A Novel Mutation of Human Liver Alanine:Glyoxylate Aminotransferase Causes Primary Hyperoxaluria Type 1: Immunohistochemical Quantification and Subcellular Distribution

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A novel alanine:glyoxylate aminotransferase (AGT) mutation involved in primary hyperoxaluria type 1 (PH1) was studied in Japanese patients. Two mutations in exon 7, c.751T>A and c.752G>A, lead to a W251K amino acid substitution. Proband 1 (patient 1) was homozygous for the W251K mutation allele (DDBJ Accession No. AB292648), and AGT-specific activity in the patient's liver was very low. To reveal the cause of the low enzymatic activity, the intracellular localization of AGT (W251K) was studied using immunohistochemistry and immunoelectron microscopy. The latter analysis showed that patient 2 had only one-fifth of the normal AGT expression per catalase, suggesting impairment of AGT (W251K) dependent transport into peroxisomes. Peroxisomal transport of human AGT is believed to be dependent on the presence of the type 1 peroxisomal targeting sequence. The C-terminal tripeptide of AGT, KKL is necessary for peroxisomal targeting. In cultured cells, EGFP-AGT (W251K) localized both in the peroxisome and cytosol. These results were consistent with the data obtained from liver analysis of patient 2. The subcellular distribution of AGT (W251K) and the results from a random mutagenesis study suggest that KKL is necessary for peroxisomal targeting of human AGT, but additional signal other than KKL may be necessary.

Key words: human liver alanine:glyoxylate aminotransferase (AGT), AGT (W251K), peroxisomes, immune-gold double staining, primary hyperoxaluria type 1

I. Introduction

Human alanine:glyoxylate aminotransferase (AGT; EC2.6.1.44), localized to liver peroxisomes, has a broad substrate specificity and uses glyoxylate as the preferred amino acceptor [11]. AGT catalyzes the formation of glycine and prevents the formation of oxalate from glyoxylate. In humans, hereditary insufficiency of AGT results in a rare disorder in which excess amounts of glyoxylate is converted to oxalate. This disorder, known as primary hyperoxaluria type 1 (PH1; MIM259900), is characterized by hyper-oxalemia and hyperoxaluria with massive calcium oxalate deposition throughout the entire body [2].

PH1 is a rare autosomal-recessive disorder caused by a deficiency of the liver-specific pyridoxal-phosphate-dependent enzyme AGT. The AGT gene (AGXT) mutation that underlies this disorder has been reported [7, 8, 13], including studies of PH1-specific mutations (G41R, F152I, G170R, and I244T) in European and North American patients [3, 17], and a PH1-specific mutation (S205P) in Japanese patients [10]. Over 140 mutations have been identified in PH1 patients worldwide [17].

In the present study, we describe a new mutation in AGXT that was identified in five Japanese PH1 patients.

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This new AGXT mutation involved two mutations in exon 7: c.751T>A mutation and c.752G>A mutation, resulting in a Trp (W) 251 and a Lys (K) amino acid substitution. These PH1 patients had low AGT-specific activity. We examined the subcellular distribution of immunoreactive AGT protein in the livers of the patients.

We also describe a pEGFP-AGT random mutagenesis system by which randomly mutated AGT proteins are created, and their localization was determined in human fibroblast cells. Data obtained from these experiments suggest the presence of unknown signals other than the type 1 peroxisomal targeting sequence (PTS1) in AGT.

II.   Materials and Methods

Chemicals and antibodies

Rabbit anti-chicken IgG-gold colloidal particles (5 nm) and goat anti-rabbit IgG, F(ab')2 specific-gold colloidal particles (15 nm) were purchased from EY Laboratories Inc. (San Mateo, CA, USA). Chicken anti-AGT antibody was prepared by Sawady Technology Co., Ltd. (Tokyo, Japan). The synthesized N-terminal 20-amino acid peptide of AGT was used as the antigen. LR White reagent (TAAB Laboratories Equipment Ltd., Reading, UK) was used as the hydrophilic embedding agent. Uranylacetate dihydrate, trisodium citrate dihydrate and lead (II)-nitrate were purchased from Merck Japan Inc. (Tokyo, Japan). A Prolong Antifade Kit was purchased from Molecular Probes Inc. (Eugene, OR, USA) and goat serum was purchased from Vector Laboratories Inc. (Burlingame, CA, USA). All other chemicals used for immunochemistry and electron microscopy were of ultra-pure grade.

L-Alanine, glyoxylate and pyridoxal 5'-phosphate were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), NADH was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan) and L-lactate dehydrogenase was purchased from Boehringer Mannheim GmbH (Ingelheim, Germany). All other chemicals were of ultra-pure grade and were obtained from Nacalai Tesque Inc. (Kyoto, Japan).

Subjects

This study protocol was approved by both Ethics Committees at the Medical School of Fukuoka University and Gunma University Graduate School of Medicine. Informed consent was obtained from all patients for the collection of tissue samples.

Family 1: Proband 1 (patient 1) was a 45-year-old woman who was diagnosed with nephrolithiasis at the age of 25, and had been maintained on dialysis therapy for 6 years prior to the study. The patient underwent liver transplantation, and received her daughter's liver. Liver biopsy samples from proband 1 and her daughter were examined for AGXT gene mutations and for AGT-specific catalytic activity.

Family 2: The other liver samples were obtained from members of another family, including a 6-year-old girl (patient 2) who was the proband, her 9-year-old sister (patient 3), their 33-year-old mother (patient 4), and their 29-year-old father (patient 5). Analysis of the AGXT gene in this family will be reported elsewhere. We examined liver AGT protein expression and AGT-specific catalytic activity in these liver samples, and performed immunohistochemical and immunoelectron microscopic analyses.

Normal control: A human liver was obtained at autopsy from a 59-year-old male who died from lung cancer and who had no history of urolithiasis. This sample was used as a normal control sample.

RNA isolation

Total RNA was extracted and isolated from patient liver samples with TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

First-strand cDNA synthesis

Full-length first-strand cDNA was generated from the mRNA templates with Oligo (dT) primers using Ready-To-Go™ You-Prime First-Strand Beads (GE Healthcare Bio-Science Corp., Piscataway, NJ, USA) according to the manufacturer's instructions.

PCR amplification

cDNA samples were amplified by PCR using specific sense (NF3) and antisense (CR3) primers. The primer sequences used are as follows: sense (NF3), 5'-TGCTGGG AAATATTCCAGGCTTTG-3', and antisense (CR3), 5'-GC CAGGCCCTTTATTAAACATTCCC-3'. KOD-Plus DNA polymerase (TOYOBO, Osaka, Japan) was used. The PCR products were subjected to electrophoresis on a 1% agarose gel, and the expected size of the amplified PCR product was 1540 bp.

Cloning of the PCR products and sequencing

The PCR products were cloned using Target Clone™ Plus (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. The plasmids were purified using a QIAprep Spin Miniprep Kit (QIAGEN, Tokyo, Japan). The PCR products were confirmed by DNA sequencing of the amplified products using primers flanking each region as follows: sense (NF3), 5'-TGCTGGGAAATATTCCAGGCT TTG-3; sense (SF1), 5'-ATCCAGTACGTGTTCCAGACC A-3'; sense (SF2), 5'-GCTGGCTATGACTGGAGAGACA-3'; and sense (SF4), 5'-GTGGATTCGGTGGCATCCCTG GG-3', using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Warrington, UK) with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Genomic DNA isolation

Genomic DNA was extracted from peripheral blood samples using a MagExtractor-Genome (TOYOBO, Tokyo, Japan), according to the manufacturer's instructions. Exon 7 of the AGXT gene was amplified as previously described [16]. The genomic DNA sequences were determined as follows: sense, 5'-GGGGAGAGAAAGGGGCACACAGA

GT-3', and antisense, 5'-TGGGGCTVTAGTTGGGGTTCT TGAG-3', using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Immunohistochemistry

Liver specimens were obtained from patients 2 and 4 by surgical biopsies. They were fixed in 4% paraformalde-hyde and 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer at pH 7.4, then paraffin-embedded and sectioned. Sections were deparaffinised, blocked with 2% goat serum, incubated with chicken anti-AGT (1:100) at 4°C overnight, and washed three times with 0.1% Triton/PBS for 5 min. Subsequently, sections were incubated with biotinylated anti-chicken IgG antibody (1:200) for 1 hr at room tem-perature, washed as described above, and stained with DAB (3,3'-diaminobenzidine tetrahydrochloride) using an ABC Elite Kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Before mount-ing, sections were counter-stained with haematoxylin.

Immunoelectron microscopy

The biopsied liver specimens were fixed for 12 hr at 4°C in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer at pH 7.4. The fixed sections were embedded in acrylic resin LR-white. Thin sections (70–80 nm thick) were cut on a Leica Ultracut S (Leica Microsystems Japan, Tokyo, Japan), and the ultra-thin sections were mounted on nickel grids. The grids were placed on a drop of PBS to rinse the mounted sections, and then transferred onto a drop of 10% H_2O_2 for 20 min at room temperature for etching. The grids were washed with PBS, and incubated on a drop of 1% bovine serum albumin in PBS (1% BSA/PBS buffer) for blocking. The sections were incubated for double-immune staining simultaneously with 25-fold diluted chicken anti-human AGT serum in 1% BSA/PBS and 100-fold diluted rabbit anti-human catalase IgG (0.03 mg/ml 1% BSA/PBS) overnight at 4°C in a humidified chamber. The sections were then washed with 1% BSA/PBS, and incubated with rabbit anti-chicken IgG colloidal gold (5-nm particles; 1–2 µg/ml) and goat anti-rabbit IgG colloidal gold (15-nm particles; 1–2 µg/ml) for 1 hr at room temperature. Pre-immune chicken serum and rabbit IgG were used for control experiments. After the sections were washed with PBS, the sections were fixed for 10 min at room temperature in 2% glutaraldehyde in 1/15 M sodium phosphate buffer at pH 7.4. The sections were then washed with distilled water and stained with 2% uranyl acetate. Next, they were subjected to adsorption staining with lead citrate. The stained sections were viewed under a JEM-2000EXII electron microscope (JEOL, Tokyo, Japan) at an acceleration voltage of 80 kV.

Enzyme preparation

The biopsied liver specimens from PH1 patients 1 to 5, the donor and the autopsy control were immediately frozen in liquid nitrogen after biopsy. All samples were kept on dry ice until homogenization, which was performed on ice in nine volumes of 10 mM potassium phosphate buffer at pH 8.2 containing 0.05% Triton X-100 (homogenization buffer) in a Potter-Elvehjem glass homogenizer with a Teflon pestle. The homogenates were centrifuged at 100,000×g for 30 min at 4°C. The supernatants were collected for analysis as the liver extracts, and stored at −30°C until use.

Alanine:glyoxylate aminotransferase assay

Alanine:glyoxylate aminotransferase was assayed using the method of Okuno et al. [11]. The standard assay mixture contained 40 mM L-alanine, 5 mM glyoxylate, 0.05 mM pyridoxal 5'-phosphate, 100 mM potassium phos-phate buffer at pH 8.2, and 50 µl of 10-fold diluted liver extract, unless otherwise described. The enzyme reaction was carried out at 37°C for 30 min or 1 hr in a total vol-ume of 800 µl. The reaction was stopped by adding 100 µl of 25% trichloroacetic acid. Denatured protein was removed by centrifugation at 1,500×g for 5 min. The supernatant (450 µl) was transferred to a fresh tube and neutralized with 50 µl of 2 M KOH, and the neutralized solution was added to 450 µl of 2 M Tris/HCl at pH 8.2, followed by addition of 0.25 µmol NADH and 12.5 µg of lactate dehydrogenase. The mixture was incubated for 30 min at room temperature, and the resultant decrease in NADH absorption was meas-ured with a Shimadzu Spectrophotometer UV-160A (Kyoto, Japan) at 340 nm and compared with a blank sample. Glyoxylate or enzyme (10-fold diluted liver extracts) was added to the blank sample after stopping of the reaction.

Protein assay

Protein concentrations were determined by the method of Bradford [1] using a Bio-Rad Protein Assay™ or a RCDC protein assay™ (Bio-Rad Laboratories, Hercules, CA, USA). Bovine serum albumin was used to prepare the standard curve.

pEGFP-SKL, pEGFP-AGT, pEGFP-AGT (W251K) expression plasmid constructs

The pEGFP-SKL expression plasmid used in this study has been previously described [6]. The pEGFP-AGT, pEGFP-AGT (W251K) and AGT mutation constructs were prepared from pEGFP-SKL. In addition to these naturally occurring mutants, over 100 AGT mutants were developed using a Diversity PCR Random Mutagenesis Kit (BD Biosciences, Tokyo, Japan), according to the manufacturer's instructions.

Cell culture, transfection and analysis of transfectants

The human fibroblast cell line, HUC-F2, was cultured in GlutaMax medium (GIBCO) supplemented with 10% fetal calf serum under 5% CO_2 in air. DNA transfection into cells was performed using the Nucleofector System (Amaxa Biosystems, Cologne, Germany) for normal human adult dermal fibroblasts with Program U-23. After 2 days of culture, cells were fixed in 4% paraformaldehyde in PBS at pH 7.4. Cells transfected with GFP constructs were also fixed and the expressed protein was visualized under

an immunofluorescent light microscope (Olympus DP70, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using Dr. SPSS Ver 8 (SPSS Inc., Chicago, IL, USA). One-factor ANOVA was used to analyse the number of gold-labelled peroxisomes in patients 2 and 4, and the control. Tukey's test was used to identify statistically significant differences. P-values less than 0.05 were regarded as statistically significant.

III. Results

AGXT gene analysis

The molecular basis of PH1 was examined by analyzing the entire coding sequence of AGXT. Patient genotypes are shown in Table 1. Mutation analysis of the AGXT gene in patient 1 (Japanese PH1 patient) revealed three novel mutations. One of these novel mutations, c.676C>A, is considered to be a silent mutation. The other two novel mutations, c.751T>A and c.752G>A, lead to homozygous mutations with Trp (W) 251 Lys (K) amino acid replacements. Gene analysis of the AGXT gene from mRNA and genomic DNA yielded identical results. The W251K mutation found in the Japanese PH1 patient was novel, and was registered as DDBJ Accession No. AB292648.

AGT-specific activity in the liver of PH1 patients

Table 1 shows the AGT-specific activity of the biopsied liver extracts. When one allele of the AGXT gene was W251K and the other was wild-type (Patient 1's daughter), the specific AGT enzymatic activity was about 60% that of the normal control sample. Similar to patient 1's daughter, patients 4 and 5 were heterozygotes of AGXT with one mutated allele, resulting in activities half of that of the normal control. When one allele of the AGXT gene was W251K and the other was a premature stop codon (Patients 2 and 3), AGT-specific activity was about 16% and 11% that of the normal control, respectively. When the two alleles were homozygous for W251K (Patient 1), the specific activity was about 22% of that of the normal control. These results were in agreement with the AGXT genotype. Therefore, the AGXT gene mutation resulted in the production of AGT protein with low AGT-specific activity, leading to PH1.

Intracellular localization of AGT protein in liver specimens from PH1 patients

To determine the intracellular localization of AGT in liver specimens from PH1 patients, we performed immuno-histochemical staining using anti-AGT antibody. Figure 1 shows the localization of AGT in specimens obtained from the normal control (Fig. 1A and B), patient 4 (Fig. 1C and D) and patient 2 (Fig. 1E and F). In the normal control sample, AGT protein was expressed by the wild-type heterogeneous AGXT gene and clearly was localized to the peroxisome. In patient 4, the AGT protein was expressed by the AGXT gene (wild-type and W251K) and localized predominantly to the punctuates (possibly peroxisomes) with weak staining throughout the cytosol. In contrast, in patient 2, the AGT protein was expressed by the AGXT gene (W251K and premature stop) and did not clearly localize to the punctuates (peroxisomes), and was instead scattered faintly throughout cytosol.

Immunoelectron microscopy analysis

To quantitatively determine the distribution of AGT, we performed double immunoelectron staining for AGT and catalase in liver specimens from the normal control, and patients 2 and 4. Catalase was stained with 15-nm gold particles and AGT was stained with 5-nm gold particles. Both types of gold particles were localized to the peroxisomes, as shown in Figure 2. The number of catalase gold particles (large particles) in each peroxisome was counted and determined to be 6.8±3.9, 6.5±3.5 and 5.6±2.6 for the normal control subject, patient 4 and patient 2, respectively (Table 2). ANOVA analysis showed that these numbers were not significantly different, indicating that peroxisome proliferation was not affected by the W251K AGT mutation. This showed that gold staining for catalase could be used as a quantitative marker of peroxisomes. The number of AGT gold particles (small particles) per peroxisome was 93.3±43.4, 42.1±23.3 and 12.9±7.7 for the normal control subject, patient 4 and patient 2, respectively. These numbers were significantly different based on the ANOVA and Tukey's test (Table 2). We also calculated the number of AGT gold particles per catalase gold particle, and found it to be 16.2±9.2, 7.2±3.4 and 3.0±1.9 for the normal control subject, patient 4 and patient 2, respectively. These numbers were significantly different based on the ANOVA and Tukey's test (Table 2). These results clearly suggest that

Table 1. *AGT-specific activity in the liver extracts*

| Family	Subject	Age (years)	AGT-specific activity (μmol/h/g liver)	Relative activity (%)	AGXT genotype	
	Normal control	59	810	100	Wild-type	Wild-type
1	Patient 1	45	178	22	W251K	W251K
1	Patient 1's daughter (donor)	—	478	59	W251K	Wild-type
2	Patient 2	6	130	16	W251K	premature stop
2	Patient 3	9	87	11	W251K	premature stop
2	Patient 4	33	452	56	W251K	Wild-type
2	Patient 5	29	405	50	Premature stop	Wild-type
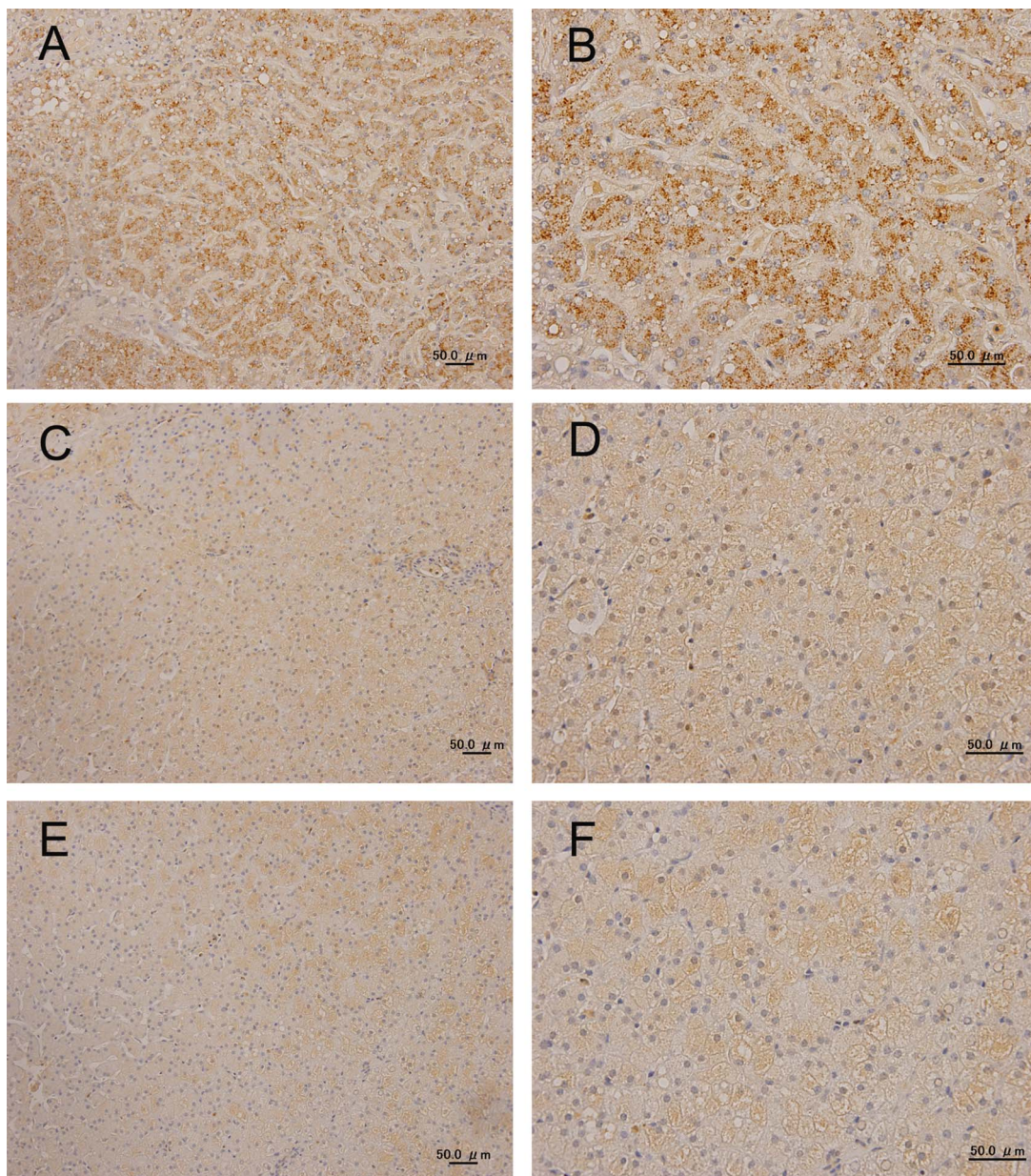
Fig. 1. Immunohistochemical staining with anti-AGT antibody of liver specimens obtained from the normal control and patients with PH1. Liver specimens from the normal control (**A** and **B**), patient 4 (**C** and **D**) and patient 2 (**E** and **F**) were stained with anti-AGT antibody by immunohistochemical method and counterstained with haematoxylin. **B, D** and **F** are enlarged images respectively for **A, C** and **E**. Bar=50 μm.

targeting of AGT to peroxisomes is impaired in patients 2 with W251K mutation.

Intracellular localization of transfected mutant AGT protein

Expression vectors containing mutant AGXT fused with the GFP gene were transfected into the human fibroblast cell line, HUC-F2. The intracellular distribution of wild-type or mutant AGT was determined by fluorescence microscopy (Fig. 3). The proteins from pEGFP-SKL (Fig. 3A) and pEGFP-AGT (wild-type) correctly localized to the peroxisomes (Fig. 3B). However, EGFP fused with AGT-W251K, the gene with one amino acid substitution,

seemed to localize both in the peroxisomes and the cytosol (Fig. 3C). While many distinctive punctuates, which might correspond to the peroxisomes, could be observed, smear signals were also detected throughout the cytoplasm, suggesting that some population of the AGT-W251K proteins failed to correctly localize to peroxisomes.

Random mutagenesis of the AGT gene

Next, the AGT gene was randomly mutated, transfected into HUC-F2 cells, and the localization of the resultant AGT protein was examined (Fig. 4). Nearly half of the mutated AGT proteins were truncated due to a stop codon, and were

Table 2. *Number of gold-labelled catalase and AGT proteins per peroxisome. The numbers of catalase-gold and AGT-gold particles were counted in each peroxisome by double immune-gold electron microscopy.*

	Age (years)	Catalase-gold	AGT-gold	AGT-gold/Catalase-gold	N
Control	59	6.8±3.9	93.3±43.4	16.2±9.2	29
Patient 4	33	6.5±3.5	42.1±23.3*	7.2±3.4*	67
Patient 2	6	5.6±2.6	12.9±7.7*, **	3.0±1.9*, **	36

Values represent means±SD; N: number of peroxisomes counted.
*: significantly different ($p < 0.05$) from the control.
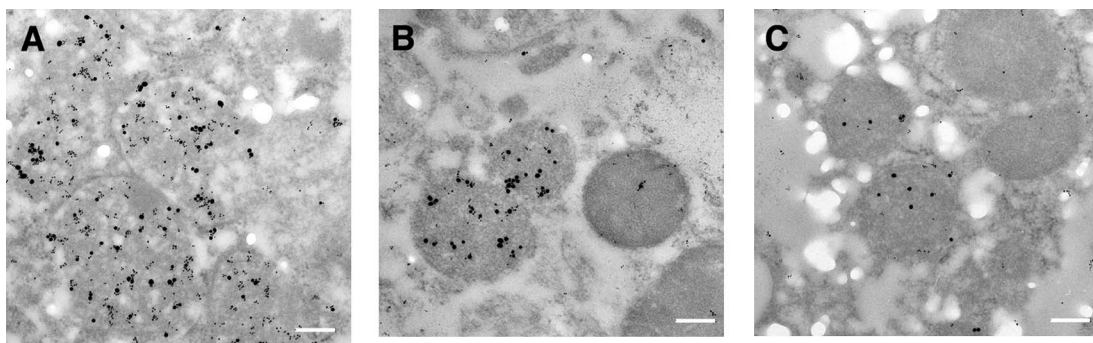**: significantly different ($p < 0.05$) from patient 4.

Fig. 2. Peroxisomal localization of AGT and catalase determined by immunoelectron microscopy. AGT was stained by 5-nm gold particles (small particle) and catalase by 15-nm gold particles (large particle). Both types of gold particles were localized in peroxisomes. Both types of gold particles were counted in each peroxisome, and numbers of each particle are listed in Table 2. **A**: normal liver; **B**: AGT heterozygous liver (patient 4); **C**: compound heterozygous liver (patient 2). Bar=200 nm.
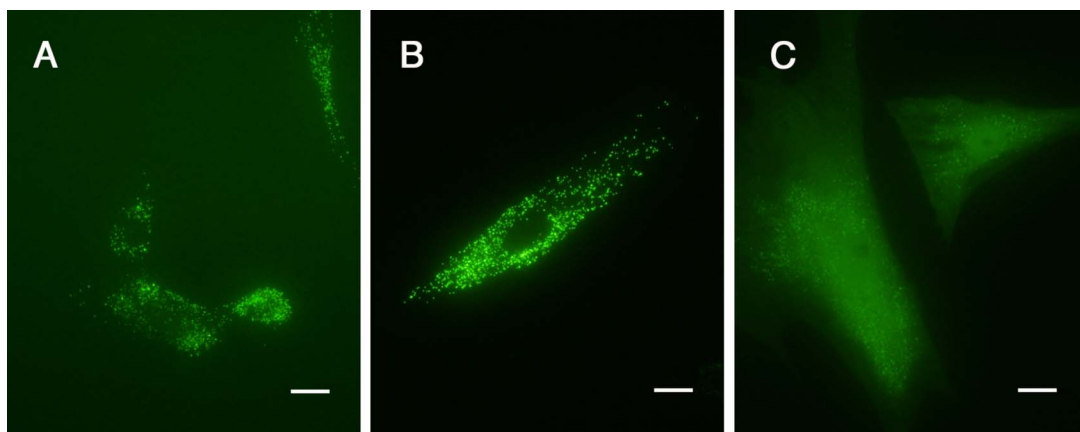

Fig. 3. Immunofluorescence analysis of the intracellular distribution of EGFP-fusion protein. EGFP-fusion protein was transfected to human fibroblast cells with the following constructs. **A**: pEGFP-SKL; **B**: pEGFP-AGT (wild-type); **C**: pEGFP-AGT (W251K). Bar=2 μm.

excluded from the analysis. Four targeting patterns, peroxisome only (Fig. 4A), peroxisome and cytosol (Fig. 4B), no targeting (Fig. 4C), or unknown (Fig. 4D), were observed in this experiment (Table 3, Fig. 4). The clones M11-15 (V93A, K225R) and M11-21 (S19T, Y134C), which had two amino acid substitutions, had full-length sequences including the peroxisomal targeting sequence PTS1 (KKL), and targeted correctly to the peroxisomes (Table 3). Moreover, although the clone M11-12 had five amino acid substitutions (I37V, D58E, S81P, P130H, K227R), the expressed protein correctly localized to peroxisomes (Fig. 4A). The clone M14-21 had four amino acid substitutions (K209R, L219R, E281V, L298P), and although it expressed full-length AGXT and had an intact PTS1 (KKL), the fusion protein failed to correctly localize to the peroxisomes (Fig. 4B). The clone M10-19 had two amino acid replacements (I244V, L302P) (Table 3). Although this clone expressed full-length AGXT and had an intact
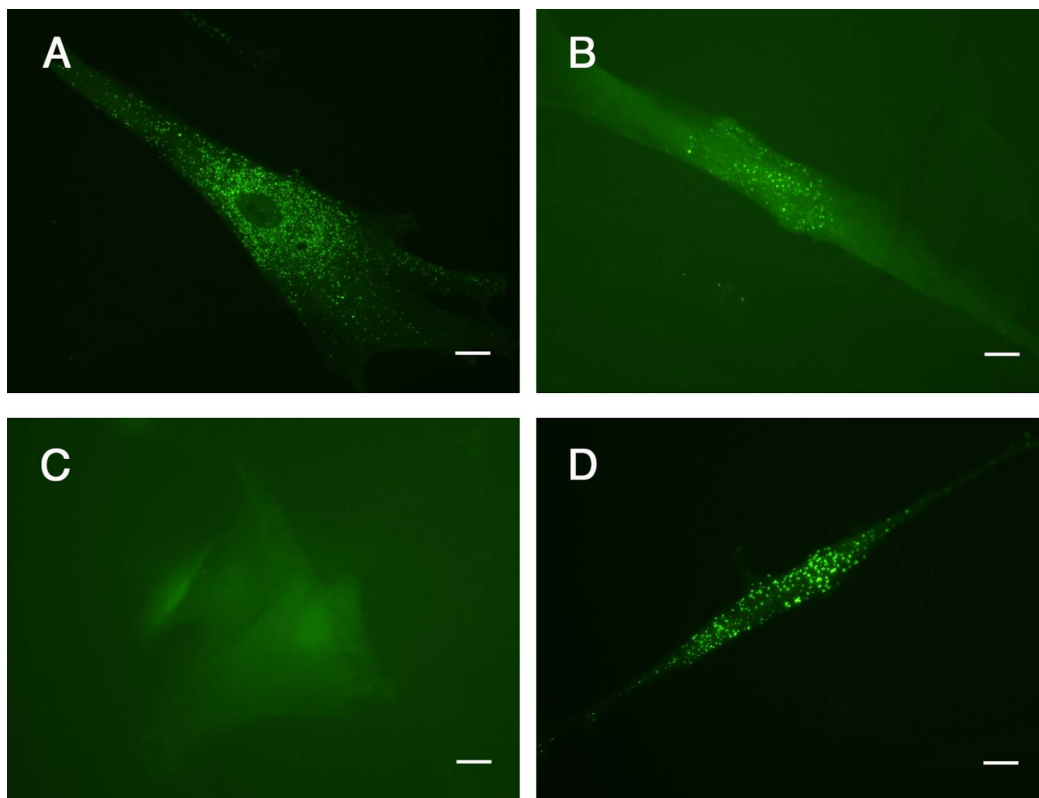
Fig. 4. The intracellular distribution pattern of mutant AGT made from random mutagenesis proteins in transfected human fibroblast cells. Human fibroblast cells were transfected with the constructs clones M11-12 (**A**), M14-21 (**B**), M10-19 (**C**) and M14-7 (**D**) (please refer to Table 3) and after 48 hr the cells were visualized for autofluorescence of GFP (**A, B, C, D**). Bar=2 μm.

Table 3. *Subcellular distribution of GFP-AGT fusion proteins from random AGT mutagenesis*

Clone name	Mutation number	C-terminal amino acids	Amino acid change	Subcellular distribution
M11-3	1	KKL	F68L	Px
M11-12	5	KKL	I37V, D58E, S81P, P130H, K227R	Px
M11-15	2	KKL	K225R, V93A	Px
M11-21	2	KKL	S19T, Y134C	Px
M14-14	4	KKL	S271C, E285G, R289H, V358A	Px
M14-7	4	KKL	P10S, L166P, F238S, K245E	unknown
M14-21	4	KKL	K209R, L219R, E281V, L298P	Px and cytosol
M11-18	3	KKL	A2T, F100S, M230V	Px and cytosol
M10-19	2	KKL	I244V, L302P	notargeting

Px: peroxisomes.

peroxisomal targeting sequence PTS1 (KKL), it did not localize to any specific organelle (Fig. 4C). The clone M14-7 (P10S, L166P, F238S, K245E) also had a full-length AGXT sequence and had the KKL motif, but this mutated protein localized in particles different from peroxisomes (Fig. 4D).

IV. Discussion

In the present study, mutation analysis of the AGXT gene in Japanese PH1 patients revealed a novel mutation,

W251K (c.751T>A; c.752G>A). PH1 patient 1 was homozygous for W251K. The cDNA sequencing data were consistent with the genomic DNA analysis. PH1 patient 4 was a heterozygote with a normal allele and an abnormal W251K allele. PH1 patients 2 and 3 were heterozygotes with W251K and a premature stop. Other mutations in PH1 have also been reported in Japanese people [10]. Because PH1 patients have a W251K mutation in the AGXT gene, AGT-specific activity in their livers was very low. As patient 4 showed approximately half of the activity found in the normal control subject, patient 4 was not considered to be

hyperoxaluric. Patients 1, 2 and 3 had approximately 10% to 20% of the AGT-specific activity seen in the normal control subject, and they were considered to have typical hyperoxaluria.

AGT protein localization was studied by immuno-histochemical microscopy and immunoelectron microscopy. AGT (W251K) was partially localized to the liver peroxisomes (Figs. 1 and 2), but the number of the molecules was low (Fig. 2B and 2C, Table 2). The number of catalase gold particles was not different between the normal control subject, patient 2 and patient 4 (Table 2). This data indicate that the quantity of catalase per peroxisome is not different between normal control subjects and PH1 patients. However, the ratio of AGT gold particles versus catalase gold particles was 16.2, 7.2, and 3.0 for the normal control subject, patient 4, and patient 2, respectively (Table 2). The number of AGT gold particles localized to peroxisomes in patient 4 was approximately half that of the normal control subject; that in patient 2 was approximately one-fifth of that in the normal control subject (Table 2). These data are consistent with those on AGT enzymatic activity (Table 1).

Peroxisome-targeting signals (PTS) have been reported as PTS1 [4, 14] and PTS2. PTS1 is a C-terminal amino acid sequence motif that contains SKL (Ser-Lys-Leu), and PTS2 is a N-terminal sequence motif. AGT belongs to the PTS1 group of proteins [5, 9], but instead of having the typical C-terminal motif, it has a KKL (Lys-Lys-Leu) [15]. After the fusion protein (pEGFP-KKL) was constructed and expressed, the KKL motif did not function as a PTS (data not shown), but GFP-SKL did (Fig. 3). This demonstrated that the KKL motif is essential, but insufficient in targeting AGT to peroxisomes, and that proteins containing the KKL motif may contain an additional, but yet unknown, amino acid motif. The 251W mutation is located far from the KKL C-terminal motif, but it appears to affect AGT peroxisomal targeting. Substitution of 251W with K might lower the affinity for targeting machineries. In the hepatocytes of patient 4, normal and mutated AGT (W251K) might compete for the machinery involved in peroxisomal targeting, and the normal AGT may be more effectively transported by the machinery, resulting in AGT levels half that of the normal control subject in patients with the 251W mutation. On the other hand, patients 2 and 3 only expressed the W251K AGT protein, which may have low affinity for the targeting machinery, and may be slowly or inefficiently transported, resulting in the low peroxisomal localization of AGT (W251K) (Table 2). The inadequate targeting of AGT (W251K) to peroxisomes might be responsible for the hyperoxalosis.

The fusion proteins expressed from the pEGFP-AGTwild and pEGFP-SKL constructs localized to peroxisomes. The EGFP-AGT (W251K) protein was targeted to peroxisomes and the cytosol. These results were consistent with the results from the immunoelectron microscopic analysis of patients 2 and 4. The results obtained from the random mutagenesis experiment demonstrated that the PTS1 motif KKL might be essential, but insufficient in targeting

AGT to peroxisomes. Two of the three amino acids, KKL of the AGT C-terminus were same with those of the three amino acids, SKL necessary for peroxisomal targeting. However, KKL is insufficient for correct peroxisomal targeting, because KKL fused with EGFP failed to localize to peroxisomes (data not shown). Moreover, in the random mutagenesis study, an AGT protein with five mutations was transported successfully into peroxisomes (Fig. 4A), while those with only two mutations failed to localize correctly (Fig. 4B). These results suggest that the portion between residues 238 and 270 is important in AGT transport into peroxisomes.

AGT protein has a pentapeptide motif, WXXXF/Y, which is known to be critical for the import of peroxisomal matrix proteins by the PTS1 receptor Pex5 [12]. This motif exists at 246 WLANF 250 in the AGT protein. It is possible that amino acid mutations between positions 244 and 251, which is in close proximity to this motif, could affect targeting to peroxisomes.

V. Acknowledgments

VI. References

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