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Stop codon mutations in the flavin-containing monooxygenase 3 (*FMO3*) gene responsible for trimethylaminuria in a Japanese population

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Abstract

The reduced capacity of flavin-containing monooxygenase 3 (FMO3) to *N*-oxidize trimethylamine (TMA) is believed to cause a metabolic disorder. The aim of this study was to investigate the inter-individual variations of FMO3. Genomic DNA of case subjects that showed only 10–20% of FMO3 metabolic capacity among self-reported trimethylaminuria Japanese volunteers was sequenced. Functional analysis of recombinant FMO3 proteins was also performed. One homozygote for a novel single nucleotide substitution causing a stop codon at Arg500 was observed. The biological parents of this Proband A were heterozygous and showed >90% TMA *N*-oxygenation metabolic capacity. Another Proband B had the Arg500Stop and Cys197Stop codons. The TMA *N*-oxygenation metabolic capacities of the father and brother of this Proband B were apparently observed by possessing Arg205Cys mutant that coded for decreased TMA *N*-oxygenase. Recombinant Arg500Stop FMO3 cDNA expressed in *Escherichia coli* membranes and a series of highly purified truncation mutants at different positions of the C-terminus of FMO3 showed no detectable functional activity toward typical FMO3 substrates. The results suggest that individuals homozygous for either of the nonsense mutations, Arg500Stop and/or Cys197Stop alleles, in the *FMO3* gene can possess abnormal TMA *N*-oxygenation.
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Introduction

The flavin-containing monooxygenase (FMO, EC 1.14.13.8) is an NADPH-dependent enzyme that catalyzes the oxygenation of a wide variety of nucleophilic compounds containing sulfur, nitrogen or phosphorus atoms [1,2]. To date, eleven *FMO* genes have been identified in humans (*FMO1* to *FMO11p*) but only FMO1–5 are functionally active [3]. FMO3 is considered a prominent functional form expressed in adult human liver although FMO5 is also present [4,5]. In humans, a 20-fold inter-individual variation in FMO3 expression levels have been reported [6,7]. FMO3 may also play a role in processing some types of drugs such as the anticancer drug tamoxifen, the pain medication codeine, the antifungal drug ketoconazole, the addictive chemical nicotine found in tobacco, and diet-derived trimethylamine (TMA) [1,2]. Wild-type human FMO3 has 532 amino acids but there are genetic polymorphisms in the *FMO3* gene that code for naturally truncated forms that have no or almost no detectable amount of functional enzymatic activity [1,8–10]. Mutations in the *FMO3* gene are summarized in a Web-database using systematic and trivial names [8].

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Trimethylaminuria, or fish-like odor syndrome, is a genetic disease characterized by excretion of excessive unmetabolized TMA [11,12]. Individuals suffering from trimethylaminuria have a decreased capacity to oxygenate free malodorous TMA to non-odorous trimethylamine *N*-oxide (TMAO) by the FMO3 [13,14] and this is the case for individuals with causative nonsense *FMO3* gene mutations found in North American and European populations [15,16]. Unpleasant and/or pungent malodor caused by excess TMA present in various bodily fluids of some affected individuals may lead to profound social problems [11,12].

Due to its strong linkage with trimethylaminuria, considerable work has been done related to the contribution of genetic polymorphisms of the *FMO3* gene of the coding region to inter-individual differences in FMO3 phenotype [17–19]. In order to identify novel mutations of FMO3 and/or haplotypes of the *FMO3* gene found in Japanese individuals suffering from trimethylaminuria, we resequenced the entire coding region of the *FMO3* gene using genomic DNA from individuals that, judged by self-reported analysis were suspected to be positive for trimethylaminuria and later showed low FMO3 metabolic capacity on the basis of urine testing of TMAO levels. In a preliminary report [20], there were some FMO3 variants like Cys197Stop, Asp198Glu or Arg205Cys observed in a Japanese population, but the characterization of these FMO3 mutants were not examined in detail.

Herein, we report data supporting the involvement of two novel deleterious *FMO3* gene mutations causative of abnormal TMA *N*-oxygenation and trimethylaminuria in self-reporting Japanese individuals that were diagnosed with low FMO3 metabolic capacity based on urine testing. Subjects homozygous for either of the nonsense mutations, Arg500Stop and Cys197Stop alleles, in the *FMO3* gene suffered from trimethylaminuria.

Materials and methods

Chemicals

TMA and TMAO were obtained from Wako Pure Chemicals (Osaka, Japan). The tertiary amine substrate 10-[(*N*,*N*-dimethylaminopentyl)-2-(trifluoromethyl)]phenothiazene (5-DPT) and its *N*-oxide were synthesized as described previously [1,21]. The other chemicals and reagents used were obtained in the highest grade available commercially.

Subjects

The Ethics Committees of Showa Pharmaceutical University and Hokkaido University approved this study. Volunteer subjects who responded to an Internet article for screening of urinary TMA and TMAO levels and for sequencing the *FMO3* gene included 90 males and 74 females ranging from 1 to 64 years of age. Informed consent was obtained from each subject or parent. The study participants collected their urine samples as described previously [22]. Urinary TMA and TMAO concentrations were determined by gas chromatography using a flame ionization detector as described previously [23]. Urinary concentrations of free TMA or total TMA (μmol/mL of urine) were corrected for creatinine excretion (mmol/mL) [22]. Individuals that showed impaired FMO3 metabolic capacity, defined as the ratio of TMAO to total TMA (% of TMAO/(TMA + TMAO)), lower than 40% were considered to constitute abnormal TMA metabolism and possibly suffering from severe trimethylaminuria [16,22,24]. The values of urinary TMA and TMAO were shown as the average of at least three determinations obtained from first morning void urine.

DNA analysis

Genomic DNA prepared from peripheral lymphocytes [20] or buccal cells [25] of the study participants were analyzed. The sequence of the complete human *FMO3* gene described in GenBank (Accession Number AL021026) was used as a reference. Polymerase chain reaction (PCR) for the all exons and exon–intron junctions of the human *FMO3* gene was conducted in a 25 μL reaction mixture containing 50 ng of genomic DNA, 1.0 U LA-*Taq* DNA polymerase (Takarabio, Shiga, Japan), LA-PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 5.0 pmol of each sense and antisense primer reported previously [23]. The PCR conditions consisted of an initial denaturation at 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s. The PCR products were directly sequenced on both strands using an ABI bigdye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with the sequencing primers [20]. The purified PCR products were analyzed on an ABI PRISM 3730*xl* DNA analyzer (Applied Biosystems).

Genotyping analysis for the novel g.30398 C > T mutation (Arg500Stop FMO3) in exon 9 was also carried out by a PCR-restriction fragment length polymorphism (RFLP) method with amplified DNA with the primers FMO3-9S and FMO3-9AS [23] and digestion by *Bss*SI at 37 °C for 2 h.

Recombinant wild-type and modified FMO3 protein preparations

The FMO3 cDNA used was previously modified by a PCR procedure [23] using a 5′-primer (5′-AAAAAGCTTACCATGGGGAAGAAAG-3′, that introduced an *Nco*I site prior to the start codon) and a 3′-primer (5′-CTAGAGAAGCTTATGATTAGGTCAACAC-3′, that introduced a *Hin*dIII site downstream of the stop codon). The full-length DNA sequence was confirmed again using DNA re-sequencing of both strands. To produce Arg205Cys FMO3, site-directed mutagenesis was performed by the primer-directed enzymatic amplification method [23]. Briefly, G-base at position 706 bp in the FMO3 cDNA was substituted by a T-base using the primers, 5′-AGAACTCAGC**t**GCACAGCAGA-3′ and 5′-TCTGCTGTGC**a**GCTGAGTTCT-3′, to introduce the single nucleotide substitution, that coded for Arg205Cys in exon 5. Similarly, a C-base at position 1590 bp was substituted by a T-base for preparation of Arg500Stop FMO3. The resultant cDNAs were amplified by KOD polymerase (Toyobo, Osaka, Japan). The wild-type and modified FMO3 cDNAs were introduced into the pTrc99A expression vector (Pharmacia Biotechnology, Milwaukee, MI, USA) and then transformed into *E. coli* strain JM109 as described previously [23]. The entire coding regions of the wild-type and mutagenized FMO3 cDNAs including the mutated sites were verified by re-sequencing of both strands.

Membrane fractions were prepared from the bacterial pellets that the FMO3 cDNAs had been introduced into by a series of fractionations and high-speed centrifugation steps as described previously [23]. Briefly, *E. coli* JM109 transformed by the FMO3 expression vector was grown overnight at 37 °C in Luria–Bertani medium containing 50 μg/mL ampicillin. A 1.0 mL aliquot of the starter culture was inoculated into 100 mL of Terrific Broth medium containing 50 μg/mL ampicillin, and 100 mM potassium phosphate buffer (pH 7.4) in a 300-mL triple-baffled flask and cultivated at 120 rpm at 30 °C. After the absorbance of the culture broth at a wavelength of 600 nm reached 0.3, 1 mM isopropyl-β-D-thiogalactoside was added and shaking was continued further for 24 h at 30 °C. The cells were then harvested by centrifugation at 10,000*g* for 20 min. All subsequent steps were carried out at 4 °C. The cells were resuspended (*ca.* 0.03 g/mL) in 50 mM Tris–acetate buffer (pH 7.5) containing 0.25 mM EDTA, 0.25 M sucrose, and 0.1 mg/mL lysozyme. The cell suspension was kept on ice for 30 min and then centrifuged at 9000*g* for 10 min. The pellet was resus-

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