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Contribution of two missense mutations (G71R and Y486D) of the bilirubin UDP glycosyltransferase (*UGT1A1*) gene to phenotypes of Gilbert's syndrome and Crigler–Najjar syndrome type II

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Abstract

In our mutation analyses of bilirubin UDP glycosyltransferase (*UGT1A1*) gene, we encountered six patients with Crigler–Najjar syndrome type II who were double homozygotes for G71R and Y486D, a patient with Gilbert's syndrome who was a single homozygote for G71R and six patients with Gilbert's syndrome who were single heterozygote for G71R. To clarify the role of each mutation in the occurrence of the two syndromes, we made four mutant expression models. Relative UGT1A1 activity of a single homozygous model of G71R was $32.2 \pm 1.6\%$ of normal, that of a single homozygous model of Y486D was $7.6 \pm 0.5\%$, that of a double homozygous model of G71R and Y486D was $6.2 \pm 1.6\%$ and that of a heterozygous model of G71R was $60.2 \pm 3.5\%$. The decreased activities of the single homozygous model of G71R and the double homozygous model were at an appropriate level to be diagnosed as Gilbert's syndrome and CN-II, respectively. The activity of a single heterozygous model of G71R was somewhat high to develop to the phenotype of Gilbert's syndrome, suggesting the presence of additional factors for the etiology of Gilbert's syndrome.

Keywords: Crigler–Najjar syndrome type II; Gilbert's syndrome; UGT1A1; Double mutation

1. Introduction

Gilbert's syndrome, first described by Gilbert and Lereboullet [1] in 1901, is characterized by mild elevation of serum total bilirubin concentration (~ 68 μM). The population incidence of Gilbert's syndrome is about $3 \sim 10\%$ [2,3]. The hepatic bilirubin UDP glycosyltransferase (UGT1A1) activity (measured from liver tissue) of these individuals is about 30% of normal [4,5]. The more severe phenotype of Crigler–Najjar syndrome type II (CN-II), described by Arias [6], is characterized by notable elevation of serum total bilirubin concentration ($102 \sim 340$ μM). The hepatic UGT1A1 activity of these individuals is about 10% of normal [5,7].

The cDNA of UGT1A1 and *UGT1A1* gene encoding of the enzyme have been recently identified [8,9], and there has been progress in mutation analysis of the *UGT1A1* gene in patients with Gilbert's syndrome and those with CN-II. In CN-II, five types of mutations have been reported [10–14]. In three of these

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five mutations, it was shown that the mutated enzymes of CN-II had reduced activity in expression studies [13,14]. On the other hand, we reported six patients with Gilbert's syndrome who had heterozygous missense mutations [15]. After that, we demonstrated that the mutation of P229Q, which was detected in Gilbert's individuals, reduced the UGT1A1 activity in expression study [16]. In addition to these missense mutations as a cause of Gilbert's syndrome, it has been shown that there are individuals with a homozygous insertion mutation in the TATA box (TAA(TA)7) [17,18], which resulted in reduced expression of UGT1A1 [17].

In our mutation analyses of patients with CN-II and Gilbert's syndrome, we encountered six patients with CN-II who were double homozygotes for G71R + Y486D (an allele which has both G71R and Y486D, simultaneously) [12,19], a patient with Gilbert's syndrome who was a single homozygote for G71R [20] and six patients with Gilbert's syndrome who were a single heterozygote for G71R [21]. The double homozygous mutation was the most abundant in our mutation analyses of eight Japanese patients with CN-II. But it was not clear how each mutation contributes to the phenotypes as Gilbert's syndrome and CN-II. In this report, we expressed normal and mutant UGT1A1s in COS-7 cells and clarified the contribution of each mutation to these syndromes.

2. Materials and methods

2.1. Materials

An ExSite PCR-Based Site-directed Mutagenesis Kit and Mammalian Transfection Kit (DEAE-dextran method) were purchased from Stratagene (La Jolla, USA), [^{14}C]UDP-glucuronic acid from Dupont-NEN (Wilmington, USA), and ECL Western Blotting Kit from Amersham (Buckinghamshire, England).

2.2. Construction of expression vectors

The cDNA of human liver UGT1A1 was amplified by PCR and inserted into pcDL expression vector, as previously described [16]. Mutations were introduced using the ExSite PCR-Based Site-directed Mutagenesis Kit. Precise instructions were followed as specified by the manual provided with the kit. Primer sets to introduce mutations were as follows (mutation points are indicated by underline): 5′-CAGAGCATTTTACACCTTGAAGAC-3′ and 5′-TCTCTGATGTACAACGAGGCGTCA-3′ were used for G71R (G to A at nucleotide 211). 5′-GACCATTCCTTGGACGTGATTGGTTTCC-3′ and 5′-CTGGTACCAGGTGAGGTCGTGGGCTGCG-3′ for Y486D (T to G at nucleotide 1456). Using these primer sets, three independent mutant versions of the cDNA in the pcDL vector were made. They were a single mutant of G71R, a single mutant of Y486D, and a double mutant of G71R + Y486D. Mutation sites and other parts of the cDNAs of the UGT1A1s were confirmed by sequencing.

2.3. Expression of cDNAs in COS-7 cells

Twenty-four hours before transfection, 6×10^5 COS-7 cells were seeded in 100-mm-diameter culture plates. DNA was transfected using the Mammalian Transfection Kit. At transfection, 340 μl of PBS solution containing DEAE-dextran and 1.0 μg of DNA was poured onto the culture cells. Four mutant expression models were generated as follows: a single homozygote for G71R, a single homozygote for Y486D, a double homozygote for G71R + Y486D and a single heterozygote for G71R. In the heterozygous model of G71R, pcDL vector with normal UGT1A1 cDNA (0.5 μg/plate) and pcDL vector with mutated UGT1A1 cDNA (0.5 μg/plate) were co-transfected to the cells. Forty-eight hours after transfection, these COS-7 cells were harvested and frozen at -80°C until subsequent analyses. Protein content was measured according to the method of Lowry et al. [22].

2.4. Preparation of antibody and measurement of expressed protein

A peptide (37 amino acid residues) was synthesized, as described earlier [23]. A polyclonal antibody directed against the synthetic peptide was generated by immunizing rabbits. Immunoaffinity antibody purification was carried out by coupling 6 mg of synthetic peptide to agarose gel. The final titer of purified antibody was 1:34,000.

2.5. Western blotting

Cell homogenates were subjected to the SDS-PAGE. The protein was transferred to Immunobilone Transfer Membrane (Millipore, Bedford MA). Detection was done using the ECL blotting system. The membrane was incubated for 12 h in blocking solution, and for 8 h in a solution of anti-UGT1A1 antibody (1:1000), and for 1 h in a solution of anti-rabbit antibody (1:1000). Then, the detection solution was added and exposed to a film for 3 ~ 60 min. The relative amounts of expressed UGT1A1s were determined from the peak areas of protein bands measured by a densitometer, Shimadzu TCL Scanner CS-910 (Shimadzu, Kyoto).

2.6. Assay of UGT1A1 activity

An assay of UGT1A1 activity was performed as reported earlier [24,25], with a slight modification as follows. The incubation mixture contained the following: cell homogenate, unconjugated bilirubin (86 μM), UDP-glucuronic acid (10 μM), 0.25 μCi (9.25 kBq) of [^{14}C]UDP-glucuronic acid (8.75 μM), bovine serum albumin (36 μM), $MgCl_2$ (10 mM), and 100 mM Tris–maleate buffer (pH 7.4) in a final volume of 100 μl. Incubations were carried out at 37°C for 30 min, or for a period of time indicated. The reaction was terminated by adding 200 μl of ice-cold pure alcohol. These mixtures were centrifuged at 12,000 rpm for 15 min. Supernatants were reduced to 20 μl by vacuum centrifugation. The bilirubin–glucuronide was separated by thin layer chromatography (TLC) of TLC plastic sheets 5748 (Merck, Darmstadt). The spots containing the bilirubin–glucuronide were measured by Molecular Imager GS 525 (Bio-Rad, Hercules). TLC plates were also exposed to BioMax films (Kodak, Rochester) for 7 days.

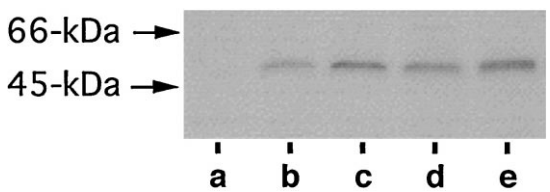

Fig. 1. Western blotting of expressed UGT1A1s. A total of 3 μg of cell homogenates were subjected to the SDS-PAGE in each lane. The peak areas of the protein bands on the film were measured by a densitometer. (a) Mock transfection (transfection of pcDL vector without cDNA), (b) homozygous model of G71R + Y486D, (c) homozygous model of Y486D, (d) homozygous model of G71R, and (e) normal UGT1A1.

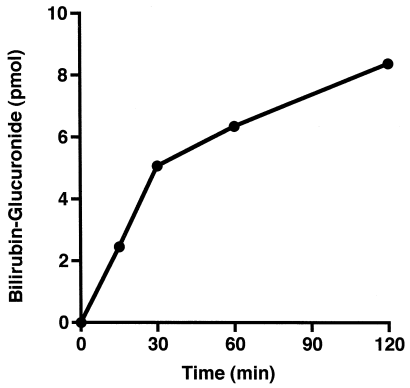

Fig. 2. Time course of UGT1A1 reaction. The amounts of expressed cell homogenates used for the reactions were 240 μg. Incubations were carried out for 15, 30, 60 and 120 min.

3. Results

3.1. Verification of UGT1A1 expression by Western blotting

In all expression models, a 54-kDa protein band was detected by western blotting (Fig. 1). The protein band was not detected in the mock transfection. The molecular weight of the band was almost the same as that reported [23]. Relative amounts of expressed

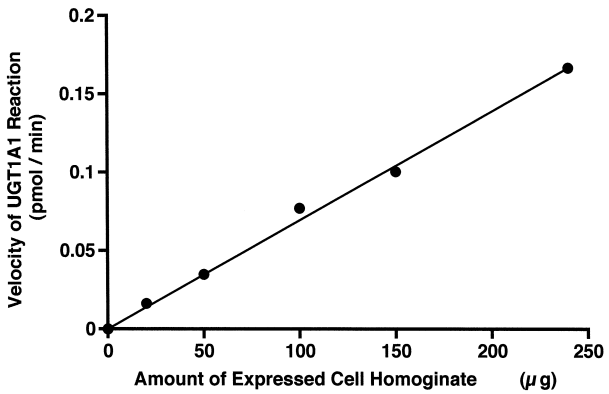

Fig. 3. Correlation between the velocity of UGT1A1 reaction and amount of the expressed cell homogenate.

UGT1A1s were computed as described in Section 2. The relative amounts of mutated UGT1A1s were within 0.5 ~ 1.3-fold of normal UGT1A1.

3.2. Assay conditions of UGT1A1 activity

As shown in Fig. 2, at up to 30 min, linearity of reaction product (bilirubin–glucuronide) with time was observed, so the velocity at 30 min incubation was adopted as the initial velocity. Subsequent incubations were carried out for 30 min. Next, the relationship between the velocity of UGT1A1 reaction and the amount of expressed cell homogenate was examined. When protein concentration in each assay tube was not equalized, linearity could not be obtained. Therefore, the total amounts of protein in the assay tubes were standardized by the addition of non-expressed cell homogenate (each assay tube con-

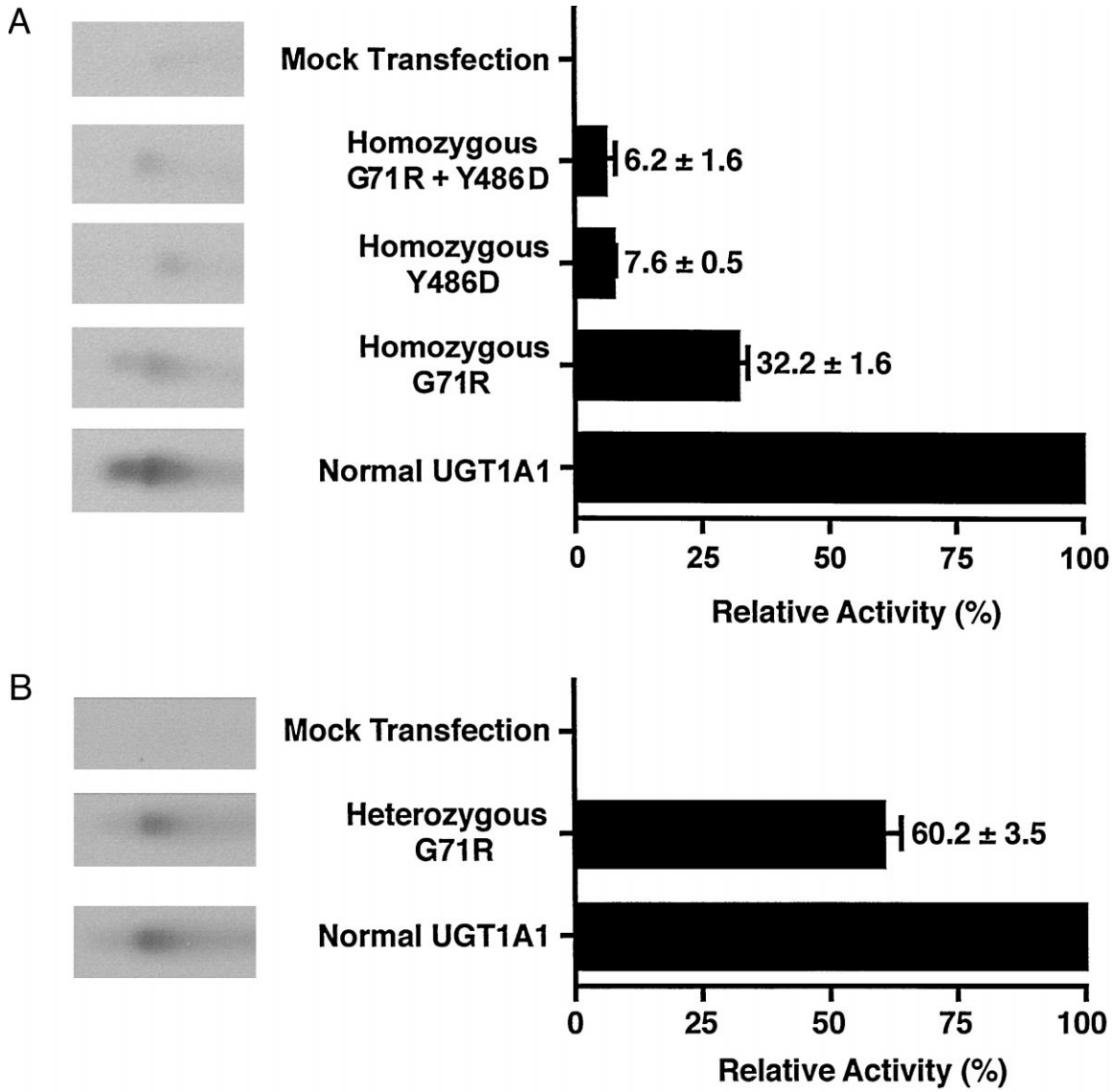

Fig. 4. Relative activities of homozygous mutation models (A) and a heterozygous mutation model of G71R (B). The amount of expressed cell homogenates used for the reaction was 200 μg. The activities of UGT1A1s were normalized by the relative amounts of expressed UGT1A1s, which were determined by Western blotting. Means ± S.D. of three independent experiments are indicated. A typical case of a spot of bilirubin–glucuronide separated by TLC is shown at the left side of the figure.

tained 240 μg of protein). By the correction, the velocity of the UGT1A1 reaction became proportional to the amount of expressed cell homogenate up to 240 μg of protein (Fig. 3). In the following study, 200 μg of expressed cell homogenates were used for the assay of UGT1A1 activity.

3.3. Enzyme activities of UGT1A1s with missense mutations

The expression experiments were performed in triplicate. The specific activity of expressed normal UGT1A1 was 0.62 ± 0.16 pmol/min/mg protein. The activities of UGT1A1s were normalized by the relative amounts of the expressed UGT1A1s. Relative activity of a single homozygous model of G71R was $32.2 \pm 1.6\%$ of normal, that of a single homozygous model of Y486D was $7.6 \pm 0.5\%$, and that of a double homozygous model of G71R + Y486D was $6.2 \pm 1.6\%$ and that of a heterozygous model of G71R was $60.2 \pm 3.5\%$ (Fig. 4A,B).

4. Discussion

It has been reported that hepatic UGT1A1 activity (measured from liver tissue) of CN-II is about 10% of normal [5,7], and that of Gilbert's syndrome is about 30% of normal [4,5]. Our six patients with CN-II, who were double homozygote for G71R + Y486D, were homozygote for normal TATA box (TAA(TA)6) [12,19]. The patient with Gilbert's syndrome, who was a single homozygote for G71R, was also homozygote for TAA(TA)6 [20]. Therefore, it was expected that the model of a single homozygote for G71R (detected in an individual with Gilbert's syndrome) would have about 30% of normal activity, and the model of double homozygote for G71R + Y486D (detected in CN-II individuals) would have about 10% of normal activity in an expression study. As we anticipated, the decreased UGT1A1 activities of the single mutation of G71R and the double mutation were at an appropriate level to be diagnosed as Gilbert's syndrome and CN-II, respectively (Fig. 4A). These findings indicate that these homozygous mutations are the genetic cause of the two syndromes. Furthermore, the reduction of UGT1A1 activity caused by the double mutation mainly resulted from Y486D.

In mutation analyses of patients with CN-II, genotypes of R209W/R209W [10], Q331R/Q331R [11], G71R + Y486D/G71R + Y486D [12], L175Q/973delG [13] and Q331X/wild [14] have been reported. Expression studies for those defective enzymes showed that a homozygous model of R209W had $4.4 \pm 2\%$ of normal activity [13] and a heterozygous model of Q331X (co-transfection of normal and mutated cDNAs) had 6% of normal activity [14]. The decreased activities of the two previous studies and present experiment for G71R + Y486D were enough to cause the phenotypes of CN-II. However, in the case of L175Q/973delG, only the L175Q allele was expressed in vitro, and the expressed activity ($38 \pm 2\%$ of normal) was somewhat high to be considered the cause of CN-II [13]. We think that the transcription and translation level of both alleles as well as the subunit structure of the enzyme [14,26] should be analyzed to fully explain the decreased activity of the single heterozygote (Q331X/wild) or the compound heterozygote (L175Q/973delG).

It has been shown that six patients with Gilbert's syndrome had heterozygous missense mutations [15]. On the other hand, there are patients with Gilbert's syndrome who had a homozygous insertion mutation in the TATA box (TAA(TA)7) [17,18]. In our ongoing study of 19 Japanese patients with Gilbert's syndrome, six had homozygous TAA(TA)7, six had a heterozygous G71R, and one had a homozygous G71R (four of the remaining cases were the other heterozygous missense mutations) [21]. The TAA(TA)7 was the most common mutation detected in patients with Gilbert's syndrome in studies by Bosma et al. [17] and Monaghan et al. [18]. The reason for the discrepancies in predominant mutation between our work and theirs can be attributed to a racial difference between Caucasians and Asians, as shown in alcohol dehydrogenase [27]. Eleven of 19 patients with Gilbert's syndrome had mutations in the coding region in our data [21]. More than one half of the missense mutations detected in our study of Japanese patients was heterozygous G71R. Therefore, it was important to clarify how much the G71R reduced the activity of UGT1A1 in the heterozygous state. The activity of the heterozygous model of G71R ($60.2 \pm 3.5\%$) was somewhat high to develop

the phenotype of Gilbert's syndrome (Fig. 4B). Additional factors might co-exist which lead to development of Gilbert's syndrome. One of the additional factors is TAA(TA)7 [28]. In the six heterozygotes for G71R of our study, two cases were compound heterozygote for G71R and TAA(TA)7. In such cases, the two abnormalities would contribute additionally to development of the phenotype of Gilbert's syndrome. But the other two of the six heterozygote for G71R were homozygote for TAA(TA)6. (The remaining two were not sequenced at TATA box). Therefore, other additional factors such as occult hemolysis may exist which lead to development of Gilbert's syndrome in the case of heterozygous G71R.

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