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

Jin-woo Park
Korea University Medical Center

In-Hwan Park
Korea University - Seoul Campus: Korea University

Jong-Min Kim
Korea University - Anam Campus: Korea University

Kyoung-Ah Kim
Korea University Medical Center

Ji-Young Park (jypark21@korea.ac.kr)
Korea University Medical Center

Research Article

Keywords: Pyrosequencing, c.855C>T, c.441C>T, c.923A>G, c.472G>A, FMO3

Posted Date: February 11th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-164091/v1

License: ☕️ This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
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 (rs2266782), 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 \textit{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

\textbf{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).

\textbf{Polymerase chain reaction (PCR) conditions and \textit{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 \textit{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 \textit{FMO3} genotyping and pyrosequencing are listed in Table 1. PCR reactions were performed to amplify sequences to identify each \textit{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).
| SNP                      | Primer | Sequences                        | Size (bp) | PCR (Tm; °C) |
|-------------------------|--------|----------------------------------|-----------|--------------|
| *FMO3* (c.855C > T, rs909530) | Forward | B 5'-TTGGGTCATTTTTTCTTCTTAT-3'   | 261       | 60           |
|                         | Reverse | 5'-ACCCCTGGTGGAAAGATTACACAGT-3'  |           |              |
|                         | Sequencing | 5'-TTGCTGGGAGGCTCAT-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'-AGCATTCTGTGTGCCATTGT-3'      | 144       | 60           |
|                         | Reverse | B 5'-AAGGAAGGGTAAGCAAAACTAT-3' |           |              |
|                         | Sequencing | 5'-CGTGAAGGAATTACAG-3'         |           |              |
| *FMO3* (c.472G > A, rs2266782) | Forward | B 5'-ATGGTAAAGAGGATCGGATGTC-3' | 132       | 60           |
|                         | Reverse | 5'-TTTTGTACGTTATGTGGCTAGCAG-3' |           |              |
|                         | Sequencing | 5'-GCCTACCTGGAAAGGACT-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 mM), 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 (\( \chi^2 = 0.1843, 0.1201, 0.0318, \) 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 | $\chi^2$ | $p$-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.

Acknowledgments: Not applicable.

References

1. Phillips, I.R. and Shephard, E.A. (2017) Drug metabolism by flavin-containing monooxygenases of human and mouse. Expert Opin Drug Metab Toxicol 13 (2), 167-181.

2. Phillips, I.R. and Shephard, E.A. (2020) Flavin-containing monooxygenase 3 (FMO3): genetic variants and their consequences for drug metabolism and disease. Xenobiotica 50 (1), 19-33.

3. Krueger, S.K. and Williams, D.E. (2005) Mammalian flavin-containing monooxygenases: structure/function, genetic polymorphisms and role in drug metabolism. Pharmacology & therapeutics 106 (3), 357-387.

4. Ceriello, A. et al. (2019) The Unique Pharmacological and Pharmacokinetic Profile of Teneligliptin: Implications for Clinical Practice. Drugs 79 (7), 733-750.

5. Tang, Y.-J. et al. (2017) Effects of FMO3 Polymorphisms on Pharmacokinetics of Sulindac in Chinese Healthy Male Volunteers. BioMed research international 2017, 4189678-4189678.

6. Xu, M. et al. (2017) Genetic and Nongenetic Factors Associated with Protein Abundance of Flavin-Containing Monooxygenase 3 in Human Liver. The Journal of pharmacology and experimental therapeutics 363 (2), 265-274.

7. Treacy, E.P. et al. (1998) Mutations of the Flavin-Containing Monooxygenase Gene (FMO3) cause Trimethylaminuria, a Defect in Detoxication. Human Molecular Genetics 7 (5), 839-845.

8. Dolphin, C.T. et al. (1997) Structural organization of the human flavin-containing monooxygenase 3 gene (FMO3), the favored candidate for fish-odor syndrome, determined directly from genomic DNA.
9. Park, S. et al. (2014) Effects of single-nucleotide polymorphisms of FM03 and FM06 genes on pharmacokinetic characteristics of sulindac sulfide in premature labor. Drug Metab Dispos 42 (1), 40-3.

10. Shimizu, M. et al. (2014) Relationships between flavin-containing mono-oxygenase 3 (FM03) genotype and trimethylaminuria phenotype in a Japanese population. Br J Clin Pharmacol 77 (5), 839-51.

11. Shimizu, M. et al. (2012) Variants in the flavin-containing monooxygenase 3 (FM03) gene responsible for trimethylaminuria in a Japanese population. Mol Genet Metab 107 (3), 330-4.

12. Shimizu, M. et al. (2019) Novel variants and haplotypes of human flavin-containing monooxygenase 3 gene associated with Japanese subjects suffering from trimethylaminuria. Xenobiotica 49 (10), 1244-1250.

13. Bushueva, O.Y. et al. (2015) Association of Flavin Monooxygenase Gene E158K Polymorphism with Chronic Heart Disease Risk. Bull Exp Biol Med 159 (6), 776-8.

14. Robinson-Cohen, C. et al. (2016) Association of FM03 Variants and Trimethylamine N-Oxide Concentration, Disease Progression, and Mortality in CKD Patients. PLoS One 11 (8), e0161074.

15. D’Angelo, R. et al. (2013) FM03 allelic variants in Sicilian and Sardinian populations: trimethylaminuria and absence of fish-like body odor. Gene 515 (2), 410-5.

16. Fennema, D. et al. (2016) Trimethylamine and Trimethylamine N-Oxide, a Flavin-Containing Monooxygenase 3 (FM03)-Mediated Host-Microbiome Metabolic Axis Implicated in Health and Disease. Drug Metab Dispos 44 (11), 1839-1850.

17. Yang, S. et al. (2019) Gut Microbiota-Dependent Marker TMAO in Promoting Cardiovascular Disease: Inflammation Mechanism, Clinical Prognostic, and Potential as a Therapeutic Target. Front Pharmacol 10, 1360.

18. Sung, J.W. et al. (2020) Population Pharmacokinetics of Sulindac and Genetic Polymorphisms of FM03 and AOX1 in Women with Preterm Labor. Pharm Res 37 (3), 44.

19. Hoffmeyer, S. et al. (2000) Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo. Proceedings of the National Academy of Sciences 97 (7), 3473-3478.

20. Cascorbi, I. et al. (2001) Frequency of single nucleotide polymorphisms in the P-glycoprotein drug transporter MDR1 gene in white subjects. Clinical Pharmacology & Therapeutics 69 (3), 169-174.

21. Du, F.X. et al. (2007) Characterizing linkage disequilibrium in pig populations. Int J Biol Sci 3 (3), 166-78.

22. Kim, K.A. et al. (2005) Genetic polymorphisms and linkage disequilibrium of sulfotransferase SULT1A1 and SULT1A2 in a Korean population: comparison of other ethnic groups. Eur J Clin Pharmacol 61 (10), 743-7.
23. Shimizu, M. et al. (2019) 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 Metab Pharmacokinet 34 (5), 334-339.

24. Bae, S.Y. et al. (2006) Effects of genetic polymorphisms of MDR1, FMO3 and CYP1A2 on susceptibility to colorectal cancer in Koreans. Cancer Sci 97 (8), 774-9.

25. Chuwongwattana, S. et al. (2020) Impact of CYP2C19, CYP3A4, ABCB1, and FMO3 genotypes on plasma voriconazole in Thai patients with invasive fungal infections. Pharmacol Res Perspect 8 (6), e00665.

26. D’Angelo, R. et al. (2014) Fish odor syndrome (trimethylaminuria) supporting the possible FMO3 down expression in childhood: a case report. J Med Case Rep 8, 328.

27. Sanger, F. et al. (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A 74 (12), 5463-7.

28. Ronaghi, M. et al. (1998) A sequencing method based on real-time pyrophosphate. Science 281 (5375), 363, 365.

29. Siqueira, J.F., Jr. et al. (2012) Pyrosequencing as a tool for better understanding of human microbiomes. J Oral Microbiol 4.

30. Ren, L. et al. (2017) Donors FMO3 polymorphisms affect tacrolimus elimination in Chinese liver transplant patients. Pharmacogenomics 18 (3), 265-275.

31. Teitelbaum, A.M. et al. (2018) Nicotine dependence is associated with functional variation in FMO3, an enzyme that metabolizes nicotine in the brain. Pharmacogenomics J 18 (1), 136-143.

32. Xu, M. et al. (2017) Genetic and Nongenetic Factors Associated with Protein Abundance of Flavin-Containing Monooxygenase 3 in Human Liver. J Pharmacol Exp Ther 363 (2), 265-274.

33. Chenoweth, M.J. et al. (2014) Variation in P450 oxidoreductase (POR) A503V and flavin-containing monooxygenase (FMO)-3 E158K is associated with minor alterations in nicotine metabolism, but does not alter cigarette consumption. Pharmacogenetics and genomics 24 (3), 172-176.

Figures
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

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.