

## LETTER TO JMG

Rare polymorphic variants of the *AGTR2* gene in boys with non-specific mental retardation

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**M**ental retardation (MR) is a frequent cause of serious handicap in children and young adults. It is defined as an overall intelligence quotient (IQ) lower than 70 associated with functional deficits in adaptive behaviour (such as daily living skills, social skills, and communication), with an onset before 18 years.<sup>1,2</sup> Moderate to severe MR (IQ<50) is estimated to affect 0.4–0.8% of the population and the prevalence increases to 2% if mild MR (50<IQ<70) is included, although these estimates vary widely between different epidemiological studies.<sup>3</sup> The underlying causes of MR are extremely heterogeneous. They include non-genetic factors, which act prenatally or during early infancy and cause brain injury, as well as established genetic causes. The prevalence of X linked mental retardation (XLMR) has been estimated as 1.8/1000 males with a carrier frequency of 2.4/1000 females.<sup>3</sup> Historically, XLMR is classified as syndromic (MRXS) or as non-specific forms (MRX). Recently, absence of expression of the angiotensin II specific receptor (*AGTR2*) gene was found in a female patient with moderately severe mental retardation (IQ=44) and a balanced X;7 chromosome translocation.<sup>4</sup> Additionally, eight of 590 unrelated male patients with MR were found to have sequence changes in the *AGTR2* gene. A deletion of one thymine (T) within a string of eight Ts has been identified in a familial form and in a sporadic case with MR, and three different missense mutations have been found in seven sporadic patients with MR (G21V, R324Q, and I337V). Five of these patients had seizures, two showed autistic behaviour, and only one patient suffered from hypertension. Some patients were reported to have additional clinical findings, but these were not further specified.<sup>4</sup> These findings prompted us to analyse extensively the *AGTR2* gene in X linked MR families collected by the European XLMR Consortium and in sporadic cases of MR, in order to make progress in elucidating the aetiology of mental handicap.<sup>5</sup>

The human angiotensin II type 2 receptor is composed of three exons and spans at least 5 kb of genomic DNA. Exons 1 and 2 encode 5' untranslated mRNA sequence and exon 3 harbours the entire uninterrupted open reading frame of *AGTR2*.<sup>6</sup> This gene encodes a 363 amino acid protein, which is involved in several functions of nerve cells, including ionic fluxes, cell differentiation, and axonal regeneration, but also in apoptosis.<sup>7–11</sup>

The aim of this study was to test a large sample of mentally retarded subjects for mutations in the coding region of the *AGTR2* gene in order to evaluate its involvement in non-specific mental retardation and to assess the frequency of mutations associated with this condition.

**METHODS AND RESULTS**

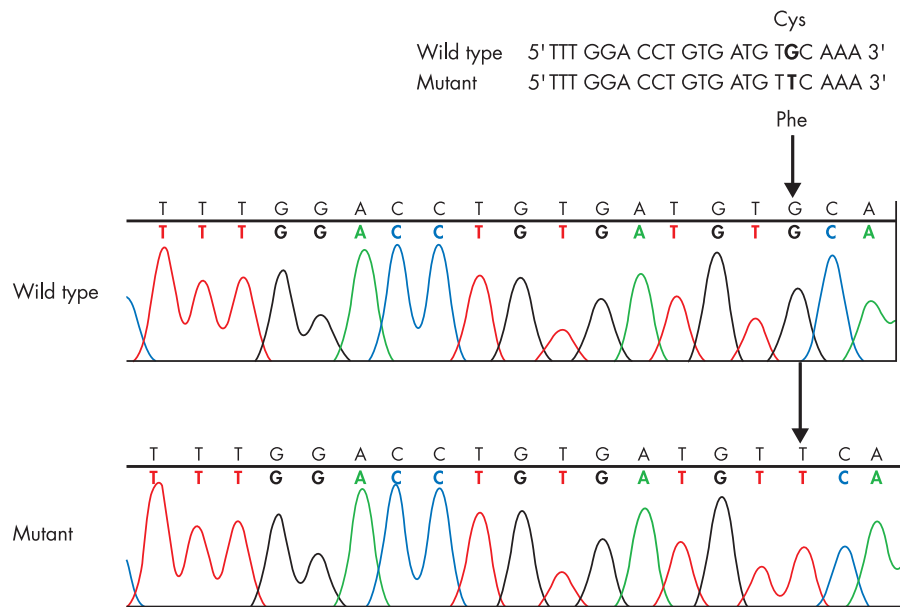
We analysed 15 large families with XLMR linked to Xq24 according to the recent version of the Euro-MRX database. Cognitive impairment is the only common feature between patients of these families. We also studied a panel of 101 clinically well characterised small families with at least two boys

**Key points**

- Mental retardation (MR) is a frequent cause of serious handicap in children and young adults. Recently, four different point mutations of the angiotensin II specific receptor (*AGTR2*) gene were found to be responsible for MR in eight out of 590 unrelated male patients.
- We performed mutation analysis of the *AGTR2* gene in 15 large families with MR linked to Xq24, in a panel of 101 clinically well characterised small families with at least two affected boys with MR, and in 244 sporadic cases of non-specific MR.
- Mutation analysis was performed using denaturing high pressure liquid chromatography (DHPLC). To limit false negative results, 20 familial cases were also screened for mutations by direct sequencing of the whole coding region of *AGTR2*.
- DHPLC analysis of the entire coding region detected no deleterious mutations in the 360 patients. A novel C117F amino acid substitution was identified as a non-pathogenic rare genetic variant.
- These observations suggest that *AGTR2* is rarely involved in non-specific MR but could be involved in more specific forms.

affected with MR and 244 sporadic cases of non-specific MR. All patients were of European origin. CGG expansion involved in fragile X syndrome, assessed by Southern blot analysis using DNA digested with *EcoRI/EagI* restriction enzymes and StB12-3 probe corresponding to the FRAXA locus, as well as cytogenetic abnormalities, were excluded.

Genomic DNA was extracted from peripheral blood lymphocytes according to standard protocols and was used to amplify the coding exon and the flanking sequences of the *AGTR2* gene. The coding exon of *AGTR2* was amplified in two overlapping fragments 1 and 2. The two PCR products were 468 and 724 bp, respectively. Primer sequences were: AGTR21F 5' gcaataagaactaggagctg 3'; AGTR21R 5' cagatttggtacatcaaac 3' and AGTR22F 5' ttatcactgcatgagtgtt 3'; and AGTR22R 5' agccatgttgattacatgca 3'. Reactions were performed in a volume of 50 µl containing 50 mmol/l Tris-HCl (pH 8.4), 1.5 mmol/l MgCl<sub>2</sub>, 200 mmol/l of all four deoxynucleotides, 0.5 mmol/l each of the primers, 2.5 units of *Taq* polymerase (Platinum, Invitrogen), and 200 ng template DNA. Forty cycles were then performed with denaturation for 30 seconds at 94°C, annealing for 30 seconds at 54°C, and elongation for 30 seconds at 72°C. The DNA synthesis step of the final cycle was extended to seven minutes. For DHPLC analysis, the amplified DNA was then heated at 94°C for seven minutes, and at 55°C for four minutes to favour formation of heteroduplexes. Mutation analysis of the MR patients was performed by using



**Figure 1** Fluorescence sequence analysis of exon 3 of the *AGTR2* gene. The arrow indicates the position of the C117F mutation.

denaturing high pressure liquid chromatography (DHPLC; Wave DNA fragment analysis system, DNASep column; Transgenomics). DHPLC conditions were chosen according to the Wavemaker program (Transgenomics, Santa Clara, CA, USA) (PCR product 1 59°C and 61°C; PCR product 2 59°C and 61°C). PCR products were subjected to chromatography using appropriate temperatures and acetonitrile gradients. PCR products were eluted with a linear acetonitrile gradient at a flow rate of 0.9 ml/minute, and those showing an abnormal DHPLC profile were directly sequenced on an automated sequencer (ABI 377, Perkin-Elmer) using the Dye Terminator method. To limit the false negative results, 20 familial cases were also screened for mutations by direct sequencing of two PCR products covering the whole coding region of *AGTR2*.

Both DHPLC and direct sequencing methods allowed the detection in two mentally retarded male patients of the R248K (G>A 742) mutation which was described previously as a polymorphism.<sup>4</sup> In addition, we also identified a novel G to T transversion in exon 3 of the *AGTR2* gene resulting in a C117F amino acid substitution in one patient (fig 1). This patient is an affected subject of a large four generation family with mental retardation, isolated growth hormone deficiency, and infantile behaviour but without other consistent phenotypic features. This family has already been described clinically by Hamel *et al.*<sup>12</sup> To determine if the novel missense mutation was the cause of mental retardation in this patient, his relatives were analysed. Ninety-nine subjects including seven affected patients were analysed for this variation. Although no unaffected subjects (including five males) had the sequence variation, two affected patients did not carry the C117F mutation.

## DISCUSSION

The lines of evidence supporting the pathogenic role of C117F are its low frequency, its absence in 100 normal chromosomes from normal male subjects, and its conserved position in the amino acid sequence of human, rat, mouse, *Oryzotolagus*, and *Meriones*. This amino acid at position 117 is located in the first extracellular loop of the *AGTR2* receptor which belongs to the seven transmembrane (TM) domain G protein coupled receptor subfamily. Surprisingly, by using BLAST programs, we identified the motif including the cysteine at position 117 in a family of human G protein coupled receptor related to the

growth hormone (GH) secretagogue and neurotensin receptors (GPR38 and GPR39).<sup>13</sup> In these GH secretagogue receptors, the cysteine is located in the TM3 domain. The overall conservation of critical amino acid residues in the TM domains of the puffer fish (puffer fish clone 78B7) and human receptors is remarkable given the 400 million years of evolution that separates these two species. TM3 appears to be essential for binding and activation of ligands. These TM3 residues are hypothesised to form part of the binding pocket and to make contact with the ligand.<sup>14</sup> These findings suggest that the cysteine at position 117 in the receptor plays an important role in the binding of the ligand(s) of *AGTR2*.

However, these features are not sufficient to define this amino acid change as the disease causing mutation. To determine if the novel missense mutation was the cause of mental retardation in this patient, his relatives were analysed. Although no unaffected subjects (including five males) had the sequence variation, two affected patients did not carry the C117F mutation, which rules out the involvement of *AGTR2* in the aetiology of the phenotype of the patient. Nevertheless, as an adult, one of these two patients with no C117F mutation was the tallest member of the family (1.59 m in height) and had the highest concentration of somatomedin C (10.3 nmol/l).<sup>12</sup> Therefore, we cannot completely rule out that this sequence variation modulates stature. However, recently an in frame duplication of 33 bp encoding for an additional tract of 11 alanines within the transcription factor *SOX3* was identified in all affected members of this family, suggesting that dysfunction of the *SOX3* protein is the primary cause of the mental retardation in this family.<sup>15</sup>

Our report clearly shows the importance of genotyping parental and grandparental DNA to avoid misleading interpretations. In addition, the ethnic origin of the patients should be considered in reporting nucleotide changes and a suitable control population should be investigated for the presence of the corresponding variation. Our findings highlight the need for extreme caution in the clinical interpretation of sequence variation in the *AGTR2* gene, especially when these are missense mutations that fall outside well characterised functional domains. Up to now, only three missense mutations have been described in males with mental retardation. The G21V mutation is located within the extracellular domain and the missense mutations R324Q and I337V are localised in the intracellular domain of the *AGTR2* protein.<sup>4</sup>

In contrast to the previous report that identified eight mutations out of 590 patients with mental retardation, no deleterious mutation was detected in our study.<sup>4</sup> This suggests that *AGTR2* is rarely involved in non-specific mental retardation but could be involved in specific forms which should be better delineated in the future.

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