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TaqMan Genotyping Assay Method for Single Nucleotide Polymorphisms (SNPs) detection in promoter region of N-Acetyltransferase 2 (NAT2) gene

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ABSTRACT *The N-acetyltransferase 2 (NAT2) polymorphism in coding region has been studies intensively. However, there are limited studies for promoter region of NAT2 gene. Several reported study showed that the promoter region polymorphism of NAT2 gene is genotyped by PCR-sequencing approach. In this paper, we describe TaqMan based assays for the NAT2 polymorphism genotyping in promoter region with following SNP: rs4646243 [T>C], rs4646244 [T>A], rs4646267 [A>G], rs4345600 [A>G], and rs4646246 [A>G]. Our result showed a good separation cluster, trailing cluster and some mix cluster. TaqMan genotyping assay has shown a sensitivity and specificity to detect polymorphism in NAT2 promoter region.*

INTRODUCTION

Human arylamine N-acetyltransferase 2 (NAT2) is responsible for the acetylation of numerous arylamine and hydrazine xenobiotics including drugs and carcinogens which consequently relates to drug effects or toxicity level (Walraven et al. 2008). The NAT2 enzyme is encoded by NAT2 gene located in human chromosome 8 (Fuselli et al. 2007). Polymorphism at the N-acetylation level is an example of a pharmacogenetic trait that implies on inter-individual and interethnic differences in response to xenobiotics (Hein et al. 2000).

Previous reported study showed that NAT2 polymorphism in coding region can be categorized into rapid, intermediate and slow groups based on the acetylation rate. Several studies have shown that the slow-acetylator NAT2 phenotype is strongly associated with a higher risk of AT-DILI consistently across different ethnic groups (Ohno et al. 2000; Fountain et al. 2005; Khalili et al. 2011; Yuliwulandari et al. 2016). A previous study on NAT2 in individuals within the Javanese and Sundanese ethnic groups of Indonesia reveals that slow-acetylator NAT2 variants are frequently observed (Yuliwulandari et al. 2008). Another study in Indonesia population also reveals that slow acetylator allele has a strong association with AT-DILI (Yuliwulandari et al. 2016). However, most studies focusing on NAT2 have

been based on the genotyping of nucleotide variants in the coding region and ignore the potential effects of the role of promoter polymorphisms in determining NAT2 phenotypes. Promoter region is the regulator region for gene expression and initiate transcription of a particular gene (Li & Zhang 2014; De Vooght et al. 2009). However, the best of our knowledge, the genotyping method for NAT2 promoter region was using sequencing approach. Therefore, we tried to find another approach to genotype single SNP with affordable price and good result.

The TaqMan allelic discrimination assay is a very popular and widely used medium-throughput genotyping format: the assays use two short minor groove binder (MGB) hydrolysis probes, each annealing to one allelic variant of the genotyped single nucleotide polymorphism (SNP) amplified within a short PCR product (Malkki & Petersdorf 2012; Shen et al. 2009). The TaqMan allelic discrimination is supported by most of real-time PCR platforms, however their proprietary software solutions utilize mainly the endpoint analyses, largely overlooking the potential of fluorescence data collected over the whole course of a PCR run (Konopac et al. 2011). In the present paper, we describe TaqMan based assays for the NAT2 polymorphism in promoter region, which illustrate the general principle of TaqMan based genotyping.

METHOD

Sample Collection

Samples was obtained from Sample bank of YARSI Research Institute, Universitas YARSI. All of protocol in this study was approved by Universitas YARSI Ethics Committee.

DNA Extraction

Genomic DNA (gDNA) was extracted using the QIAamp Blood mini kit (Qiagen) according to manufacturer's instructions. Isolated gDNA was analyzed by 0.8% agarose gel electrophoresis to evaluate DNA quality. DNA quantity was assessed by using the Infinite 200 Pro NanoQuant (TECAN).

SNP genotyping in NAT2 promoter region

SNP genotyping was performed with TaqMan® SNP genotyping assays (Applied Biosystems Inc) to detect the specific NAT2 promoter region that contain the following SNPs: rs4646243 [T>C], rs4646244 [T>A], rs4646267 [A>G], rs4345600 [A>G], and rs4646246 [A>G] (Yuliwulandari et al. 2008).

SNP genotyping was performed on Real-time PCR thermal cycler LightCycler 480 (Roche) in 384 well plates pre-loaded with 20x TaqMan genotyping Assays in 5 µL reaction volume including 1x TaqMan genotyping master mix (Life Technologies), nuclease free water (Promega), and 10 ng DNA template. PCR program the following cycling conditions at 95°C for 10 min, followed by 40 cycles at 92 °C for 15 s and at 60 °C for 1 min. The amplification analysis was performed using allelic discrimination with automated allele calling settings for the Light Cycler Software 4.0 (Roche).

RESULT

An allelic discrimination cluster plot is shown in Fig. 1 to Fig. 5 from SNP amplification in promoter region. The cluster plot diagram was analyzed using EndPoint Genotyping Analysis

on Light Cycler Software 4.0 software (Roche). A fluorescent signal from only the VIC dye indicates homozygosity for Allele A; the presence of only FAM dye fluorescence indicates homozygosity for Allele B, and the presence of both fluorescent signals indicates Allele A and Allele B (A/B) heterozygosity.

Ideally, a cluster plot shows one, two, or three clusters and the no template controls (NTCs). The points in each cluster are grouped closely together and each cluster is located well away from the other clusters. Based on TaqMan Assay manual (Applied Biosystem 2005), allele A will be located on lower right corner, Allele B will be located on upper left corner and allele A/B will be located approximately on midway between allele A and allele B cluster. No templete control (NTC) always located on the bottom left corner, as well as no-amplification sample. Meanwhile undetermined samples or cluster will be located anywhere outside the region of fix cluter.

A cluster plot from rs4646243 (Fig. 1), rs4646244 (Fig. 2), rs4646267 (Fig. 3), and rs4646246 (Fig. 5) showed a good separation. Good separation means the NTC are distant from any clusters and the separation of the three clusters is clear. However, the cluster in rs4646243 (Fig. 1) and rs4646267 (Fig. 3) was shown little bit trailing. A cluster plot from rs4345600 (Fig. 4) are not well separated between undetermined cluster and major cluster. Those cluster also shown a trailing cluster as well as no amplification sample. The undetermined sample also shown in rs4646243 cluster (Fig. 1)

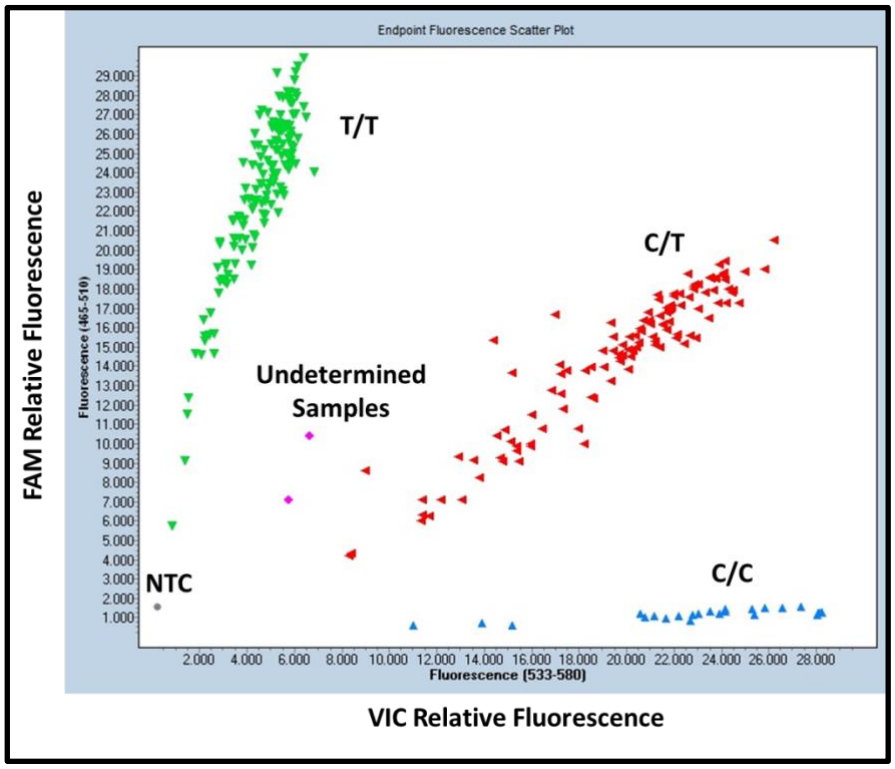

Figure 1. TaqMan assay genotype result for SNP rs4646243

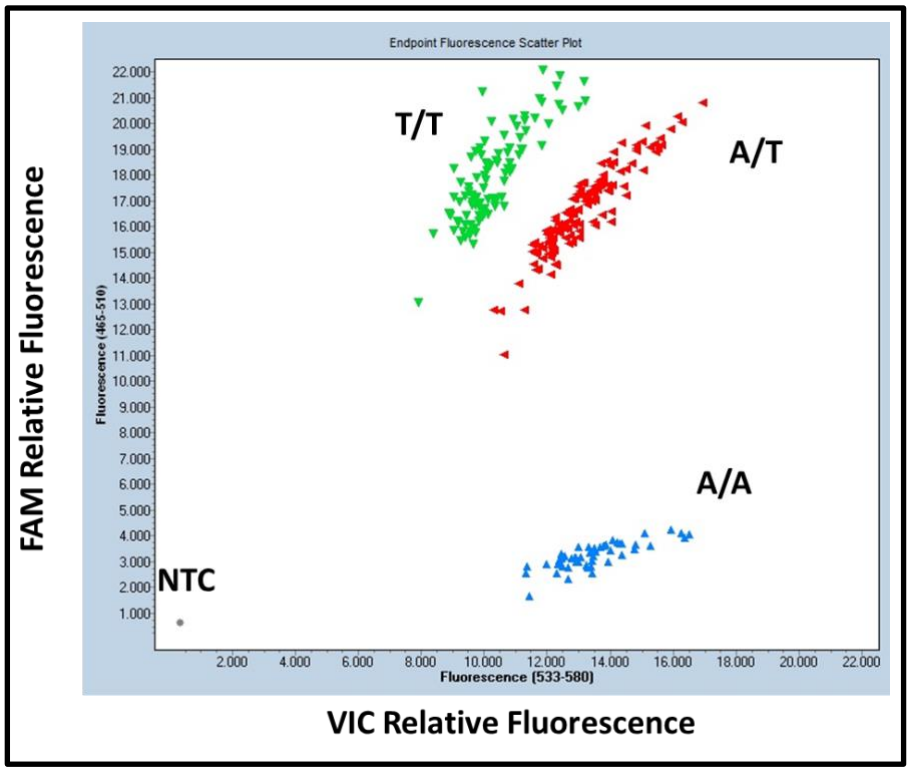

Figure 2. TaqMan assay genotype result for SNP rs4646244

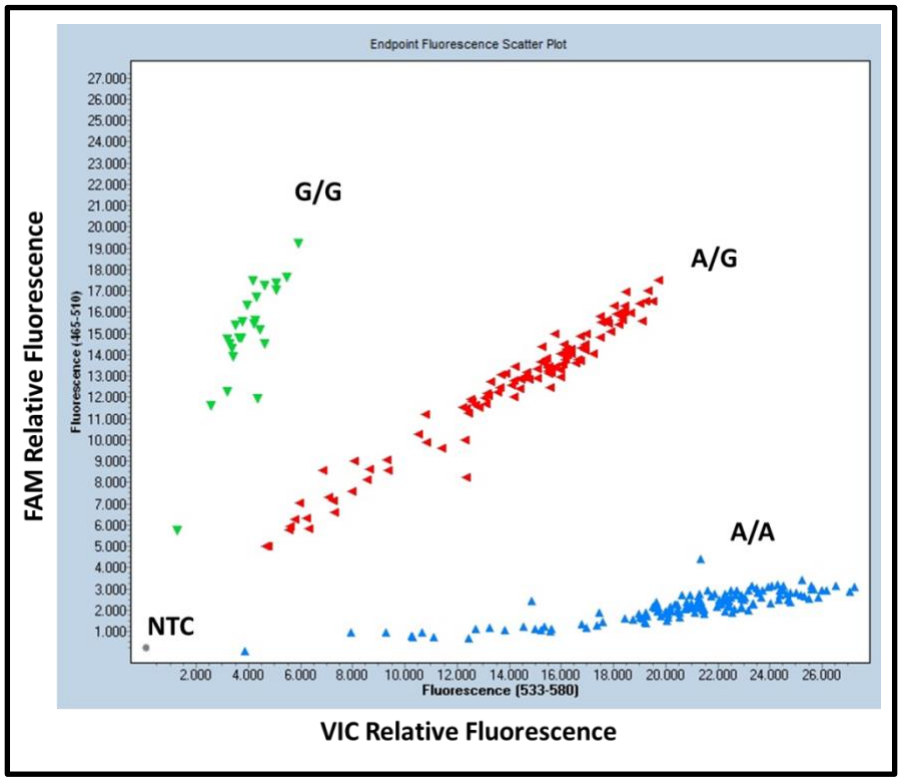

Figure 3. TaqMan assay genotype result for SNP rs4646267

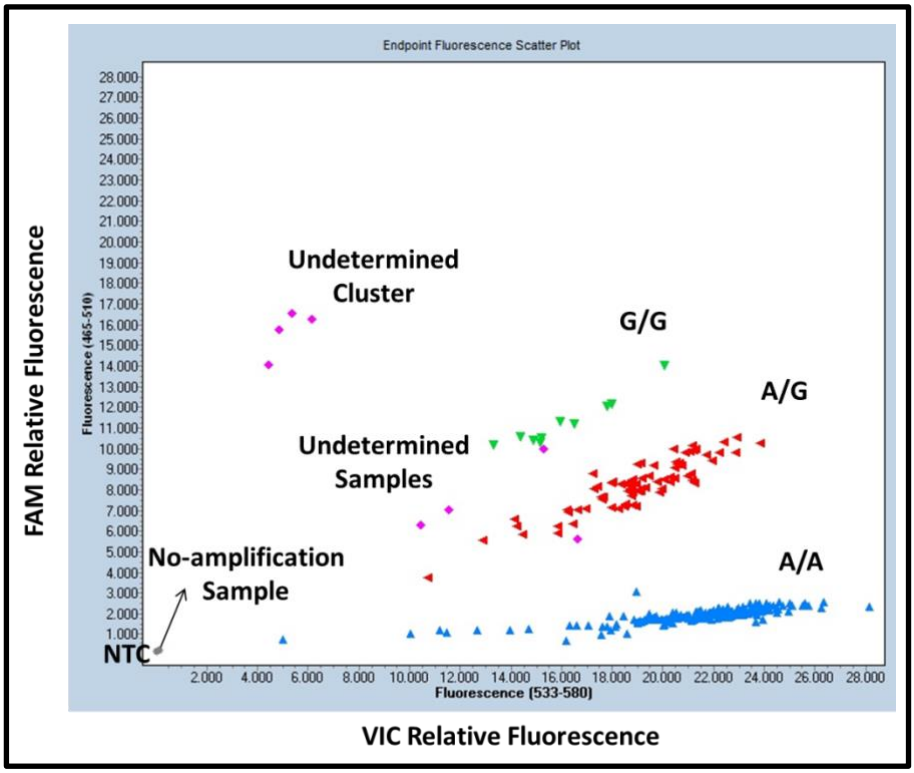

Figure 4. TaqMan assay genotype result for SNP rs4345600

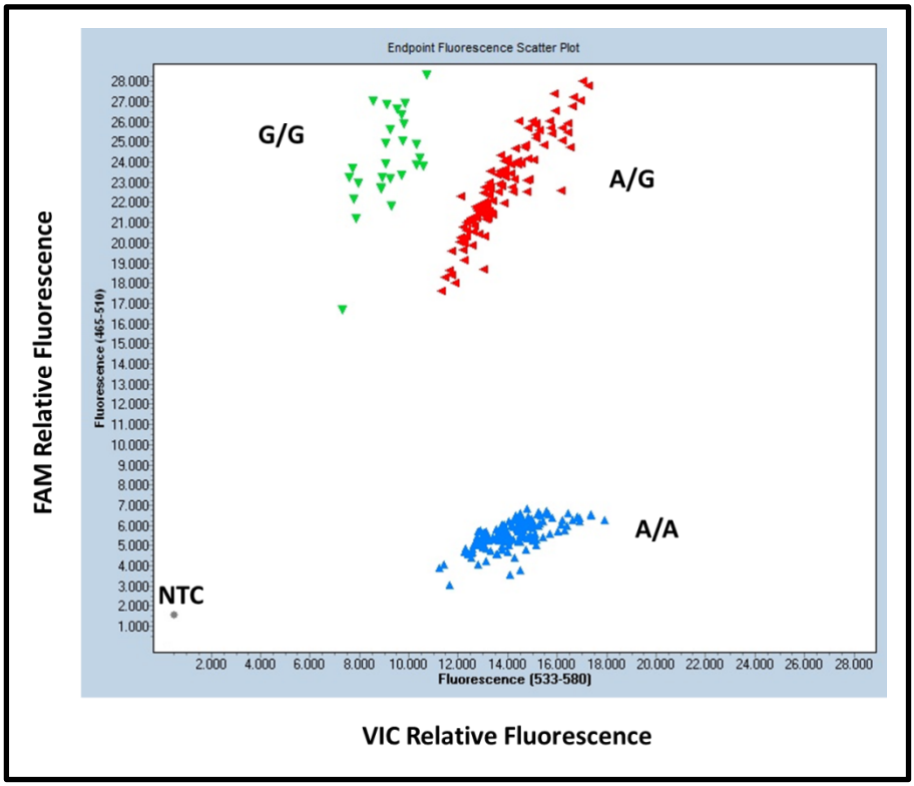

Figure 5. TaqMan assay genotype result for SNP rs4646246

DISCUSSION

Study of NAT2 polymorphism in promoter region is still rarely done. Most of study are still focusing in coding region polymorphism. Whereas, promoter region has an important role as a regulatory region for gene expression and initiate transcription of a NAT2 gene. Previous reported study regarding promoter region has been conducted by Yuliwulandari et al (Yuliwulandari et al. 2008) and Kang et al (Kang et al. 2009) in normal population with PCR-direct sequencing approach to genotyped the polymorphism. Therefore in this study we used TaqMan assay genotyping approach in AT-DILI cases and control to genotyped the polymorphism. To the best of our knowledge, it is the first study in Indonesia.

Among the most robust assays for SNP genotyping, TaqMan SNP Genotyping Assays are provides a more economic and easy to handle procedure to efficiently generate thousands of genotypes for genetic association studies (Schleinitz et al. 2011). Each assay allows genotyping of individuals for a single nucleotide polymorphism (SNP) (Applied Biosystem 2014). The TaqMan SNP Genotyping Assay involves the use of forward and reverse primers to amplify the polymorphic sequence of interest and two dye-labeled probes for allele-specific detection. At the 5′ site, one probe is labeled with VIC dye, which detects the "Allele 1" sequence, while the other one is labeled with 6FAM dye, which detects the "Allele 2" sequence. VIC and 6FAM are also referred to as reporter dyes. Based on the principle of fluorescence resonance energy transfer, at the 3′ site the probes are labeled with a nonfluorescent quencher (NFQ), which absorbs the energy of the reporter dyes as long as both are bound to the probe and therefore are in spatial proximity. Further, TaqMan probes incorporate a minor groove binder (MGB) at the 3′ end. The MGB molecule (e.g., dihydrocyclopyrroloindole tripeptide [DPI3]) binds to the minor groove of the DNA helix and improves hybridization-based assays by stabilizing the probe/ template complex (Schleinitz et al. 2011).

In general, our result showed a good separation cluster between allele A homozygous, allele B homozygoes and allele A/B heterozygous for rs4646243 (Fig. 1), rs4646244 (Fig. 2), rs4646267 (Fig. 3), and rs4646246 (Fig. 5) except for rs4345600 (Fig. 4). However, some of good separation cluster shown a trailing clusters (Fig. 1 & Fig 3). Trailing cluster describes the variation of fluorescence signals among a given genotype that has the appearance of being spread across an imaginary line that extends from the no template control (NTC). This phenomenon is often caused by variability in concentration of individual genomic DNA (Malkki & Petersdorf 2012; Applied Biosystem 2014). The quality of genomic DNA concentration is critical in TaqMan Assay approach. Furthermore, because of the quantitative nature of the assay, every effort should be made to ensure a uniform concentration of DNA across samples. DNA concentration should be determined using a fluorescent method as this is more accurate than that determined by measuring absorbance at 260 nm (Hui et al. 2008). Fluorescence assays are less prone to interference

than A260 measurements. Fluorophore excitation and subsequent emission produce higher sensitivity, and many fluorophores provide specificity by preferentially binding to double stranded DNA (Gallagher & Desjardins 2007).

An undetermined samples as shown in rs4646243 (Fig. 1) and rs4345600 (Fig. 4) are considers as outliers. Generally, it caused by a variable DNA quality or pipetting issues (Applied Biosystem 2014). Because of low confidence in assigning the correct genotype, this small number of samples should not be used in data analysis and should remain undetermined (Hui et al. 2008). A modification of PCR annealing temperature is one of best approach to prevent an outliers samples and make a better separation clusters (Malkki & Petersdorf 2012).

Besides an undetermined samples, there are no-amplification sample showed in rs4345600 clusters (Fig. 4). There are some possibilities that cause this problem. The presence of polymerase inhibitors can decrease PCR efficiency. Potential PCR inhibitors can originate from the tissue source of the DNA sample or from the purification method. Degraded DNA also can affect PCR efficiency due to the presence of fewer template copies, which will affect the success of TaqMan SNP Genotyping Assay. Degradation can result from using very old DNA samples freezing and thawing DNA samples repeatedly and leaving DNA samples at room temperature (Applied Biosystem 2014).

CONCLUSION

TaqMan genotyping assay method has shown a sensitivity and specificity to detect polymorphism in NAT2 promoter region. It is still comparable to other available techniques used to genotype single SNPs or small numbers of SNPs. It is also economical and time-saving.

An equal concentration of genomic DNA, modification of PCR annealing temperature, and prevent a potential inhibition of PCR amplification has to be tried in further research to prevent a trailing cluster, an outliers cluster and no amplification samples.

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