A generalized deficiency in the mitochondrial enzyme, ornithine aminotransferase (OAT: EC 2.6.1.13), is the hallmark of gyrate atrophy (GA), a hereditary degenerative disease of the choroid and retina of the eye that leads to blindness. A human OAT cDNA, previously constructed and characterized in our laboratory, and anti-human OAT antibody were used as probes to examine the OAT gene, mRNA and protein of GA patients. A blot analysis of the genomic DNAs, RNAs and proteins of 14 GA patients identified a case with a partial heterozygous deletion of the functional OAT gene located on chromosome 10, no detectable OAT mRNA, and a barely detectable level of OAT antibody-reactive protein. The rest of the cases showed grossly normal OAT gene, mRNA, and variably reduced levels of OAT protein. A restriction fragment length polymorphism (RFLP) was identified in the functional OAT gene sequence with EcoRI which may be useful for prenatal diagnosis of GA. RFLPs were also identified in the OAT-related gene sequences located on the X chromosome with Hind III and Pst I which may potentially show linkage to X-linked retinitis pigmentosa locus. The finding of an OAT gene, mRNA, and protein defect in a GA case constitutes the first real demonstration of the molecular genetic defect of OAT in GA.

MATERIALS AND METHODS

SOUTHERN BLOT ANALYSIS

High-molecular-weight genomic DNA was isolated from the patient or normal human leukocytes or skin fibroblasts by the method of Blin and Stafford, digested with appropriate restriction enzyme (EcoRI, Hind III, Pst I; Bethesda Research Laboratories, Gaithersburg, MD), electrophoresed in 0.8% agarose gel, transferred onto nitrocellulose filter, hybridized with the 2.1 Kbp OAT cDNA labeled with 32P to approximately 108 cpm/µg by random priming, washed with 0.1 X SSC (15 mM NaCl, 1.5 mM Na Citrate) at 55°C, and autoradiographed as described before.

NORTHERN BLOT ANALYSIS

RNA was isolated from the patient or normal human fibroblasts by the guanidine thiocyanate
method,\textsuperscript{11} electrophoresed under denaturing condition in a formaldehyde gel,\textsuperscript{12} transferred onto nitrocellulose filter, hybridized with \textsuperscript{32}P-labeled human OAT cDNA probe or actin cDNA probe, and autoradiographed as described before.\textsuperscript{13} Actin-probing was performed to check the quality and quantity of RNA actually present on the blot for each lane.

Western Blot Analysis

Extracts containing the cellular proteins were obtained from the patient and normal human fibroblasts, electrophoresed in 10\% SDS-polyacrylamide gel, transferred onto nitrocellulose filter, reacted with the anti-human OAT rabbit antibody,\textsuperscript{5} and processed for immunodetection as described.\textsuperscript{14}

Results

High-molecular-weight genomic DNAs of leukocytes or skin fibroblasts from 14 GA patients and normal subjects were subjected to a southern analysis using a radioactively labeled OAT cDNA probe. The results for four representative cases and a control are shown in Figure 1. All 14 patients tested had a documented diagnosis of GA on the basis of chorioretinal degeneration, hyperornithinemia and zero to minimal OAT activity in their cells. Hybridization of the OAT probe to human genomic DNA digested with EcoRI, Hind III, or Pst I yields a complex pattern consisting of multiple fragments (at least 71 Kbp total) containing the OAT gene sequence (Figs. 1, 2). We have previously mapped the human OAT gene sequence to chromosomes 10 and X, and demonstrated that the OAT gene is most likely a gene family consisting of multiple members.\textsuperscript{15} The hybridization analysis of the OAT gene sequences in 14 GA patients identified a case containing a novel 5.4 Kbp EcoRI genomic fragment, not found in normal subjects or other GA patients (Fig. 1, Patient 35). The 5.4 Kbp fragment appears to be a truncated form of one of the two 6.2 Kbp allelic fragments containing functional OAT gene sequences\textsuperscript{15} since the latter shows a 50\% decrease in relative hybridization intensity. That this case represents a partial heterozygous deletion of the OAT gene was supported by the additional demonstration of a truncated form of a Hind III, Bgl II, Sph I, and Msp I functional OAT gene fragment in this patient (data not shown) and the failure to detect

Fig. 1. Southern blot analysis of genomic DNA from GA patients. The hybridization patterns of EcoRI OAT genomic fragments are shown for four representative GA patients (35, 37, 39 and 43) and normal control (N). Arrow points to the novel 5.4 Kbp fragment in patient 35.

Fig. 2. Restriction fragment length polymorphisms of OAT gene sequences. For each enzyme digestion, (A) represents the restriction fragment pattern usually observed, and (B) represents a less common variant pattern containing a restriction fragment length polymorphism (RFLP). RFLPs with EcoRI (6.5 Kbp and 5.7 Kbp fragments), Pst I (5.5 Kbp and 5.7 Kbp fragments), and Hind III (14.3 Kbp and 12 Kbp fragments) are shown.
any of the altered forms in up to 30 subjects, making it unlikely for them to be restriction fragment length polymorphism (RFLP). The rest of the cases showed grossly normal patterns of OAT gene fragments (data not shown). RFLPs were observed with EcoRI (6.5 Kbp and 5.7 Kbp fragments), Hind III (14.3 Kbp and 12 Kbp fragments), and Pst I (5.5 Kbp and 5.7 Kbp fragments) in the OAT gene sequences (Fig. 2). The approximate frequency of occurrence of the less common variant in each of the RFLP sets was 13% for the 5.7 Kbp EcoRI fragment, 5% for the 12 Kbp Hind III fragment, and 13% for the 5.7 Kbp Pst I fragment among subjects. The EcoRI polymorphism is present in the putative functional OAT gene sequence located on chromosome 10 whereas the Hind III and Pst I polymorphisms are present in the OAT-related gene sequences located on the X chromosome, as judged by the previous chromosomal mapping of these gene sequences.15

Skin fibroblasts were obtained from the GA patients, and mRNAs were isolated and subjected to a northern analysis. The results for five representative cases and two controls are shown in Figure 3. The OAT activity in these fibroblasts ranged from 0-5% of normal control. Patient 35 is the case with the putative partial heterozygous deletion of the OAT gene described above. Patient 36 is a B6-responder in that administration of high dose of vitamin B6, a coenzyme required for OAT, resulted in a lowering of the serum ornithine level and improvement in ERG response.16 The rest of the cases shown are B6-nonresponders.16 The northern blot analysis of the fibroblast RNAs with the OAT probe indicated the presence of OAT mRNA, similar in size and amount to that found in normal control, in all cases except one (patient 35) in whom no OAT mRNA was detectable (Fig. 3A). The analysis was repeated three times to confirm the result, especially the complete absence of mRNA in patient 35, and the actual quality and quantity of RNA present on the northern blots were checked by probing with actin cDNA (Fig. 3B).

The level of OAT protein had been shown to be markedly decreased in patient 14 by immunoaassay previously, as mentioned.5 The level of OAT protein present in fibroblasts from patients 35, 36, and 37 was also determined by western blot analysis using the anti-human OAT antibody. The result indicated the presence of variably reduced levels of immunoreactive OAT protein in these cases with patient 35 showing the lowest level, if any, of immunoreactive protein (data not shown).

Discussion

The discovery of hyperornithinemia in GA patients by Simell and Takki in 1973 led to the speculation of a defect in an ornithine-metabolizing enzyme and the actual demonstration of OAT deficiency in these patients in 1977.2-4 There has been ample evidence to assume that OAT is the primary defect in GA, including the uniform deficiency of this enzyme activity in GA patients, half-normal activity of the enzyme in obligate heterozygotes, and markedly decreased concentration of the enzyme in cells from GA patients.3,56 A presumed defect in OAT, however, has not been demonstrated at the gene level. Our finding of an OAT gene, mRNA, and protein defect in one GA case constitutes the first real demonstration of the OAT defect in GA at the gene level and provides
confirmation for the primary role the OAT gene is thought to play in this disease. The absence of OAT mRNA in patient 35 (patient 4 in ref. 16) is most likely due to the partial deletion of one of the OAT gene alleles and a mutation in the regulatory region of the other OAT gene allele which results in nontranscription of the gene and/or a mutation in the gene itself, which in turn results in lability or defective processing and rapid degradation of the transcribed mRNA. Examples of both types of gene mutation have been demonstrated in thalassemia.\textsuperscript{17} The barely detectable level of OAT antibody-reactive material in patient 35 may be an extremely small amount of OAT protein made from undetectably low level of OAT mRNA or some cross-reacting material that is not OAT. The determination of the exact nature of the gene defect will require cloning and sequencing analysis of this patient's OAT gene.

The presence of apparently normal OAT mRNA and a variable amount of immunoreactive OAT protein in three GA patients' fibroblasts despite the lack of OAT activity in these cells indicates that a subtle defect, such as a point mutation, is most likely present in the mRNA, which may result in poor translation of the message, lability or defective transport of the translated protein to the mitochondria, or inactive OAT protein. Patient 14 is especially interesting in this regard in that the OAT concentration has been shown to be markedly decreased (1.9% of normal) in his cells by immunoassay.\textsuperscript{5} Thus, the possible nature of the mRNA defect in this patient may be narrowed down to poor translation of the message, defective transport and/or lability of the translated protein. Cloning and characterization of this patient's OAT mRNA would elucidate the exact nature of the defect, and this is in progress in our laboratory. Finding of normal-appearing mRNA as seen here is apparently more often the rule in inherited diseases, as demonstrated in patients with ornithine transcarbamylase deficiency,\textsuperscript{18} argininosuccinate synthetase deficiency,\textsuperscript{19} juvenile form of Sandhoff disease,\textsuperscript{20} partial adenosine deaminase deficiency,\textsuperscript{21} and thalassemia.\textsuperscript{17} Some of the types of subtle mutations in mRNA mentioned above have been identified in \(\beta\)-thalassemias.\textsuperscript{17}

The finding of a normal pattern of OAT gene sequences in most of the GA patients tested by genomic southern analysis is consistent with the likely presence of a subtle mutation, such as a point mutation, instead of gross deletions or rearrangements, in most cases of GA. This has also been found to be the case in patients with ornithine transcarbamylase deficiency\textsuperscript{22} and \(\beta\)-thalassemias.\textsuperscript{17} A subtle mutation in the OAT gene would also be more likely to allow for synthesis of OAT mRNAs from it, as actually demonstrated and discussed above. The EcoRI RFLP found in the OAT gene may be useful for a prenatal diagnosis of GA since this polymorphism is located within the functional OAT gene on chromosome 10.\textsuperscript{13} The Hind III and Pst I RFLPs which are present in the OAT-related gene sequences on the X chromosome may also become very useful as markers for another important retinal degenerative disease, the X-linked retinitis pigmentosa (XLRP), since we have demonstrated that the X-chromosome OAT-related gene sequences map to the same region of X as the L1.28, a linkage marker for XLRP.\textsuperscript{12,23}

Finally, our results on the status of OAT gene expression in GA patients appear to correlate well with the clinical heterogeneity that is observed for GA. It is likely that a complete lack of OAT gene expression as essentially found in patient 35 represents those cases that have no detectable OAT activity, nonresponsiveness to \(B_6\) therapy, and a more rapid progression to blindness. Expression of the OAT gene and synthesis of mRNA and a decreased amount of OAT protein as found in patients 14, 15, 36 and 37 may represent those cases that have residual OAT activity (up to 19% of normal), responsiveness to \(B_6\) therapy, and a milder course of the disease.

Key words: gyrate atrophy, ornithine aminotransferase, mRNA, gene, deletion, restriction fragment length polymorphism

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