DEVELOPMENT AND VALIDATION OF A PROBE-FREE REAL-TIME SYBR GREEN ARMS-PCR FOR UGT1A1*6 GENOTYPING IN THE VIETNAMESE KINH POPULATION

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*Irinotecan-induced toxicities, such as neutropenia and diarrhea, affect up to 36% of East Asian colorectal cancer patients due to impaired UGT1A1 glucuronidation, primarily from the UGT1A1*6 variant (c.211G>A, rs4148323). We developed a real-time SYBR Green ARMS-PCR assay using three parallel reactions: internal control with outer primers (OF/OR, 353 bp amplicon), G-specific (OF/IR_G, 176 bp), and A-specific (IF_A/OR, 232 bp). The assay was validated on 30 NGS-confirmed samples (10 GG, 10 GA, 10 AA) and applied to 100 healthy Kinh individuals. It achieved 100% NGS concordance, with ΔCt thresholds (≤ -8 for GG, -3 < ΔCt < 5 for GA, ≥ 8 for AA) yielding 100% sensitivity/specificity. In the Kinh cohort, genotypes were 75 GG (75%), 23 GA (23%), and 2 AA (2%), with G allele frequency 86.5% and A allele 13.5%, conforming to Hardy-Weinberg equilibrium (p=0.877). This probe-free assay enables rapid (<2 hours), low-cost genotyping for personalized irinotecan dosing in low-resource settings.*

Keywords: Real-time SYBR Green ARMS-PCR, SNP genotyping, *UGT1A1*6*, irinotecan toxicity, pharmacogenetics, Kinh population.

I. INTRODUCTION

Irinotecan is a key treatment for colorectal cancer, but its active metabolite SN-38 causes severe toxicities like neutropenia and diarrhea in up to 36% of East Asian patients due to reduced UGT1A1 enzyme activity, mainly from the UGT1A16 variant (c.211G>A, rs4148323).[1-3] In East Asians, including the Kinh Vietnamese with ~13% A allele frequency, heterozygous (GA) and homozygous (AA) carriers face 2- to 4-fold higher risks, leading to FDA/CPIC guidelines for dose reductions.[4] However, in low-resource settings like Vietnam, next-generation sequencing (NGS) is costly and slow, while traditional methods like tetra-primer ARMS-PCR require post-PCR electrophoresis and are prone to allele dropout.[5-7] We developed a real-time SYBR Green ARMS-PCR assay adapting tetra-primer principles into three parallel reactions, using outer primers as internal control for reaction quality and inhibition monitoring, to enable rapid, affordable genotyping. This study validates the assay on 30 known samples and estimates UGT1A16 frequencies in 100 Kinh individuals.

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II. MATERIALS AND METHODS

1. Type of Study and Study design

This study was a methodological, crosssectional study aimed at developing and validating a probefree realtime SYBR Green ARMSPCR assay for UGT1A1*6 genotyping.

The diagnostic performance of the assay was evaluated by comparison with Sanger sequencing as the reference method.

2. Sample collection and DNA extraction

A total of 130 genomic DNA samples from healthy volunteers of the Kinh ethnic group were analyzed. The participants consented to be involved in the study. For assay development and validation, 30 DNA samples (10 per genotype: GG, GA, AA at *UGT1A1*6* [c.211G>A, rs4148323]) were selected; these had been previously genotyped by Next Generation Sequencing (NGS) as the standard. An additional 100 unknown DNA samples were used for allele frequency estimation. Peripheral blood was collected in EDTA tubes at Chemedic Vietnam Laboratory Center during 2023-2024. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Germany) or an automated DNA extraction system Miracle AutoXT (LiliF; iNtRON Biotechnology, Korea) according to the manufacturer's protocol.[8] DNA purity and concentration were assessed by NanoDrop spectrophotometry (Thermo Fisher Scientific, USA), with A260/A280 ratios of 1.8-2.0 indicating high purity, and DNA integrity was verified by agarose gel electrophoresis (NIPPON Genetics EUROPE, Lot.No. D00557). Samples were stored at -20°C until use.

3. Primer design

Primers for the Real-time SYBR Green ARMS PCR assay were designed based on the *UGT1A1* reference sequence (NC_000001.11) using the web-based Tetra-primer ARMS-PCR design tool (https://primer1.soton.ac.uk/primer1.html), previously validated for ARMS-PCR applications.[9,10] The primer set included two outer primers and two allele-specific inner primers to target the *UGT1A1*6* variant (c.211G>A, rs4148323). The forward outer primer (OF: 5'-CCCATGCTGGGAAGATACTGTTGATCCC-3') and reverse outer primer (OR: 5'-TGGGAACAGCCAGACAAAAGCATAGCAG-3') were used to amplify a 353 bp internal control product (CtIC). Two allele-specific inner primers were designed with primary and secondary mismatches to enhance specificity: the forward inner primer for allele A (IF_A: 5'-CTGACGCCTCGTTGTACATCAGAG*CC***A**-3', where bold indicates the primary mismatch, and italic/underlined bases indicate the secondary mismatch) paired with OR to produce a 232 bp product specific for the A allele (CtA); and the reverse inner primer for allele G (IR_G: 5'-GGGTACGTCTTCAAGGTGTAAAATGC*GC***C**-3', with mismatches similarly marked) paired with OF to generate a 176 bp product specific for the G allele (CtG). Predicted melting temperatures (Tm) were calculated using uMelt (https://dna-utah.org/umelt/). Primer specificity and PCR product identity were further evaluated by sequence alignment. The primer sequences and representative PCR amplicons were reanalyzed by BLAST against the reference *UGT1A1* gene using UGEN software, and using the NCBI's nucleotide BLAST tool to avoid non-specific amplification of *UGT* family gene members (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

All primers were synthesized by Phusagenomics (Vietnam, Lot: 062623_A01_D6]).

4. Real-time SYBR Green ARMS-PCR and data analysis

Real-time SYBR Green ARMS-PCR was performed on the 30 development samples (10 per genotype: GG, GA, AA), which served as positive controls previously genotyped by Next Generation Sequencing (NGS). Duplicate reactions for a subset (n=10) were performed to assess repeatability (intra-assay coefficient of variation [CV] <2% for Ct values). For each sample, three parallel reactions were conducted

simultaneously in separate tubes to enable direct comparison of amplification efficiencies: (i) internal control reaction using outer primers OF and OR (CtIC, 353 bp amplicon); (ii) G allele-specific reaction using OF and IR_G (CtG, 176 bp amplicon); and (iii) A allele-specific reaction using IF_A and OR (CtA, 232 bp amplicon). No-template controls (NTC) were included in each run to monitor contamination, showing no amplification (Ct ≥ 40). Reactions were conducted in a 20 µL volume containing 10 µL 2X PCR Master Mix (iNtRON, Biotechnology, Korea, Lot #: 251620456), final concentration of 0.25 µM each primer (total primer amount: 5 pmol per reaction), 5 µL of diluted genomic DNA (10-40 ng/µL), and nuclease-free water. PCR was carried out on a DTprime 5 Real-Time PCR System (DNA-Technology, Russia) with the following protocol: initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s and 58°C for 30 s with single fluorescence measurement at the end of each cycle, and a final elongation at 72°C for 5 min. PCR efficiencies were estimated from amplicon sizes and melting curves (~100%; complying with MIQE guidelines).[11] Baseline was set automatically, and threshold at 0.2 ΔRn. Average cycle threshold (Ct) and melting temperature (Tm) values were calculated for data analysis.

Melting curve analysis was performed by increasing the temperature from 72°C to 95°C at a rate of 0.5°C/15 s, with fluorescence measurements every 5 s. The negative derivative of fluorescence intensity versus temperature (-dF/dT) was used to determine Tm for each PCR product (internal control, allele A-specific, allele G-specific). Predicted Tm values were calculated using web-based software to confirm product specificity.

Data analysis involved calculating ΔCt (CtG - CtA) for each sample. Ct values ≥ 40 were considered 'no amplification' based on the instrument noise threshold and NTC performance (mean Ct NTC undetermined >40), allowing unbiased ΔCt calculation. Genotypes were called using an empirical algorithm optimized from the 30 known development samples: thresholds for ΔCt were set to distinguish homozygous (GG or AA) from heterozygous (GA), with a defined gray zone (e.g., overlapping ΔCt values) requiring NGS confirmation for ambiguous calls. Thresholds were further optimized by receiver operating characteristic (ROC) analysis in Origin 2024 (OriginLab, USA), using ΔCt as the predictor and genotype as the outcome (one-vs-rest approach for multi-class classification to determine sensitivity/specificity at optimal cutoffs). Samples were classified into three groups based on ΔCt values: *Group 1* (GA genotype): ΔCt not significantly different from zero (balanced amplification). *Group 2* (GG genotype): ΔCt significantly negative (preferential G allele amplification). *Group 3* (AA genotype): ΔCt significantly positive (preferential A allele amplification). Statistical analysis was performed using SPSS v22 (IBM). The ΔCt ranges for Group 1 were reported as the 10th to 90th percentiles, while those for Groups 2 and 3 were reported as the 90th and 10th percentiles, respectively. Differences in ΔCt between groups were assessed using one-way ANOVA.

5. Validation of real-time SYBR Green ARMS-PCR

The real-time SYBR Green ARMS-PCR assay was validated using the 30 development samples (10 per genotype: GG, GA, AA at *UGT1A1*6*), which had been previously genotyped by NGS as the standard. Genotypes were called based on empirical ΔCt thresholds

derived from the known samples and confirmed by melting curve analysis (distinct Tm values for allele-specific products and internal control). Amplification curves and cycle threshold (Ct) distributions were analyzed to assess allele-specific performance. Concordance between ARMS-PCR calls and NGS results (DNBSEQ G200, MGI, China) was evaluated using a 3×3 contingency table. Cohen's Kappa statistic was calculated for agreement, and diagnostic metrics (sensitivity, specificity, positive predictive value [PPV], and negative predictive value [NPV]) were computed for each genotype, with 95% confidence intervals (Wilson score method) using SPSS v22 (IBM). A full confusion matrix is provided in S5 Table.

6. Determination of *UGT1A1*6* allele frequencies in the Kinh population

Allele frequencies of *UGT1A1*6* were determined by applying the real-time SYBR Green ARMS-PCR assay to 100 unknown DNA samples from healthy Kinh individuals. Genotype frequencies were calculated as the proportion of each genotype (GG, GA, AA) among the 100 samples, with 95% confidence intervals (Wilson score method). Allele frequencies were derived from the total number of alleles (n=200), with exact binomial 95% confidence intervals. The population was tested for Hardy-Weinberg Equilibrium (HWE) using the Chi-square test and the exact test (Monte Carlo simulation with 10,000 iterations) in SPSS v22 (IBM) to account for small expected counts. Allele frequencies were compared with those reported in other East Asian populations from the Genome Aggregation Database (gnomAD) to assess the genetic profile of the Kinh population.[4]

7. Ethical Considerations

This study complied with the ethical principles of biomedical research. All participants were healthy volunteers who provided written informed consent after being informed of the study objectives. Personal data were kept confidential. All procedures were performed under sterile conditions to ensure biosafety.

III. RESULTS

1. Development of the real-time ARMS PCR assay for *UGT1A1*6* genotyping

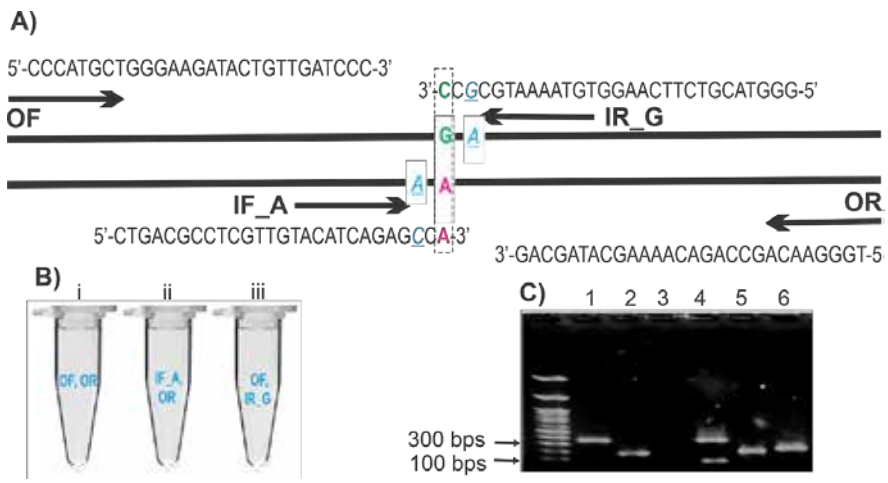

Figure 1. Primer Design and Allele-Specific Amplification for *UGT1A1*6* detection by Real-time SYBR Green ARMS PCR

(**A**) Schematic diagram of primer design targeting the *UGT1A1*6* variant. The outer primers (OF and OR) amplify a 353 bp control product. Two allele-specific inner primers are used: IF_A (specific for the A allele) and IR_G (specific for the G allele), yielding amplicons of 232 bp and 176 bp, respectively, when paired with OR or OF. The SNP site (boxed) and mismatch-enhancing bases (underlined) are indicated. (**B**) Reaction tube setup for allele-specific PCR assays: (i) OF + OR (control); (ii) IF_A + OR (specific for A allele); (iii) OF + IR_G (specific for G allele). (**C**) Agarose gel electrophoresis of PCR products using outer (OF/OR) and allele-specific inner primers. First lane: 100 bp DNA ladder; Lane 1: GG sample amplified with outer primers (OF/OR); Lane 2: GG sample with G-allele inner primers (OF/IR_G); Lane 3: GG sample with A-allele inner primers (IF_A/OR); Lane 4: GA sample with outer primers (OF/OR); Lane 5: GA sample with G-allele inner primers (OF/IR_G); Lane 6: GA sample with A-allele inner primers (IF_A/OR). Clear bands at expected product sizes confirm allele-specific amplification and successful discrimination between GG and GA genotypes.

The Real-time SYBR Green ARMS PCR assay was developed to genotype the *UGT1A1*6* variant (c.211G>A, rs4148323). Primer design and allele-specific amplification were optimized as shown in Fig1. The outer primers (OF and OR) amplified a 353 bp control product, serving as an internal control (CtIC). Two allele-specific inner primers, IF_A (specific for the A allele) and IR_G (specific for the G allele), produced amplicons of 232 bp and 176 bp, respectively, when paired with OR or OF (Fig1A). Primary and secondary mismatches were incorporated to enhance specificity, with the SNP site and mismatch bases clearly indicated (Fig1A). Reaction setups for allele-specific PCR assays were established: (i) OF + OR (control), (ii) IF_A + OR (A allele-specific), and (iii) OF + IR_G (G allele-specific) (Fig1B). Agarose gel electrophoresis confirmed allele-specific amplification, with clear bands at the expected sizes: 353 bp (outer primers), 232 bp (A allele-specific), and 176 bp (G allele-specific). Lanes from GG and GA samples consistently showed the expected patterns, confirming reproducibility and successful discrimination between the G and A alleles (Fig1C).

Performance of real-time SYBR Green ARMS PCR: Ct, ΔCt, and Tm analysis

The performance of the real-time ARMS-PCR assay was evaluated using 30 samples (10 per genotype: GG, GA, AA) for the *UGT1A1*6* variant, with duplicate reactions for a subset (n=10) yielding intra-assay repeatability (CV <2% for Ct values). No-template controls (NTC) showed no amplification (Ct ≥ 40), confirming absence of contamination. The input gDNA of all control samples in the range of normal gDNA concentration isolated from whole-blood EDTA. The cycle threshold values of internal controls (CtIC) for all genotypes (AA, GA, GG) ranged from 19.5 to 23.3, confirming the consistency and quality of genomic DNA (gDNA) extracted from EDTA whole blood (Fig2A).[12]

Melting curve analysis revealed Tm values for the internal control at 86.89-87.62°C, matching predicted values based on product sequences (Fig 2C). Tm values for G allele-specific products (CtG) were 87.00-87.61°C, and for A allele-specific products (CtA) were 81.73-83.29°C, consistent with predicted ranges.

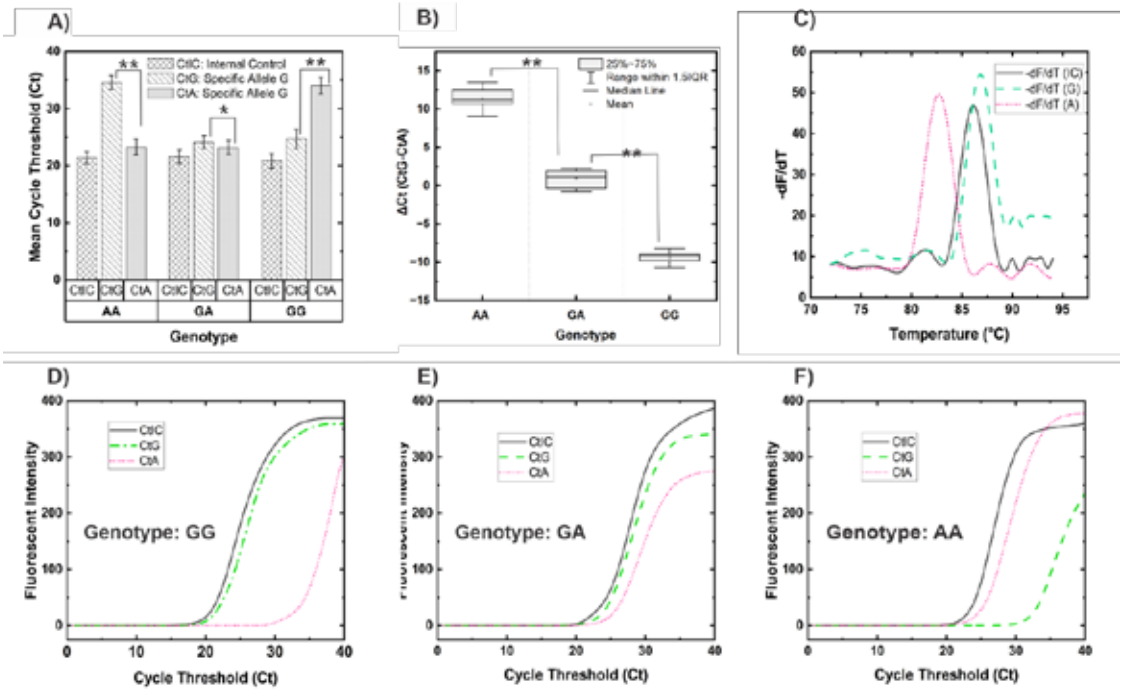

Figure 2. Comprehensive Analysis of Real-time SYBR Green ARMS PCR for *UGT1A1*6* Genotyping

(**A**) Bar chart showing mean cycle threshold (Ct) values for internal control (CtIC), G allele-specific (CtG), and A allele-specific (CtA) reactions across AA, GA, and GG genotypes (n=10 per group). Error bars represent standard deviation. * indicates no significant difference between CtA and CtG ($p>0.05$); ** indicates significant difference ($p<0.05$). (**B**) Box plot illustrating ΔCt (CtG - CtA) distributions for the same genotypes, with boxes indicating interquartile range (IQR), median lines, whiskers extending to 1.5 × IQR, and mean ΔCt values marked by square markers. ** indicates significant differences between ΔCt values across genotypes ($p<0.05$). (**C**) Melting curve showing -dF/dT versus temperature for CtIC, CtG, and CtA products, confirming Tm values. (**D-F**) Amplification curves showing fluorescent intensity versus cycle for genotypes GG, GA, and AA, respectively, demonstrating allele-specific amplification patterns.

Allele-specific amplification was determined using ΔCt (CtG - CtA), and samples were classified into three groups based on ΔCt distributions (Fig2B). Receiver operating characteristic (ROC) analysis optimized the ΔCt thresholds, yielding area under the curve (AUC) values of 1.00 for GG vs. non-GG and AA vs. non-AA, with optimal cutoffs of $\Delta Ct \leq -8$ for GG, $-3 < \Delta Ct < 5$ for GA, and $\Delta Ct \geq 8$ for AA; no samples fell in the gray zone requiring additional NGS confirmation.

Group 1 (GA genotype): Both allele-specific reactions amplified efficiently, with mean CtG of 24.12 ± 0.35 (range: 22.64-25.70) and mean CtA of 23.18 ± 0.39 (range: 21.00-24.68), resulting in a mean ΔCt of 0.94 ± 0.35 (range: -0.73 to 2.34). The difference between CtG and CtA was not significant ($p=0.150$; Fig 2A, *), confirming balanced amplification of both alleles. Tm values for G and A allele-specific products were 87.00-87.61°C and 81.73-83.29°C, respectively

(Fig 2C, S3 Table). Amplification curves showed comparable fluorescence intensity for both alleles (Fig 2E).

Group 2 (GG genotype): Amplification was significantly more efficient for the G allele, with a mean CtG of 24.67 ± 0.53 (range: 22.05-26.60) compared to a mean CtA of 34.01 ± 0.46 (range: 31.34-35.61). The difference between CtG and CtA was significant ($p<0.001$; Fig2A, **), with a mean ΔCt of -9.35 ± 0.23 (range: ≤ -8.4), reflecting preferential G allele amplification. Tm values were consistent with predicted ranges (Fig 2C), and amplification curves confirmed dominant G allele amplification (Fig 2D).

Group 3 (AA genotype): Amplification specified the A allele, with a mean CtA of 23.23 ± 0.46 (range: 21.12-25.67) and a mean CtG of 34.57 ± 0.38 (range: 31.23-35.70). The difference between CtG and CtA was significant ($p<0.001$; Fig 2A, **), with a mean ΔCt of 11.34 ± 0.38 (range: ≥ 9.11), confirming preferential A allele amplification. Tm values aligned with predicted ranges (Fig 2C, S3 Table), and amplification curves showed dominant A allele amplification (Fig 2F).

Comparative analysis across genotypes further validated the assay's specificity. CtA values were not significantly different between AA and GA genotypes ($p=0.936$; S2 Table) but were significantly higher in GG ($p<0.001$; Fig 2A, **). Similarly, CtG values were comparable between GA and GG genotypes ($p=0.411$; Fig 2A, *) but significantly higher in AA ($p<0.001$; Fig 2A, **). The ΔCt values showed significant differences across all genotypes ($p<0.001$; Fig 2B, **), confirming that ΔCt reliably distinguishes GG, GA, and AA genotypes. The distinct amplification patterns for each genotype (Figs 2D-F) and consistent Tm values (Fig 2C) further support the assay's accuracy and specificity.

Method validation

The real-time SYBR Green ARMS-PCR assay was validated using the 30 known development samples (10 per genotype: GG, GA, AA at *UGT1A1*6*, with genotypes previously confirmed by NGS sequencing as the standard. The assay demonstrated 100% concordance with NGS sequencing (30/30 samples; 95% CI 88.1-100%, Wilson score method), accurately identifying all GG, GA, and AA genotypes. Cohen's Kappa statistic indicated perfect agreement (κ=1.00). Diagnostic metrics for each genotype were: sensitivity 100% (95% CI 88.1-100%), specificity 100% (95% CI 88.1-100%), positive predictive value (PPV) 100% (95% CI 88.1-100%), and negative predictive value (NPV) 100% (95% CI 88.1-100%). These results, supported by the clear separation in Ct, ΔCt, and Tm values (Fig 2), confirm the assay's high accuracy and reliability for *UGT1A1*6* detection.

2. *UGT1A1*6* genotype and allele frequencies in the Kinh population

Table 1. Genotype and allele frequencies of the *UGT1A1*6* G>A mutation in a Vietnamese cohort (N=100)

Frequency	Genotype (N=100)			Allele (n=200)	
	*1/*1 (GG)	*1/*6 (GA)	*6/*6 (AA)	*1 (G)	*6 (A)
Observed	75 (75%)	23 (23%)	2 (2%)	173 (86.5%)	27 (13.5%)
Expected	74.82 (74.82%)	23.36 (23.36%)	1.82 (1.82%)	-	-

Frequency	Genotype (N=100)	Allele (n=200)	
Hardy-Weinberg Chi-square (χ^2)	$\chi^2 = 0.0238$, $p = 0.877$	-	-

Note: N = number of individuals; n = number of alleles. 95% CIs calculated using the Wilson score method for genotypes and exact binomial for alleles. HWE assessed by Chi-square and exact test (Monte Carlo simulation with 10,000 iterations) in SPSS v22.

Genotype and allele frequencies of *UGT1A1*6* were determined in 100 unknown samples from a Kinh population using the real-time SYBR Green ARMS-PCR assay. The genotype distribution was 75 GG (75%), 23 GA (23%), and 2 AA (2%). Allele frequencies were calculated as 86.5% for the G allele (173/200) and 13.5% for the A allele (27/200) (Table 1). The population was in Hardy-Weinberg Equilibrium (HWE) ($\chi^2 = 0.0238$, exact $p = 0.877$), indicating no significant deviation from genetic equilibrium. The observed A allele frequency of 13.5% aligns with reported values in East Asian populations from the Genome Aggregation Database (gnomAD), ranging from 10-16%.

Population frequency comparison with public genomic databases. The allele frequency data obtained in this study were highly consistent with publicly available reference data from the NCBI genomic databases. Specifically, the frequency of the UGT1A1 *6 A allele in the Vietnamese population has been reported to be approximately (A= 10.6%; 65/614 Vietnamese), which is comparable to the A allele frequency observed in our Kinh cohort. This frequency is notably lower than those reported in other East Asian populations, such as the Japanese population (A = 18.04% 13,974/77,442 individuals, 38KJPN dataset) and the Korean population (A = 19.81%; 1,433/7,234 individuals, Korea4K dataset) (https://www.ncbi.nlm.nih.gov/snp/rs4148323). These interpopulation differences highlight ethnic variability in UGT1A1 *6 allele distribution and further support the relevance of populationspecific pharmacogenetic screening strategies.

IV. DISCUSSION

This study presents a real-time SYBR Green ARMS-PCR assay for genotyping the UGT1A1*6 variant (c.211G>A, rs4148323), a clinically relevant polymorphism associated with irinotecan-induced toxicities in East Asian populations. Using 30 NGS-confirmed reference samples representing all three genotypes (GG, GA, AA), the assay demonstrated complete concordance with the sequencing standard and clear discrimination based on ΔCt (CtG − CtA) values. The empirically defined ΔCt thresholds enabled unambiguous genotype assignment without a gray zone, supporting the robustness of this approach for routine laboratory use.

A key strength of the proposed method lies in its adaptation of tetra-primer ARMS-PCR principles into a real-time SYBR Green format. Traditional tetra-primer ARMS-PCR is cost-effective but often requires post-PCR electrophoresis, increasing turnaround time and susceptibility to interpretation errors due to nonspecific bands or allele dropout.[13,14] By transferring allele discrimination to kinetic (Ct) and melting-curve analysis, the present assay eliminates the electrophoresis step while retaining probe-free affordability. The use of allele-specific primers with additional mismatches further enhances specificity, an approach that has been shown to improve

discrimination of single-nucleotide variants in ARMS-based systems.[15]

The melting-curve profiles provided an additional layer of validation, with distinct Tm ranges observed for A- and G-specific amplicons. Although SYBR Green binds nonspecifically to double-stranded DNA, melt-curve analysis is widely accepted as an effective safeguard to confirm product identity in SNP genotyping assays.16 In this study, the consistent separation of melting peaks across genotypes supports the reliability of SYBR Green chemistry for targeted pharmacogenetic testing when combined with appropriate primer design and analysis criteria.

Application of the assay to 100 healthy Kinh individuals revealed an A allele frequency of 13.5%, consistent with reports from other East Asian populations.[4] This finding reinforces the clinical relevance of UGT1A1*6* screening in Vietnam, where a substantial proportion of individuals may carry at least one reduced-function allele. Given evidence linking UGT1A1*6* to increased risk of severe neutropenia and diarrhea during irinotecan therapy, pre-treatment genotyping has been recommended to guide dose adjustment and improve treatment safety.[1-4,7]

Several limitations should be acknowledged. First, the validation cohort was relatively small, and larger multi-center studies are needed to confirm reproducibility and performance across diverse laboratory settings. Second, the assay was evaluated on a single real-time PCR platform and reagent system, and inter-platform robustness was not assessed. Third, the current workflow requires three parallel reactions per sample, which may limit throughput compared with multiplex or probe-based assays; however, this trade-off may be acceptable in settings where simplicity and low cost are prioritized. Finally, clinical outcome data were not included, and future studies correlating genotypes with irinotecan toxicity in Vietnamese patients would strengthen the translational value of this assay.

V. CONCLUSION

In conclusion, we developed and validated a probe-free real-time SYBR Green ARMS-PCR assay for UGT1A1*6 genotyping with complete concordance to NGS in reference samples. The assay demonstrated clear ΔCt-based discrimination of GG, GA, and AA genotypes and was successfully applied to determine allele frequencies in a Kinh Vietnamese cohort. Owing to its low cost, short turnaround time, and minimal technical requirements, this method represents a feasible and reliable laboratory method for UGT1A1*6 genotyping, which may facilitate future pharmacogenetic studies related to irinotecan use in resourcelimited settings..

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