Regular Article

A Mutation in the Flavin-containing Monooxygenase 3 Gene and its Effects on Catalytic Activity for N-oxidation of Trimethylamine In Vitro

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Summary: To clarify the mutation of the flavin-containing monooxygenase (FMO) 3 gene causing fish-odor syndrome, we analyzed the FMO3 gene of a Thai subject who possibly suffered from fish-odor syndrome. A novel mutation, a single-base substitution from G to A at the position of 265 (G265A), was identified in exon 3. The mutation caused an amino acid substitution from valine to isoleucine at residue 58 (V58I). The mutated FMO3 protein with V58I exhibited the reduced trimethylamine N-oxidase activity when it was expressed in E. coli. The $V_{\text{max}}/K_m$ value for the activity of the mutant-type FMO3 was about 5 times lower than that for the wild-type FMO3.

Key words: FMO3; fish-odor syndrome; trimethylamine; trimethylaminuria

Introduction

The flavin-containing monooxygenase (FMO) catalyzes the oxidation of a wide variety of compounds containing a sulfur, nitrogen or phosphorus atom.¹,² The proteins and cDNAs of FMO have been isolated from several mammalian species including humans.³⁻²¹ Based on the sequence similarity, mammalian FMOs are classified into five isoforms (FMO1⁻FMO5).²² Human FMO3 is considered as a major form in adult livers, while human FMO1 is detectable in the kidney and fetal livers, but not in adult livers.²³ It seems that functional proteins of FMO4 are not expressed in humans.²⁴,²⁵ There is only a limited knowledge of the physiological roles of human FMOs.

Trimethylamine (TMA) is one of the tertiary amines that are the typical substrates of FMO.²,²⁶ TMA is derived from dietary sources such as choline, carnitine, lecithin and TMA N-oxide (TMAO).²⁷ This chemical exists as a vapor under standard conditions (boiling point, 3°C) and exhibits low olfactory threshold. The reduced capacity to N-oxidize TMA is believed to cause a metabolic disorder known as fish-odor syndrome or trimethylaminuria in which patients excrete higher amounts of TMA in the urine, sweat and breath.²⁸,²⁹ To clarify the molecular form(s) of FMO responsible for the N-oxidation of TMA, Lang et al. expressed the forms of the enzyme in E. coli and proved that the human FMO3 catalyzed the N-oxidation of TMA,³⁰ which was in accordance with the previous concept that the deficiency of FMO3 was the cause of fish-odor syndrome.³¹,³² Recently, patients of fish-odor syndrome were reported in Orientals in addition to Caucasians.³³ The frequency of several mutations in FMO3 gene was analyzed in some Asians.³⁴,³⁵ However, the mutation(s) of the FMO3 gene causing the fish-odor syndrome in Orientals has not been fully understood. In this paper, we analyzed the FMO3 gene of a possible patient of fish-odor syndrome in Thailand, and found a novel mutation in the gene causing the reduced capacity of TMN N-oxidation.

Materials and Methods

Materials

TMA hydrochloride was purchased from Wako Pure Chemicals (Osaka, Japan). TMAO dihydrate was obtained from Aldrich Chemical (Milwaukee, WI). NADPH, NADP⁺, glucose 6-phosphate and glucose 6-
phosphate dehydrogenase were purchased from Oriental Yeast (Tokyo, Japan). The pTrc99A vector was obtained from Pharmacia Biotechnology (Milwaukee, WI). Restriction endonucleases were purchased from New England Biolabs (Beverly, MA) and Takara Shuzo (Kyoto, Japan). Human liver microsomes, rabbit polyclonal antibodies raised to human FMO3, and microsomal standard for human FMO3 were obtained from Pharmacia Biotechnology (Milwaukee, WI). Restriction endonucleases were purchased from New England Biolabs (Beverly, MA) and Takara Shuzo (Kyoto, Japan). Human liver microsomes, rabbit polyclonal antibodies raised to human FMO3, and microsomal standard for human FMO3 were from Daiichi Pure Chemicals (Tokyo, Japan). All other chemicals were of the highest grade commercially available.

Subjects
Possible patients of fish-odor syndrome were judged from the fish-like odor of exhalation by the doctor in Thailand (Department of Laboratory Medicine, Faculty of Medicine, Chulalongkorn University). The patient had no symptoms of other diseases. The study was approved by the ethics committee of Hokkaido University and Chulalongkorn University. Informed consent was obtained from all participants.

Urinary excretion of TMA and TMAO
Urine samples were stored at −80°C until analysis. TMA in the urine was directly analyzed by a head-space gas chromatography, and TMAO was determined indirectly after reduction of TMAO with TiCl₃ to generate TMA in the urine was directly analyzed by a head-space gas chromatography, and TMAO was determined indirectly after reduction of TMAO with TiCl₃ to generate TMA. The amount of TMAO was calculated from the increase in TMA after the chemical reduction.

The mutation, G265A, was introduced into the FMO3 cDNA by PCR with Pfu DNA polymerase. An Nco I-Sac I fragment from the pTHF3 was subcloned into the pBluescript II SK (−) (Stratagene). This plasmid was used as a template. The sequences of the oligonucleotide primers used for mutagenesis are as follows: FMO3m265 (S), 5'-TTACAAATACATCTTTTCAC-3'; FMO3m265 (AS), 5'-TGGAAGATTTGAATTGTAA-3'; M13-21, 5'-TGTAACACGCCTGAGCAGT-3'; M13Rev, 5'-CAGGAAACAGCTATGAC-3'. Two PCR products were amplified using two pairs of primers: FMO3m265 (S) and M13Rev, FMO3m265 (AS) and M13-21. The fragment containing the mutated sequences was generated by a second PCR using the two PCR products as templates and the primers M13-21 and M13Rev. This fragment was digested with NcoI and SacI, and then was replaced with the NcoI-SacI fragment of the pTHF3. This plasmid was designated as pTHF3m265. The introduction of mutation was verified by DNA sequencing.

Expression of human FMOs in E. coli
E. coli JM105 cells transformed with a plasmid, pTrc99A, pTHF3 or pTHF3m265 were grown with shaking at 37°C in modified Terrific Broth containing ampicillin (50 μg/mL). When cell density monitored by absorbance at 600 nm reached 0.8, induction of the protein expression was initiated by addition of isoprpyl-β-D-thiogalactopyranoside (1 mM). After a further in-

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5’ to 3’)</th>
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<tbody>
<tr>
<td>Ex1-S</td>
<td>ATCCATCAGAGGTTGGGC</td>
</tr>
<tr>
<td>Ex1-AS</td>
<td>GCACACTACGCTGGCTATAAT</td>
</tr>
<tr>
<td>Ex2-S</td>
<td>ACAGGGCGTACTCCATAC</td>
</tr>
<tr>
<td>Ex2-AS</td>
<td>GATCTATCAAGGGAGACCTGTG</td>
</tr>
<tr>
<td>Ex3-S</td>
<td>GAACTGCCGCTACCTGTG</td>
</tr>
<tr>
<td>Ex3-AS</td>
<td>GTTGAAGATTAGAAAGGGGAAC</td>
</tr>
<tr>
<td>Ex4-S</td>
<td>TCATACTGTATCTGGCAAACCA</td>
</tr>
<tr>
<td>Ex4-AS</td>
<td>TAGATGGCCTACAAATATGGT</td>
</tr>
<tr>
<td>Ex5-S</td>
<td>GCAGGTAAATATACATACATTTC</td>
</tr>
<tr>
<td>Ex5-AS</td>
<td>CCCACATTTCATACACACTCT</td>
</tr>
<tr>
<td>Ex6-S</td>
<td>GCTGGGCTTAAGATACCTTC</td>
</tr>
<tr>
<td>Ex6-AS</td>
<td>TGGGCTTACGGACATTAAGG</td>
</tr>
<tr>
<td>Ex7-S</td>
<td>ATAGAGGGGAAATATTACACTCC</td>
</tr>
<tr>
<td>Ex7-AS</td>
<td>CAAGGTTATGGTCACGTGCAATTCTC</td>
</tr>
<tr>
<td>Ex8-S</td>
<td>GGAAATTACAGGCGGTTCCT</td>
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<tr>
<td>Ex8-AS</td>
<td>GGCTAAACTGTCTATGTAATG</td>
</tr>
<tr>
<td>Ex9-S</td>
<td>CTACACAGGTTGGGATCC</td>
</tr>
<tr>
<td>Ex9-AS</td>
<td>CCTCTCTGGATTGTCAG</td>
</tr>
</tbody>
</table>
cubation at 30°C for 24 h with shaking, the bacterial membrane fraction was prepared as reported by Atta-Asafo-Adjei et al.\textsuperscript{11)}

**Western blot analysis**

Western blot analysis was performed as described by Itoh et al.\textsuperscript{36)} Briefly, membrane proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (7.5% gel) according to the method of Laemmli.\textsuperscript{39)} Separated proteins were transferred onto an Immobilon membrane (Millipore, Bedford, MA). Rabbit polyclonal antibodies raised to human FMO3, goat anti-rabbit IgG antibody and peroxidase-conjugated rabbit anti-peroxidase antibody were sequentially added to the membrane. Then the proteins were stained with 3,3’-diaminobenzidine and hydrogen peroxide to visualize the expressed FMO.

**Determination of FAD Content**

The FAD content was determined fluorometrically by the method of Faeder and Siegel.\textsuperscript{40)}

**Kinetics of TMA N-oxidation**

TMA N-oxidation was analyzed as described previously\textsuperscript{36)} with minor modifications. Briefly, a typical reaction mixture consisted of 50 mM Tris-HCl buffer (pH 8.4), an NADPH generating system (10 mM MgCl\textsubscript{2}, 10 mM glucose 6-phosphate, 0.4 mM NADP\textsuperscript{+} and 0.5 U/mL glucose 6-phosphate dehydrogenase) and human liver microsomes (0.4 mg/mL protein) or the membrane fraction of *E. coli* (0.5 mg/mL protein) in a final volume of 0.5 mL. The reaction mixture was preincubated at 37°C for 3 min. The reaction was initiated by the addition of TMA. After incubation for 10 min, 25% (w/v) ZnSO\textsubscript{4} (50 μL) and a saturated aqueous solution of Ba(OH)\textsubscript{2} (50 μL) was added to stop the reaction. The mixture was centrifuged at 1,300 × g for 5 min. An aliquot (0.4 mL) of the supernatant was transferred to a glass vial, made alkaline with 5 M NaOH (20 μL), and then evaporated to dryness in vacuo at 80°C. The residue was dissolved in 1 M HCl (0.4 mL) followed by the addition of 20% (w/v) TiCl\textsubscript{4} (0.1 mL). The TMAO in the sample was reduced in the capped vial at room temperature for 1 h. Immediately after the addition of 10 M NaOH (5 mL), the vial was capped with a teflon-lined septum and kept at 95°C for 30 min. The headspace gas (0.8 mL) was subjected to a gas chromatograph. To determine kinetic parameters, the TMA concentration ranged from 6.25 to 200 μM for the assay using *E. coli* membranes. Lineweaver-Burk plots were adapted for the determination of kinetic parameters.

**Gas chromatography**

GC-17A series gas chromatograph (Shimadzu, Kyoto, Japan) with a flame thermionic detector was used for the analysis of TMA as described previously.\textsuperscript{36)}

**Results**

**Urinary excretion of TMA and TMAO in Thai**

We investigated the urinary excretion of TMA and TMAO in a Thai subject who possibly suffered from fish-odor syndrome. The percentage of total TMA excreted as TMAO in this individual was lower than that of a healthy Thai subject (Table 2). This result was in accordance with the fact that the patient had the fish-like odor of exhalation.

<table>
<thead>
<tr>
<th>Subject</th>
<th>% Total TMA excretion as TMAO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Possible patient</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>90.8</td>
</tr>
<tr>
<td>Healthy volunteers</td>
<td></td>
</tr>
<tr>
<td>Male (n = 3)</td>
<td>97.9</td>
</tr>
<tr>
<td>1 M</td>
<td>97.9</td>
</tr>
<tr>
<td>2 M</td>
<td>96.7</td>
</tr>
<tr>
<td>3 M</td>
<td>97.5</td>
</tr>
<tr>
<td>Female (n = 3)</td>
<td></td>
</tr>
<tr>
<td>1F</td>
<td>96.0</td>
</tr>
<tr>
<td>2F</td>
<td>98.8</td>
</tr>
<tr>
<td>3F</td>
<td>98.9</td>
</tr>
</tbody>
</table>

**Sequence analysis of the FMO3 gene of a possible patient**

The sequences of all of the nine exons and the intron/exon boundaries of the *FMO3* gene were analyzed, because of the possibility that this individual was a heterozygous carrier of the allele for the fish-odor syndrome. Thus, we found a single base substitution from G to A at the position of 265 (G265A) in exon 3 (Fig. 1). This is a novel mutation which has not been reported. The mutation was detected only in an allele of this individual, but not in alleles of normal healthy Thai volunteers. The mutation caused an amino acid substitution from valine to isoleucine at the residue 58 (V58I).

**TMA N-oxidase activity of the mutant-type FMO3 expressed in E. coli**

To evaluate the effects of the substitution of V58I on the TMA N-oxidase activity of FMO3, the mutant-type FMO3 was expressed in *E. coli* after the mutation was introduced into a wild-type FMO3 cDNA. The expression of the mutant-type FMO3 in *E. coli* was confirmed by Western blot analysis (Fig. 2). The mutant-type protein was detected at the same mobility with an expression level similar to that of the wild-type protein. Immunoreactive protein was undetectable in the membranes of *E. coli* transformed with a vector. The expression level of FMO in the *E. coli* membranes was estimat-
Table 3. Kinetic parameters for trimethylamine N-oxidation by a wild-type FMO3-58V and a mutant-type FMO3-58I expressed in E. coli

<table>
<thead>
<tr>
<th></th>
<th>$V_{max}$ (nmol/min/nmol FMO)/(nmol/min/mg prot.)</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type FMO3-58V</td>
<td>55.1 ± 10.9/517 ± 176</td>
<td>50.9 ± 4.9</td>
<td>1.09 ± 0.24</td>
</tr>
<tr>
<td>Mutant-type FMO3-58I</td>
<td>10.5 ± 3.0/53.1 ± 19.9</td>
<td>46.2 ± 12.5</td>
<td>0.23 ± 0.04</td>
</tr>
</tbody>
</table>

*The values were calculated from Lineweaver-Burk reciprocal plots. Each value represents the mean ± SD (n = 3).

Fig. 1. Nucleotide sequences around the nucleotide 265 of the FMO3 gene. Electropherograms of the FMO3 gene (A) and the FMO3 gene with the substitution from G to A at the position of 265 (B) are shown. The nucleotide number is indicated according to the sequence of FMO3 cDNA.23)

Fig. 2. Western blot analysis for human FMO3 expressed in E. coli. Each lane contains 20 μg of protein, except for the lane of human FMO3 microsome standard (0.6 μg). Membranes prepared from E. coli were separated by SDS-PAGE and then transferred onto a nylon membrane. Rabbit polyclonal antibodies raised to human FMO3 were used for the immunoreaction.

Analysis of the G265A mutation in Thai subjects

The G265A mutation was genotyped by sequencing the FMO3 gene in six normal healthy Thai volunteers and twelve Thai subjects who possibly suffered from fish-odor syndrome. Another heterozygous carrier of this mutant allele was found among 12 possible patients of fish-odor syndrome.

Discussion

Recently, it was reported that a patient who suffered
from fish-odor syndrome had an FMO3 gene with a mutation or deletion that resulted in reduced activity for TMA oxidation. The Thai subject, a possible patient of fish-odor syndrome, excreted 90.8% of TMA as TMAO, while the percentage of total TMA excreted as TMAO in the urine of patients with fish-odor syndrome was reported to be less than 55% under normal dietary conditions in the previous studies. The reported mutations, G291A (M66I), TMA oxidation. The Thai subject, a possible dietary conditions in the previous studies. The North American with fish-odor syndrome. Dolphin V58I mutation exhibited about 5 times lower the enzyme activities. In fact, the FMO3 carrying the mutation A52T, the N61S and the M66I, suggest- ing a possibility that the mutation caused a reduction of TMA oxidation under normal physiological conditions. The mutation may alter the protein structure (Table 3). The mutation may alter the protein structure of FMO3, though V58 is located neither in the FAD-containing monooxygenase expressed in pig liver: primar- y sequence, distribution, and evidence for a single gene. Biochemistry, 29: 119–124 (1990).

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References


35) Mushiroda, T., Yokoi, T., Takahara, E., Nagata, O., Kato, H. and Kamataki, T.: The Suncus (Suncus muri-
A New Mutation in Flavin-containing Monoxygenase 3

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(1993).


