture emerges indicating that some genes are mutated in both syndromic and nonsyndromic mental retardation.[4] However, mutations in such genes account for only a small proportion of XLMR-affected families and males with sporadic mental retardation.[1] The *FMR1* gene, mutated in the fragile X syndrome, is the most noticeable exception, with a prevalence of 2%–2.5% in cohorts of mentally retarded males[5] and affecting roughly one-quarter of XLMR-affected families.[6] Therefore, many genes involved in XLMR still await identification.

Here, we report a nonsense mutation in *UBE2A,* which encodes a ubiquitin-conjugating enzyme (E2) in the proteasome pathway of protein degradation, as the cause of a novel XLMR syndrome. Ubiquitination of proteins and their degradation constitute a major mechanism in the regulation of protein levels in mammalian cells. In addition, ubiquitination is recognized to have pleiotropic functions in the regulation of various cellular processes, such as control of transcription factor activity,[7] receptor internalization,[8] and histone modifications, which modulate chromatin structure.[9]

The described family includes three mentally retarded males in two generations, related through their clinically normal mothers (fig. 1 and table 1). Informed consent was obtained from every participating individual or from his or her guardian(s), and the study was approved by The Ethics Committee on Research on Human Subjects of the Institute of Biosciences, University of São Paulo, São Paulo. Physical examination was performed on the three affected males. The family provided information about the patients (i.e., pregnancy and condition at birth, developmental milestones, intellectual and adaptive functioning) and made medical records available. The patients' mothers were clinically unaffected and did not show any overt intellectual or adaptive impairment; I-2 is a housewife, II-2 is the head of a school for mentally impaired children, and II-4 is a nutritionist. At age 46 years and 9 mo, II-3 developed acute myeloid leukemia. Chromosome studies of cultured blood lymphocytes—prometaphase G-banding of individuals II-3, III-2, and III-3 and in situ hybridization of subtelomeric probes (Chromoprobe Multiprobe-T System [Cytocell]) of individual II-3—did not reveal any alterations. The result of molecular testing of patient III-2 for fragile X syndrome was negative.

On the basis of the family pedigree, we assumed an X-linked pattern of inheritance for this previously undescribed mental retardation syndrome (fig. 1). This assumption was further strengthened by our finding that the presumptive obligate carriers had completely skewed X inactivation in leukocytes, as demonstrated by the methylation status of the CAG repeat of the androgen receptor gene[10] (data not shown). Indeed, skewed X-chromosome inactivation appears to be characteristic of carriers of many gene mutations involved in XLMR.[11] These observations prompted us to search for the X-linked gene involved in the syndrome. Given the small size of the family, an exclusion-mapping strategy was performed. Using DNA extracted from peripheral blood leukocytes, we genotyped 46 microsatellite loci throughout the X chromosome (fig. 2), to locate regions of common descent in the three patients. In the initial mapping, 18 microsatellite loci ~10 cM apart (ABI PRISM Linkage Mapping Set MD-10 [Applied Biosystems]) were amplified by PCR with fluorescent-

From the Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, São Paulo (R.M.P.N.; P.A.O. A.M.V.-M.); and Department of Human Genetics, Radboud University Medical Centre, Nijmegen, The Netherlands (A.P.M.d.B.)

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Address for correspondence and reprints: Dr. Angela M. Vianna-Morgante, Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, Caixa Postal 11461, 05422-970 São Paulo, SP, Brazil. E-mail: avmorgan@ib.usp.br

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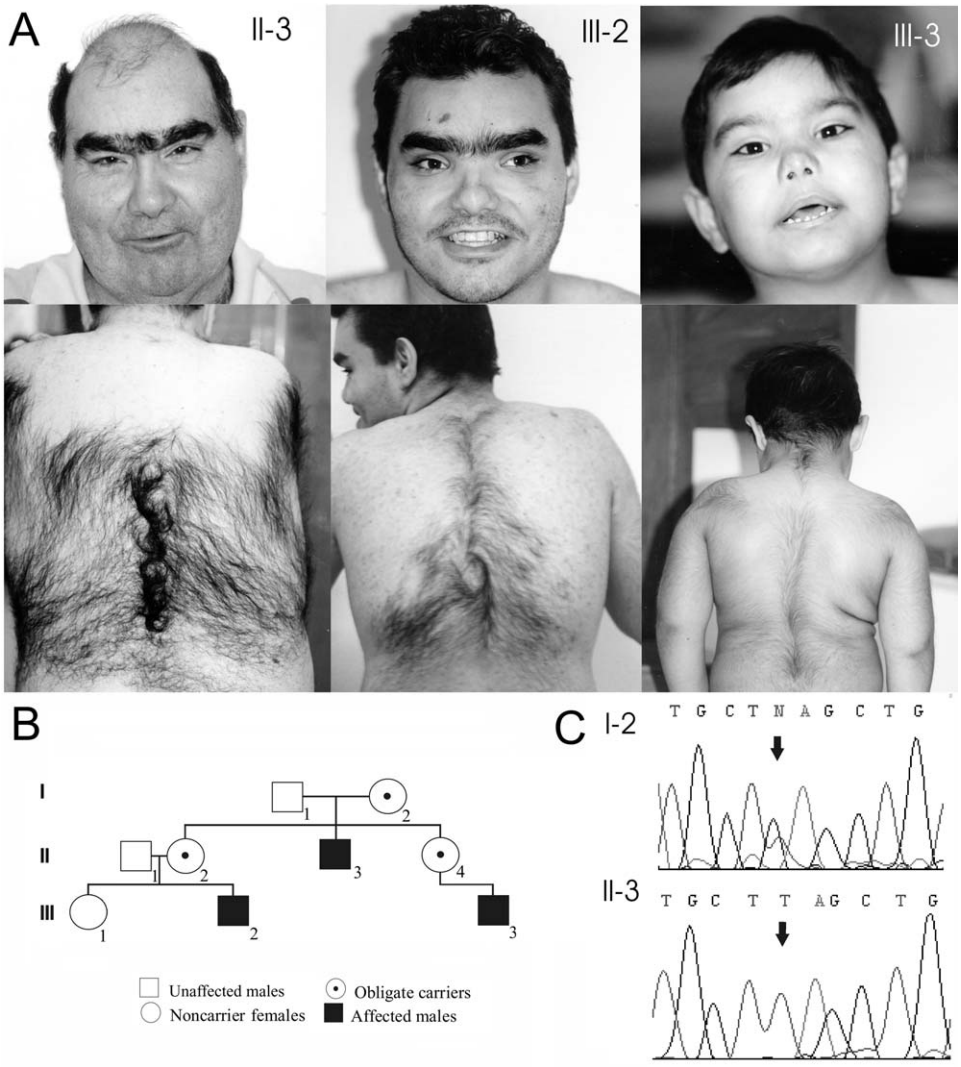

Figure 1. *A,* The three affected males, II-3 (aged 46 years and 7 mo), III-2 (aged 19 years and 11 mo), and III-3 (aged 5 years and 4 mo). *B,* Two-generation genealogy of the study family, showing affected males related through their clinically normal mothers. *C,* Chromatograms of the sense-strand sequence from I-2 (obligate carrier) and II-3 (affected male). A c.382C→T mutation in *UBE2A* exon 6 was identified as the cause of the syndrome.

labeled primers, and the amplified fragments were analyzed on a MegaBACE 1000 automated sequencer with the MegaBACE Genetic Profiler software (Amersham Bioscience–GE Healthcare). The other 28 markers were selected from the National Center for Biotechnology Information (NCBI) database, with regard to their location on the X chromosome and level of heterozygosity; PCR was performed according to standard conditions, and, after electrophoresis on 6% denaturing polyacrylamide gels, the amplified products were visualized by silver staining. Genotyping of 36 markers spaced at ~5 cM disclosed three loci, at Xq23-25, harboring alleles shared by all three affected males, but their inheritance from a common ancestor could not be determined. A further 10 loci 1 Mb apart within the defined Xq23-q25 region were analyzed. An ~15-Mb segment could be delimited by the excluded markers *DXS8088* (Xq23) and *DXS1047* (Xq26.1); all alleles within this defined region were shared by the affected males, and alleles at *DXS8053, DXS8081,* and *DXS8057* were proven to be identical by descent (fig. 2). This candidate segment contained 86 known genes (NCBI); three of them had been previously associated with mental re-

The figure is available in its entirety in the online edition of *The American Journal of Human Genetics.*

Figure 2. Pedigree of the affected males and genotypes of the 46 analyzed markers, from Xpter to Xqter. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Table 1. Clinical Findings for the Mentally Retarded Patients

Characteristic	Patient		
	II-3	III-2	III-3
Age at examination	46 years and 7 mo	19 years and 11 mo	5 years and 4 mo
Birth weight (percentile)	50th	90th–97th	> 97th
Height (percentile)	<3rd	10th–25th	10th
Weight (percentile)	>97th	90th	>97th
Head circumference (percentile)	>98th	>98th	50th
Hair whorls	+	+	+
Wide face	−	+	+
Midface hypoplasia	−	+	+
Synophris	+	+	+
Up-slanted palpebral fissures	+	+	+
Ocular hypertelorism	−	+	−
Low nasal bridge	−	+	+
Large mouth with down-turned corners and thin lips	+	+	+
Short, broad neck	+	+	+
Low posterior hairline	+	+	+
Widely spaced nipples	+	+	+
Small penis	+	+	+
Small, flat feet, with dorsum swelling	+	+	+
Onychodystrophy[a]	+	+	−
Marked generalized hirsutism	+	+	+
Myxedematous appearance	+	+	+
Dry skin	+	+	+
Seizures	+	+	+
Severe speech impairment	+	+[b]	+[b]
White matter hypodensity[c]	Not examined	+	+

[a] After puberty.
[b] Absent speech.
[c] Determined by magnetic resonance imaging.

tardation: *LAMP2* (lysosomal associated membrane protein 2), mutated in patients with multisystem glycogen storage disease—Danon disease (MIM 300257), an X-linked dominant disorder affecting predominantly cardiac and skeletal muscles—and also found to be mutated in primary cardiomyopathy[12,13]; *GRIA3* (glutamate receptor, ionotrophic, AMP 3), interrupted in a female carrier of a balanced chromosomal translocation t(X;12)(q24;q15) who presented with bipolar disorder and mental retardation[14]; and *AGTR2* (angiotensin II receptor, type 2), disrupted in a balanced translocation t(X;7)(q24;q22) in a female with moderate mental retardation[15] and also mutated in males with variable mental retardation.[16] On the basis of the clinical features associated with these mutations and those present in our patients, we considered *AGTR2* the best candidate gene. Mutation screening was performed by direct sequencing on a MegaBACE 1000. The *AGTR2*-only coding exon was amplified with primers described elsewhere,[16] and no mutations were found. We then performed a candidate-gene search (Genatlas) and found 30 genes within the candidate interval that were expressed in brain. However, only one of those genes, *UBE2A/HR6A,* was expressed in both brain and lymphocytes. Because of the skewed X-inactivation in the patients' mothers, which likely represents the survival/proliferation advantage of lymphocytes with the active normal allele in a woman with mutation, we sequenced this gene directly. The six coding exons of *UBE2A* were amplified, with flanking intronic primer pairs (table 2) that we designed using Primer3 software,[17] and were sequenced. A c.382C→T substitution leading to a premature UAG stop codon (Q128X) was detected in all three affected males and in their mothers (fig. 1). The sister of one of the affected males (III-1, with a rather random X-inactivation pattern, as documented by the methylation status of the *AR* gene [data not shown]) did not carry this mutation.

We then screened for *UBE2A* mutations in 19 affected

Table 2. Primer Pairs Designed to Amplify the Coding Sequence of the *UBE2A* Gene, with Use of Primer3 Software

Exon	Primer Sequence (5′→3′)
UBE2A-1F	cgtggggctttaatgacata
UBE2A-1R	aaccttcgggaagacagaca
UBE2A-2F	catgcgggacttcaagaggt
UBE2A-2R	ccaaacattttcccctaccc
UBE2A-3F	ccgggacatccatttgtagt
UBE2A-3R	cagaggcaggttcctaagca
UBE2A-4F	cctctctaccctgtatctttgcat
UBE2A-4R	ggcaccacaaaatacacagga
UBE2A-5F	tgggaagcaacataggaatctt
UBE2A-5R	aggtgtgagcgactgtaccc
UBE2A- 6F	tgttttgcattaaggaactgaca
UBE2A-6R	gggaggtgacaaacacatca

males from XLMR-affected families that were collected by the Euro-MRX Consortium, previously mapped to intervals encompassing this gene. Four families had syndromic and 15 families had nonsyndromic idiopathic XLMR. The phenotypes, linkage intervals, and maximum LOD scores of the Euro-MRX families are summarized in table 3. No mutations were detected in these patients.

UBE2A/HR6A is a ubiquitination pathway gene that encodes E2. The E2 conjugases, in conjunction with ligating enzymes (E3), mediate the attachment of ubiquitin molecules to proteins, thus targeting them for degradation by the proteasome complex. *UBE2A* is one of the two human orthologues of the *Sacharomyces cerevisiae RAD6/UBC2* gene. In humans and other mammals, the gene is duplicated with one X-linked (*UBE2A*) copy and one autosomal (*UBE2B*) copy.[23] The coding regions of human *UBE2A* and *UBE2B* paralogues share 80% identity and produce proteins with 96% amino acid identity and a seven-residue difference in their 152 amino acids. The *UBE2A* mutation in our patients introduces a premature stop codon and abolishes the 25 C-terminal amino acids of the protein. The great importance of this sequence for UBE2 function can be inferred from its high conservation in both vertebrates and *Drosophila* (fig. 3).

The high conservation of UBE2A and UBE2B amino acid sequences raises the question of function specificity or redundancy of these proteins, which are ubiquitously expressed, although the ratios between these proteins vary significantly in different cells and tissues.[24] The fact that double *Ube2a/Ube2b* (*hr6a/hr6b*) knockout mice are not viable indicates that these genes are crucial for development, and viability was demonstrated to depend on the presence of at least one functional allele, by the construction of different knockout mice.[25] However, the *Ube2a*- and *Ube2b*-knockout mice differ at least in reproductive performance. Male-limited sterility is exhibited by *Ube2b*-knockout[26] but not *Ube2a*-knockout[25] mice. In contrast, whereas *Ube2b*-knockout females are fertile,[26] *Ube2a*-knockout females fail to produce offspring in spite of normal ovulation; the absence of the UBE2A/HR6A protein in oocytes prevents embryonic development beyond the two-cell stage.[25] Since UBE2B protein levels, compared with those of UBE2A,[24] are high in spermatids of wild animals and the opposite is observed in oocytes,[25] the infertility phenotypes might result from a dose-dependent effect in the germ cells. However, other observations point to different functional properties of UBE2A and UBE2B. The polyubiquitination of the cyclophilin CYC4/hCyP-60 requires UBE2B (but not UBE2A),[27] and UBE2A (not UBE2B) was found to interact with Rfp14 (ret finger protein-like 4) in a yeast two-hybrid screen.[28]

The affected males present a neurodevelopmental disorder. A number of studies have addressed the function of ubiquitination during neuronal development. Nerve growth factor (NGF)–induced neurite outgrowth from rat pheochromocytoma cells (PC12) is concurrent with increased levels of ubiquitin-protein conjugates and coincides with up-regulated activities of ubiquitin-conjugating enzymes but not with enhanced ubiquitin-dependent proteolysis; neurite outgrowth is accelerated by blocking ubiquitin-dependent proteolysis, and such outgrowth is inhibited by a dipeptide inhibitor of E3-dependent ubiquitination. These data imply that ubiquitination and ubiquitin-dependent proteolysis are positive and negative regulators of neurite outgrowth, respectively.[29] Down-regulation of *UBE2B* mRNA in PC12 cells leads to a reduction

Table 3. Families from the Euro-MRX Consortium Screened for Mutations in *UBE2A*

Family	Phenotype[a] (MRX Number)	Maximum LOD Score	Flanking Markers	Reference
D004	MRX	1.20	*DXS993* and *DXS8043*	...
L022	MRX (MRX35)	2.41	*DXS178* and *HPRT*	Gu et al.[18]
L025	MRX	1.50	*DXS424* and Xqter	Claes et al.[19]
L037	MRX (MRX70)	2.10	*DXS8063* and *DXS1047*	Claes et al.[20]
L048	MRX	1.30	*DXS991* and *DXS1047*	...
N005	MRX	1.03	*DXS424* and *DXS292*	...
N043	MRX	1.14	*DXS8076* and *DXS1108*	...
N108	MRX	1.51	*DXS1169* and *DXS8067*	...
P004	MRX	.68	*DXS1217* and *DXS1062*	...
T011	MRX (MRX61)	3.51	*DXS135* and *DXS737*	...
T013	MRX (MRX62)	2.23	*DXS458* and *DXS737*	Raynaud et al.[21]
T014	MRX	1.20	*MAOB* and *DXS425*	Raynaud et al.[21]
T025	MRX	1.00	*DXS1214* and *DXS1212*	...
T048	MRX	.60	*DXS993* and *DXS737*	...
T052	MRX	2.20	*DXS990* and *DXS8057*	...
L056	MRXS (spastic paraplegia, macrocephaly, hypotonia, and developmental delay)	2.18	*DXS8054* and *DXS1001*	...
N032	MRXS (hypotonia, ataxia, and areflexia)	6.97	*DXS1231* and *DXS1001*	...
P014	MRXS (microcephaly, epilepsy, and developmental delay)	.60	*DXS986* and *DXS1047*	...
T019	MRXS (short stature, microcephaly, and facial dysmorphy)	2.96	*DXS178* and *DXS292*	Raynaud et al.[22]

NOTE.—All families have males with mental retardation in at least two generations. Obligate carrier females are not affected.

[a] MRX and MRXS = nonsyndromic and syndromic XLMR, respectively.

Figure 3. Alignment of UBE2 protein sequences, showing the high amino acid sequence identity between the human protein and those of mouse (100%), zebrafish (96%–100%), and *Drosophila* (85%–87%). The consensus ubiquitin-binding cysteine- and serine-phosphorylation sites are underlined. In the box, the highly conserved segment of the protein corresponds to the sequence abolished by the c.382C→T mutation (Q128X), which creates a premature stop codon. The identical amino acid sequence of UBE2A in the vertebrates is shown in bold. (Information is based on the NCBI database and GenBank.)

of NGF-induced neurite length, and pharmacological inhibition of ubiquitin-dependent protein degradation was shown to significantly reduce axonal length and branching of adult sensory neurons in vitro.[30] In *Drosophila,* synaptic development and function have been shown to be regulated by ubiquitin-dependent mechanisms.[31] Taken together, these data point to an important role of the ubiquitin proteasome pathway in neuronal differentiation.

A few other human disorders have been recognized to result from mutations in genes involved in ubiquitination and proteasome function.[32] Mutations in ligase genes from the ubiquitin enzymatic pathway were identified as causative for Angelman syndrome (MIM 105830) (*UBE3A*), recessive juvenile Parkinson disease (MIM 600116) (*PARK2*), autoimmune polyendocrinopathy syndrome type 1 (MIM 240300) (*AIRE*), and von Hippel-Lindau disease (MIM 193300) (*VHL*). To our knowledge, the *UBE2A* mutation described here is the first in a ubiquitin-conjugating enzyme gene to be associated with a human disease. As in the case of *UBE3A* mutations causing Angelman syndrome,[33,34] mutation of *UBE2A* leads to neurodevelopmental anomalies.

UBE2A mutations may be exclusive to the novel mental retardation syndrome described here or may also cause different clinical pictures, including nonsyndromic mental retardation, as reported for other genes involved in XLMR.[4] However, the failure to detect *UBE2A* mutations in 19 idiopathic XLMR-affected families mapped to intervals encompassing *UBE2A* suggests that mutations in this gene are not a common cause of XLMR, in keeping with most XLMR genes identified to date.

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Web Resources

The accession numbers and URLs for data presented herein are as follows:

Euro-MRX Consortium, http://www.euromrx.com/
Genatlas, http://www.genatlas.org/
GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *UBE2A* [accession number NP_003327], *UBE2B* [accession number NP_003328], *Ube2a* [accession numbers NP_062642 and NP_033484], *ube2a* [accession number NP_958430], *ube2b* [accession number NP_956013], and *UbcD6* [accession number NP_524130])
Greenwood Genetic Center, http://www.ggc.org/xlmr.htm (for the X-linked Mental Retardation Database [August 2005])
NCBI, http://www.ncbi.nlm.nih.gov/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for Danon disease, Angelman syndrome, recessive juvenile Parkinson disease, autoimmune polyendocrinopathy syndrome type 1, and von Hippel-Lindau disease)
Primer3, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

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