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Regular Article

Genetic variants of flavin-containing monooxygenase 3 (*FMO3*) in Japanese subjects identified by phenotyping for trimethylaminuria and found in a database of genome resources

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ABSTRACT

The oxygenation of food-derived trimethylamine to its *N*-oxide is a representative reaction mediated by human flavin-containing monooxygenase 3 (FMO3). Impaired FMO3 enzymatic activity is associated with trimethylaminuria (accumulation of substrate), whereas trimethylamine *N*-oxide (metabolite) is associated with arteriosclerosis. We previously reported *FMO3* single-nucleotide and/or haplotype variants with low FMO3 metabolic capacity using urinary phenotyping and the whole-genome sequencing of Japanese populations. Here, we further analyze Japanese volunteers with self-reported malodor and interrogate an updated Japanese database for novel *FMO3* single-nucleotide and/or haplotype variants. After 3 years of follow up, seven probands were found to harbor the known impaired *FMO3* variant p.(Gly191Cys) identified in the database or novel variants/haplotypes including p.(Met66Val), p.(Arg223Gln), p.(Glu158Lys;Glu308Gly;Arg492Trp), and p.(Glu158Lys;Glu308Gly;Pro496Ser). The known severe mutation p.(Cys197Ter) (a TG deletion) and four variants including p.(Tyr269His) and p.(Pro496Ser) were first detected in the updated genome panel. Among previously unanalyzed FMO3 variants, the trimethylamine/benzydamine *N*-oxygenation activities of recombinant p.(Met66Val), p.(Arg223Gln), p.(Tyr269His), p.(Glu158Lys;Glu308Gly;Arg492Trp), and p.(Glu158Lys;Glu308Gly;-Pro496Ser) FMO3 variant proteins were severely decreased (V_{max}/K_m <10% of wild-type). Although the present novel mutations or alleles were relatively rare, both in self-reported Japanese trimethylaminuria sufferers and in the genomic database panel, three common *FMO3* missense or deletion variants severely impaired FMO3-mediated *N*-oxygenation of trimethylamine.

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1. Introduction

Flavin-containing monooxygenases (FMOs; EC 1.14.13.8) form a complementary enzyme system to the cytochrome P450 (P450) enzyme family, and both must be taken into account during drug development [1,2]. FMOs are a family of well-conserved NADPH-dependent enzymes that oxygenate a range of heteroatom-containing substances, including many drugs [3–5]. Among the five active forms of human FMO (FMO1–FMO5), FMO3 is the prominent form in adult livers [6–8] and it exhibits marked individual variability in oxygenation activities [9]. FMO3 expression commences at the time of birth and increases over time [7], reaching adult levels during adolescence [10]. Moreover, genetic mutations in *FMO3* sometimes cause trimethylaminuria (or fish-odor syndrome) [11,12] as a result of the impaired *N*-oxygenation of food-derived trimethylamine [13]. Odorous trimethylamine is extensively metabolized to odorless trimethylamine *N*-oxide; however, there is a reported association between the

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concentrations of trimethylamine *N*-oxide in blood and atherosclerotic cardiovascular disease [14].

More than 30 different mutations and around 40 polymorphisms of *FMO3* have been reported to date [5,13]. Many of the impaired polymorphic *FMO3* variants associated with the metabolic disorder trimethylaminuria were determined [13]. Genetic testing in combination with traditional urinary phenotyping assays is a useful approach to understanding the molecular basis of the condition and to detect heterozygous carriers. We previously reported phenotype–gene relationship analyses in 428 [15], 787 [16], 171 [17], 640 [18], and 164 [19] Japanese subjects using traditional assays. Additionally, the Tohoku Medical Megabank Project brings together population genomics, medical genetics, and prospective cohort studies to support the establishment of personalized healthcare in Japan [20]. We recently identified rare novel single-nucleotide substitutions in human *FMO3*, e.g., p.(Gly191Cys) and p.(Arg492Gln) variants with extremely low frequencies (<~0.1%), among the whole-genome sequences of the approximately 3500 members of the Japanese population reference panel (3.5K JPN) curated by the Tohoku Medical Megabank Organization [15]. However, the extent of overlap of these newly detected rare *FMO3* alleles between our urinary phenotyped population and the Japanese 3.5K JPN population reference panel is currently unknown.

Against this background, the purpose of the present study was to further investigate both previously reported and novel *FMO3* single-nucleotide variants in Japanese volunteers with self-reported trimethylaminuria and those identified among the updated 4.7K JPN whole-genome sequences of the enlarged Japanese population reference panel (with 1200 new panel members) [20]. Using these two methodologies in different Japanese cohorts, i.e., in self-reported trimethylaminuria sufferers analyzed using urinary phenotyping assays and in the panel of an extensive whole-genome sequence database, the present follow-up study adds to our knowledge of the common functional *FMO3* polymorphisms in populations. In this study, novel variants found in the seven probands with impaired trimethylamine metabolism, novel variants identified in the 4.7K JPN database, and previously unanalyzed variants were evaluated. We report herein three common *FMO3* single-nucleotide or allele variants, namely *FMO3* p.(Gly191Cys), p.(Cys197Ter), and p.(Glu158Lys;Glu308Gly;Pro496Ser) variants, having impaired trimethylamine *N*-oxygenation capacities in combination with phenotyping and whole-genome sequence data in different Japanese cohorts.

2. Materials and methods

2.1. Phenotype/genotype analyses of individuals and a survey of genetic FMO3 variants in an extensive database

Between April 2018 and December 2020, 979 unrelated Japanese subjects (367 male and 612 female subjects) responded to our Internet article seeking volunteers with fish-like body odor and were recruited. The current study follows up our previous investigations of the phenotype–gene relationship in a total of 2190 Japanese subjects as described in five previous reports covering 428 [15], 787 [16], 171 [17], 640 [18], and 164 [19] subjects. Of these 2190 subjects, 1040 were genotyped based on their phenotype. Informed consent was obtained from the subjects and/or from their parents. The current study was approved by the Ethics Committee of Showa Pharmaceutical University. Phenotyping of the newly recruited 979 potential trimethylaminuria sufferers was carried out by evaluating the ratio of the amount of trimethylamine *N*-oxide in urine compared with the total amount of trimethylamine and trimethylamine *N*-oxide as measured by gas chromatography [16,21]. Briefly, the head-space gas was subjected to gas chromatograph Nexis GC-2030 equipped with a headspace sampler HS-20 and a flame ionization detector (Shimadzu, Kyoto, Japan) using a capitally column (Inertcap for amines, 0.32 mm i.d. x 60 m, GL Sciences, Tokyo, Japan) under a carrier helium gas (at a flow rate of 20 mL/min). Genotyping of 71 subjects with impaired phenotypes (around 7% of the new recruits) was done by sequencing analysis of both strands of the polymerase chain reaction products from eight coding *FMO3* exons (2–9) and some intronic, flanking, and 3′-untranslated regions of *FMO3* (a total of 2.9 kb) in DNA prepared from buccal cells, as described previously [15–19]. The human *FMO3* reference used in the current study was the complete gene sequence described in GenBank (accession number NC_000001.10 and AL021026); for example, 171086469, and g. 30386 for *FMO3* p.(Pro496Ser), respectively. In the current study, seven subjects with FMO3 metabolic activity toward trimethylamine *N*-oxygenation <90% of the wild type [15–19] were identified among the 979 newly recruited subjects.

Previously reported and novel *FMO3* single-nucleotide variants were also identified in the whole-genome sequences of the Japanese population reference panel (4.7K JPN) of the Tohoku Medical Megabank Organization, now updated to include around 4700 subjects, as described previously [15,20].

2.2. Expression of recombinant FMO3 variant proteins and enzyme assays

Variant and wild-type FMO3 cDNAs were prepared as previously described [15]. To produce the variant FMO3 proteins, site-directed mutagenesis using a QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) was carried out with the designated primers shown in Table 1. The complete coding regions of the mutagenized and wild-type FMO3 cDNAs were confirmed by repeat sequencing of both strands with primers [15]. Modified and wild-type FMO3 cDNAs were inserted into the pTrc99A expression vector (Pharmacia Biotechnology, Milwaukee, WI, USA) and then transformed into *Escherichia coli* strain JM109. Bacterial membrane fractions expressing FMO3 were prepared from bacterial pellets after centrifugation, as previously described [19]. The amounts of recombinant FMO3 (0.010–0.27 nmol FMO3/mg bacterial protein, Supplemental Table 1) were determined by immunochemical quantification (Supplemental Fig. 1) using an anti-FMO3 antibody (ab126790, Abcam, Cambridge, UK) and comparing the results with those of a recombinant human FMO3 standard (Corning, Woburn, MA, USA), as reported previously [15].

Trimethylamine and benzydamine *N*-oxygenation rates were evaluated as described previously [15–19]. Briefly, a typical

Table 1
Sequences of eight sets of primers used for mutagenesis of *FMO3*.

Primer	Sequence
Met66Val-S	5′-CAACTCTTCCAAAGAGGTGATGTGTTTCCCAG-3′
Met66Val-AS	5′-CTGGGAAACACATCACCTCTTTGGAAGAGTTG-3′
Arg223Gln-S	5′-GGGTGATGAGCCAGGTCTGGGACAATG-3′
Arg223Gln-AS	5′-CATTGTCCCAGACCTGGCTCATCACCC-3′
Tyr269His-S	5′-CAAGCATGAAAACCATGGCTTGATGCC -3′
Tyr269His-AS	5′-GGCATCAAGCCATGGTTTTCATGCTTG -3′
Tyr269Phe-S	5′-CAAGCATGAAAACTTTGGCTTGATGCC -3′
Tyr269Phe-AS	5′-GGCATCAAGCCAAAGTTTTCATGCTTG-3′
Val299Ile-S	5′-GTGGCATTGTGTCCATAAAGCCTAACGTG-3′
Val299Ile-AS	5′-CACGTTAGGCTTTATGGACACAATGCCAC-3′
Cys397Ser-S	5′-GTAATAAAGGGAACTAGTACTTTGCCTTCTA-3′
Cys397Ser-AS	5′-TAGAAGGCAAAGTACTAGTTCCCTTTATTAC-3′
Ile426Thr-S	5′-GCAAAAGCGAGACCACACAGACAGATTACAT-3′
Ile426Thr-AS	5′-ATGTAATCTGTCTGTGTGGTCTCGCTTTTGC-3′
Pro496Ser-S	5′-CCGGTCGTTGAAATCCATGCAGACACG-3′
Pro496Ser-AS	5′-CGTGTCTGCATGGATTTCAACGACCGG-3′

incubation mixture consisted of bacterial membranes (5–10 pmol equivalent FMO3) fortified with an NADPH-generating system (0.25 mM $NADP^+$, 2.5 mM glucose 6-phosphate, and 0.25 units/mL glucose 6-phosphate dehydrogenase) and 20–1000 μM trimethylamine (Fujifilm Wako Pure Chemical, Osaka, Japan) or benzydamine (Sigma-Aldrich, St. Louis, MO, USA) in a final volume of 0.10 mL of 50 mM potassium phosphate buffer (pH 8.4) as recommended previously [9]. The oxygenation reactions were carried out at 37 °C for 10 min and then terminated by adding 0.30 mL of 0.1% formic acid in methanol (15%) and acetonitrile (85%). After centrifugation (4 °C, 20,000×*g*, 10 min), the metabolite concentrations were determined using liquid chromatography/tandem mass spectrometry or liquid chromatography/fluorescence spectrometry as described previously [15–19]. Deuterium-labeled trimethylamine-d_9 and trimethylamine *N*-oxide-d_9 (Sigma-Aldrich) were used as internal standards. Kinetic parameters for the *N*-oxygenations of trimethylamine and benzydamine catalyzed by recombinant human FMO3 proteins were calculated from a curve fitted by nonlinear regression (mean ± standard error, n = 6 substrate concentrations, in triplicated) with Michaelis-Menten equations, using Prism software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Genetic FMO3 variants found in individuals and identified in the 4.7K JPN database

Among the 979 newly recruited unrelated Japanese subjects tested during a further three years of follow-up investigation after previous phenotype–gene relationship analyses of a total of 2026 Japanese subjects [15–19], seven additional probands were found to have less than ~75% of wild-type FMO3 metabolic capacity (Table 2). These seven probands underwent *FMO3* sequence analyses (Fig. 1). Probands 1 and 5 (a 53-year-old man and a 14-year-old boy), with FMO3 metabolic capacities of 26% and 34% (Table 2), respectively, harbored a novel g.16906 A>G substitution that resulted in p.[(Met66Val)] *FMO3* (Fig. 1A) and a g.23869 G>A substitution that resulted in p.[(Arg223Gln)] *FMO3* (Fig. 1C). These *FMO3* p.[(Met66Val)] and p.[(Arg223Gln)] alleles were in the *trans* configuration with *FMO3* p.[(Cys197Ter)] [19]. Probands 6 and 7 (a 3-year-old girl and a 26-year-old woman) with FMO3 metabolic capacities of 66% and 15% (Table 2), respectively harbored a novel g.29270 T>A substitution that resulted in p.[(Cys397Ser)] *FMO3* (Fig. 1D) and a g.30386 C>T substitution that resulted in p.[(Pro496Ser)] *FMO3* (Fig. 1F). These *FMO3* p.[(Cys397Ser)] and p.[(Pro496Ser)] alleles were in the *cis* configuration with *FMO3* p.[(Glu158Lys;Glu308Gly)], which has a moderate trimethylamine *N*-oxygenation activity, as established previously [15,18].

Probands 3 and 4 (a 3-year-old boy and a 29-year-old woman), with 74% and 19% FMO3 metabolic capacity, respectively (Table 2), harbored the p.[(Gly191Cys)] *FMO3* variant that was recently discovered in the 3.5K JPN database [15]. Pedigree analysis of proband 2, who had a low FMO3 metabolic capacity of 48%, found that this 8-year-old girl had compound heterozygous mutations and had inherited the *FMO3* p.(Gly148Ter) mutation (Fig. 1B) from her father, who was heterozygous for the stop codon but had a normal FMO3 metabolic capacity of 98% (Fig. 1G). The *FMO3* p.[(Gly148Ter)] allele in proband 2 was in the *trans* configuration with the known p.[(Arg205Cys)] *FMO3* variant, which was previously found to have moderate trimethylamine *N*-oxygenation activity [19]. In addition, among the 979 most recently recruited subjects, a 3-year-old boy with an FMO3 metabolic capacity of 97% harbored p.[(Glu158Lys;Glu308Gly;Arg492Trp)] *FMO3* (data not shown). This novel *FMO3* p.[(Glu158Lys;Glu308Gly;Arg492Trp)] allele was in the *trans* configuration with the known p.[(Glu158-Lys;Glu308Gly)] *FMO3* variant. The apparent *FMO3* allele frequencies of p.[(Met66Val)], p.[(Arg223Gln)], p.[(Glu158Lys;Glu308Gly;Arg492Trp)], and p.[Glu158Lys;Glu308Gly;Pro496Ser] were low (~1% and ~0.05%) in the 71 of 979 newly recruited unrelated Japanese subjects and in a total of 1111 subjects previously genotyped and phenotyped in combination [15–19], respectively.

In our recent study, we identified eleven novel *FMO3* single nucleotide substitutions in the 3.5K JPN database [15]. In the current study, we interrogated the enlarged 4.7K JPN database and identified an additional four new single nucleotide substitutions in *FMO3*, namely p.(Tyr269His), p.(Tyr269Phe), p.(Ile426Thr), and p.(Pro496Ser), and two known *FMO3* variants, p.(Cys197Ter) and p.(Val299Ile) with rs numbers (i.e., Reference SNP cluster IDs in The National Center for Biotechnology Information) (Table 3). The allele frequencies of these variants other than p.(Cys197Ter) were extremely low (0.01%, Table 3). A synonymous *FMO3* variant, p.(Glu281Glu), was also newly found in the 4.7K JPN database.

3.2. Catalytic function of recombinant variant FMO3 proteins identified by phenotyping or whole-genome sequence data in Japanese cohorts

The ten novel or previously unanalyzed FMO3 variants Met66Val, Arg223Gln, Tyr269His, Tyr269Phe, Val299Ile, Glu158Lys;Glu308-Gly;Cys397Ser, Ile426Thr, Glu158Lys;Glu308Gly;Arg492Trp, Pro49 6Ser, and Glu158Lys;Glu308Gly;Pro496Ser and wild-type FMO3 protein were recombinantly expressed in bacterial membranes. Tyr269Phe, Val299Ile, Glu158Lys;Glu308Gly;Cys397Ser, Ile426Thr, and Pro496Ser variant FMO3 proteins exhibited almost normal or slightly modulated trimethylamine and benzydamine *N*-oxygenation activities (Table 4). In contrast, recombinant Met66Val, Arg223Gln, Tyr269His, Glu158Lys;Glu308Gly;Arg492Trp, and Glu158Lys;-Glu308Gly;Pro496Ser FMO3 proteins had much lower trimethylamine and benzydamine *N*-oxygenation activities than those of wild-type FMO3 protein (V_{max}/K_m <10% that of the wild-type, Table 4).

Table 2
In vivo FMO3 metabolic capacity determined from urine tests of seven probands harboring four novel and five known *FMO3* variants.

Proband	Age (years)/gender	*FMO3* genotype	FMO3 metabolic capacity (%)
1	53/Male	p.[(Met66Val)];[(Cys197Ter)]	26
2	8/Female	p.[(Gly148Ter)];[(Arg205Cys)]	48
3	3/Male	p.[(Val58Ile)];[(Gly191Cys)]	74
4	29/Female	p.[(Gly191Cys)];[(Cys197Ter)]	19
5	14/Male	p.[(Cys197Ter)];[(Arg223Gln)]	34
6	3/Female	p.[(Glu158Lys;Glu308Gly)]; [(Glu158Lys;Glu308Gly;Cys397Ser)]	66
7	26/Female	p.[(Glu158Lys;Glu308Gly;Pro496Ser)]; [(Cys197Ter)]	15

Four novel variants of *FMO3*, i.e., p.[(Met66Val)], p.[(Arg223Gln)], p.[(Glu158Lys;Glu308Gly;Cys397Ser)], and p.[(Glu158Lys;Glu308Gly;Pro496Ser)], and five known mutations of *FMO3*, i.e., p.[(Val58Ile)], p.[(Gly191Cys)], p.[(Cys197Ter)], p.[(Arg205Cys)], and p.[(Glu158Lys;Glu308Gly)], were detected.

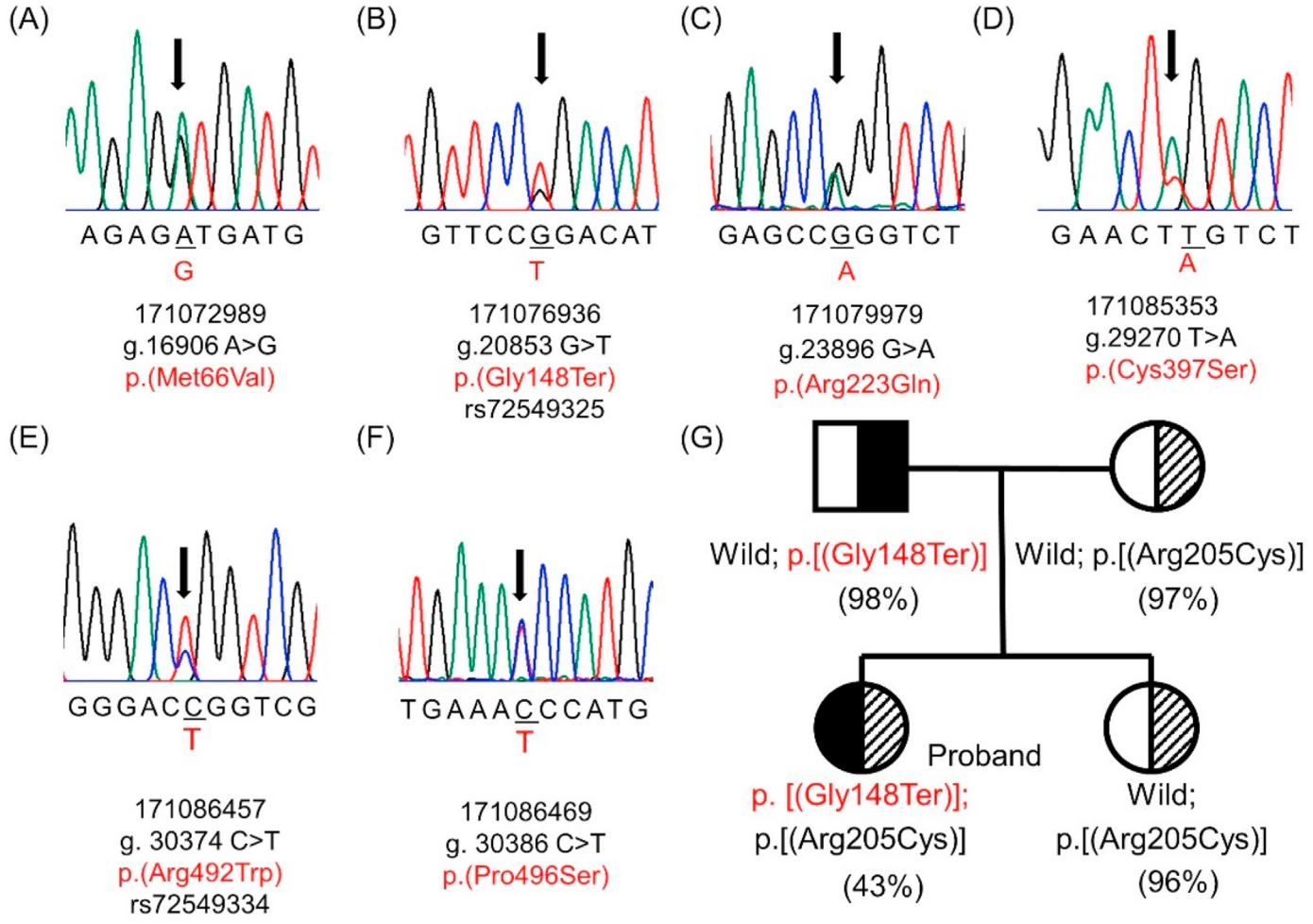

Fig. 1. Nucleotide sequences of novel and known *FMO3* variants (A–F) found in seven probands and a pedigree analysis of proband 2 (G) for the presence of *FMO3* variants. Both strands were sequenced and the sequences are shown only for sense strands of genomic DNA from probands harboring one of four novel single-nucleotide mutations of *FMO3*, i.e. (A) p.(Met66Val), (C) p.(Arg223Gln), (D) p.(Cys397Ser), and (F) p.(Pro496Ser), or one of two known single-nucleotide mutations of *FMO3*, i.e., (B) p.(Gly148Ter) and (E) p.(Arg492Trp). (G) Proband 2 had a low FMO3 metabolic capacity of 43% and inherited a g.20853 G>T mutation in *FMO3* [that resulted in p.(Gly148Ter)] from his father, who was heterozygous for the stop codon mutation but had a normal FMO3 metabolic capacity of 98%. Familial studies were not possible for the other probands. The complete human *FMO3* gene sequence given in GenBank (Accession Number NC_000001.10 and AL021026) was used as the reference.

Table 3
Novel and known *FMO3* variants, rs numbers, and allele frequencies identified in the updated 4.7K JPN database of the Tohoku Medical Megabank Organization.

Position	Reference allele	Altered allele	Exon	Amino acid change	dbSNP rs number	Allele frequency, %
I. Frameshift or missense *FMO3* variants						
171077323	c.590CTG	C	5	Cys197fs[a]	rs3832024	0.23
171080116	c.805T	C	7	Tyr269His		0.01
171080117	c.806A	T	7	Tyr269Phe		0.01
171083214	c.895G	A	7	Val299Ile	rs774663327	0.01
171086260	c.1277T	C	9	Ile426Thr		0.01
171086469	c.1486C	T	9	Pro496Ser		0.01
II. Synonymous *FMO3* variant						
171083162	c.843G	A	7	Glu281Glu	rs756543605	0.01

A TG deletion with a T/A substitution in *FMO3* exon 5 (… TG(TG/-)AT/AAT…) would cause the known frameshift p.(Cys197Ter;Asp198Glu) variant. The complete human *FMO3* gene sequence given in GenBank [Genome Reference Consortium Human Build 37 (GRCh37) p13 chr 1, Accession Number NC_000001.10] was used as the reference.

4. Discussion

In humans, FMO3 mediates the *N*-oxygenation of therapeutic drugs and food-derived trimethylamine [1,2]. The recent clinical impacts of trimethylamine *N*-oxide and trimethylamine in the blood should be noted in relation to atherosclerotic cardiovascular disease [14] and trimethylaminuria, respectively [11,12]. In the current study, novel variants found in the seven probands with impaired trimethylamine metabolism, novel variants identified in the 4.7K JPN database, and previously unanalyzed variants were evaluated. We identified four novel mutations of *FMO3*, i.e., *FMO3* p.(Met66Val), p.(Arg223Gln), p.(Cys397Ser), and p.(Pro496Ser) (Fig. 1) in Japanese subjects phenotyped for FMO3 using urine tests (Table 2). The heterozygous *FMO3* p.(Gly148Ter) mutation (see in

Table 4
Kinetic analyses of trimethylamine and benzydamine *N*-oxygenations by recombinant FMO3 proteins (wild-type and ten variants).

	Trimethylamine *N*-oxygenation			Benzydamine *N*-oxygenation		
Amino acid change	K_m (μM)	V_{max} (min^{-1})	V_{max}/K_m, (%)	K_m (μM)	V_{max} (min^{-1})	V_{max}/K_m, (%)
Wild type	23 ± 1	100 ± 1	4.4 (100)	71 ± 8	170 ± 5	2.4 (100)
Met66Val[a]	45 ± 4	5.6 ± 0.1	0.12 (3)	31 ± 12	6.2 ± 0.5	0.20 (8)
Arg223Gln[a]	28 ± 3	0.2 ± 0.1	0.01 (1)	52 ± 18	0.5 ± 0.1	0.01 (1)
Tyr269His[b]	35 ± 8	2.4 ± 0.1	0.07 (2)	110 ± 24	7.8 ± 0.5	0.07 (3)
Tyr269Phe[b]	23 ± 1	93 ± 1	4.1 (92)	54 ± 5	140 ± 4	2.6 (110)
Val299Ile[c]	19 ± 1	92 ± 1	4.8 (110)	59 ± 8	150 ± 5	2.5 (100)
Glu158Lys;Glu308Gly;Cys397Ser[a]	24 ± 2	89 ± 1	3.7 (84)	56 ± 6	120 ± 2	2.1 (88)
Ile426Thr[b]	57 ± 5	110 ± 3	1.9 (43)	84 ± 14	150 ± 7	1.8 (75)
Glu158Lys;Glu308Gly;Arg492Trp[c]	28 ± 13	0.7 ± 0.1	0.03 (1)	25 ± 13	3.8 ± 0.4	0.15 (6)
Pro496Ser[b]	26 ± 1	120 ± 1	4.7 (110)	64 ± 7	200 ± 6	3.1 (130)
Glu158Lys;Glu308Gly;Pro496Ser[a]	41 ± 9	13 ± 1	0.32 (7)	47 ± 18	15 ± 1	0.32 (13)

Kinetic parameters were calculated from a fitted curve by nonlinear regression (mean ± standard error, n = 6 substrate concentrations in the range 20–1000 μM) with Michaelis-Menten equations: $v = V_{max}[S]/(K_m + [S])$.
[a] Novel variants found in the seven probands with impaired trimethylamine metabolism.
[b] Novel variants identified in the 4.7K JPN database.
[c] Previously unanalyzed variants.

proband 2) was previously reported in one Korean subject identified using caffeine urine tests [22]. In the present study, we describe the novel *FMO3* variants p.(Met66Val) (Fig. 1A) and p.(Arg492Trp) (Fig. 1E), in which the amino acid substitutions are in the identical positions as the previously identified *FMO3* p.(Met66Ile) [23] and p.(Arg492Gln) [15] variants. The *FMO3* allele p.[(Glu158Lys;Arg492Trp)] reportedly has low trimethylamine *N*-oxygenation activity in Europeans [24]. The new *FMO3* p.[(Glu158Lys;Glu308Gly;Arg492Trp)] allele resulted in a much lower trimethylamine *N*-oxygenation activity (7%) than wild-type FMO3 (Table 4). The *FMO3* p.(Gly191Cys) mutation, newly identified in the 3.5K JPN database, exhibited severely decreased FMO3 activities (V_{max}/K_m <10% of wild-type FMO3) [15] and was detected in two independent probands 3 and 4 in the present phenotyping study (Table 2). Four further novel *FMO3* mutations were identified in the enlarged 4.7K JPN genome database (Table 3). Although single-nucleotide *FMO3* variations resulting in stop codons, e.g., p.(Trp41Ter), p.(Gln470Ter), and p.(Arg500Ter), were previously detected in the 3.5K JPN database [15], a known severe *FMO3* mutation [a TG deletion with a T/A substitution, p.(Cys197Ter;Asp198Glu)] and four new variants, including *FMO3* p.(Tyr269His) that results in diminished FMO3 activities, were first detected in the updated 4.7K JPN genome panel.

The recombinant p.(Met66Val), p.(Arg223Gln), p.(Tyr269His), p.(Glu158Lys;Glu308Gly;Arg492Trp), and p.(Glu158Lys;Glu308Gly;Pro496Ser) variant FMO3 proteins expressed in *E. coli* membranes exhibited lower trimethylamine and benzydamine *N*-oxygenations than those of wild-type FMO3 in *in vitro* systems (Table 4). It should be noted that the *FMO3* p.(Pro496Ser) variant was identified in the database, whereas the *FMO3* p.(Glu158Lys;-Glu308Gly;Pro496Ser) haplotype was identified in a self-reported trimethylaminuria subject with a low-FMO3-activity phenotype. Interestingly, the single-nucleotide substitution p.(Pro496Ser) did not alter the catalytic function of the recombinantly expressed FMO3 protein; however, the combined *FMO3* p.(Glu158Lys;-Glu308Gly;Pro496Ser) haplotype had a greatly decreased function as measured using recombinantly expressed FMO3 protein (Table 4). We reported several p.(Glu158Lys;Gln470Ter), p.(Val257Met;Trp388Ter), and p.(Arg500Ter) variant FMO3 proteins with no detectable activity and p.(Glu158Lys;Thr201Lys;-Glu308Gly) FMO3 protein with a much reduced trimethylamine *N*-oxygenation activity [13]. Although we recently reported that limited drug interactions could occur between drugs that are FMO3 substrates and food-derived trimethylamine [25], it should be noted that haplotype analysis for *FMO3* will be important to establish personalized healthcare in the future. In addition to those previously identified human variant *FMO3* alleles [15], homozygous or compound heterozygous mutations of any of the known and new frameshift, nonsense, or missense variant *FMO3* alleles may lead to reduced or seriously impaired trimethylamine *N*-oxygenation activity *in vivo*.

The *FMO3* variants p.(Gly191Cys) and p(Cys197Ter) and the *FMO3* haplotype p.(Glu158Lys;Glu308Gly;Pro496Ser) were rare in both Japanese self-reported trimethylaminuria sufferers (a total of ~1000 individuals) and in the updated 4.7K JPN database with 1200 new panel members (0.01–0.23%). In conclusion, using two different analytical approaches (phenotype analysis of self-reported trimethylaminuria sufferers and whole-genome sequence data from a Japanese cohort) we found common missense or deletion *FMO3* variants that severely impaired FMO3-mediated *N*-oxygenation of malodorous trimethylamine.

Author contributions

M. Shimizu, N. Koibuchi, and A. Mizugaki mainly carried out traditional urinary FMO3 metabolic capacity analysis and genotyping. E. Hishinuma, S. Saito, and M. Hiratsuka mainly analyzed the updated 4.7K JPN database. H. Yamazaki designed the research and mainly wrote the manuscript. All authors gave final approval of the manuscript.

Declaration of competing interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dmpk.2021.100387.

References

[1] Cashman JR. Role of flavin-containing monooxygenase in drug development. Expet Opin Drug Metabol Toxicol 2008;4(12):1507–21.

[2] Taniguchi-Takizawa T, Kato H, Shimizu M, Yamazaki H. Predicted contributions of flavin-containing monooxygenases to the N-oxygenation of drug candidates based on their estimated base dissociation constants. Curr Drug Metabol 2020. https://doi.org/10.2174/1389200221666201207195758. In press.

[3] Krueger SK, Williams DE. Mammalian flavin-containing monooxygenases: structure/function, genetic polymorphisms and role in drug metabolism. Pharmacol Ther 2005;106(3):357–87.

[4] Cashman JR, Zhang J. Human flavin-containing monooxygenases. Annu Rev Pharmacol Toxicol 2006;46:65–100.

[5] Phillips IR, Shephard EA. Flavin-containing monooxygenase 3 (FMO3): genetic variants and their consequences for drug metabolism and disease. Xenobiotica 2020;50(1):19–33.

[6] Koukouritaki SB, Hines RN. Flavin-containing monooxygenase genetic polymorphism: impact on chemical metabolism and drug development. Pharmacogenomics 2005;6(8):807–22.

[7] Shimizu M, Denton T, Kozono M, Cashman JR, Leeder JS, Yamazaki H. Developmental variations in metabolic capacity of flavin-containing mono-oxygenase 3 in childhood. Br J Clin Pharmacol 2011;71(4):585–91.

[8] Hernandez D, Janmohamed A, Chandan P, Phillips IR, Shephard EA. Organization and evolution of the flavin-containing monooxygenase genes of human and mouse: identification of novel gene and pseudogene clusters. Pharmacogenetics 2004;14(2):117–30.

[9] Nagashima S, Shimizu M, Yano H, Murayama N, Kumai T, Kobayashi S, et al. Inter-individual variation in flavin-containing monooxygenase 3 in livers from Japanese: correlation with hepatic transcription factors. Drug Metabol Pharmacokinet 2009;24(3):218–25.

[10] Koukouritaki SB, Simpson P, Yeung CK, Rettie AE, Hines RN. Human hepatic flavin-containing monooxygenases 1 (FMO1) and 3 (FMO3) developmental expression. Pediatr Res 2002;51(2):236–43.

[11] Shephard EA, Treacy EP, Phillips IR. Clinical utility gene card for: trimethylaminuria – update 2014. Eur J Hum Genet 2015;23(9).

[12] Yamazaki H, Shimizu M. Genetic polymorphism of the flavin-containing monooxygenase 3 (FMO3) associated with trimethylaminuria (fish odor syndrome): observations from Japanese patients. Curr Drug Metabol 2007;8(5):487–91.

[13] Yamazaki H, Shimizu M. Survey of variants of human flavin-containing monooxygenase 3 (FMO3) and their drug oxidation activities. Biochem Pharmacol 2013;85(11):1588–93.

[14] Sheng Z, Tan Y, Liu C, Zhou P, Li J, Zhou J, et al. Relation of circulating trimethylamine N-oxide with coronary atherosclerotic burden in patients with ST-segment elevation myocardial infarction. Am J Cardiol 2019;123(6):894–8.

[15] Shimizu M, Yoda H, Nakakuki K, Saso A, Saito I, Hishinuma E, et al. Genetic variants of flavin-containing monooxygenase 3 (FMO3) derived from Japanese subjects with the trimethylaminuria phenotype and whole-genome sequence data from a large Japanese database. Drug Metabol Pharmacokinet 2019;34(5):334–9.

[16] Shimizu M, Yoda H, Igarashi N, Makino M, Tokuyama E, Yamazaki H. Novel variants and haplotypes of human flavin-containing monooxygenase 3 gene associated with Japanese subjects suffering from trimethylaminuria. Xenobiotica 2019;49(10):1244–50.

[17] Shimizu M, Origuchi Y, Ikuma M, Mitsuhashi N, Yamazaki H. Analysis of six novel flavin-containing monooxygenase 3 (FMO3) gene variants found in a Japanese population suffering from trimethylaminuria. Mol Genet Metab Rep 2015;5:89–93.

[18] Shimizu M, Kobayashi Y, Hayashi S, Aoki Y, Yamazaki H. Variants in the flavin-containing monooxygenase 3 (FMO3) gene responsible for trimethylaminuria in a Japanese population. Mol Genet Metabol 2012;107(3):330–4.

[19] Yamazaki H, Fujita H, Gunji T, Zhang J, Kamataki T, Cashman JR, et al. Stop codon mutations in the flavin-containing monooxygenase 3 (FMO3) gene responsible for trimethylaminuria in a Japanese population. Mol Genet Metabol 2007;90(1):58–63.

[20] Yasuda J, Kinoshita K, Katsuoka F, Danjoh I, Sakurai-Yageta M, Motoike IN, et al. Genome analyses for the Tohoku medical Megabank Project towards establishment of personalized healthcare. J Biochem 2019;165(2):139–58.

[21] Zhang AQ, Mitchell SC, Ayesh R, Smith RL. Determination of trimethylamine and related aliphatic amines in human urine by head-space gas chromatography. J Chromatogr 1992;584(2):141–5.

[22] Park CS, Chung WG, Kang JH, Roh HK, Lee KH, Cha YN. Phenotyping of flavin-containing monooxygenase using caffeine metabolism and genotyping of FMO3 gene in a Korean population. Pharmacogenetics 1999;9(2):155–64.

[23] Akerman BR, Forrest S, Chow L, Youil R, Knight M, Treacy EP. Two novel mutations of the FMO3 gene in a proband with trimethylaminuria. Hum Mutat 1999;13(5):376–9.

[24] Dolphin CT, Janmohamed A, Smith RL, Shephard EA, Phillips IR. Compound heterozygosity for missense mutations in the flavin-containing monooxygenase 3 (FMO3) gene in patients with fish-odour syndrome. Pharmacogenetics 2000;10(9):799–807.

[25] Shimizu M, Uehara S, Suemizu H, Yamazaki H. *In vivo* drug interactions of itopride and trimethylamine mediated by flavin-containing monooxygenase 3 in humanized-liver mice. Drug Metabol Pharmacokinet 2021;37:100369.