Correlation Between FMO3 Genotypes and FMO Activity Measured by Using Trimethylamine N-Oxidation and Ranitidine N-Oxidation[†]

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국문초록

Trimethylamine 및 Ranitidine의 N-산화대사능을 이용하여 측정 한 FMO 활성도와 FMO3 유전자형의 상관성

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연구배경 : 사람 flavin-containing monooxygenase 3(FMO3)의 활성도를 비관혈적으로 측정하기 위해 trimethylamine(TMA)이 이용될 수 있으나 TMA는 많은 식이중에 존재하여 활성도 측정에 어려움이 있어, 본 연구진은 최근에 생체내 FMO 활성도를 측정하기 위한 비관혈적 ranitidine N-산화대사능 검사를 개발하였다. 본 연구에서는 생체내 TMA N-산화대사능과 ranitidine N-산화대사능에 미치는 FMO3 E₁₅₈K 및 E₃₀₈G 돌연변이의 효과를 교차시험으로 조사함으로써, FMO3 유전자형에 대한 두 가지 FMO 표현형 검색방법의 민감도를 비교하였다.

방 법 : FMO3 유전자의 K₁₅₈ 및 G₃₀₈ 돌연변이에 대해 유전자형 검색을 시행한 36명의 건강한 한국인 자원에서 TMA N-산화대사능과 ranitidine N-산화대사능을 이용하여 FMO 활성도를 측정하였다. TMA N-산화대사능은 24시간 소변중의 TMA N-oxide(TMAO)와 total TMA(TTMA)의 비로 측정하였으며, ranitidine N-산화대사능은 150 mg ranitidine을 경구투약후 8시간까지 배설된 소변중의 ranitidine N-oxide와 ranitidine의 비로 측정하였다.

결 과 : TMAO/TTMA 비에 대해서는, K₁₅₈ 돌연변이 대립형질에 대해 동형접합자이거나 이형접합자인 13명이 wild type E₁₅₈ 대립형질에 대해 동형접합자인 23명과 유의한 차이가 없었으며, G₃₀₈ 돌연변이 대립형질에 대해 동형접합자이거나 이형접합자인 12명 역시 wild type E₃₀₈ 대립형질에 대해 동형접합자인 24명과 유의한 차이가 없었다. 그러나, ranitidine N-oxide/ranitidine 비에 대해서는, K₁₅₈ 돌연변이 대립형질에 대해 동형접합자이거나 이형접합자인 13명이 E₁₅₈ wild type 대립형질에 대해 동형접합자인 23명에 비해, 그리고 G₃₀₈ 돌연변이 대립형질에 대해 동형접합자이거나 이형접합자인 12명이 wild type E₃₀₈ 대립형질에 대해 동형접합자인 24명에 비해 각각 통계적으로 유의하게 낮았다.

결 론 : 한국인 FMO3 유전자에서 흔하게 발견되는 두 가지 돌연변이인 E₁₅₈K and E₃₀₈G 유전자형은 TMA N-산화대사능과는 상관관계가 없었으나 ranitidine N-산화대사능으로 측정 한 FMO 활성도와는 통계적으로 유의한 상관관계를 나타내었다.

중심단어 : FMO · FMO3 유전자형 · 유전적 다형성 · TMA N-산화대사 · Ranitidine N-산화대사.

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INTRODUCTION

Activity of most drug-metabolizing enzymes is known to be influenced by their genetic make up. Variations of drug metabolizing enzyme activities may have an impact on the duration of action and the efficacy of drugs. Furthermore, such variations of foreign compound metabolism are known to significantly modulate the toxicity and mutagenicity of environmental chemicals. This was studied frequently in association with the genetic polymorphism of hepatic cytochrome P450 monooxygenases, but not with the hepatic flavin-containing monooxygenase 3 (*FMO3*) which also shows extensive genetic variability in human population.

The mammalian *FMOs* (EC 1.14.13.8) are microsomal enzymes that catalyze the NADPH-dependent oxygenation of many substances which include endogenous amines, tyramine, nicotine, clinically useful drugs (eg, tricyclic antidepressants, ranitidine), and trimethylamine (TMA)¹⁾. *FMOs* oxidize soft nucleophiles with an electron-rich center, typically the chemicals containing a nitrogen, sulfur, phosphorus or selenium atom²⁻⁴⁾. An inherited defect in the *FMO*-catalyzed *N*-oxidation of the diet-derived amine, TMA, results in an autosomal recessive disorder named trimethylaminuria or "fish-odor syndrome"^{5, 6)}. Individuals affected with trimethylaminuria have a markedly reduced capacity to oxidize the malodorous volatile TMA to its more polar, odorless, non-volatile metabolite TMA *N*-oxide (TMAO), and display a strong, unpleasant, fish-like odor⁷⁻⁹⁾.

While TMA (*N,N*-dimethylmethanamine) has

been available for a non-invasive determination of the *in vivo* *FMO* activity in man^{5, 6, 10)}, it is contained in many foods like fish, cheese, egg and liver as choline derivatives¹¹⁾ and thus, it is not suitable as a sensitive and specific probe compound to determine the *in vivo* *FMO* activity. Recently, we developed a non-invasive method for determination of the *in vivo* *FMO* activity in human by assessing urinary molar concentration ratio of ranitidine and ranitidine *N*-oxide, and used this "ranitidine *N*-oxidation test" to phenotype a Korean population for their *in vivo* *FMO* activity¹²⁾. In our previous "ranitidine *N*-oxidation test", we found a significant correlation between the ranitidine *N*-oxidation phenotypes and *FMO3* mutant genotypes determined by the presence of two mutant alleles (*K*₁₅₈ and *G*₃₀₈ alleles) which are found commonly in our Korean population. The "ranitidine *N*-oxidation test" showed that volunteers who were homozygous or heterozygous for either one or both of the *K*₁₅₈ and *G*₃₀₈ mutant alleles had significantly lower *in vivo* RA *N*-oxidation capacities than those with homozygous wild type *FMO3* *E*₁₅₈ and *E*₃₀₈ alleles¹²⁾. However, there is no report that relates the *in vivo* *FMO* activities determined by conventional TMA test with these mutant *FMO3* genotypes. Furthermore, no data comparing the relative sensitivities of "ranitidine *N*-oxidation test" and conventional "TMA *N*-oxidation test" for the subjects carrying the common *FMO3* mutant genotypes are available.

In the present study, we have examined (1) the relationship between TMA *N*-oxidation capacity and ranitidine *N*-oxidation capacity, (2) the effect of *FMO3* genotypes determined by the

Table 1. Demographics and *FMO3* genotypes of 36 Subjects

	Range		Median	Mean $\pm$ SD	
Age (yrs)	19-28		23	23 $\pm$ 2	
Weight (kg)	45-90		68	68 $\pm$ 10	
Creatinine clearance (mL/min)	77-250		134	140 $\pm$ 38	
FMO3 genotypes	<i>HHDD</i>	<i>HhDD</i>	<i>HHdD</i>	<i>HhDd</i>	<i>hhdd</i>
Number of subjects	22	2	1	10	1
(%)	61.1	5.5	2.8	27.8	2.8

presence of two common mutant alleles (K_{158} and G_{308} alleles) on the TMA *N*-oxidation capacity, and (3) the sensitivities of conventional "TMA *N*-oxidation test" and the newly developed "ranitidine *N*-oxidation test" in the Korean subjects carrying the common *FMO3* mutant genotypes by cross-over design.

MATERIAL AND METHODS

Subjects

Thirty-six healthy Korean volunteers (34 men and 2 women) were recruited from the students at Medical College of INHA University, Incheon, Korea. Their ages ranged between 18 and 28 years (mean age $\pm$ SD, 22.8 $\pm$ 2.1 years) (Table 1). No subject had any history of major diseases and although 19 regularly smoked cigarettes, none consumed more than moderate amounts of alcohol. None of the volunteers have been exposed to any recent drug treatment and none were taking any concurrent medication at the time of the study. During the entire study period, undue physical activity was avoided.

Full informed consent was obtained from all of the participants before starting the study and

appropriate ethical approval was granted by the Institutional Review Board of Inha University Hospital.

Certain precautions must be taken during the selection of volunteers because spuriously high levels of free TMA may be observed in women during menstruation¹³⁾ and in those subjects who have vaginal or urinary tract infections¹⁴⁾. Such individuals were identified and excluded from the study.

Study protocol

All of the volunteers were tested for their TMA *N*-oxidation capacity (*in vivo* FMO activity) firstly. They were asked not to eat foods like fish, cheese, eggs and liver for 2 days before the test and also during the urine collection day. Each volunteer was requested to collect a 0 to 24-hour total urine sample in a 2 L container to which 15 mL hydrochloric acid (6 mol/L) had already been added. This ensured that any TMA excreted in the form of volatile free base would be converted to the nonvolatile hydrochloride salt. The volume of total urine was recorded and an aliquot (20 mL ; pH 2.0) was stored at -70°C until headspace GC analy-

sis, which was carried out as soon as possible. Blood samples were obtained from all the volunteers to extract DNA for analysis of *FMO3* E₁₅₈K and E₃₀₈G mutations.

One week later, *in vivo* ranitidine *N*-oxidation capacities (FMO activities) were measured in the same 36 volunteers. All volunteers were asked not to take any methylxanthine (known FMO substrate) containing drinks, foods, or drugs for 2 days before and during the study period. After an overnight fasting (10 hr), each volunteer voided control urine and was given a single tablet of 150 mg [477 μ mol] ranitidine (Zantac, GlaxoWellcome Korea, Seoul, Korea) with 200 mL of water for oral administration. Subjects refrained from all foods and drinks (except plain mineral water *ad libitum*) until 4 hr after the RA administration. Subjects remained seated for the first 4 hr (morning classes) and blood samples (10 mL) were obtained at this time point to prepare the serum for measurement of creatinine concentration¹⁵⁻¹⁷⁾. Thereafter, normal lunch was served without restricting fluid intake. Serum was obtained by centrifugation (6 min at 3,600g) immediately after the blood collection and was stored at -70 °C until the creatinine concentration was determined by the Jaffe method¹⁸⁾ at a later time. All urine produced by each volunteers for 8 hr after the ranitidine administration was bulked and the volume was measured. After saving 20 mL aliquot for storage at -70 °C for later HPLC analysis, the remainder was discarded.

Genotyping for *FMO3* mutations

Venous blood samples, drawn in ethylenediaminetetraacetic acid (EDTA) as an antico-

agulant, were centrifuged to obtain the buffy coat layer. Genomic DNA was isolated from the buffy coat layer using the general phenol-chloroform purification method and amplified with PCR using the oligonucleotides (for the *FMO3* E₁₅₈K mutation in exon 4, 5'-GCT AGC ATA GAA AAG AGG GA-3' sense and 5'-CGA GAG TCA CCC GAG TAC CCG-3' antisense oligonucleotides and for the *FMO3* E₃₀₈G mutation in exon 7, 5'-GTC TCT GTT TTC CAT AGA G-3' sense and 5'-CTT CGC AAT CCA TGA GCC TC-3' antisense oligonucleotides) designed from respective intronic sequences of human *FMO3* gene as described by Park et al¹⁹⁾.

These two *FMO3* gene mutations (K₁₅₈ and G₃₀₈ mutant alleles) which are found commonly in our Korean population were probed by digestion with specific restriction enzymes, *Hinf* I for the exon 4 and *Dra* II for the exon 7 mutation sites, respectively. From these results, the homo- and heterozygotic status of each mutant allele present in *FMO3* gene could be determined. To detect the presence of *FMO3* K₁₅₈ mutation, the PCR products of exon 4 (615 bp) were digested with *Hinf*I restriction endonuclease, cleaving the wild type (*FMO3* E₁₅₈) product into four fragments (217, 205, 127, and 66 bp) but cleaving the mutant type (*FMO3* K₁₅₈) into 3 fragments (283, 205 and 127 bp). Similarly, for detection of *FMO3* G₃₀₈ mutation, the exon 7 PCR products of 408 bp size were digested with *Dra* II restriction endonuclease, cleaving only the mutant type (*FMO3* G₃₀₈) product into two fragments (293 and 115 bp) but leaving the wild-type (*FMO3* E₃₀₈) product uncut.

GC analysis of TMA in urine samples

TMA present in the urine was determined by

a modification of the head-space GC technique as described previously in the literature²⁰). Urine (5 mL) and 0.2% v/v aqueous isopropylamine (100 μ L; internal standard) were placed in a 15 mL screw-top septum glass vial. Potassium carbonate (2 g) and potassium hydroxide (2 g) was added to the urine and the vial was sealed with air-tight polytetrafluoroethylenelined septum cap. All of these procedures were carried out on ice. The vial was then vortex mixed and heated at 90°C for 20 minutes in an aluminum heating block and an aliquot (1 mL) of the generated head-space gas was injected directly onto the GLC column with an air-tight syringe.

TMAO was quantitated as TMA after chemical reduction²¹). Urine (2 mL) was reduced by titanium (III) chloride (0.2 mL 8.6% [w/v] in 28% [w/v] aqueous HCl) in a septum vial at 90°C for 2 minutes. An aliquot of the reduced urine was then diluted with water and analyzed as described above. All urine samples were analyzed twice.

The chromatography was performed on a HP 5890 series II model gas chromatograph (Hewlett-Packard, CA, U.S.A.) with a "flame ionization" detector, the injector port being held at 200°C and the detector at 250°C. The column (30 m by 0.247 mm inside diameter) was DB-1701 (J&W Scientific, CA, U.S.A.) and was placed in an oven with gradient temperatures which consisted of initial 5 min at 70°C and following a linear gradient increase by 32°C/min until the final 1 min at 180°C with a nitrogen carrier gas flow rate of 50 mL/min. According to the conditions described for the assay, the retention time obtained for TMA was 2.1 minutes.

The area under the relevant peak was calcu-

lated by using the Millennium version 2.15 program (Waters, MA, U.S.A.) and the result was expressed in terms of micromoles of TMA or TMAO excreted in 0 to 24-hour urine samples. In addition, the percentage of TMAO over the total TMA-related material (TTMA) excreted was calculated as follows: $[\text{TMAO (in micromoles)}/\text{TTMA (in micromoles)}] \times 100$.

A metabolic ratio (MR) for TMA *N*-oxidation was also calculated as TMA (in micromoles)/TMAO (in micromoles) excreted in the 0 to 24-hour urine samples.

HPLC analysis of ranitidine and its metabolites in urine samples

Amounts of RA and its *N*-oxide, *S*-oxide and desmethylranitidine metabolites present in the urine were determined by using a reversed-phase high performance liquid chromatography (HPLC). The HPLC system consisted of a Novapak C-18 column (3.9 $\times$ 300 mm, 4 μ m; Waters Corporation, MA, USA), Waters model 510 programmable pump and computerized Waters 996 photodiode array detector. Aliquots (100 μ L) of the thawed urine samples were spiked with 200 nmol of quinine (internal standard) dissolved in 100 μ L methanol and centrifuged for 20 min at 100,000g to remove the proteins which may have been present. Subsequently, supernatant (40 μ L) was injected into the HPLC system. The mobile phase used for initial 7 min isocratic elution consisted of 1 : 9 (v/v) mixture of acetonitrile and phosphate buffer (5 mM, pH 8.0) (solution A). This was followed by 3 min linear gradient elution with solution B (7 : 3 (v/v) acetonitrile and phosphate buffer) and then continued for 8 min isocratic elution with 100% solution B. The flow rate was 0.8 mL

/min. The eluate was screened between 280 nm to 340 nm for qualitative identification of metabolite peak positions and then monitored at 320 nm for quantitative analysis. Ranitidine and its metabolites were identified by their absorption spectra, specific retention times and comparison with their respective metabolite standards using the photodiode array detector. Detection limits for ranitidine and its *N*-oxide using this HPLC method were 0.7 ng (about 2 pmoles).

The quantities of ranitidine and its *N*-oxide obtained from HPLC chromatogram were multiplied by dilution factors and converted to total amounts excreted. The molar ranitidine *N*-oxide/ranitidine ratio in the bulked 8 hr urine was used as the index of FMO activity.

Statistical analyses

The nonparametric Kruskal-Wallis test was applied for analysis of genotype-dependent differences in the metabolic ratios and the difference between the values of the two groups was determined by Mann-Whitney U test.

RESULTS

None of the volunteers experienced any adverse effects after the oral administration of ranitidine.

FMO3 genotypes

Distribution of each *FMO3* genotypes carrying the wild (E_{158} : 'H') or the mutant type (K_{158} : 'h') allele in exon 4 (*Hinf* I site) and the wild (E_{308} : 'D') or the mutant type (G_{308} : 'd') allele in exon 7 (*Dra* II site) among 36 Korean sub-

jects is shown in Table 1. The allele frequencies for the wild type (E_{158}) and mutant type (K_{158}) in exon 4 were 0.81 and 0.19, respectively. The allele frequencies of the wild (E_{308}) and mutant (G_{308}) types in exon 7 were also 0.82 and 0.18, respectively. Therefore, the genotype frequencies for both mutation sites showed were the same as described previously in Korean population¹²⁾.

Determination of TMA *N*-oxidation capacity from urinary ratio of TMA and TMAO

The results obtained for 36 subjects investigated are given in Table 2. The mean values found for 0 to 24-hour urinary excretion of TMA (mean $\pm$ SD) were 58.2 ± 37.1 μ mol (3.4 ± 2.2 mg) with an inter-individual variation range of 20.6 to 170.5 μ mol (8.3-fold difference) and the mean value for excretion of TMAO (mean $\pm$ SD) were 567.0 ± 433.4 μ mol (42.6 ± 32.6 mg) with a variation range of 108.3 to 1986.6 μ mol (18.3-fold difference). The percent of TMAO in total TMA excreted varied from 75.2 to 97.0 with a mean SD of 88.8 ± 5.7 (Table 2). When the data were expressed in terms of a metabolic ratio (TMA/TMAO), the values varied from 0.03 to 0.33 (11 fold maximal individual variation) with a mean $\pm$ SD of 0.13 ± 0.07 (Table 2).

To evaluate the effect of cigarette smoking on *in vivo* TMA *N*-oxidation, the TMAO/TTMA (%) and TMA/TMAO (MR) obtained from 17 nonsmokers (15 men and 2 women) and 19 smokers (all men) were compared (Table 2). Median TMAO/TTMA (%) for the nonsmokers was 90.7% with an inter-individual variation range of 75.2% to 96.5% and for the smokers, it was 87.4% with a variation range of 81.6% to

Table 2. 24hr urinary excretion of trimethylamine *N*-oxide (TMAO) and trimethylamine (TMA) in 36 subjects

	Range	Median	Mean $\pm$ SD
TMAO (mol / 24 hr)			
in all subjects (n=36)	108.3-1986.6	404.8	567.0 $\pm$ 433.4
in nonsmokers (n=17)	108.3-1556.2	396.5	487.5 $\pm$ 363.6
in smokers (n=19)	203.9-1986.6	413.1	624.2 $\pm$ 499.3
TMA (μ mol / 24 hr)			
in all subjects (n=36)	20.6-170.5	49.5	58.2 $\pm$ 37.1
in nonsmokers (n=17)	20.6-135.1	38.9	51.0 $\pm$ 33.7
in smokers (n=19)	20.6-170.5	52.9	64.6 $\pm$ 39.7
TMAO / TTMA* (%)			
in all subjects (n=36)	75.2-97.0	88.4	88.8 $\pm$ 5.7
in nonsmokers (n=17)	75.2-96.5	90.7	88.7 $\pm$ 6.2
in smokers (n=19)	81.6-97.0	87.4	88.1 $\pm$ 5.1
TMA / TMAO (MR)			
in all subjects (n=36)	0.03-0.33	0.13	0.13 $\pm$ 0.07
in nonsmokers (n=17)	0.04-0.33	0.10	0.13 $\pm$ 0.08
in smokers (n=19)	0.03-0.23	0.14	0.12 $\pm$ 0.06

*TTMA (total TMA) = TMAO + free TMA

97.0%. And the median TMA/TMAO ratio (MR) for nonsmokers was 0.10 with a variation range of 0.04 to 0.33 and for the smokers, it was 0.14 with a range of 0.03 to 0.23. Their median ratios (TMAO/TTMA [%] and TMA/TMAO [MR]) between nonsmokers and smokers were not different ($P=.987$, Mann-Whitney U test) and indicated that the *in vivo* TMA *N*-oxidation capacity (FMO activity) was not influenced by cigarette smoking.

Determination of ranitidine *N*-oxidation capacity from urinary ratio of ranitidine *N*-oxide and ranitidine

Amounts of ranitidine and its *N*-oxide excret-

ed in urine were determined from the bulked 8 hr urine and their ratios (ranitidine *N*-oxide/ranitidine [%]) were used to assess the individual ranitidine *N*-oxidation capacity (FMO activity). The mean value found for 0 to 8-hour urinary excretion of ranitidine (mean $\pm$ SD) was 113.9 $\pm$ 31.0 μ mol with an inter-individual variation range of 59.5 to 194.4 μ mol (3.3-fold difference) and the mean value for excretion of ranitidine *N*-oxide (mean $\pm$ SD) was 9.9 $\pm$ 4.0 μ mol with a variation range of 4.4 to 22.7 μ mol (5.2-fold difference). The range of ranitidine *N*-oxide/ranitidine (%) varied from 4.5% to 13.1% (2.9-fold individual variation) with a mean $\pm$ SD of 8.6 $\pm$ 1.9% (Table 3).

To evaluate the effect of cigarette smoking on

Table 3. 8 hr urinary excretion of ranitidine and its *N*-oxide in 36 subjects after oral administration of 150 mg (477 μ mol) ranitidine.

	Range	Median	Mean $\pm$ SD
Ranitidine <i>N</i> -oxide (mol/8 hr)			
in all subjects (n=36)	4.4-22.7	9.0	9.9 $\pm$ 4.0
in nonsmokers (n=17)	5.5-22.7	8.2	10.1 $\pm$ 4.5
in smokers (n=19)	4.4-18.1	9.1	9.7 $\pm$ 3.5
Ranitidine <i>N</i> -oxide (mol/8 hr)			
in all subjects (n=36)	59.5-194.4	111.1	113.9 $\pm$ 31.0
in nonsmokers (n=17)	59.5-175.7	114.2	118.1 $\pm$ 28.1
in smokers (n=19)	70.4-194.4	97.5	110.2 $\pm$ 33.8
Ranitidine <i>N</i> -oxide/ranitidine (%)			
in all subjects (n=36)	4.5-13.1	8.8	8.6 $\pm$ 1.9
in nonsmokers (n=17)	4.9-13.1	8.3	8.5 $\pm$ 2.4
in smokers (n=19)	4.5-10.8	8.8	8.7 $\pm$ 1.4

in vivo ranitidine *N*-oxidation capacity, the ranitidine *N*-oxide/ranitidine (%) obtained from 17 smokers (15 men and 2 women) and 19 smokers (all men) were compared (Table 3). Median ranitidine *N*-oxide/ranitidine (%) for nonsmokers was 8.3% with an inter-individual variation range of 4.9% to 13.1% and for the smokers, it was 8.8% with a variation range of 4.5% to 10.8%. Results showed that the median ratios between nonsmokers and smokers were not different ($P=.987$, Mann-Whitney U test) and indicated that the *in vivo* ranitidine *N*-oxidation capacity was not influenced by cigarette smoking.

Correlation between TMA *N*-oxidation capacity and ranitidine *N*-oxidation capacity

There is no statistically significant correlation between the TMA *N*-oxidation capacity (TMAO

/TTMA [%] or TMA/TMAO [MR]) and the ranitidine *N*-oxidation capacity (ranitidine *N*-oxide/ranitidine [%]) in the same subjects (Fig. 1; $P>.05$).

Impact of *FMO3* mutant genotypes on TMA *N*-oxidation

Among the 36 subjects genotyped, 22 (61.1%) had homozygous wild type alleles ('HHDD') on both exon 4 (*Hinf*I site) and exon 7 (*Dra* II site) and their mean TMAO/TTMA (%) was $88.8 \pm 5.4\%$ (median=90.7%). Genotypes of 10 subjects (27.8%) were heterozygous for both *FMO3* K₁₅₈ and G₃₀₈ alleles ('HhDd') and they had the mean TMAO/TTMA (%) of $88.0 \pm 6.8\%$ (median=87.4%). Only one subject was homozygous for both *FMO3* K₁₅₈ and G₃₀₈ ('hhdd') mutant alleles and he had TMAO/TTMA ratio of 95.3%. Because the K₁₅₈ and G₃₀₈ mutations represent prominent *FMO3* variants in the nor-

Fig. 1. Correlation analyses between the TMA *N*-oxidation capacity and the ranitidine *N*-oxidation capacity in 36 subjects. A, TMAO/TTMA (%) to ranitidine *N*-oxide/ranitidine (%), Pearson correlation coefficient = -0.159, not significant ($P = .355$). B, TMA/TMAO (MR) to ranitidine *N*-oxide/ranitidine (%), Pearson correlation coefficient = 0.147, not significant ($P = .394$).

mal Korean adults¹⁹, functional effects of these mutations on TMA *N*-oxidation capacities (TMAO/TTMA [%]) were compared for all 36 Korean subjects genotyped. The following differences were tested: (1) TMAO/TTMA (%) as a function of *FMO3* E₁₅₈K (Fig. 2, A) and *FMO3* E₃₀₈G (Fig. 2, B) mutant genotypes, respectively, and (2) TMAO/TTMA (%) as a function of the observed genotype constellation of subjects, as shown in Table 4. None of the differences was significant by Kruskal-Wallis comparison.

Impact of *FMO3* mutant genotypes on ranitidine *N*-oxidation

We analyzed how much of the individual variation of ranitidine *N*-oxidation capacity might be explained by the *FMO3* mutant genotypes.

The urinary ranitidine *N*-oxide/ranitidine ratios (*FMO* activities) were correlated with *FMO3* genotypes. The ranitidine *N*-oxide/ranitidine ratios showed significant differences between the five different *FMO3* genotype groups (Table 4; $P = .031$, Kruskal-Wallis test). Among the 36 subjects genotyped, 22 individuals who were homozygous for the wild type *FMO3* allele ('HHDD') had the mean ranitidine *N*-oxide/ranitidine (%) of 9.2 ± 1.8 (median = 8.9%) and this was significantly higher than the mean 7.5 ± 1.5 (median = 7.8%) observed in the 10 individuals who were heterozygous for both *FMO3* K₁₅₈ and G₃₀₈ mutant alleles ('HhDd') (Table 4; $P = .005$, Mann-Whitney U test). One subject who was homozygous for both *FMO3* K₁₅₈ and G₃₀₈ mutant alleles ('hhdd') had the lowest ranitidine *N*-oxide/ranitidine (7.1%).

Fig. 2A TMAO/TTMA (%) of 13 subjects with homozygous ('hh') or heterozygous ('Hh') *FMO3* K₁₅₈ mutant alleles were not different from those of 23 subjects with wild type alleles ('HH'). B, The percents of 12 subjects with homozygous ('dd') or heterozygous ('Dd') *FMO3* G₃₀₈ mutant alleles were not different from those of 24 subjects with wild type alleles ('DD'). Horizontal solid bars denote median values and dotted bars indicate the mean values.

With regard to the effect of *FMO3* K₁₅₈ mutation in exon 4 on ranitidine *N*-oxidation capacity, 13 volunteers who carried either heterozygous ('Hh') or homozygous ('hh') mutant alleles had lower ranitidine *N*-oxide/ranitidine (%) than that observed in the 23 subjects with homozygous wild type allele ('HH') (Fig. 3, A ; $P=0.10$, Mann-Whitney U test). And regarding the effect of *FMO3* G₃₀₈ mutation in exon 7, 12 volunteers who carried either heterozygous ('Dd') or homozygous ('dd') mutant alleles had lower ranitidine *N*-oxide/ranitidine (%) than that

observed in the 24 subjects with homozygous wild type allele ('DD') (Fig. 3, B ; $P=0.001$, Mann-Whitney U test).

DISCUSSION

Genetic polymorphism of cytochrome P450 has been regarded as the major source of individual variability in pharmacokinetics of drugs^{22,23}. Although much biochemical data suggest participation of FMO in the metabolism of many drugs, effect of FMO

Table 4. *In vivo* FMO activity in relation to *FMO3* genotype patterns

	HHDD (n=22)	HhDD (n=2)	HHdd (n=1)	HhDd (n=10)	hhdd (n=1)
TMAO/TTMA (%)					
Range	81.3-96.8	83.8-94.4		75.2-97.0	
Median	90.7	89.1		87.8	
Mean SD	88.8±5.4	89.1±7.4	86.9	88.0±6.4	95.3
Ranitidine N-oxide/ranitidine (%)					
Range	1.9-13.1	8.3-10.7		4.5-9.3	
Median	8.9	9.5		7.8*	
Mean SD	9.2±1.8	9.5±1.7	7.9	7.3±1.6	7.4*

*Significant difference from 'HHDD' genotype group ($P<.05$, KruskalWallis test)

polymorphism on the variability of pharmacokinetics has not been studied extensively.

When occurring homozygously, the rare *FMO3* I₆₆, L₁₅₃, X₃₀₅, and W₄₉₂ mutations are known to cause complete inactivation of the enzyme and have been demonstrated to be responsible for fish-odor syndrome^{7, 8, 24}. However, two common *FMO3* mutations, namely, K₁₅₈ and G₃₀₈, have recently been reported to be associated only with reduced TMA *N*-oxidation capacity *in vivo*²⁵. According to their TMA phenotyping data, individuals homozygous for the wild type sequence (*FMO3* E₁₅₈) or heterozygous for *FMO3* K₁₅₈ mutation had normal TMAO/TTMA ratios (>94 %) but the one individual who was homozygous for both *FMO3* K₁₅₈ and G₃₀₈ mutations had a decreased TMA *N*-oxidation capacity (TMAO/TTMA ratio : 46%). Previously, we reported that the Korean subjects with homozygous wild type (*FMO3* E₁₅₈ and E₃₀₈) alleles ('HHDD') had higher RANO/RA ratio (higher FMO activity) than the subjects who had the *FMO3* K₁₅₈ and G₃₀₈ mutant alleles either in heterozygotic ('HhDd')

or homozygotic ('hhdd') manners¹².

In this study, we have compared the *in vivo* ranitidine *N*-oxidation capacities and *in vivo* TMA *N*-oxidation capacities in the same individuals by comparing their urinary molar ratios of parent compounds (TMA and ranitidine) to their *N*-oxide metabolites (TMAO and ranitidine *N*-oxide which are known to be catalyzed specifically by the hepatic *FMO3* in human)^{26, 27}. In addition, the impact of the two *FMO3* mutations (K₁₅₈ and G₃₀₈), which have been demonstrated to occur commonly in our Korean population¹⁹, on TMA and ranitidine *N*-oxidation capacities have been compared by cross-over design.

Results indicated that there is no significant correlation between the *N*-oxidation capacities determined by using these two FMO phenotyping probe substances, and that the two common *FMO3* mutations (K₁₅₈ and G₃₀₈) affects only *in vivo* ranitidine *N*-oxidation activity but not *in vivo* TMA *N*-oxidation activity. Subjects who were homozygous for the wild type *FMO3* se-

Fig. 3.A. Ranitidine *N*-oxide/ranitidine (%) of 13 subjects with homozygous ('hh') or heterozygous ('Hh') *FMO3* K₁₅₈ mutant alleles were lower than those of the 23 subjects with wild type alleles ('HH'). B, The percents of 12 subjects with homozygous ('dd') or heterozygous ('Dd') *FMO3* G₃₀₈ mutant alleles were also lower than those of the 24 subjects with wild type alleles ('DD'). Horizontal solid bars denote median values and dotted bars indicate the mean values. *P < .05 versus wild type group.

quences ('HHDD') had significantly higher RA *N*-oxidation capacity than those who were heterozygous for both *FMO3* K₁₅₈ and G₃₀₈ mutations ('HhDd'). Moreover, although we could identify only one subject with homozygous *FMO3* mutations for both K₁₅₈ and G₃₀₈ alleles ('hhdd'), he had the lowest ranitidine *N*-oxidation capacity. This finding is consistent with our previous report¹²⁾ and suggests a gene dose effect may exist for the *in vivo* FMO activity measured by

ranitidine *N*-oxidation.

When the ranitidine or methimazole was metabolized with recombinant human *FMO3*, in pH 8.4, the *K_m* value obtained for ranitidine *N*-oxidation (2mM) was higher than that observed for methimazole *S*-oxidation (35 μ M) which is also known to be metabolized primarily by the hepatic *FMO3* in human²⁷⁾. Despite this much higher *K_m* value observed for ranitidine *N*-oxidation,

there was a close correlation between the ranitidine *N*-oxidation activity and the *FMO3* content of human liver microsomes²⁷. The ability of liver microsomes isolated from 5 adult males producing ranitidine *N*-oxide at pH 8.4 varied from 12.5 to 117 pmol/mg microsomal protein and this was closely correlated with the specific contents of *FMO3* in these human liver microsomes²⁷. *FMO* is known to be present in a reactive state, ready to oxidize available substrates³, and because of this unusual reaction mechanism, the rate of *in vivo* oxidation for low *K_m* substrates like TMA (*K_m*=28 μ M at pH 7.4²⁶) and *K_m*=724 μ M at pH 8.5²⁸) or methimazole is limited apparently by the mass transfer rate³. Thus, 5 to 10 fold changes of *FMO* activity created by genetic defects, for example, would not produce recognizable differences in the metabolic rate for these low *K_m* substrates. Therefore, rather poor substrates of *FMO*, like ranitidine with low affinity (high *K_m*) for *FMO3*, would serve as more sensitive indicator of the changes in *FMO* activity.

Excretion of parent drug and its metabolite (ranitidine and its *N*-oxide) in urine is known to be influenced both by their metabolism and renal clearance²⁹ and thus, it is possible that the renal clearance factor may have contributed to the observed individual differences of *FMO3* activity. However, we did not find any significant correlation between the creatinine clearance and the urinary ranitidine (Pearson correlation coefficient=0.029, *P*=.865) or ranitidine *N*-oxide excretions (Pearson correlation coefficient=-0.044, *P*=.801) or the ranitidine *N*-oxide/ranitidine ratio (Pearson correlation coefficient

=-0.110, *P*=.522). From these results we could infer that the observed individual variability of ranitidine *N*-oxidation capacity is caused primarily by the metabolic differences in the liver and not by the differences in renal clearance rate. This further indicated that the individual differences of ranitidine *N*-oxidation capacity observed in our Korean population are caused by the *FMO3* genetic polymorphism in the liver.

In conclusion, (1) no significant correlation between TMA *N*-oxidation capacity and ranitidine *N*-oxidation capacity is observed; (2) *FMO3* mutations (*K*₁₅₈ and *G*₃₀₈) have no impact on the *in vivo* TMA *N*-oxidation rate but affect the ranitidine *N*-oxidation rate; (3) individual differences of ranitidine *N*-oxidation rate is more sensitively correlated with the *FMO3* mutations (*K*₁₅₈ and *G*₃₀₈) which are commonly observed in our Korean population.

SUMMARY

Background :

While trimethylamine (TMA) has been available for a non-invasive determination of flavin-containing monooxygenase 3 (*FMO3*) activity in man, it is contained in many foods and thus insensitive. We recently developed a non-invasive ranitidine *N*-oxidation test to determine the *in vivo* *FMO* activity. In this study, we compared the sensitivities of these two *FMO* phenotyping methods in reference to the *FMO3* mutant genotypes by examining the functional effects of *FMO3* *E*₁₅₈*K* and *E*₃₀₈*G* mutations on the rates of *in vivo* TMA *N*-oxidation and ranitidine *N*-oxidation by cross-over design.

Methods :

In 36 Korean healthy volunteers who were genotyped for the presence of *K*₁₅₈ and *G*₃₀₈ mutations in their *FMO3* gene, FMO activities were determined by using ranitidine *N*-oxidation and TMA *N*-oxidation. Ratios of TMA *N*-oxide (TMAO) over total TMA (TTMA) in 24-hour urine and of ranitidine *N*-oxide versus ranitidine in 8-hour urine after an oral ingestion of 150 mg ranitidine were determined.

Results :

Ratios of TMAO/TTMA obtained in 13 subjects with homo- or heterozygous *FMO3* *K*₁₅₈ mutant alleles were not different from those of 23 subjects with wild type *FMO3* *E*₁₅₈ allele, and also, the ratios in 12 subjects with homo- or heterozygous *FMO3* *G*₃₀₈ mutant alleles were not different from those of 24 subjects with wild type *FMO3* *E*₃₀₈ allele. However, the ranitidine *N*-oxide/ranitidine ratios of 13 subjects with homo- or heterozygous *FMO3* *K*₁₅₈ mutant alleles were significantly lower than those of the 23 subjects with *FMO3* *E*₁₅₈ wild type alleles, and also, the ratios of 12 subjects with *FMO3* *G*₃₀₈ mutant alleles were lower than those of the 23 subjects with wild type *FMO3* *E*₃₀₈ alleles.

Conclusion :

FMO activities determined by ranitidine *N*-oxidation, but not by TMA *N*-oxidation, were sensitively correlated with the common *FMO3* *E*₁₅₈*K* and *E*₃₀₈*G* mutant genotypes in our Korean subjects.

KEY WORDS :

FMO · *FMO3* genotype · Polymorphism · TMA *N*-oxidation · Ranitidine *N*-oxidation.

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