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Alteration of Substrate Specificity by Common Variants, E158K/E308G and V257M, in Human Hepatic Drug-metabolizing Enzyme, Flavin-containing Monooxygenase 3

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Our earlier studies found a significant correlation between the activities of ranitidine *N*-oxidation catalyzed by hepatic flavin-containing monooxygenase (FMO) and the presence of mutations in exon 4 (E158K) and exon 7 (E308G) of the *FMO3* gene in Korean volunteers. However, caffeine *N*-1 demethylation (which is also partially catalyzed by FMO) was not significantly correlated with these *FMO3* mutations. In this study, we examined another common mutation (V257M) in exon 6 of *FMO3* gene. The V257M variant, which is caused by a point mutation (G769A), was commonly observed (13.21% allele frequency) in our subjects (n=159). This point mutation causes a substitution of Val^{257} to Met^{257}, with transformation of the secondary structure. The presence of this mutant allele correlated significantly with a reduction in caffeine *N*-1-demethylating activity, but was not correlated with the activity of *N*-oxidation of ranitidine. In a family study, the low FMO activity observed in a person heterozygous for a nonsense mutation in exon 4 (G148X) and heterozygous for missense mutation in exon 6 (V257M) of *FMO3* was attributed to the mutations. Our results suggest that various point mutations in the coding regions of *FMO3* may influence FMO3 activity according to the probe substrates of varying chemical structure that correlate with each mutation on the *FMO3* gene.

Key Words: Flavin-containing monooxygenase, Polymorphism, Caffeine, Ranitidine, Substrate specificity, Phenotype, Genotype, Human

INTRODUCTION

Flavin-containing monooxygenase (FMO; EC 1.14.13.8) is a family of isozymes involved in the oxidative metabolism of many clinically useful drugs, plant alkaloids and endogenous chemicals containing N, S and P atoms (reviewed by Cashman, 2002a). Among the five FMO isozymes identified in humans, FMO3 is the major enzyme present in adult human liver and is thought to be responsible for most of the *in vivo* hepatic metabolism in human (Lomri et al, 1992; Dolphin et al, 1996; Overby et al, 1997).

Recently, several important mutations in the human *FMO3* gene responsible for fish-odor syndrome (FOS) or trimethylaminuria (TMAU) have been found (reviewed by Cashman, 2002b). In addition to these mutations causing TMAU, we have identified several other FMO mutations, including E158K and E308G as variants (Park et al, 1999; Kang et al, 2000), which are not the causes of TMAU. FMO3 oxidizes many substrates other than trimethylamine (TMA). In addition, we have demonstrated that the production of theobromine (TB) from caffeine (CA) by *N*-1 demethylation is catalyzed in the most part by the FMO contained in adult human liver microsomes (Chung & Cha, 1997; Chung et al, 1998). Based on this information, we developed a "coffee test" for determining the *in vivo* FMO activity by comparing the urinary molar concentration ratio of TB to CA and used this FMO phenotyping method to determine the *in vivo* activities of normal Korean volunteers (Chung et al, 1999). In our previous study, we identified several missense mutations, including E158K and E308G, and a nonsense mutation, G148X, in those with low CA *N*-1 demethylation activities (Park et al, 1999). However, attempts to explain the reduced FMO activity determined by the "coffee test" with these missense mutations did not reveal significant correlation, suggesting that the reduced FMO activity catalyzing the *N*-1 demethylation of CA may be explained by other mutations or these identified *FMO* variant mutations may affect the metabolism of other FMO substrates. Thus, we have developed a new method for determining the *in vivo* FMO3 activity by comparing the urinary molar concentration ratio of ranitidine *N*-oxide to ranitidine (RANO/RA) (Kang et al, 2000). The phenotypes of FMO activities determined by RANO/RA provided significant correlation with *FMO3* genotypes identified by E158K and E308G.

In an effort to identify the specific *FMO3* mutations

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ABBREVIATIONS: FMO, flavin-containing monooxygenase; TMA, trimethyamine; TMAU, trimethyaminuria; FOS, fish-odour syndrome; CA, caffeine; TB, theobromine; RA, ranitidine.

associated with the FMO phenotypes determined by N-1 demethylation of CA (TB/CA), we have compared whether a newly found FMO3 mutation, V257M, could be matched with the FMO phenotypes determined by TB/CA ratio.

In this article, we report that the V257M mutation in *FMO3* provides significant correlation with the FMO phenotypes determined by the "coffee test" (TB/CA), but not with those determined by the *N*-oxidation of RA (RANO/RA). Furthermore, we report that the low TB/CA FMO activities, heretofore-unable to explain either by the presence of nonsense mutation (G148X) or by the missense mutations (E158K and E308G) (Park et al, 1999), may now be explained with the newly found V257M mutation.

METHODS

Subjects and study protocol

A total of 159 young healthy volunteers participated in this study. All volunteers provided written informed consent and were judged healthy according to their medical history, physical examination and routine laboratory analyses. They were asked not to take any beverages containing caffeine or foods containing choline (such as egg, liver and fish) for at least two days before the study. None of the subjects were using any concurrent medications. This study was approved by the Institutional Review Board of Inha University Hospital (Inchon, Korea).

Results of the FMO phenotyping study obtained with CA metabolism, and the FMO3 genotyping study for E158K and E308G mutations in 71 volunteers in our previous study (Park et al, 1999), were used for comparison. Briefly, the volunteers provided control urine early in the morning after an overnight fast (10 h) and were given a cup of instant coffee (200 mL; 110 mg CA *in toto*). A 1 h urine sample was collected between 4 h and 5 h after the oral administration of coffee, and an aliquot was analyzed for metabolites of CA, using HPLC as described in our previous study (Park et al, 1999).

Results obtained from the FMO phenotyping study using RA metabolism in 88 volunteers (a different set from the CA study) were used for comparison. Briefly, the volunteers provided control urine after an overnight fast and were given a tablet containing 150 mg RA (Zantac®, Glaxo Wellcome Korea, Ltd.) with 200 mL water. After collecting 10 mL of peripheral blood, the subjects were asked to collect 08 h bulked urine, as described by Kang et al. (2000). The volume of bulked urine was measured, RA metabolites were then analyzed using HPLC by the method described previously (Kang et al, 2000).

FMO3 genotype analysis for V257M mutation

Genomic DNA from 159 volunteers used in the previous (Park et al, 1999) and the present studies were used. The oligonucleotides used for the initial PCR amplification, which leads to the detection of the exon 6 mutation (V257M) in *FMO3*, were designed from the intronic sequences (sense: 5′ −GTC CAC CAG AAT ATC CAC TAA C−3′ and antisense: 5′ −GCC AGC AGG CAT ATC ACG TT−3′). Amplified PCR product (296 bp) was used as a template for the site-specific second PCR to detect the presence of G769A substitution producing FMO3/Met^{257} mutant allele. In this second PCR, two site-specific antisense oligonucleotides (5′−GCA TTC ATC TGC TTC AC−3′ and 5′ −GCA TTC ATC TGC TTC AT −3′) were adopted for the detection of wild type and mutant alleles. The conditions for the site-specific second PCR were 1 min pre-denaturation at 94°C, 15 cycles (30 cycles in the first PCR) of 1 min at 94°C, 1 min at 57°C and 1 min at 72°C, followed by 10 min at 72°C for the post-synthesis step. The DNA fragment (204 bp) obtained from this second PCR was analyzed for the presence of the V257M (G769A) mutation on a low melting 2% agarose gel (NuSieve GTG, FMC, Rockland, ME) and was viewed on an ultraviolet trans-illuminator after staining with ethidium bromide.

Pedigree study for one family

One individual with very low FMO activity (TB/CA=0.64) was found to be heterozygous for a premature stop codon (G148X) mutation and for the VM mutant genotype. Thus, we recruited his family members and examined them for the presence of the V257M mutation (genotyping) and determined their FMO activities using the CA metabolism (phenotyping). All members of his family had normal renal and hepatic functions and were in good health without apparent TMAU. For the exon 4 (E158K) and exon 7 (E308G) mutations in *FMO3*, all of his family members were found to have wild type alleles (i.e., FMO3/Glu^{158} and FMO3/Glu^{308}).

Statistical analysis

Data in Figs. 1 and 2 indicate the median values of each group or subgroup for comparison. Statistical significances were analyzed by the Mann-Whitney *U* test, or Kruskal-Wallis test with Dunn's post-hoc test set with significance at $p<0.05$. Data presented in the text are expressed in terms of mean±S.D.

RESULTS

Correlation between TB/CA phenotypes and FMO3 V257M mutation

The 71 volunteers who had been phenotyped for their TB/CA FMO activities and genotyped for the presence of mutations in exon 4 and exon 7, in our previous study (Park et al, 1999), were genotyped again for the presence of the FMO3/Met^{257} mutant allele in exon 6. The mean TB/CA FMO activities for the groups that were homozygous wild type (VV) and heterozygous for the mutant (VM) genotypes were 3.12 ± 2.43 and 1.59 ± 1.18, respectively. As shown in Fig. 1A, the difference was statistically significant ($p<0.001$, Table 1). There were no subjects homozygous for the mutant genotype (MM) in this group.

Presence of exon 4 and exon 7 mutations in the *FMO3* of these 71 volunteers had previously been identified by using restriction enzymes, i.e., *Hin*fI (E158K) (wild type 'H' and mutant type 'h') and *Dra*II (E308G) (wild type 'D' and mutant type 'd'). The exon 4 and exon 7 mutations were linked in *cis*-form, and the volunteers had 'HHDD', 'HhDd', 'hhdd', 'HhDD' and 'HHDd' *FMO3* genotypes (Park et al, 1999; Kang et al, 2000). When the TB/CA phenotypes were compared with the presence of exon 4 and exon 7 *FMO3* genotypes, no significant correlation was found (Fig. 1B, Table 1). Furthermore, when the TB/CA FMO activities of those in the 'HHDD' subgroup with FMO3/Met^{257} mutant

Table 1.

Genotypes	E158K/E308G				V257M	
	HHDD	HhDd	hhdd	h/d[c]	VV	VM
No. (n=71)	42	18	7	4	48	23
Caffeine[a]	2.75±2.42	2.61±2.28	2.50±1.31	1.56±0.38	3.12±2.43	1.59±1.18[e]
95% CI	1.99~3.50	1.48~3.74	1.30~3.71	0.96~2.16	2.41~3.82	1.08~2.10
No. (n=88)	59	23	1	5	69	19
Ranitidine[b]	12.10±2.90	9.19±2.75[d]	8.11	10.82±3.18	10.97±3.17	12.25±2.76
95% CI	11.35~12.86	7.99~10.37	–	6.88~14.77	10.21~11.73	10.92~13.58

Data are shown as mean±SD of FMO activities obtained from TB/CA ratio for caffeine (a) and RANO/RA for ranitidine (b) The letter of 'h/d' indicates people with HHDd (n=1) or HhDD (n=3) genotype in a total of 71 persons (caffeine), and hhDD (n=1), HhDD (n=3) or HHDd (n=1) in a total of 88 persons (ranitidine) (c) $P<0.001$ compared with the HHDD genotype group for ranitidine (d) $P<0.001$ compared with the VV genotype group for caffeine (e).

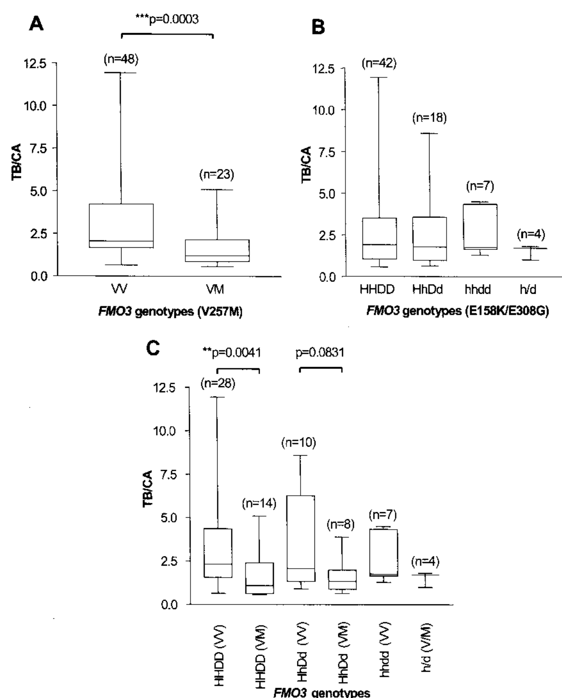

Fig. 1. Correlation between TB/CA FMO phenotypes and *FMO3* genotypes. FMO phenotypes were determined by urinary TB/CA ratio. They significantly correlated with *FMO3* genotypes analyzed by the presence or absence of V257M mutation ($p<0.001$) (A). FMO activities (TB/CA) did not have any significant correlation with the *FMO3* genotypes determined by E158K ('H' and 'h' alleles) and E308G ('D' and 'd' alleles) mutations (B). The decreased TB/CA FMO activities were significantly correlated with the presence of the V257M mutation ($p<0.01$) within the 'HHDD' wild type, but were not significantly different ($p>0.05$) within the 'HhDd' heterozygous mutant type (C). Numbers in parentheses indicate the number of subjects in each *FMO3* genotype subgroup, and horizontal bars within box represent the median values in Fig. 1 and 2.

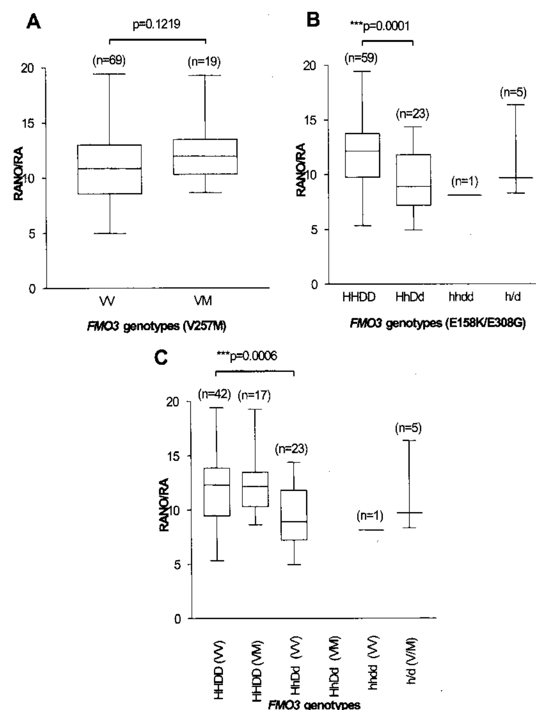

Fig. 2. Correlation between RANO/RA FMO phenotypes and *FMO3* genotypes. FMO phenotypes were determined by the urinary RANO/RA ratio. They did not correlate with the *FMO3* genotypes analyzed by the presence or absence of the V257M mutation (A). FMO activities (RANO/RA) showed a significant correlation (Kruskal-Wallis with Dunn's post-hoc test; $p<0.01$) with the *FMO3* genotypes determined by the presence of both E158K ('H' and 'h' alleles) and E308G ('D' and 'd' alleles) mutations (B). The RANO/RA FMO activities were not correlated.

allele were compared, significant correlation was found (Fig. 1C, Table 2). Among the 42 subjects with wild type 'HHDD', the subgroup with the homozygous wild type exon 6 genotype (Val^{257}/Val^{257}, n=28) had a significantly higher mean TB/CA FMO activity (3.31±2.65) than the subgroup that was heterozygous for the exon 6 genotype (V^{257}/M^{257}, n=14, 1.61±1.31) ($p<0.01$). Within the group with the heterozygous 'HhDd' genotype, the subgroup homozygous for the wild genotype for exon 6 (V^{257}/V^{257}, n=10) had the higher mean TB/CA FMO activity of 3.40±2.71 than the subgroup with the heterozygous V^{257}/M^{257} genotype (n=8; 1.62±1.08), but this was not significantly different (p=0.0831, Fig. 1C). These results indicate that the V257M mutation has a significant effect on the TB/CA FMO3 activity.

Table 2.

Genotypes	E158K/E308G/V257M					
	HHDD/VV	HHDD/VM	HhDd/VV	HhDd/VM	Hhdd/VV	h/d (V/M)[c]
No. (n=71)	28	14	10	8	7	4
Caffeine[a]	3.31±2.65	1.61±1.31[d]	3.40±2.71	1.62±1.08	2.50±1.31	1.56±0.38
95% CI	2.28~4.34	0.85~2.37	1.46~5.34	0.72~2.52	1.30~3.71	0.96~2.16
No. (n=88)	42	17	23	0	1	5
Ranitidine[b]	11.97±2.94	12.43±2.84	9.19±2.75[e]	–	8.11	10.82±3.18
95% CI	11.06~12.89	10.97~13.89	7.99~10.37	–	–	6.88~14.77

Data are shown as mean±SD of FMO activities obtained from TB/CA ratio for caffeine (a) and RANO/RA for ranitidine (b) The letter of 'h/d (V/M)' indicates people with HHDd/VM (n=1) or HhDD/VV (n=3) genotype in a total of 71 persons (caffeine), and hhDD/VV (n=1), HhDD/VV (n=2), HhDD/VM (n=1) or HHDd/VV (n=1) in a total of 88 persons (ranitidine) (c) $P<0.01$ compared with the HHDD/VV genotype group for ranitidine (d) $P<0.001$ compared with the HHDD/VV genotype group for caffeine (e).

Correlation between RANO/RA FMO phenotypes and FMO3 V257M mutation

The 88 volunteers, who had been phenotyped for their RANO/RA FMO activities and genotyped for the presence of exon 4 and exon 7 mutations in a population, were genotyped again for the presence of the exon 6 mutation (V257M). The mean (RANO/RA) FMO activities for the wild type (V^{257}/V^{257}) and heterozygous mutant (V^{257}/M^{257}) groups were 10.97±3.17 (n=69) and 12.25±2.76 (n=19), respectively (Fig. 2A, Table 1). They were not significantly different (p=0.1219). Volunteers with wild genotypes for exon 4 and exon 7 mutations showed significantly higher RANO/RA activity (12.10±2.90) than those with heterozygous 'HhDd' genotype (9.19±2.75, p<0.01, Fig. 2B). Among the volunteers with the wild 'HHDD' genotype (n=59), the subgroup with the wild genotype for exon 6 (V^{257}/V^{257}) (n=42) had a mean RANO/RA FMO activity of 11.97±2.94 and the heterozygous subgroup (V^{257}/M^{257}) (n=17) had 12.43±2.84, which is not significantly different (p=0.7696) (Fig. 2C, Table 2). Within the group with the heterozygous 'HhDd' genotype (n=23), the subgroup with the wild genotype for exon 6 (V^{257}/V^{257}) (n=23) had a mean RANO/RA FMO activity of 9.19±2.75, and the activities were significantly lower than those with 'HHDD' with a genotype of exon 6 V^{257}/V^{257} (p<0.001, Fig. 2C, Table 2). The one individual homozygous for the exon 4 and exon 7 mutant alleles ('hhdd') had low RANO/RA FMO activities, even though homozygous for the wild type exon 6 allele (V^{257}/V^{257}) (8.11). Thus, as shown in our previous study (Kang et al, 2000), although the mean RANO/RA FMO activity was significantly affected by exon 4 and exon 7 mutations (E158K and E308G, respectively), the RANO/RA FMO activities of the subgroups carrying exon 6 mutant alleles were not significantly different. These results indicate that the V257M mutation does not affect RANO/RA FMO3 activity.

Pedigree study of a person with FMO3/Stop[148] and FMO3/Met[257] mutant alleles

Family members of the person who had low TB/CA FMO activity (0.64), with a heterozygous G148X mutation, were recruited and their TB/CA FMO activities were determined. In addition, their *FMO3* genes were analyzed with respect to the status of the V257M mutation. As indicated in Fig. 3, the subject had inherited the FMO3/Stop[148] mutant allele from his mother, who had very low TB/CA FMO activity (0.24), even though she was homozygous for the wild type exon 6 allele. All the family members, including the propositus, had low TB/CA FMO activities (0.12~1.69). Although his grandfather and father did not carry the nonsense FMO3/Stop[148] mutant allele, they were heterozygous for the V257M mutation and had extremely low TB/CA FMO activities (0.12 and 0.30, respectively). His sister did not inherit the nonsense mutant allele (FMO3/Stop[148]), but had low TB/CA FMO activities. He and his sister inherited the FMO3/Met[257] mutant allele from his father and had low TB/CA FMO activities (0.64 and 0.50, respectively). However, his brother, who was homozygous for the wild type allele (VV), had a relatively high TB/CA

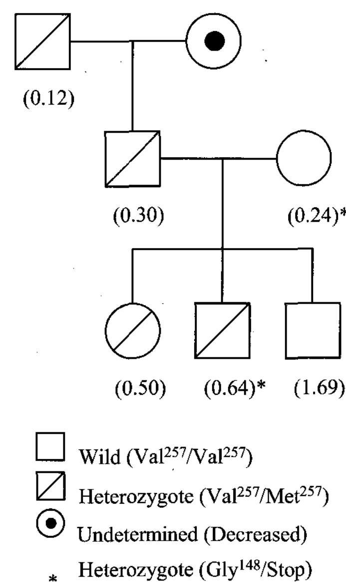

Fig. 3. Pedigree analysis for the presence of the V257M mutation in the *FMO3* gene. Subjects with hatches were heterozygous (Val^{257}/Met^{257}) and had very low TB/CA FMO activities (parentheses). The propositus for the V257M missense mutation as well as the G148X nonsense mutation is indicated by an arrow. His mother, who was a heterozygote for the G148X mutation, is indicated by a star.

FMO activity (1.69).

Allelic frequencies of V257M mutation in Korean population

A total of 159 individuals have been genotyped for the presence of the V257M mutation in exon 6 of their *FMO3* gene. Among these individuals, 117 (73.6%) were homozygous wild type (V^{257}/V^{257}) and 42 (26.4%) were heterozygous (V^{257}/M^{257}) (Table 1). The frequency of the FMO3/Met^{257} mutant allele was 13.21% and its distribution in our Korean population was compatible with the Hardy-Weinberg equilibrium.

DISCUSSION

In our previous studies, we found that production of theobromine (TB) by *N*-1 demethylation of caffeine (CA), a relatively non-toxic compound contained in coffee, is catalyzed primarily by the FMO contained in adult human liver microsomes (Chung & Cha, 1997; Chung et al, 2000). Subsequent efforts to identify a probe substrate for FMO phenotyping and to correlate these with the genotypes of E158K or E308G mutations in the *FMO3* gene, led us to identify ranitidine (RA) (Kang et al, 2000). Interestingly, the TB/CA FMO activities were significantly correlated with the presence of the V257M mutation, but not with E158K or E308G mutation, in *FMO3*. Furthermore, although the low TB/CA FMO activities obtained in the previous pedigree study could not be completely explained by the presence of a nonsense mutation (G148X) (Park et al, 1999), they could be explained by the presence of the V257M mutation (Fig. 3). There were, however, some differences in the TB/CA FMO activities between the grandfather, father and sister, who were all heterozygous for the exon 6 genotype (V^{257}/M^{257}). The extremely low TB/CA FMO activity (0.12) observed in the grandfather, as well as the lower FMO activity (0.30) observed in the father, when compared with that of the sister (0.50), could be due to the general age-dependent depression of FMO activity (Chung et al, 2000). In any case, these results clearly indicate that the presence of the V257M mutation affects the FMO activity which catalyzes *N*-1 demethylation of CA. Our results also suggest that the substrate specificity can be altered by mutations in the coding region of an enzyme. Although this is the first report indicating that functional mutations in human FMO may alter the *in vivo* substrate specificity, some mutations in human CYPs have been shown to alter the binding and metabolic capacities for a given substrate and change the substrate specificity (Oliver et al, 1997; Oscarson et al, 1997; Woods et al, 1998).

As mentioned earlier, the production of TB from CA (*N*-1 demethylation) is catalyzed primarily by the FMO3 and in a minor part by the CYP1A2 contained in adult human liver microsomes. Conversely, the production of paraxanthine (PX) from CA through *N*-3 demethylation is catalyzed primarily by CYP1A2, but in a minor part by FMO (Chung & Cha, 1997). Thus, when we examined if CYP1A2 activity determined by the urinary molar ratio of (PX+17U) to CA could have been affected by the presence of the V257M mutation in *FMO3*, no correlation could be found (data not shown). This suggested that the minor contribution provided by CYP1A2 in producing TB from CA by *N*-1 demethylation (*in vitro* FMO activity) has no *in vivo* significance and does not affect the genetic influence of the V257M mutation on TB/CA FMO activity.

Fig. 2B indicates clearly that the RANO/RA FMO activities are significantly correlated with genotypes for E158K and E308G mutations. However, the RANO/RA FMO activities for subjects with or without the FMO3/Met^{257} mutant allele were not different (Fig. 2A). This made it clear that, although the RA *N*-oxidation *in vivo* is causatively affected by the presence of FMO3/Lys^{158} (exon 4) and FMO3/Gly^{308} (exon 7) mutant alleles, the *N*-1 demethylation of CA producing TB is affected by the FMO3/Met^{257} (exon 6) mutant allele.

As mentioned above, point mutations in the coding region of a given enzyme may bring about changes in the secondary structure, which in turn, alters the tertiary structure and function of the expressed enzyme. If the change in the secondary structure caused by the three *FMO3* mutations under study is minor, the expressed FMO3 enzyme may still retain the catalytic activity, but have modified substrate-binding affinity or reduced activity due to modification of the substrate access channel to the catalytic site. The tertiary structure of FMO3 has been suggested to have the longer and narrower substrate access channel, compared with that of FMO1 (Nagata et al, 1990; Lomri et al, 1993; Cashman, 1995). Thus, smaller substrates like TMA and methimazole and a substrate with longer aliphatic side chain (RA) may have easier access to the catalytic site and a higher affinity to FMO3, therefore, would be oxidized faster than bulky substrates like CA. Furthermore, FMO is known to be present in a reactive state, ready to oxidize the available substrates (Ziegler, 1990). Because of this unusual reaction mechanism, *in vivo* oxidation of small and low *Km* substrates like TMA and methimazole is limited apparently by the mass transfer rate (Ziegler, 1988). Thus, five- to ten-fold changes in FMO activity created by mutations in *FMO3* would not produce recognizable differences in the metabolic rate for these low *Km* substrates. Only a major non-conservative change in the secondary structure of FMO3, like that created by P153L mutation in *FMO3* gene, might abolish the catalytic activity for a small substrate like TMA, thereby causing TMAU (Dolphin et al, 1997b).

Comparing the changes in the secondary structure of FMO3 brought about by the functional (missense) mutations in *FMO3* gene, the alteration caused by the V257M mutation is much less extensive than that caused by the E158K, E308G (Park et al, 1999) or P153L (Dolphin et al, 1997a,b) mutations. In the presence of the V257M mutation, the sheet structure was transformed into a helical structure, and such a minor change in the secondary structure of FMO3 may only lead to a slight modification in the substrate access channel and prevent the access of bulky substrate like CA but not of a small substrate like TMA. In support of this hypothesis, Treacy et al. (1998) reported that five subjects among 40 Australian volunteers were found to carry the V257M mutation without apparent TMAU, which is inconsistent with our studies. Thus, depending on the nature and degree of structural modifications put on to FMO3 by the mutation in *FMO3* gene, accessibility of bulky substrates may be denied or modified, and this may lead to alteration of the substrate specificity. Such an allelic variation, in turn, may be responsible for the inter-individual and ethnic polymorphism in the disposition of drugs metabolized by FMO in human.

In conclusion, the V257M mutation in the *FMO3* gene occurs commonly in Korean population and may be

responsible for the decreased N-1 demethylation of CA in affected individuals. Other apparently linked *FMO3* gene mutations which are also common in our Korean population (E158K and E308G) affect the N-oxidation of RA. These allelic polymorphisms may explain some of the underlying reasons for the individual differences observed in the *in vivo* FMO activities oxidizing different probe drugs used to phenotype the FMO in human.

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