Rapid Pyrosequencing Method for *FMO3* Non-Synonymous Genetic Variant Evaluation in A Korean Population

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Abstract

Background: The aim of this study was to develop a feasible pyrosequencing method to detect non-synonymous single nucleotide polymorphisms (SNPs) of the flavin-containing monooxygenase 3 (*FMO3*) gene and compare the ethnic differences in the frequencies of these alleles.

Methods and Results: This pyrosequencing method was used to identify four non-synonymous *FMO3* SNPs, including c.855C>T (rs909530), c.441C>T (rs1800822), c.923A>G (rs2266782), and c.472G>A (rs2266782). The allele frequencies of these SNPs in 122 unrelated Korean subjects were analyzed, and were as follows: 44.7% for c.855C>T, 23.4% for c.441C>T, 23.0% for c.923A>G, and 27.1% for c.472G>A. Linkage disequilibrium (LD) analysis showed that c.923A>G and c.472G>A were in strong LD (D' = 0.8289, r^2 = 0.5332).

Conclusions: The designed pyrosequencing method was successfully applied to identify the c.855C>T, c.441C>T, c.923A>G, and c.472G>A SNPs. The frequencies were similar to those reported previously in a Japanese population. However, in general, large differences between ethnicities were found.

1. Introduction

Flavin-containing monooxygenases (FMOs) are a family of microsomal antioxidant defense enzymes responsible for the nicotinamide adenine dinucleotide phosphate-dependent oxygenation of soft nucleophiles [1–3]. Among them, FMO3 is primarily located in the human liver and metabolizes various nitrogen- and sulfur-containing drugs with a broad range of substrates (e.g., teneligliptin, sulindac, amphetamine, and tamoxifen) [4–6]. The *FMO3* gene contains nine exons ranging from 80 to 705 bp [7], and seven non-synonymous and two synonymous genetic polymorphisms have been identified [8]. Among them, *FMO3* c.855C > T (rs909530), c.441C > T (rs1800822), c.923A > G (rs2266780), and c.472G > A (rs2266782) are known to be common in East Asian populations [9–12].

These mutations have shown frequent functional inter-individual and inter-ethnic variability, and their significance is associated with the pharmacokinetics of certain chemicals (e.g., sulindac and ranitidine) [9]. Related disease manifestations have been described previously [13, 14]. One of the most commonly studied topics is the impact of *FMO3* gene mutations in trimethylaminuria (also known as fish odor syndrome) patients [12, 15]. The FMO3 enzyme is known to increase plasma trimethylamine *N*-oxide (TMAO) levels by converting trimethylamine (TMA) derived from the gut microbiome [16]. Therefore, the single nucleotide polymorphisms (SNPs) responsible for FMO3 loss-of-function may result in increased plasma TMA levels [10]. Moreover, increased levels of TMAO are associated with greater risk of cardiovascular disorders because they affect atherosclerosis pathogenesis [17]. Recently, it was reported that a mutation in *FMO3* was responsible for sulindac pharmacokinetics in Korean and Chinese populations [9, 18]. Because the pharmacogenetics of the *FMO3* gene play a crucial role in its substrate disposition, it is necessary to develop a feasible method to detect SNPs and validate the analysis for future research.

Therefore, this study aimed to develop a rapid, feasible pyrosequencing method to detect non-synonymous *FMO3* SNPs c.855C > T (rs909530), c.441C > T (rs1800822), c.923A > G (rs2266780), and c.472G > A (rs2266782), all of which have been reported to be functional and commonly present in the Korean population [9, 18], and compare allele frequencies with those reported in other ethnic groups.

2. Materials And Methods

Subjects and methods

Genomic DNA samples were obtained from 122 unrelated Korean subjects who provided written informed consent. The protocol for the assay was approved by the institutional review board of Anam Hospital, Korea University Medical Center (Seoul, Korea).

Polymerase chain reaction (PCR) conditions and FMO3 genotyping using the pyrosequencing method

Genomic DNA was isolated from peripheral blood leukocytes as previously described [19, 20]. We developed a pyrosequencing method to identify the functional SNPs of the *FMO3* gene: c.855C > T (rs909530), c.441C > T (rs1800822), c.923A > G (rs2266780), and c.472G > A (rs2266782). The primers used in the PCR analysis for *FMO3* genotyping and pyrosequencing are listed in Table 1. PCR reactions were performed to amplify sequences to identify each *FMO3* SNP using newly developed primer sets after biotin was attached to the 5' end of each forward (or reverse) primer using the PSQ Assay Design software (Biotage AB, Uppsala, Sweden).

Table 1
Oligonucleotide primers used for polymerase chain reaction (PCR) and pyrosequencing to detect *FMO3* polymorphisms

SNP	Primer	Sequences	Size (bp)	PCR (Tm; °C)
FMO3 (c.855C > T, rs909530)	Forward	B 5'-TTGGGTCATTTTTTCCTTCCTTAT-3'	261	60
	Reverse	5'-ACCCTGTTGCAAAGATTACACAGT-3'		
	Sequencing	5'-TTGCTGGGAGCTCAT-3'		
FMO3 (c.441C > T, rs1800822)	Forward	B 5'-CCACTGAAAGGGATGGTAAAAA-3'	125	60
	Reverse	5'-AGCAGCTTAAATTTTGGCCTTAC-3'		
	Sequencing	5'-TGGGATACACATGATGTC-3'		
FMO3 (c.923A > G, rs2266780)	Forward	5'-AGCATTCTGTGTGGCATTGT-3'	144	60
	Reverse	B 5'-AAGGAAGGGGTAGGCAAAACTAT-3'		
	Sequencing	5'-CGTGAAGGAATTCACAG-3'		
FMO3 (c.472G > A, rs2266782)	Forward	B 5'-ATGGTAAAAAAGAATCGGCTGTC-3'	132	60
	Reverse	5'-TTTTGTCAGTTATGTGGCTAGCAG-3'		
	Sequencing	5'-GCCTTACCTGGAAAGGACT-3'		

SNP, single nucleotide polymorphism; PCR, polymerase chain reaction; B, biotinylated at the end of the primer; Tm, melting temperature

The PCR reaction volume was 30 μL, which contained genomic DNA (30 ng), PCR buffer (10·), dNTPs (0.25 mM), 10 pmol primers (1 μL each), and 5U Taq polymerase (iNtRON, Seongnam, Korea). PCR reactions were carried out with an initial denaturation step at 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The final termination step was performed at 72°C for 5 min. For pyrosequencing reactions, 25 μL of the PCR template in a single well was immobilized by incubation (with shaking at 1,400 rpm for 10 min at room temperature) with a mixture of 5 μL streptavidin beads (Streptavidin Sepharose™ High Performance, GE Healthcare Bio-Science AB, Uppsala, Sweden) and 40 μL annealing buffer containing 0.4 μM sequencing

primer incorporated into each well. For strand separation, all liquids were removed using a vacuum prep workstation (Pyrosequencing AB, Uppsala, Sweden). The beads captured on probes were incubated in 70% ethanol, and the solution was flushed through the filters for 5 s. The beads were then treated with a denaturing solution (0.2 M NaOH) that was flushed through the filters for 5 s. A wash buffer (10 mM Tris-acetate, pH 7.6) was used to rinse the beads for 5 s. All liquid was completely drained from the probes, and the beads were released into a PSQ 96 Plate Low (Pyrosequencing AB) containing the sequencing primer. The PSQ 96 Plate Low was heated at 85°C for 2 min, and the reactions were allowed to cool to room temperature. The resulting mixture was analyzed using a PSQ 96MA Pyrosequencer (Pyrosequencing AB). The pyrosequencing accuracy was validated by direct DNA sequencing of randomly selected samples using the same genomic DNA.

Statistical analysis

Genetic equilibrium and linkage disequilibrium (LD) were tested according to the Hardy-Weinberg equation using SNPAnalyzer version 9.0 (DYNACOM Co., Ltd., Yokohama, Japan). The chi-square test was used to assess the consistency of the pyrosequencing method. $p < 0.05$ (two-tailed) was considered to be statistically significant. D' and r^2 are standard measurements for LD [21]. D' values were calculated as D/D_{max}, where D is the coefficient of LD ranging from − 0.25 to 0.25. Generally, the standardized value D' is preferred because D is often affected by allele frequencies [22].

3. Results

Each SNP for c.855C > T, c.441C > T, c.923A > G, and c.472G > A was identified and compared to the pre-designed pyrosequencing histogram (Fig. 1). Representative peaks for each SNP analysis are shown in Fig. 2. The sequenced data obtained from the pyrosequencing method were randomly selected and validated by direct DNA sequencing, which showed 100% concordance, thereby indicating 100% specificity and sensitivity.

The observed allele frequencies for *FMO3* genetic analysis in the Korean population (n = 122) using our pyrosequencing method were as follows: 44.7% for c.855C > T, 23.4% for c.441C > T, 23.0% for c.923A > G, and 27.1% for c.472G > A (Table 2). The allele frequencies in the analyses did not deviate from Hardy-Weinberg equilibrium (χ^2 = 0.1843, 0.1201, 0.0318, and 0.4729, respectively; p = 0.6677, 0.7290, 0.8584, and 0.4917, respectively). LD analysis showed that c.923A > G and c.472G > A were in strong LD (D' = 0.8289, r^2 = 0.5332).

Table 2
Genotyping and allele frequencies of *FMO3* single nucleotide polymorphisms (SNPs) in this study

SNP	Genotype	Counts	Frequency	Allele	Frequency	χ^2	*p*-value
c.855C > T	G/G	36	0.2951	G	0.5533	0.1843	0.6677
	G/A	63	0.5164	A	0.4467		
	A/A	23	0.1885				
c.441C > T	G/G	70	0.5738	G	0.7664	0.1201	0.7290
	G/A	47	0.3852	A	0.2336		
	A/A	5	0.041				
c.923A > G	A/A	72	0.5901	A	0.7705	0.0318	0.8584
	A/G	44	0.3607	G	0.2295		
	G/G	6	0.0492				
c.472G > A	C/C	63	0.5164	C	0.7295	0.4729	0.4917
	C/T	52	0.4262	T	0.2705		
	T/T	7	0.0574				
The expected and observed frequencies were compared using the Hardy-Weinberg equation.							

The analyzed allele frequencies were compared to those investigated in other ethnicities and those reported in the HapMap database (Table 3). The data were very limited, especially in European and African populations; however, the trend of frequencies in *FMO3* polymorphisms in our study was most similar to those published previously in the Japanese population. Only the frequencies of c.923A > G and c.472G > A appeared to be similar in the Chinese population. The occurrence of the c.923A > G polymorphism showed some similarity to the minor allele frequency (MAF) of the HapMap data on Utah residents with Northern and Western European ancestry from the CEPH collection reported by the National Center for Biotechnology Information SNP database (https://www.ncbi.nlm.nih.gov/snp), while others showed substantial differences.

Table 3
Comparisons of *FMO3* allele frequencies in this study with those in other ethnic groups

SNP	Population	Frequency (%)	Reference
c.855C > T	Korean (n = 122)	44.7	Present study
	Japanese (n = 3552)	38.8	[23]
	Chinese (n = 285)	26.1	[32]
	European (n = 226)	27.9	HapMap-CEU database
	Sub-Saharan African (n = 294)	54.4	HapMap-YRI database
c.441C > T	Korean (n = 122)	23.4	Present study
	Japanese (n = 3552)	19.9	[23]
	Chinese (n = 285)	5.8	[32]
	European (n = 226)	6.6	HapMap-CEU database
	Sub-Saharan African (n = 294)	3.7	HapMap-YRI database
c.923A > G	Korean (n = 122)	23.0	Present study
	Japanese (n = 3552)	19.8	[23]
	Chinese (n = 285)	19.8	[32]
	European (n = 170)	35.9	[31]
	Sub-Saharan African (n = 294)	1.4	HapMap-YRI database
c.472G > A	Korean (n = 122)	27.1	Present study
	Japanese (n = 3552)	21.0	[23]
	Chinese (n = 285)	16.5	[6]
	European (n = 224)	42.0	HapMap-CEU database
	African-American (n = 133)	41.9	[33]

SNP, single nucleotide polymorphism; MAF, minor allele frequency; CEU, Utah residents with Northern and Western European ancestry from the CEPH collection; YRI, Yoruba in Ibadan, Nigeria

4. Discussion

Our results indicate that the newly developed rapid pyrosequencing method for analyzing the c.855C > T, c.441C > T, c.923A > G, and c.472G > A SNPs is a feasible and accurate technique. The allele frequencies obtained by this method in 122 Korean subjects suggested that the results were generally most similar to

those reported in the Japanese population [23]. To our knowledge, this is the first study to analyze non-synonymous *FMO3* SNPs using a pyrosequencing method.

Various methods have been suggested for the analysis of the targeted SNPs. For example, *FMO3*-related SNPs were detected by PCR-restriction fragment length polymorphism [24], real-time PCR [25], or direct sequencing methods [26]. The automated sequencing method was first introduced in the 1970s by Frederick Sanger [27]. The principle of this method is the use of dideoxynucleotide triphosphates as a DNA sequence termination technique. Our pyrosequencing method for analyzing *FMO3* SNPs was designed based on the solution-based pyrosequencing method suggested by Ronaghi et al. in 1998, which is a simple method suitable for automation using apyrase, DNA polymerase, and luciferase that eventually detects light emission by pyrophosphate production during DNA synthesis [28]. The major advantages of this method are its simplicity, feasibility, sensitivity, and specificity compared to conventional sequencing systems [29]. Therefore, we assumed that our developed method was suitable for our study aims to precisely, rapidly, and cost-effectively measure the occurrence of targeted SNPs in a relatively large sample size.

SNPs are the most frequently occurring sequence variations in the human genome and often vary among different ethnic groups. Among the currently reported non-synonymous *FMO3* SNPs, we chose to analyze the c.855C > T, c.441C > T, c.923A > G, and c.472G > A SNPs, which are relatively common and known to be functionally effective in East Asian populations as described previously [9, 23]. The allele frequencies observed in this study were comparable to those reported in the Japanese population, while only c.923A > G and c.472G > A were similar to those found in the Chinese population. Among the analyzed *FMO3* SNPs, c.855C > T was the most common in the Korean population, and the result was similar to that reported in a smaller Korean population (n = 41, MAF = 0.329) [9].

Several previous studies have investigated functional SNPs in *FMO3*, although detailed pharmacokinetic studies based on *FMO3* genetic polymorphisms are scarce. For example, c.441C > T and c.855C > T are associated with fast tacrolimus elimination in Chinese patients [30]. Furthermore, c.855C > T and c.472G > A have been shown to affect the pharmacokinetics of sulindac in women who underwent preterm labor [9]. c.923A > G has been associated with reduced nicotine dependence in European Americans [31]. Considering these ethnic and inter-individual differences in SNPs and their suspected clinical roles, personalized dosing, pharmacokinetics, and pharmacodynamics studies for drugs based on *FMO3* SNPs may present a novel research direction.

In conclusion, the designed pyrosequencing method was successfully applied to identify the c.855C > T, c.441C > T, c.923A > G, and c.472G > A SNPs. In Korean subjects, c.855C > T (rs909530) was most frequently found among the four non-synonymous *FMO3* SNPs. Significant differences were observed when the frequencies of these alleles were compared to those of other ethnic groups; however, they were most similar to those reported in the Japanese population.

Declarations

Funding: No funding to declare.

Conflicts of interest: No conflicts of interest to declare.

Ethics approval: The authors have obtained appropriate institutional review board approval and followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent was obtained from all participants.

Consent to participate: Participants agreed and signed written informed consents.

Consent for publication: Participants agreed and signed written informed consents.

Availability of data and materials: Not available for participants' privacy.

Code availability: Not applicable.

Authors' contributions: JW Park and JY Park wrote the manuscript and designed the research. IW Park, JM Kim, and KA Kim performed the data analysis.

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Figures

Figure 1.

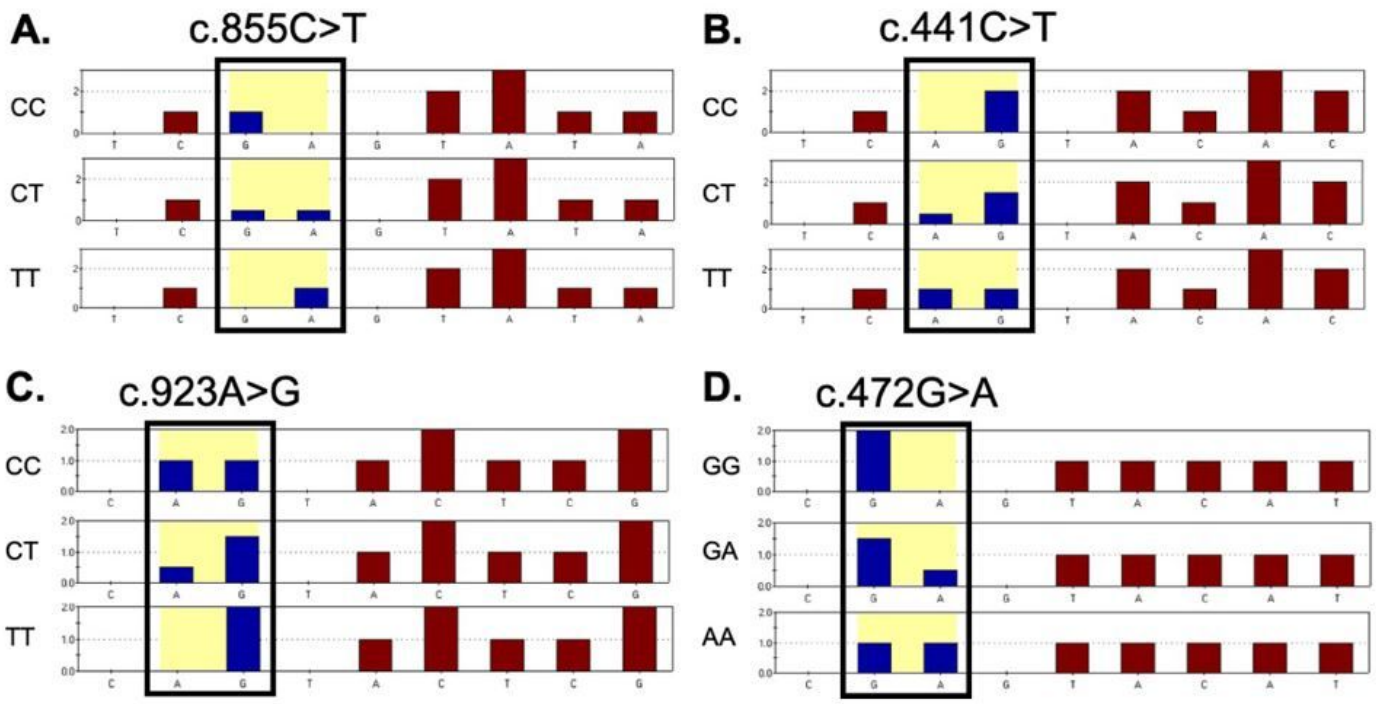

Figure 1

Designed pyrosequencing histograms for FMO3 single nucleotide polymorphisms using pyrosequencing software. Black boxed areas indicate the polymorphism site to be detected (A to D).

Figure 2.

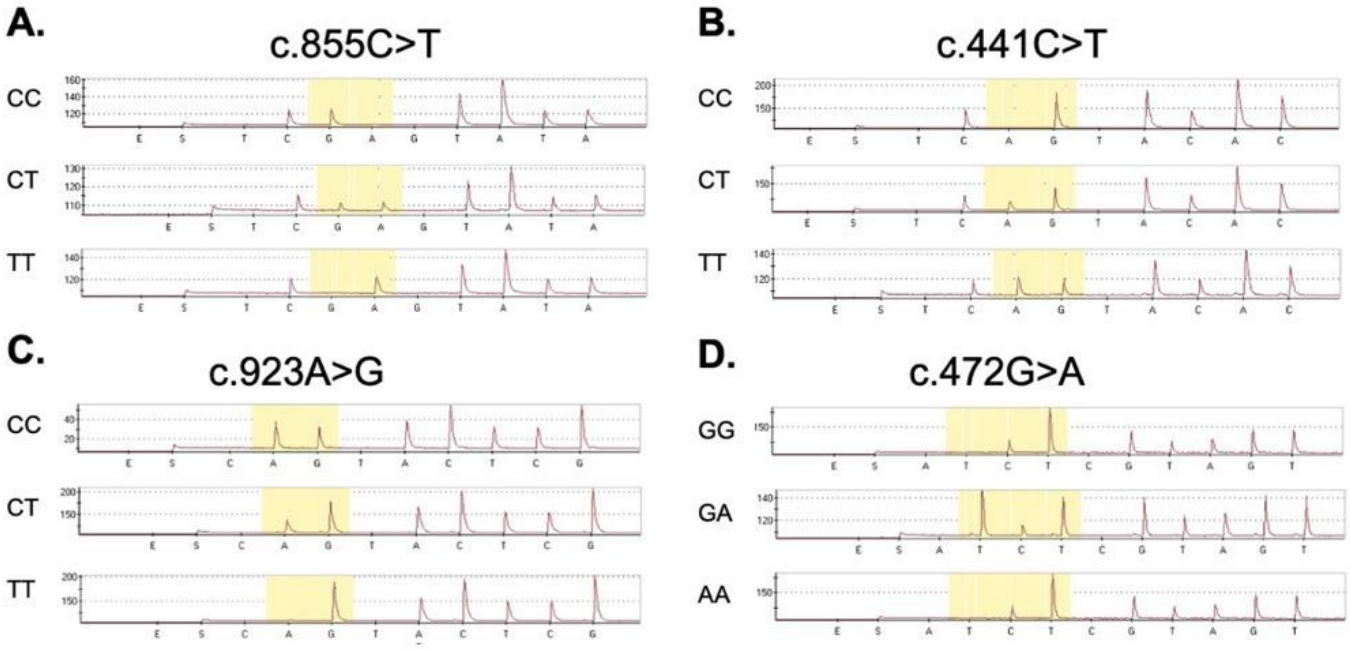

Figure 2

Representative pyrograms for identifying FMO3 single nucleotide polymorphisms (yellow highlights: c.855C>T [A], c.441C>T [B], c.923A>G [C], and c.472G>A [D]) using the established pyrosequencing method.