

A novel RFLP-ARMS TaqMan PCR-based method for detecting the BRAF V600E mutation in melanoma

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Abstract. To enable the rapid and sensitive screening of the BRAF V600E mutation in clinical samples, a novel method combining restriction fragment length polymorphism (RFLP) analysis with the popular amplification refractory mutation system (ARMS) TaqMan quantitative (qPCR) genotyping method in a single reaction tube was developed. A total of 2 primer pairs were designed to enrich for and genotype the BRAF mutational hotspot (RFLP primers and ARMS primers) and a restriction enzyme was used to remove the wild-type alleles. The analysis revealed that this method detected mutant alleles in mixed samples containing >0.1% mutant sequences. In a survey of 53 melanoma samples, this method detected 21 mutation-positive samples. This novel RFLP-ARMS TaqMan qPCR protocol may prove useful for detecting mutations in clinical samples containing only a small proportion of mutant alleles.

Introduction

BRAF is one of the most frequently mutated protein kinases in human cancer (1,2). BRAF protein kinase has been suggested to be among the most likely protein kinase genes to carry

driver mutations (3). BRAF mutations have been identified in the majority of malignant melanomas, and the frequency of BRAF mutations in malignant melanomas is 59% (1). The mutation frequency of BRAF is relatively low in other types of cancer, including papillary thyroid carcinoma and colorectal carcinoma. Research by Davies and his colleagues indicated that the BRAF mutation is a novel diagnostic and prognostic biomarker in thyroid cancer by analyzing cytological and histological thyroid samples, which occurs specifically and with a high prevalence (35.8%) in papillary thyroid carcinoma, followed by colorectal carcinoma (18%), gliomas (11%), sarcomas (9%), ovarian carcinomas (4%) and lung cancer (3%) (1). Mutations in BRAF have been associated with altered sensitivities to numerous drugs, including PLX4720, Nutlin-3a, AZ628, bortezomib, embelin, RDEA119, FH535, CI-1040, CHIR-99021, AP-24534, obatoclox mesylate, PF-562271, CEP-701, FTI-277, 17-AAG, PD-0325901, SB590885, AZD6244, PD-173074, ZM-447439, BIBW2992, temsirolimus, metformin, AZD6482 and gefitinib (<http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/>; visited March 10th, 2014). The most notable mutational hotspot for BRAF is p.V600E (c.1799T>A), which accounts for ~90% of the known cancer-associated mutations (4).

The BRAF V600E mutation is strongly associated with significantly improved treatment response (5-7). Therefore, it is considered important to screen for this mutation prior to selecting a therapeutic strategy. However, it has proven challenging to determine the status of this mutation in clinical samples (8,9). A major problem has been that the mutant cells are typically outnumbered by numerous surrounding wild-type cells at the tissue sampling site (8,9). A number of methods have been developed to overcome this problem, including those based on restriction fragment length polymorphism (RFLP) analysis (10,11), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis (12), ligase chain reaction (13), suspension array (14), amplification refractory mutation system polymerase chain reaction (ARMS PCR) (15-19), allele-specific enzymatic amplification (20), mutant-enriched PCR (11,21), pyrosequencing (22), coamplification at lower denaturation temperature PCR (COLD-PCR) (23-27), high resolution

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melting (28,29), fluorescent amplicon generation (30), locked nucleic acid/peptide nucleic acid clamp PCR (31,32), anti-primer quenching-based quantitative (qPCR) (33) and SNaPshot analysis (34-36).

Among these techniques, RFLP and ARMS PCR are widely used (37,38). Restriction enzymes specifically digest wild-type alleles, leaving the mutant alleles available for analysis. This approach has been successfully used to detect mutations in tumor protein 53, Ras and epithelial growth factor receptor (11,39-41). ARMS PCR is based on the principle that extension is efficient when the 3' terminal base of a primer matches its target, but inefficient or nonexistent when the 3' terminal base is mismatched. Therefore, when primers are designed against the mutation of interest, amplification proceeds only if the mutation is present (38). This strategy has been successfully used to screen for point mutations (42-44). However, RFLP analysis involves a number of post-PCR processing steps, which may increase the risk for contamination of the PCR product (10,11), and the usefulness of the ARMS method may be limited by inefficient amplification due to the abundance of wild-type alleles (19).

The present study describes a novel method that combines RFLP analysis and ARMS TaqMan qPCR in a one-step reaction tube, and suggests the use of this technique, termed 'RFPL-ARMS TaqMan PCR,' to screen clinical melanoma samples for the BRAF V600E mutation.

Materials and methods

Plasmid construction. Recombinant plasmids encoding wild-type and V600E mutant BRAF were constructed as described by Board *et al* (44). Briefly, corresponding outer and mutant primers were used to yield half fragments with complimentary ends using wild type tissue DNA as a template (first half primer sequences: forward, 5'-CCAGGAGTGCCAAGAGAATA-3', and reverse, 5'-CCATCGAGATTTCTCTGTAGCTAGACCA-3'; second half primer sequences, forward, 5'-TGGTCTAGCTACAGAGAAATCTCGATGG-3', and reverse, 5'-TTTCAACAGGGTACACAGAACA-3'), with each half fragment containing a mutant base. PCR was performed in a 50 μ l mixture containing 5 μ l 10X PCR Buffer (Takara Bio Inc., Otsu, Japan), 1.25 U Takara TaqTM polymerase (Takara Bio Inc.), 4 μ l dNTP mixture (Takara Bio Inc.), 0.5 μ M primers and 5 μ l DNA (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). The reaction procedure was as follows: Initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 15 sec, primer annealing at 53°C for 20 sec, and 72°C for 50 sec, final extension took place at 72°C for 5 min. The two half fragment products were mixed equally as a template for the second round PCR. The second round of PCR used inner nested primers (forward, 5'-AGCATCTTCATTCCAATG AAGAGCC-3', and reverse, 5'-CATCCACAAAATGGATCC AGACAAC-3'). The second round was performed in a 50 μ l mixture containing 5 μ l 10X PCR Buffer (Takara Bio Inc.), 1.25 U Takara TaqTM polymerase (Takara Bio Inc.), 4 μ l dNTP mixture (each 2.5 mM; Takara Bio Inc.), 0.5 μ M primers and 5 μ l template. The thermocycling conditions included initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec, 50°C for 30 and 72°C for 50 sec, the final extension took place at 72°C for 5 min. Self-priming of the complementary

half fragments and the subsequent amplification created a final product harboring the mutant base. The products were ligated into the pMD19 plasmid (Takara Bio Inc., Otsu, Japan), and recombinants containing mutant alleles were produced and confirmed by sequencing performed by Sangon Biotech Co., Ltd., (Shanghai, China). The sequencing machine used was ABI-PRISM 3,730 (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the analysis software was DNASTAR 5.0 (DNASTAR, Inc., Madison, WI, USA). Recombinant plasmid DNA was extracted using a Tiangen Plasmid DNA kit (Tiangen Biotech Co., Ltd., Beijing, China). As a positive control, the recombinants were mixed with an equal amount of human genomic DNA (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany).

Sample collection and DNA extraction. In total, 53 patients with melanoma treated at the Department of Dermatology, The Third Affiliated Hospital of Sun Yat-sen University (Guangzhou, China), were enrolled in the present study from March 2011 to December 2012. Unrelated patients diagnosed with melanoma were included. These samples were all biopsies. The median age of patients was 68 years (age range 39-84 years). A total of 33 cases (62%) were females, and 20 cases (38%) were males. A total of 10 patients (19%) exhibited Clark level II disease (penetration of melanoma into the second layer of the skin, the dermis) (45), and 18 patients (34%), 24 patients (45%) and 1 patient (2%) exhibited III, IV and V stage disease, respectively. The study protocol was approved by the Ethics Committee of The Third Affiliated Hospital of Sun Yat-sen University, and written informed consent was obtained from all patients. DNA was extracted from 10% formalin-fixed that was fixed at room temperature for 24 h, paraffin-embedded melanoma samples using a QIAamp DNA FFPE Tissue kit (Qiagen GmbH, Hilden, Germany). Briefly, formalin-fixed, paraffin-embedded blocks containing the maximum number of tumor-rich areas from the patients were selected and sliced into 3 5- μ m thick sections. Each section was processed by proteinase K digestion at 56°C for >16 h, and the obtained lysate was loaded onto a QIAamp column. Following 2 washes, the DNA was eluted with 100 μ l ddH₂O. The extracted DNA was kept at -20°C until it was used for PCR analysis.

RFLP-ARMS TaqMan PCR-based genotyping. The RFLP-ARMS TaqMan PCR assay described in the present study was a one-step PCR that used a mutation-enriching reaction and ARMS primer genotyping process to selectively eliminate wild-type genes and detect the mutant alleles. For the BRAF V600E mutation, a restriction enzyme was used to digest the wild-type genomic DNA, thereby enriching the mutant allele, and then a pair of RFLP primers: Forward, 5'-AGCATCTTCATTCCAATGAAGAGCC-3'; and reverse, 5'-CATCCACAAAATGGATCCAGACAAC-3', designed to amplify a 400-500 bp fragment containing the mutant allele [melting temperature (T_m), 65°C], were used. The ARMS primers were designed to selectively amplify a mutant allele with a lower T_m (60°C) with the following respective sequences: Forward, 5'-TAGGTGATTTTGGTCTAGCTACACA-3' (mismatched base is underlined); and reverse, 5'-CCACAAAATGGATCCAGACAAC-3'. To improve the specificity of the ARMS primers,

an additional mismatch base was introduced at the penultimate nucleotide of the mutation site (G was replaced by C), based on the principles described by Newton *et al* (19). Mutant allele enrichment and genotyping were performed in a single tube via a 3-phase reaction: i) 65°C for 30 min, allowing the restriction enzyme, TspRI, to cut the wild-type DNA; ii) enrichment of the mutant allele with the RFLP primers and thermocycling conditions of 95°C for 10 min, followed by 5 cycles of 95°C for 15 sec, 65°C for 20 sec and 72°C for 60 sec; and iii) selective amplification of the mutant allele with the ARMS primers, and 40 cycles of 95°C for 15 sec and 60°C for 35 sec (fluorescence collection). During the second phase, the ARMS primers were unable to bind at the higher temperature (65°C), while in the third phase, the RFLP primers did not function as the 400-500 bp product was incompletely synthesized during the 35 sec elongation phase.

The PCR reaction mixtures contained 12.5 μ l TaqMan universal PCR MasterMix (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 0.2 μ M probe, 0.25 μ M primer (each), 3 μ l DNA, 5 IU TspRI (New England Biolabs, Inc., Ipswich, MA, USA), and ddH₂O to 5 μ l. qPCR was performed using an ABI7300 (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). DNA samples were extracted from all clinical tissue samples using a QIAamp DNA FFPE Tissue kit (Qiagen GmbH, Hilden, Germany), and tested using our RFLP-ARMS TaqMan PCR detection system.

Specificity assay. To determine the specificity of the proposed RFPL-ARMS TaqMan PCR method, reactions were performed with 2-200 ng of wild-type genomic DNA (Sigma-Aldrich, Saint Louis, Missouri, USA) per reaction and assessed the inefficiency caused by extension from wild-type DNA. An internal control assay was used to assess the total DNA concentration from 2-200 ng in each sample. The forward primer was designed to begin at c.1798G; it amplified wild-type and V600E mutant BRAF using the aforementioned reverse primer and probe. The change in the threshold cycle (Δ Cq) [Δ Cq=(mutation Cq)-(control Cq)] was defined for each sample. The reactions were performed five times for each DNA concentration, and each reaction was repeated in triplicate to define a cut-off Δ Cq value (46).

Assessing the detection limits of RFLP-ARMS TaqMan PCR. The wild-type and mutant plasmid DNA samples were diluted 10-fold. The effective copy number of plasmids was obtained by comparing the Cq of the diluted samples with that obtained from Human Random Control DNA Panels (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) with a known concentration of 100 ng/ μ l. In a high-quality DNA sample, there was an average of 1 set genomic DNA per 3 pg.

To assess the sensitivity of our assay for the BRAF V600E mutation, it was compared with the ARMS TaqMan PCR protocol without a removed enzyme digestion step. The mutant-encoding plasmid (50,000, 5,000, 500, 50 or 5 copies) was mixed with wild-type genomic DNA (30 ng/ μ l), corresponding to 80, 8, 0.8, 0.08 and 0.008% mutation rate. For quantification, a standard curve was generated by plotting the Cq cycle numbers against the log of each corresponding DNA copy numbers for the known standards. The linear correlation coefficients (R^2) and slopes were calculated using

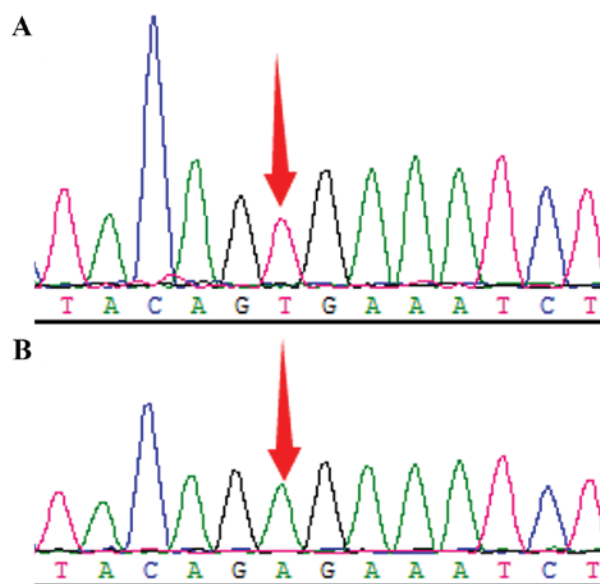


Figure 1. Comparison of results from the mutation-enriched PCR sequencing and direct PCR sequencing assays. (A) Direct PCR sequencing of the mimic human genomic DNA panel containing 1% V600E mutation, without mutant enrichment. (B) Mutation-enriched PCR sequencing of the same mimic human genomic DNA panel containing 1% V600E mutation. PCR, polymerase chain reaction. Red arrows indicate mutation base location.

ABI7300 software (v.1.3; Applied Biosystems; Thermo Fisher Scientific, Inc.).

Genotyping by DNA sequencing. All samples were re-analyzed by PCR sequencing, described as following. Kimura *et al* (47) previously demonstrated that direct sequencing failed to yield satisfactory results from samples containing mixtures of wild-type and mutant DNA. In the present study, the existing mutation-enriched PCR sequencing method was improved by restriction enzyme selectively cutting wild type alleles and leaving the mutant alleles enriched, increasing the mutation rate. This method was adapted for the detection of the BRAF V600E mutation. This method involved a first PCR amplification step, enzymatic digestion (to remove the wild-type DNA), a second PCR step for mutant enrichment and a final sequencing step. PCR was performed in a total volume of 25 μ l containing 12.5 μ l 2X Gold Fast PCR mix (Tiangen Biotech Co., Ltd., Beijing, China), 0.4 μ M each primer (round 1: forward, 5'-AGCATCTTCATTCCAATGAAGAGCC-3', and reverse, 5'-CATCCACAAAATGGATCCAGACAAC-3'; round 2: forward, 5'-CATAATGCTTGCTCTGATAGGA-3', and reverse, 5'-CCACAAAATGGATCCAGACAAC-3') and 3 μ l DNA (for the first round of PCR) or 3 μ l digested product (for the second round of PCR). The cycling conditions consisted of 95°C for 10 min, followed by 20 (round 1) or 35 (round 2) cycles of 95°C for 15 sec, 58°C for 20 sec and 72°C for 30 sec, with a final extension at 72°C for 7 min and a final hold at 4°C. Following the first round of PCR, digestion of the wild-type product was performed in a 50 μ l volume containing 10 μ l first-round PCR product, 5 μ l 10X CutSmart Buffer (New England Biolabs, Inc., Ipswich, MA, USA) and 5 IU TspRI (New England Biolabs, Inc.) at 65°C for 30 min. All of the obtained second-round PCR products were sequenced by Sangon Biotech Co., Ltd. (Shanghai, China). The sequencing

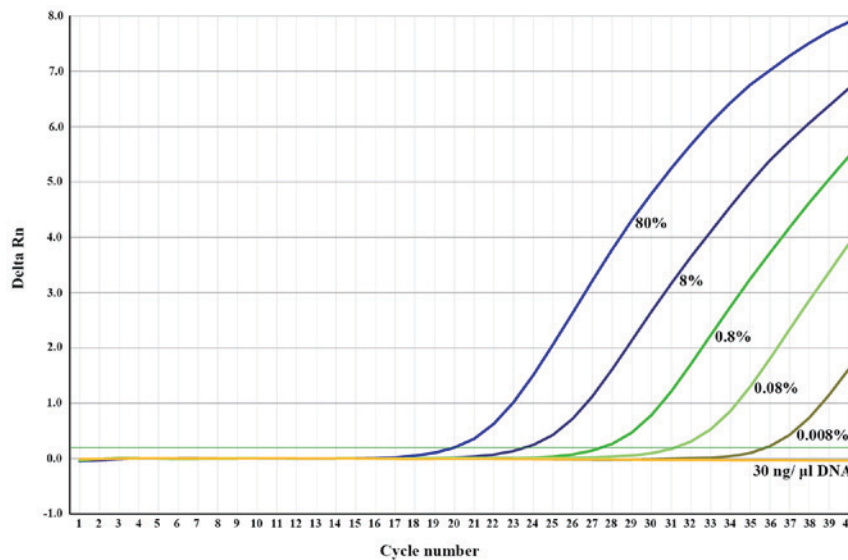


Figure 2. Sensitivity of the BRAF V600E mutation detection by RFLP-ARMS quantitative PCR. Mixing the mutant-encoding plasmid (50,000, 5,000, 500, 50 or 5 copies) with wild-type genomic DNA (30 ng/ μ l), corresponding to 80, 8, 0.8, 0.08 and 0.008% mutation rate. RFLP-ARMS, restriction fragment length polymorphism-amplification refractory mutation system; PCR, polymerase chain reaction. The horizontal green line represents the threshold.

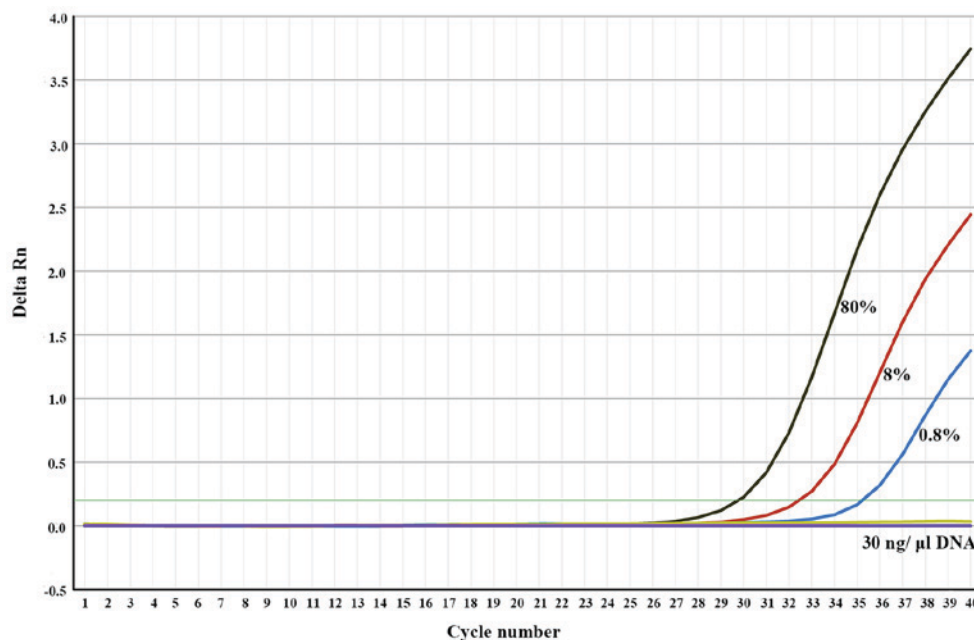


Figure 3. Sensitivity of the BRAF V600E mutation detection by ARMS TaqMan PCR. Mixing the mutant-encoding plasmid (50,000, 5,000, 500, 50 or 5 copies) with wild-type genomic DNA (30 ng/ μ l), corresponding to 80, 8, 0.8, 0.08 and 0.008% mutation rates. ARMS, amplification refractory mutation system; PCR, polymerase chain reaction. The horizontal green line represents the threshold. Mutation rates of 0.08 and 0.008% did not generate an amplified signal.

machine used was ABI-PRISM 3730 (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the analysis software was DNASTAR (v.5.0; DNASTAR, Inc.).

Statistical analysis. Fisher's exact test was used to compare the sensitivity difference of the two methods (RFLP-ARMS TaqMan PCR and PCR sequencing) in the detection of clinical samples with V600E mutation rates <5%. $P < 0.05$ was considered to indicate a statistically significant difference. SPSS for Windows was used (version 23.0; IBM Corp., Armonk, NY, USA).

Results

Specificity. To assess the specificity of the RFLP-ARMS TaqMan PCR method, RFLP-ARMS TaqMan PCR was performed using wild-type genomic DNA (2-200 ng/ μ l). By testing all the wild-type genomic DNA (2-200 ng) cases, the cut-off ΔCq value was determined to be 3 Cq below the lowest ΔCq value observed; the final cut-off ΔCt value was determined to be 14. The ΔCq values were then calculated as the difference between the mutant and control Cq values. If the difference was smaller than the cut-off ΔCt value,

the sample was classified as positive (a larger ΔCq reflected the presence of fewer mutant alleles). If the difference was larger than the cut-off point, the sample was classified as mutation-negative or beyond the limits of detection.

Sensitivity. To determine the minimal detection limit of the RFLP-ARMS TaqMan PCR method, a mimic human genomic DNA panel containing the mutant plasmid and normal wild-type human genomic DNA was used. The results revealed that the mutation-enriched PCR sequencing method exhibited increased sensitivity compared with direct PCR sequencing (Fig. 1), and was able to identify mutations making up ~1% of the total genomic DNA content. Furthermore, the RFLP-ARMS TaqMan PCR assay was demonstrated to be a sensitive and practical method to screen for the BRAF V600E mutation. As indicated in Fig. 2, this RFLP-ARMS TaqMan PCR method allowed the detection of mutants within mixed samples containing <0.01% of the V600E mutation (corresponding to <10 copies), while 0.8% V600E mutations were detected by ARMS TaqMan PCR, but 0.08 and 0.008% gave no amplification signal (Fig. 3).

Mutation analysis of clinical samples. RFLP-ARMS TaqMan PCR and PCR sequencing was performed on 53 clinical samples. Among them, 21 samples were identified to be positive for the BRAF V600E mutation by RFLP-ARMS TaqMan PCR, while only 18 positive samples were identified by PCR sequencing (Table I). The three discordant samples were then analyzed by Droplet Digital PCR (ddPCR; QX200, Bio-Rad Laboratories, Inc., Hercules, CA, USA). The results indicate that the mutation frequency of the three samples was 3.8, 1.2 and 0.6%, respectively, and the results (3.8, 1.2 and 0.6%) indicate that the samples were positive for the BRAF V600E mutation, which verified the sensitivity of RFLP-ARMS TaqMan PCR. The result of Fisher's exact test revealed a significant difference between the two methods in detecting low-frequency mutations (<5%). Future experiments will use a larger sample size, which may result in increased statistical significance.

Discussion

For mutation detection, direct sequencing is a straightforward and commonly-used method (48-50). The present study described a novel mutation-enriched PCR sequencing method and indicated that it was able to detect mutant alleles that represented >10% of the total genomic DNA content. This strategy is therefore more sensitive compared with direct PCR sequencing, which has a lower detection limit of 25-30% (10,51). The mutation-enriched PCR sequencing method of the present study was more practical compared with direct PCR sequencing of clinical samples, as it required fewer steps. It is difficult to obtain homogeneous tumor samples in the clinical setting, and the sequencing reaction may fail due to an excess of wild-type sequences (52). Therefore, it is critical to develop more sensitive genotyping methods.

Furthermore, the present study described a second novel method that combines a modified RFLP analysis and ARMS TaqMan qPCR to screen for the BRAF V600E mutation without any post-PCR processing. In our previous

Table I. Comparison of RFLP-ARMS TaqMan PCR and PCR sequencing for BRAF V600E mutation detection.

RFLP-ARMS TaqMan PCR	PCR sequencing		Total
	+	-	
+	18	3	21
-	0	32	32
Total	18	35	53

RFLP, restriction fragment length polymorphism; ARMS, amplification refractory mutation system; PCR, polymerase chain reaction; +, positive; -, negative.

investigations, we found that when we introduced an additional mismatch at the 3'-end, there was a marked decrease (The Cq value increased) in the sensitivity of the ARMS qPCR method (unpublished). In the present study, the RFLP primers were introduced into the ARMS TaqMan qPCR to improve the sensitivity. In contrast to general mutation-enriched PCR, a restriction enzyme was used to digest the wild-type DNA, enhancing the proportion of mutant alleles prior to the PCR amplification step. This protocol has the following advantages: Firstly, the restriction enzyme digestion step enhances the specificity by removing the wild-type genomic DNA and enriching the mutant allele; secondly, the sensitivity was additionally improved by using RFLP primers with a T_m that was higher (by 5°C) compared with that of the ARMS primers, which amplified a longer fragment; thirdly, the digestion and PCR reactions were performed in a single tube, avoiding the requirement for any post-PCR processing and decreasing the risk of PCR product contamination; finally, the RFLP and ARMS PCR steps were independent reactions. Together, these benefits ensure that the RFLP-ARMS TaqMan PCR assay described in the present is simple to use and amenable to high-throughput operation.

The results also revealed that the RFLP-ARMS TaqMan PCR assay was able to detect 0.1% mutant alleles in a background of ~20 copies of total genomic DNA. The sensitivity and selectivity were significantly higher compared with those achieved by the existing sequencing-based methods. Using this method, 53 melanoma samples were successfully screened for the BRAF V600E mutation. The RFLP-ARMS TaqMan PCR method of the present study identified 21 mutation-positive samples. A total of 18 of these samples were identified by direct PCR sequencing, indicating the high potential of the protocol. The mutation frequency of the three discordant samples was analyzed, which verified that the more sensitive qPCR method was able to detect mutations in samples containing only a small proportion of mutant alleles.

In summary, the novel RFLP-ARMS TaqMan PCR protocol described in the present study offers a means to improve the sensitivity and specificity of mutation detection, and may be a promising method for screening mutant alleles in cancer samples that contain relatively few mutant cells.

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Availability of data and materials

All datasets generated or analyzed in the present study are included in this published article.

Authors' contributions

YZ, WL and SCY collected tissue samples and wrote the article. SQ, TY and JH contributed to study design, the majority of the experiments, data analysis and article-writing. JZ, LG, XH and WC participated in the study design, performed the Droplet Digital PCR experiment, data analysis and interpretation and revised the manuscript.

Ethics and consent to participate

The study protocol was approved by the Ethics Committee of The Third Affiliated Hospital of Sun Yat-sen University, and written informed consent was obtained from all patients.

Consent for publication

All the study participants have approved the publication of this manuscript.

Competing interests

The authors declare that they have no competing interests.

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