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Rapid identification of the *NAT2* genotype in tuberculosis patients by multicolor melting curve analysis

Aim: *NAT2* genotype is an indicator for isoniazid dosage adjusting for tuberculosis treatment. Multicolor melting curve analysis (MMCA) was evaluated as a potential method for *NAT2* genotyping. **Materials & methods:** 352 blood samples were analyzed by MMCA kit (Zeesan Biotech Co., Xiamen, China) targeting *NAT2* SNPs at T341C, C481T, G590A and G857A, and direct sequencing was used as control. **Results:** The sensitivity, specificity and accuracy of the MMCA assay for rapid *NAT2* genotype detection were 97.9, 99.6 and 99.1% respectively, whereas for intermediate genotypes the values were 99.5, 98.7 and 99.1%, respectively, and for slow genotypes the values were 100% for the three aspects. The 24 saliva and blood for the control samples were also successfully analyzed using the MMCA assay, both produced uniform outcomes. **Conclusion:** The MMCA assay described in our study is very promising for the efficient determination of *NAT2* genotype, and can facilitate the personalized dosing of isoniazid.

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Keywords: acetylator genotype • multicolor melting curve analysis • *NAT2*

Tuberculosis (TB) is one of the most common infectious diseases and it remains a serious threat to public health despite global awareness and control programs [1]. Isoniazid (INH), the main first-line anti-TB drug, has excellent curative effect and low price. The drug is mainly metabolized by arylamine N-acetyltransferase 2 (*NAT2*) *in vivo* [2,3]. The *NAT2* gene polymorphisms, classify the individuals into three groups that is, the rapid-, intermediate- and slow acetylators [4,5]. Previous studies have demonstrated that plasma INH concentrations of patients with different *NAT2* acetylation status show significant variation. The rapid acetylation patients maintained the lowest level of plasma INH concentration which may cause treatment failure; the slow acetylation patients maintained the highest level of plasma drug concentration that can frequently cause liver injury; while the intermediate acetylation

patients maintained medium drug concentration in the plasma [3,6]. Hence, the identification of *NAT2* genotype for the TB patients is important before commencing the INH-based treatment regimen. Adjusting the INH dosage according to the *NAT2* genotype is a feasible measure for efficacy during medical care.

The *NAT2* gene encodes for a Phase II detoxification enzyme and has over 100 *NAT2* allelic variants, identified in various human populations to date according to the NAT website [7] [8]. Among other polymorphisms in the *NAT2* open reading frame, SNPs at seven different sites of the exon are considered having a most serious phenotypic effect on *N*-acetylation activity: the SNPs rs1801279 (c.191G>A), rs1041983 (c.282C>T), rs1801280 (c.341T>C), rs1799929 (c.481C>T), rs1799930 (c.590G>A), rs1208 (c.803A>G) and rs1799931 (c.857G>A). The

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NAT2 genotypes are categorized as either rapid or slow ones depending on the SNP combinations on the same chromosome and results from *in vitro* and *in vivo* experiments [3,9,10]. In turn, this categorization is used to identify slow (two slow haplotypes), intermediate (one rapid and one slow haplotype) and rapid (two rapid haplotypes) genotypes of *NAT2* [9,11–13]. In theory, the commonly used seven *NAT2* SNPs could produce 128 possible genotypes but in practice, the polymorphic loci in high linkage disequilibrium can greatly reduce the number of observed genotypes. The aforementioned seven *NAT2* SNPs define well-characterized haplotypic groups, commonly encountered in human populations. Thus, using the seven SNPs panel to infer *NAT2* genotype and phenotype is recognized as an appropriate strategy for diagnostic purposes [8].

Deriving the genotype from the seven SNPs by DNA sequencing is still considered as the gold standard for *NAT2* genotyping [5,9,14]. Despite its advantages, automated Sanger sequencing remains more complicated and expensive, compared with PCR-based genotyping methods of a limited set of SNPs. Therefore, we sought to evaluate the multicolor melting curve analysis (MMCA) method to analyze the four most common SNP sites, in *NAT2*, c.341T>C, c.481C>T, c.590G>A and c.857G>A, in Chinese individuals. The *NAT2* genotype of patients can be identified by the composite analysis of the nonvariant and variant for these four SNPs with different *T_m* values and the formation of different melting curves. The performance of the four SNPs MMCA assay was also compared with the gold standard for *NAT2* genotyping. Since the MMCA is a rapid, simple and highly efficient method for SNP detection, it can be adopted by the clinical laboratories for improved and personalized INH dose administration.

Materials & methods

Ethical statement

This study was approved by the Ethics Committee of Beijing Chest Hospital (Beijing, China). All the patients and healthy volunteers enrolled in this study signed written consent forms.

Sample collection

Peripheral blood samples were collected in heparin–lithium–anticoagulant tubes from 352 TB patients who visited Beijing Chest Hospital during April–August 2015. Both saliva samples and peripheral blood samples from 24 healthy volunteers were also collected during the study period.

DNA extraction

Genomic DNA from the peripheral blood samples was extracted using the BloodGen Mini Kit (Cwbio, Beijing, China). The extracted DNA was used as the template for PCR sequencing and the MMCA assay. Quantity and quality of DNA samples was measured using Nanodrop® 2000C (Thermo Scientific, MA, USA). DNA from saliva samples was extracted using a boiling method. More specifically, 1 ml saliva samples were centrifuged at 12,000 rpm for 2 min followed by washing once with 1 ml saline, boiling with 0.5 ml saline at 95°C for 20 min. After centrifugation, the supernatant was collected as the template DNA for MMCA assay.

PCR conditions for MMCA assay

The MMCA PCR was done using a kit developed by Zeesan Biotech Co., Xiamen, China. MMCA assay was performed for the analysis of four SNPs in rs1801280 (c.341T>G), rs1799929 (c.481C>T), rs1799930 (c.590G>A) and rs1799931 (c.857G>A) positions in an 870-bp long coding region of *NAT2*. Totally four differently colored fluorophores were used in this kit, including CY5, FAM, ROX and HEX. The PCR and melting curve analysis were performed on a lightcycler® 480 instrument (Roche Diagnostics, Basel, Switzerland). The assay was performed in a total volume of 25 µl using 19.6 µl of PCR master mix (with probe primers and dNTPs), 0.4 µl of 2U Taq and 5 µl of template DNA as recommended by the manufacturer. PCR was initiated by 10 min of denaturation–activation at 95°C, followed by 10 cycles at 95°C for 15 s (denaturation), 70°C for 15 s with 1°C decrease per cycle until the temperature reached 60°C, extension at 76°C for 20 s, then another 50 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s

Table 1. <i>T_m</i> value of each variant amplicon analyzed for the interpretation of nonvariant or variant.			
Gene	SNP site	<i>T_m</i> (°C)	
		Nonvariant	Variant
<i>NAT2</i>	c.341T>C	CY5 – 64.0 ± 1	CY5 – 69.0 ± 1
	c.481C>T	HEX – 63.0 ± 1	HEX – 54.5 ± 1
	c.590G>A	ROX – 59.0 ± 1	ROX – 54.0 ± 1
	c.857G>A	FAM – 60.0 ± 1	FAM – 55.0 ± 1

Figure 1. Melting curve of the specific SNP sites of *NAT2* in multicolor melting curve analysis assay. The change in melting curve shape caused by nucleotide exchange was demonstrated. Each line indicates the melting curve for homozygous nonvariant (red line, T_{m1}), heterozygous variant (green line, T_{m1}), homozygous variant (red line, T_{m2}), and NC (blue line).

and extension at 76°C for 20 s were performed. The melting program included three steps: denaturation at 95°C for 1 min, follow by renaturation at 35°C for 3 min and a continuous reading of fluorescence from 45 to 85°C by heating at increments of 0.03°C/s. The MMCA curve was analyzed using the Lightcycler® 480 software release 1.5.0 SP3. In this experiment, homozygous nonvariant template and pure water were used as positive and negative controls, respectively.

The sensitivity of MMCA was determined by the following procedure: a DNA sample was firstly diluted to 30 ng/μl, then the sample were serially diluted (1:2) with ddH₂O to the concentration of 0.01 ng/μl. Twelve PCR reactions were performed for each of the eight concentrations obtained, and a 5 μl aliquot was added to each PCR reactions. The sensitivity was evaluated by calculating the PCR reaction successful rate.

PCR amplification for DNA sequencing

For DNA sequencing, an 823 bp fragment of the *NAT2* gene (819–1641 bp of the gene code) was amplified using the forward primer 5'-GGGCT-GTCCCTTTGAGA-3' and reverse primer 5'-TAGTGAGTTGGGTGATAC-3'. Each 20 μl PCR mixture contained 10 μl HiFi PCR mix (Transgen, Beijing, China), 0.5 μl of each of the forward and reverse primers from 10 μM stocks, 8 μl double distilled water and 1 μl DNA template. PCR was performed with initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, with an additional extension at 72°C for 10 min. PCR products were analyzed on 1.5% agarose gels to confirm size of product which was then sequenced with the

same primers as the PCR at a commercial sequencing company (TSINGKE Biological technology, Beijing, China).

Statistical analysis

The statistical analysis was performed using SPSS v12.0 (SPSS Inc., IL, USA). The sensitivity, specificity and accuracy of MMCA method to identify the *NAT2* genotype of TB patients were verified and evaluated in comparison with PCR and direct sequencing method.

Results

MMCA method designed to analyze four SNPs of *NAT2*

The peak pattern of melting curve with different T_m values for nonvariant or variant SNPs are shown in Table 1. The MMCA assay was considered successful since the melting curve of the four channels from the positive control was presented as homozygous nonvariant whereas smooth amplified curves were observed for the negative control. One of the representative melting curves from MMCA analysis is shown in Figure 1. The deviation in the T_m value and the shape of melting curves for the tested patient samples, in contrast with the positive control, is caused by the nucleotide exchange in the specific SNP site. If only a nonvariant peak or a variant peak appeared then the tested sample was identified as a homozygous nonvariant or homozygous variant, respectively, and if both appeared in the melting curve then the tested sample was identified as a heterozygous variant in the specific SNP site. The minimal amount of DNA needed for MMCA reaction was 0.5 μg, and a curve was drew according to the sensitivity analytic assay (Figure 2).

Figure 2. The sensitivity analytic assay outcomes of multicolor melting curve analysis. x-axis indicates the logarithm of DNA concentration (ng/μl); the sensitivity was evaluated by calculating the PCR reaction successful rate for DNA samples with different concentrations. LG: log₁₀.

Four SNPs MMCA assay to identify the *NAT2* genotype of TB patients

The interpretation standard for the four SNPs MMCA assay and the frequencies of each genotype are shown in Table 2. The genotypes and the deduced phenotypes of *NAT2* were identified according to the nomenclature listed on the *NAT2* Alleles Nomenclature homepage [7,8].

Comparison of MMCA method with direct sequencing

According to the direct sequencing and analysis of the seven SNP sites, the *NAT2* genotypes of 352 TB patients consisted of 27.6% (97/352) rapid acetylators, 55.1% (194/352) intermediate and 17.3% (61/352) slow acetylators. For direct sequencing method, *NAT2* genotype analysis only considered the four SNPs included in the MMCA which had perfect con-

Table 2. *NAT2* genotype confirmed by multicolor melting curve analysis method and frequencies of different *NAT2* genotype in the present study.

Type of melting curve analysis peaks	Position of SNP	Genotype	Deduced phenotype	No. of samples	Genotype frequency (%)
Only nonvariant peaks (homozygous)		*4/*4	Rapid	96	27.3
One heterozygous variant peak	481	*11/*4 or *12/*4	Rapid	0	0
	341	*5/*4	Intermediate	1	0.3
	590	*6/*4	Intermediate	92	26.1
	857	*7/*4	Intermediate	77	21.9
Two heterozygous variant peaks	481,341	*5/*11 or *5/*12 or *5/*4	Intermediate	23	6.5
	481,590	*11/*6 or *12/*6 or *6/*4	Intermediate	1	0.3
	481,857	*11/*7 or *12/*7 or *6/*4	Intermediate	1	0.3
	341,590	*5/*6	Slow	0	0
	341,857	*5/*7	Slow	0	0
	590,857	*6/*7	Slow	28	8
Three heterozygous variant peaks	Any combination		Slow	7	2
Four heterozygous variant peaks	341,481,590,857		Slow	0	0
One homozygous variant peak	341	*5/*5	Slow	0	0
	590	*6/*6	Slow	22	6.2
	857	*7/*7	Slow	4	1.1
	481	*11/*11 or *11/*12 or *12/*11 or *12/*12	Rapid	0	0
	481 homozygous variant + any single heterozygous variant		Intermediate	0	0
	481 homozygous variant + three heterozygous variant		Slow	0	0

cordance with the seven-SNP analysis. The four-SNP MMCA assay was directly compared with the seven-SNP analysis postsequencing method to calculate the sensitivity, specificity and accuracy (Table 3). The sensitivity, specificity and accuracy of MMCA method for the rapid acetylator *NAT2* genotypes were 97.9 (95/97), 99.6 (254/255) and 99.1% (348/352), respectively, and 99.5 (193/194), 98.7 (156/158) and 99.1% (349/352) for the intermediate acetylator genotypes and 100 (61/61), 100 (291/291) and 100% (352/352) for slow acetylator genotypes, respectively. The overall accuracy of the four-SNP MMCA method for *NAT2* genotyping was 99.1% (349/352).

MMCA of *NAT2* genotype using saliva samples

Saliva samples from the 24 healthy volunteers were also successfully analyzed with the MMCA assay. The

results derived from these samples were completely consistent with the blood samples from respective individuals.

Discussion & conclusion

Early in the 1960s, the polymorphism of *NAT2* was reported to have association with INH neurotoxicity due to their effect on variability in *N*-acetylation capacity [15]. Other than INH, many aromatic amine drugs are also affected by the acetylation polymorphism [16,17]. The studies conducted in east-Asian population showed a significantly higher risk of INH-induced hepatotoxicity in slow acetylators with odds ratios of 3.10, 3.18 and 4.70 in different studies. Furthermore, many clinical and observational studies confirmed that *NAT2* genotype-guided dosing of INH resulted in lower incidences of antituberculosis drug-

Table 3. Sensitivity, specificity and accuracy of the multicolor melting curve analysis assay in comparison with direct sequencing.						
Direct sequencing	Patients (n)					
	Rapid by MMCA	Intermediate by MMCA	Slow by MMCA	Sensitivity	Specificity	Accuracy
Rapid	95	2	0	97.9%	99.6%	99.1%
Intermediate	1	193	0	99.5%	98.7%	99.1%
Slow	0	0	61	100%	100%	100%
MMCA: Multicolor melting curve analysis.						

induced hepatotoxicity (ATDH) or early treatment failure than the conventional standard regimen [18–21]. Hence, the literature indicates a straightforward correlation between *NAT2* genotype and phenotype, suggesting that determination of *NAT2* genotype could be an accurate way to infer the acetylator phenotype of individuals treated with INH [3,22].

The distribution of *NAT2* polymorphisms is highly diverse among various populations. For instance, the Western populations show a significantly higher percentage of slow acetylators genotypes (40–70%) [23–25], while the prevalence of the *NAT2* slow acetylators genotypes is 15–20% in Chinese and Japanese populations [26–28]. According to our seven-SNP analysis postsequencing outcomes, the *NAT2* genotypes of 352 TB patients consisted of 27.6% rapid acetylators, 55.1% intermediate and 17.3% slow acetylators. The frequency of rapid, intermediate, slow acetylators and the distribution of *NAT2* genotypes in our study are generally similar to previous studies conducted in Chinese population [29]. The results suggest that about 40–50% of the TB patients who are rapid acetylators or slow acetylators in China require an adjusted dosage of INH, thus determination of *NAT2* genotype before INH administration is important.

At present, many methods can be used for the identification of gene polymorphisms, such as PCR–HPLC method, reverse dot blot and direct sequencing. Despite being the gold standard for all other methods, DNA sequencing is complicated and requires expensive equipment which decreases its feasibility for a clinical laboratory. Although the PCR–HPLC is affordable and easy-to-apply, but the specificity and sensitivity of this method are not ideal, thus it is not suitable for large-scale sample testing. MMCA detects the mutations using multiple dual labeled, self-quenched probes each labeled with differently colored fluorophore [30,31], proved to be a rapid, reliable and efficient approach for the detection of multiple SNPs in a single reaction tube [32–34]. What is more, these probes-based MMCA was proven with universal cross-platform compatibility to be performed on different real-time PCR instru-

ments [31]. However, a performance evaluation of an MMCA kit for *NAT2* genotyping in TB patients of China has never been performed. Since previous studies had demonstrated that *NAT2*12* (0%), *NAT2*13* (1%) and *NAT2*14* (0%) genotypes were rare in Asian populations [24], a four-SNP panel specific to Asian populations has been assumed to be sufficient for the *NAT2* genotyping of Chinese population. Our results also demonstrated that both four- and seven-SNP assessments had same accuracy if direct sequencing method is used, thus the four-SNP genotype panels can be effectively used instead of the seven-SNP genotype panels for *NAT2* phenotype inference in Chinese population. However, it has been reported that in Africa a small amount of people have SNP at site 191 [24], which is not included in this kit. Therefore, when this technique being applied in other international communities, substitutes SNP c.481C>T for SNP c.191G>A (*14) for probe design would be appropriate in some countries, as SNP 481 only been adopted to identify a rapid haplotypes with limited clinical significance.

Four specific fluorescent probes were designed to detect the four SNPs of *NAT2* gene, and a total of 29 genotypes were identified in this assay (see Table 2). Three *NAT2* genotypes were most common: *NAT2*4* was present in about 52.63%, *NAT2*6* in about 23.45% and *NAT2*7* in about 16.15% of the tested TB patients. Compared with the genotype analysis based on the seven SNPs analysis postsequencing method, the *NAT2* phenotypes inference (using the four SNPs MMCA assay) showed high accuracy. In our study, all the rapid acetylators had *NAT2*4/NAT2*4* genotypes, while the *NAT2*6* genotypes and *NAT2*7* genotypes were most frequent in slow acetylators.

Two rapid acetylators with *NAT2*4/NAT2*4* genotype were misclassified as intermediate acetylators, and one intermediate acetylators with *NAT2*6/NAT2*4* genotype was misclassified as a rapid acetylators in this assay. The occurrence of the above misclassifications was related to mislabel, which made the MMCA method obtain perfect sensitivity and specificity comparing with direct DNA sequencing.

Besides blood samples that are commonly tested, we found that saliva samples can be used readily for the MMCA assay, since parallel tests produced highly consistent outcomes for blood and saliva samples. Non-invasive sample collection method will increase the willingness of the subjects for undergoing such tests. Additionally, as 96-well plates can be used in MMCA, the method can be adjusted for high-throughput screening of samples, facilitating its clinical application. Furthermore, the whole procedure, including DNA preparation, only takes 2.5 h, which makes a rapid *NAT2* genotype test more feasible before launching an INH containing regimen. Reproducibility of MMCA had been evaluated by repeating the assays with different samples or with same sample for multiple times, they all produced uniform outcomes (data not shown), which clarified the clinical usage potential of this method.

In conclusion, the *NAT2* genotyping method by MMCA has the advantages of being rapid, simple, high performance and high throughput. The present study demonstrates that these four SNPs MMCA assay can be easily implemented in clinical laboratories with high sensitivity, specificity and accuracy to infer the *NAT2* phenotype in TB patients in China.

Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have 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 has been obtained from the participants involved.

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Executive summary

- Introducing a rapid, simple and reliable method for *NAT2* genotyping, which can facilitate the personalized dosing of isoniazid for tuberculosis treatment.
- Multicolor melting curve analysis (MMCA) in this assay can detect both blood and saliva samples efficiently, indicating it could be readily adopted by the clinical laboratories.
- Our data demonstrated that about half of the Chinese tuberculosis patients should adjust the isoniazid doses according to their *NAT2* genotypes, so as to increase the treatment efficiency and decrease the side effect.

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