Clinica Chimica Acta 494 (2019) 123–131

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

Sensitive and selective detections of codon 12 and 13 *KRAS* mutations in a single tube using modified wild-type blocker

Ning Su[a,b,c], Kun Wei[c], Na Zhao[b], Liu Wang[c], Guang-Jie Duan[d], Xiao-Dong Ren[c], Xue-Mei Qu[b,c], Qing Huang[c,b,a,*]

[a] Institute for Clean Energy & Advanced Materials, Faculty of Materials and Energy, Southwest University, Chongqing 400715, China
[b] Department of Laboratory Medicine, Southwest Hospital, Third Military Medical University (Army Medical University), Chongqing 400038, China.
[c] Department of Laboratory Medicine, Institute of Surgery Research, Daping Hospital, Third Military Medical University (Army Medical University), Chongqing 400042, China.
[d] Institute of Pathology and Southwest Cancer Center, Southwest Hospital, Third Military Medical University (Army Medical University), Chongqing 400038, China.

ARTICLE INFO

Keywords:
Metastatic colorectal carcinomas
KRAS genes
Low-abundance mutations
Wild-type blocker
Referenced internal positive controller

ABSTRACT

It was hypothesized that in the WTB-PCR system, the greater number of cycles, associated with the thermodynamic driving force of DNA polymerase resulted in artificial introduction of mutant nucleotides in amplicons. In the current study, universal WTB-PCR was developed to overcome these limitations, in which two strategies were used: phosphorothioate modifications were made at the 5′-termini bases of the WTB oligonucleotides, and amplification of referenced internal positive controller (RIPC) fragments was performed. The results showed that universal WTB-PCR could detect single-copy *KRAS* mutant alleles with higher selectivity (i.e., 0.01%), and with greater ability to eliminate non-specific amplification of *KRAS* wild-type alleles in amounts up to 200 ng. Moreover, the introduction of referenced internal positive controller (RIPC) fragments prevented false-negative results caused by inadequate amounts of input sample DNA, and allowed for quantitative analysis of the mutation levels in each FFPE sample. In clinical application in 50 samples of FFPE tissue sections from mCRC patients, 70% (35/50) showed various mutations at codons 12 and 13 of *KRAS* genes; 30% (15/50) could be detected by traditional PCR without WTB oligonucleotides. In conclusion, universal WTB-PCR is a rapid, simple and low-cost method for detection of low-abundance *KRAS* mutations in mCRC patients.

1. Introduction

The colorectal carcinoma (CRC) is the third most commonly diagnosed cancer in males and the second in females, with an estimated 1.4 million cases and 693,900 deaths occurring in 2012 [1,2]. Cetuximab (Erbitux® ImClone Systems) and Panitumumab (Vectibix®, Amgen) are monoclonal antibody therapies against the epidermal growth factor receptor (*EGFR*) and have been used clinically for targeted treatment of human metastatic CRC (mCRC) [3]. The RAS-RAF-MAPK pathway is a major signaling pathway that triggers cell proliferation upon *EGFR* ligand binding by activating the *KRAS* and *BRAF* genes [4]. *KRAS* mutation is an established predictive biomarker of resistance to anti-*EGFR* therapy in mCRC patients. Mutations in *KRAS* are found in approximately 30% of all human cancers and about 35–45% of CRCs. Clinical research results have shown that mutations of *KRAS* genes, especially at codons 12 or 13 are associated with a patient's clinical response to either Cetuximab or Panitumumab [5].

Intratumor heterogeneity is the variation in cell phenotypes within a tumor, caused by genetic or nongenetic sources. This heterogeneity is no longer considered to be a simple tumor trait, but is now recognized as a source of prognostic and predictive biomarkers for assessing the risk of tumor progression and therapeutic resistance, respectively. This diversity represents a major obstacle to effective and personalized cancer treatment [6]. Furthermore, intratumor heterogeneity applies not only to tumor cells but also to components of the microenvironment [7]. To remedy the drift of iconography and preliminary diagnosed to instructing therapy, there is a boom in liquid biopsy which is one of the top ten breakthrough technologies released by MIT Technology Review

Abbreviation: KRAS, Kirsten rat sarcoma viral oncogene homolog; mCRC, metastatic colorectal carcinoma; WTB, wild-type blocker; RIPC, referenced internal positive controller; FFPE, formalin-fixed paraffin-embedded.

* Corresponding author at: Department of Laboratory Medicine, Institute of Surgery Research, Daping Hospital, Third Military Medical University (Army Medical University), Chongqing 400042, China.
E-mail address: qinghuang@tmmu.edu.cn (Q. Huang).

https://doi.org/10.1016/j.cca.2019.03.1618
Received 7 January 2019; Received in revised form 18 March 2019; Accepted 18 March 2019
Available online 19 March 2019
0009-8981/

in 2015. Low-abundance gene detection technology and method is used as a common detection method in the field of precision medical treatment molecular diagnosis aimed at two of liquid biopsy, circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA). Furthermore, for the CTCs and ctDNA, because of the interference of a mass of normal blood cells and normal circulation of free DNA, and the low abundance of CTC and ctDNA with mutation, there is a great demand for highly sensitive clinical genetic mutation detection. The sensitivity of clinical mutation abundance detection is generally required to reach 0.1–0.01%, and current methods are not able to reach this. The existing detection methods are even less sensitive to low-abundance mutated genes, and cannot meet the clinical requirements. Therefore, there is an urgent need to develop a new method which can fulfill the clinical sensitivity and selectivity requirements of liquid biopsy.

Currently, there are various methods targeting *KRAS* mutation analysis, which mainly include PCR restriction fragment length polymorphism mapping (PCR-RFLP), allele-specific PCR (AS-PCR), amplification refractory mutation system (ARMS), high resolution melting analysis (HRMA), dual priming oligonucleotides (DPO), allele-specific hydrolysis or dual hybridization probes, smart amplification process version 2 (SMAP 2), pyrosequencing, next generation sequencing (NGS), BEAMing, IntPlex, and droplet digital PCR (ddPCR). None of these could be used to detect low-abundance *KRAS* mutations because of their limited selectivity (i.e., 1–5%) [8–13]. Although several methods (e.g., BEAMing [14], IntPlex [15] and ddPCR [16]) showed much higher selectivity, even up to 0.0005%, they had limited clinical applications because of their complexity or high costs. To satisfy the requirement of clinically available methods, maintaining simplicity and low cost, there is a need to develop better methods for detecting low-abundance *KRAS* mutations.

Among various *KRAS* mutation analysis methods, the wild-type blocking PCR (WTB-PCR) is one of the most common assays because of its higher selectivity (i.e., up to 0.01%) for detection of low-abundance *KRAS* mutations with acceptable simplicity and cost-effectiveness [17]. In the present study, a novel method called universal WTB-PCR was developed to overcome the aforementioned disadvantages of traditional WTB-PCR, targeting mutation at codons 12 and 13 of *KRAS* genes, in which the WTB oligonucleotides were modified with phosphorothioate at their 5′-termini positions to resist the 5′ to 3′ exonuclease activities of *Taq* DNA polymerase. Further, referenced internal positive controller (RIPC) fragments (i.e., human *leptin* genes) were *co*-amplified to satisfy the thermodynamic driving force of DNA polymerase and to monitor the input sample gDNA in the reaction mixture. In most versions of WTB-PCR targeting *KRAS* or other gene mutations, the WTB oligonucleotides were overlapped with the allele-specific primers. If the WTB oligonucleotides were located between the locus-specific forward (LSF) and reverse (LSR) primers targeting *KRAS* genes, they must have abilities to resist the 5′ to 3′ exonuclease activities of *Taq* DNA polymerase, such as peptide nucleic acid (PNA) [18,19]. The method was too expensive to be acceptable in clinical applications. Without RIPC in the WTB-PCR reaction mixture, non-specific amplification of *KRAS* mutant (MT) alleles was triggered easily because of the thermodynamic driving force of DNA polymerase. Further, in these conditions, there was no ability to prevent false-negative results caused by insufficient input genomic DNA (gDNA) of clinical samples [17]. These results showed that the wild-type (WT) alleles of *KRAS* genes could be eliminated in up to 200 ng of input sample gDNA with universal WTB-PCR. Furthermore, the method could be used to quantitatively analyze the mutation levels in each formalin-fixed paraffin-embedded (FFPE) sample. Based on these strategies, the current universal WTB-PCR method detected *KRAS* MT-alleles at sensitivity up to single copy and selectivity up to 0.01%. The preliminary application results showed that universal WTB-PCR detected more mutant samples (70%) than traditional PCR (30%).

2. Material and methods

2.1. Patients and tissue samples

The FFPE tissue blocks from Chinese mCRC patients were obtained from Southwest Hospital (Chongqing, China). The ethics committee at Southwest Hospital approved this study, and written informed consent was obtained from the patients or their family members prior to sample collection. In total, there were 50 FFPE samples collected. To ensure that enough tumor DNA could be extracted from the FFPE, hematoxylin-eosin (HE) staining was first performed to select FFPE tissue blocks that harbored at least 70% neoplastic cells in order to reduce the influence from non-neoplastic cells [17].

2.2. Preparation and quantitation of gDNA and quality control (QC) plasmids

The gDNA of healthy volunteers and cell line SW480 (ATCC, Manassas, VA, USA) using the QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, served as WT- and MT-gDNA in the following sections, respectively. The gDNA from the 50 samples of FFPE tissue blocks was extracted using a Paraffin Sample DNA Extraction Wax-Free™ DNA Kit, (Cat. WF-100; TrimGen, Sparks, MD, USA) following the manufacturer's instructions. The gDNA concentrations were quantitatively determined using the *leptin* quantitative PCR (qPCR) system [20]. The WT- and MT-QC plasmids corresponding to the various mutations of codons 12 and 13 were used to evaluate the feature of the current developed universal WTB-PCR system [17].

2.3. Screening WTB oligonucleotides in the traditional WTB-PCR system

Two types of WTB oligonucleotides targeting codons 12 and 13 of the *KRAS* gene (i.e., SW-1703 and -1704 in Table 1) were designed to select the ones that were resistant to the 5′ to 3′ exonuclease activity of *Taq* DNA polymerase in the traditional WTB-PCR system. The assays were performed in 20 μL of reaction mixture containing 1 × Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, Waltham, MA, USA), 500 nM of locus-specific forward (LSF; SW-1656 in Table 1) and reverse (LSR; SW-1657 in Table 1) primers, 250 nM locus-specific TaqMan (LST; SW-1437 in Table 1) probes, certain concentrations of various WTB oligonucleotides (i.e., SW-1703 and -1704 in Table 1), and varying amounts of WT- and MT-gDNA. Reactions were performed on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) under the following cycling conditions: incubation at 50 °C for 2 min, denaturation at 95 °C for 2 min, and 60 cycles at 95 °C for 15 s and 60 °C for 30 s (with single fluorescence acquisition). The quantification cycle (C_q) values were determined automatically using CFX ManagerTM Software v3.1 (Bio-Rad).

2.4. Development and optimization of universal WTB-PCR

To eliminate the non-specific amplification observed in traditional WTB-PCR systems, universal WTB-PCR was developed to satisfy the thermodynamic driving force of thermophilic DNA polymerase and to monitor the gDNA input amount. This was accomplished by supplementing traditional WTB-PCR with RIPC. The universal WTB-PCR reactions included the same reagent mixture concentration as traditional WTB-PCR, as well as LSF, LSR and LST oligonucleotides (i.e., SW-329, −330, and − 1434; Table 1) targeting RIPC (i.e., human *leptin* genes). All reaction mixtures contained 1 × Platinum® Quantitative PCR SuperMix-UDG (Invitrogen), the specified primers, TaqMan probes, and templates at certain concentrations. The thermal cycling conditions and data analysis methods were the same as those in the traditional WTB-PCR system.

Table 1
Sequences of oligonucleotides used in the present study.

Oligo ID	Target	Description	Oligo sequence (5'>3')
SW-329	Leptin	LSF-1	CAGTCTCCTCCAAACAGAAAGTCA
SW-330	Leptin	LSR-1	GTCCATCTTGGATAAGGTCAGGA
SW-1656	KRAS	LSF-2	AGGCCTGCTGAAAATGACTGAATAT
SW-1657	KRAS	LSR-2	GCTGTATCGTCAAGGCACTCTT
SW-1434	Leptin	LST-1	(VIC)CGGTTTGGACTTCATT(MGB)
SW-1437	KRAS	LST-2	(FAM)CAACTACCACAAGT(MGB)
SW-1703	KRAS	WTB-1	CTACGCCACCAGCT(C3-Spacer)
SW-1704	KRAS	WTB-2	CTACGCCACCAGCT(C3-Spacer)

The single underlined letters in HQ-1703 and HQ-1704 indicate the LNA, and the double underlined letters in HQ-1704 indicate the PS-modified bases. The fluorescent reporter and quencher are indicated at the terminal sequences of HQ-1434 and HQ-1437.

2.5. Analysis of clinical samples using universal WTB-PCR

Following analysis of the 50 FFPE samples, both reaction assays with (i.e., universal WTB-PCR) and without (i.e., traditional PCR) were performed to amplify RIPC fragments and the targeted *KRAS* genes. Moreover, sequencing was performed to analyze the variations of *KRAS* genes. In both traditional PCR and universal WTB-PCR, the RIPC fragments (i.e., *leptin*) showed similar Cq values of about 23 cycles, which indicated sufficient input gDNA for prevention of false-negative results in all reaction mixtures.

3. Results

3.1. Features of WTB oligonucleotides with phosphorothioate modifications

In various types of WTB-PCR systems, one of the most common types of variations is the localization of WTB oligonucleotides between the positions of LSF and LSR primers [21,22]. For this type of WTB-PCR, the WTB oligonucleotides (e.g., PNA) must be able to resist 5′ to 3′ exonuclease activity [18,19]. Otherwise, it is necessary to use DNA polymerase that is deficient in both 5′ to 3′ exonuclease and strand-displacement activities (e.g., Stoffel DNA polymerase) [23]. Although it has been used in the above described WTB-PCR, the PNA was too expensive to be routinely used in clinical applications. Because the oligonucleotides have phosphorothioate modifications with resistance to the activities of various exonucleases and endonucleases [18,19] and they are much cheaper than PNA, it was hypothesized that WTBs with phosphorothioate modifications at their 5′-termini should be able to resist 5′ to 3′ exonuclease activities of the commonly used thermophilic DNA polymerases (e.g., *Taq* DNA polymerases). In the present study, in addition to the traditional unmodified WTBs (WTB-2 in Table 1), the WTB-1 oligonucleotides (Table 1) were designed to have three phosphorothioate nucleotides at their 5′-termini positions to resist 5′ to 3′ exonuclease activities of *Taq* DNA polymerases (Fig. 1A). Moreover, to monitor the amplifications in real time using WTB-PCR, the LST probes that were used in the reaction mixture did not overlap with the WTB oligonucleotides (Fig. 1B-C).

Based on our previous publications [17,20], the reaction mixtures containing 50 ng of WT-gDNA and 500 nM WTBs, either with (WTB-1 in Table 1) or without (WTB-2 in Table 1) phosphorothioate modifications were used to evaluate the ability of various WTB oligonucleotides to target codons 12 and 13 of *KRAS* genes. Compared with a traditional PCR system that contains no WTBs, the results indicated that the WTBs without phosphorothioate modifications (i.e., WTB-2 in Table 1) were hydrolyzed by the 5′ to 3′ exonuclease activity of *Taq* DNA polymerase. This was observed in the C_q values of these assays, particularly because the WTB-PCR using WTB-2 had similar C_q values to assays using no WTBs (Fig. 2). However, for the WTB-PCR using WTBs with phosphorothioate modifications (i.e., WTB-1 in Table 1), the C_q values were increased to 35–38 cycles (Fig. 2). The results indicate that WTB-1 has the ability to resist 5′ to 3′ exonuclease activities of *Taq* DNA polymerase because of its phosphorothioate modifications at its 5′-termini positions. Therefore, WTB-1 was used in the WTB-PCR assays in the following sections.

Theoretically, if WTB-1 works perfectly in the WTB-PCR system, the amplification of *KRAS* WT-alleles should be eliminated. However, as shown in Fig. 2, there were non-specific amplifications during the terminal cycles (approximately 35–38) of the 40 total thermal amplification cycles. It was speculated that the non-specific amplification was associated with the relatively low fidelity of *Taq* DNA polymerase used in the current WTB-PCR system [17]. Partial amplicons in the WTB-PCR reaction, using WTB-1 oligonucleotides were sequenced. The results show that the artificial adenosine substitutions were present in the complementary positions of WTBs (data not shown), in a manner consistent with previous publications. Therefore, it was necessary to develop additional strategies to eliminate non-specific amplifications.

3.2. Optimization of the universal WTB-PCR system and elimination of non-specific amplification

Previous publications indicate that the thermodynamic driving force of thermophilic DNA polymerase plays an important role in reducing or eliminating non-specific amplification in allele-specific PCR (AS-PCR) systems, especially for AS-PCR systems only having AS-primers and unmatched templates [24,25]. In traditional WTB-PCR, in which only the LSF and LSR targeting *KRAS* genes were present, it was easily to trigger the non-specific amplifications of the artificial mutations originated from the lower fidelity of *Taq* DNA polymerase [17]. It was speculated that, if additional fragments (e.g., RIPC) were co-amplified along with the targeted MT-alleles of *KRAS* genes, the non-specific amplification in the later cycles (i.e., 35–38 thermal cycles), commonly seen in traditional WTB-PCR might be reduced or eliminated. Therefore, additional RIPC fragments (i.e., human *leptin* genes) were co-amplified with *KRAS* genes in WTB-PCR using WTBs having phosphorothioate modifications at their 5′-termini bases (Figs. 1, 3). This method was called universal WTB-PCR in the present study. Other advantages of RIPC were that it could be used to monitor the input amount of sample gDNA, in order to avoid false-negative results.

In the preliminary experiments, increasing final concentrations of LSF and LSR oligonucleotides targeting *KRAS* (i.e., 200, 500 and

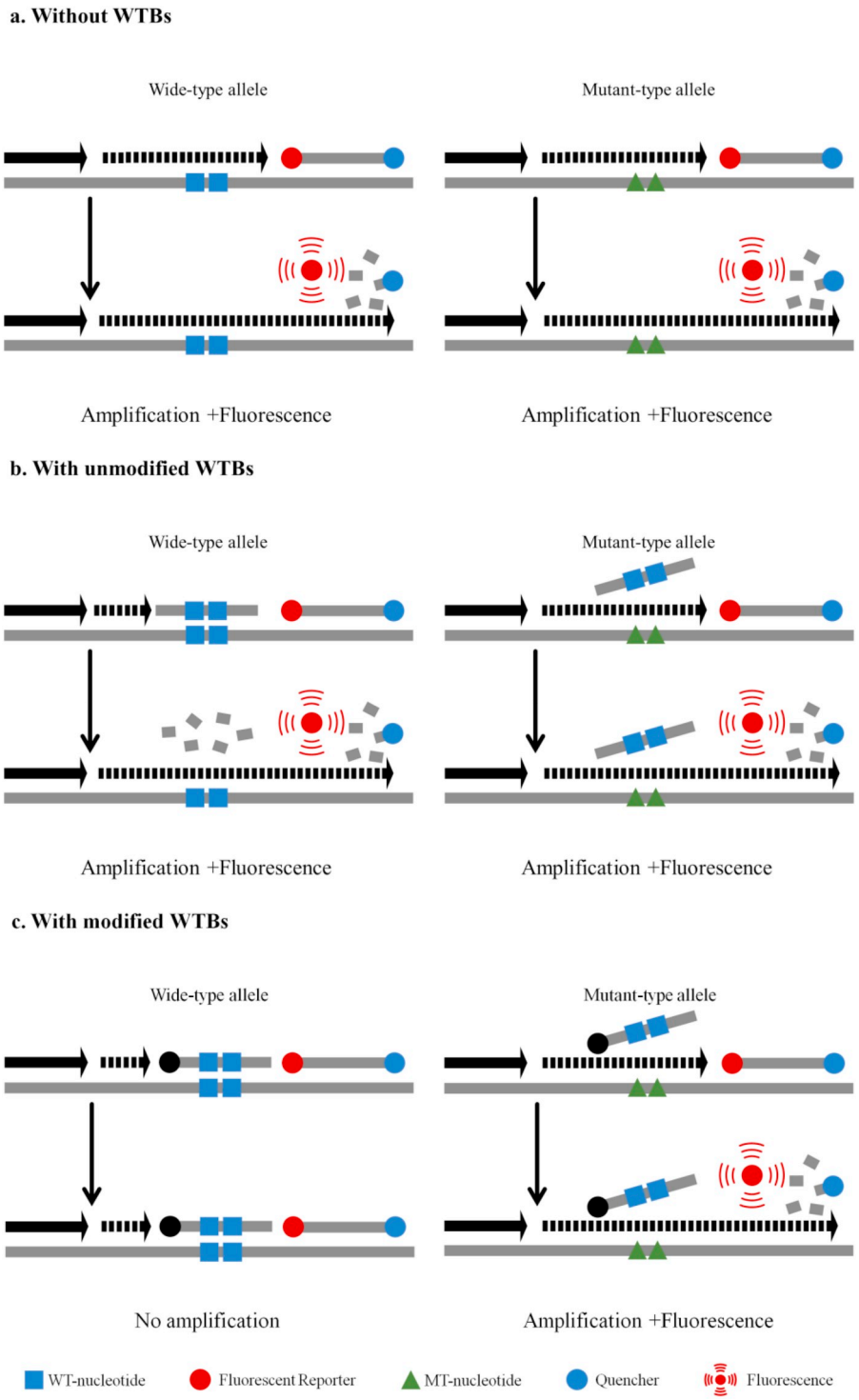

Fig 1. Principle of WTB-PCR. A. Unmodified WTBs fail to inhibit the amplification of WT-alleles because of hydrolysis, but another oligonucleotide is shown to visibly inhibit blockage of WT-alleles' amplification. B. Traditional PCR (i.e. without WTB) cannot distinguish WT- and MT-alleles of the KRAS gene because both forms release fluorescence upon amplification. C. Conversely, for WTB-PCR, only MT-alleles could be amplified because the amplification of WT-alleles was blocked by the WTB oligonucleotide.

900 nM) and *leptin* (i.e., 200 and 500 nM) genes were used in universal WTB-PCR to satisfy the thermodynamic driving force of *Taq* DNA polymerase (Table 2). The results showed that the amplification of *KRAS* WT-alleles could not be completely suppressed at LSF and LSR oligonucleotide RIPC (i.e., *leptin* gene) concentrations of only 200 nM (Table 2). Moreover, under such conditions, the universal WTB-PCR system had poor reproducibility, based on the values of standard deviation (SD) and coefficient of variance (CV; Table 2). When the LSF and LSR oligonucleotides targeting RIPC were increased to 500 nM, the amplification of *KRAS* WT-alleles was eliminated at various final concentrations of LSF and LSR oligonucleotides targeting *KRAS* genes (Table 2). The universal WTB-PCR system containing 500 nM LSF and LSR oligonucleotides targeting *leptin* and *KRAS* genes showed the lowest values of both SD and CV parameters, which indicated that the system had the best reproducibility. Therefore, the optimal reaction conditions were defined as follows: 500 nM each LSF and LSR

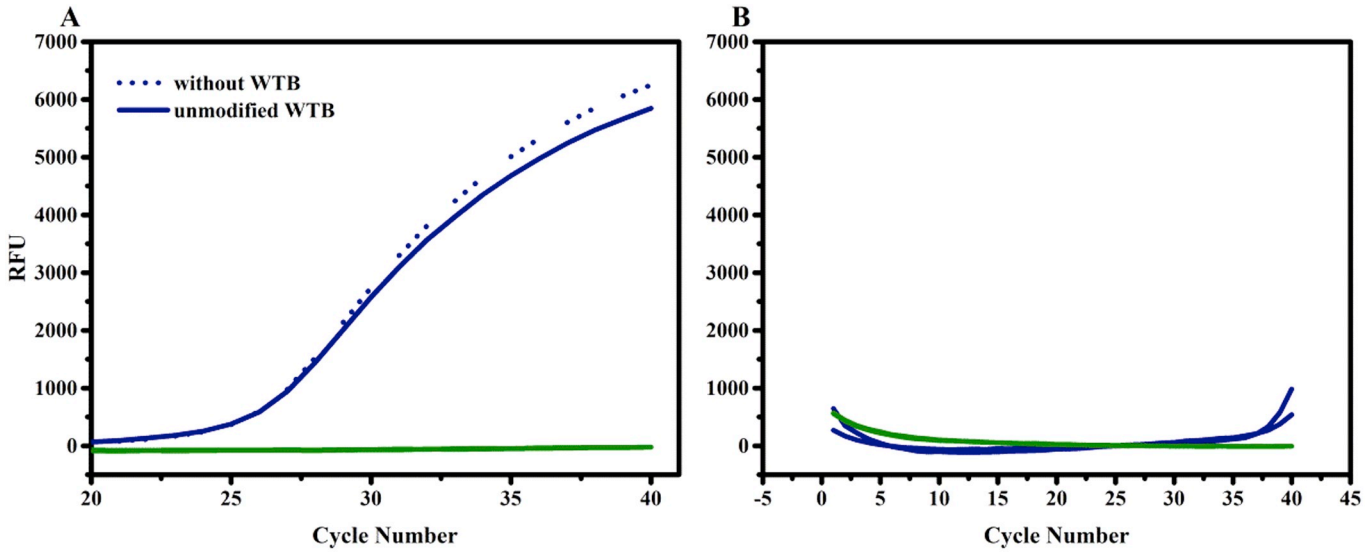

Fig. 2. Screening of various WTB oligonucleotides targeting *KRAS* WT-alleles.
Panels A and B show the amplification curves under various reaction conditions, in which the navy and olive curves indicate the amplification curves of *KRAS* and NTC, respectively. Panel A shows the results of PCR assays with traditional WTBs (solid navy lines; WTB-2 in Table 1) and without WTBs (dotted navy lines). Panel B shows the results of PCR assays with modified WTBs (WTB-1 in Table 1).

oligonucleotide targeting *leptin* and *KRAS* genes, 250 nM each LST oligonucleotide targeting *leptin* and *KRAS* genes, 500 nM WTB-1 targeting *KRAS* WT-alleles, and 40 thermal amplification cycles. Such conditions were used in the following sections.

At the above described optimal reaction conditions, the amplification of the *leptin* and *KRAS* genes showed similar amplification efficiencies. In PCR reactions lacking WTB-1, both genes exhibited similar C_q values (Fig. 3A). However, with the introduction of additional WTB-1 (i.e., universal WTB-PCR), the amplification of *KRAS* WT-alleles was eliminated (Fig. 3B). Using serially increasing amounts of *KRAS* WT-gDNA as the input template, the results showed that optimal universal WTB-PCR conditions could eliminate the amplification of *KRAS* WT-alleles in up to 200 ng of WT-gDNA, which could satisfy the requirements of clinical application (Fig. 3C). Moreover, the results showed that the amplification efficiencies of *leptin* were 95.6%, which indicates that this target could be used to monitor the total amount of input DNA (Fig. 3D).

Although the amplification of *KRAS* WT-alleles could be eliminated in universal WTB-PCR at optimal reaction conditions, there was still a need to explore whether the amplification of *KRAS* MT-alleles could be suppressed with WTB-1 because of the non-specific complementary hybridization between WTB-1 and MT-alleles. Moreover, there was a need to explore whether the amplification of *KRAS* MT-alleles could be suppressed by the RIPC fragments. Specifically, we hypothesized that the amplification of lower-abundance templates might be suppressed by the amplification of higher-abundance templates in almost any PCR system. Therefore, in further experiments, serially increasing abundances of MT-QC plasmids representing common MT-alleles at codons 12 and 13 of *KRAS* genes were spiked into *KRAS* WT-gDNA in both reaction mixtures of the PCR system with and without WTB-1 and the WTB-PCR system with and without RIPC. The results show the amplification of *KRAS* MT-alleles could not be suppressed by WTB-1 and RIPC fragments. Specifically, there were similar C_q values between universal WTB-PCR and traditional PCR or traditional WTB-PCR.

3.3. Methodology parameters of the universal WTB-PCR system

To evaluate the selectivity and sensitivity of the current developed universal WTB-PCR at optimal reaction conditions, serially diluted concentrations of *KRAS* MT-gDNA prepared from cell line SW-480, containing homozygous *KRAS* MT-alleles (i.e., c.35G > C; p.G12 V) were spiked into *KRAS* WT-gDNA to prepare templates containing serial percentages of MT-alleles (i.e., 0.01, 0.1, 1, 10, 25, 50, 100%; Fig. 4). The results showed that universal WTB-PCR had the ability to detect low-abundance MT-alleles with selectivity up to 0.01% and sensitivity up to single copy (Fig. 4A). Further data analysis results showed that the amplification efficiency of *KRAS* MT-alleles was 97.76% (R^2 = 0.999; Fig. 4B). Because the amount of total DNA was kept consistent in reaction mixtures containing the above described serially increasing percentages of MT-alleles, the C_q values of RIPC fragments (i.e., *leptin* genes) were also kept consistent (data not shown). Further data analysis results showed that the ΔC_q values between *KRAS* MT-alleles and RIPC fragments could be used to evaluate the percentages of *KRAS* MT-alleles without the need to consider the standard curves of *KRAS* MT-alleles, which were more useful in clinical applications (Fig. 4 B and C).

3.4. Clinical application of universal WTB-PCR

Following analysis of the 50 FFPE samples, both reaction assays with and without, were performed to amplify RIPC fragments and targeted *KRAS* genes. Moreover, sequencing was performed to analyze the variations of *KRAS* genes. In both traditional PCR and universal WTB-PCR, the RIPC fragments (i.e., *leptin*) showed similar Cq values of about 25.2 cycles (ranges: 21.42 to 27.09), which indicated sufficient amounts of input gDNA were used in all reaction mixtures to avoid false-negative results. Compared with traditional PCR, that showed amplification of *KRAS* genes in all samples, universal WTB-PCR showed positive amplifications of *KRAS* MT-alleles in 70.0% (35/50) of samples. Sequencing results showed that all 35 samples that were positively amplified with universal WTB-PCR were predominantly composed of *KRAS* MT-alleles (Table 3; Fig. 5). In contrast, only 15 of the 35 mutated samples could be detected following sequencing analysis of the traditional PCR products (Table 3). Therefore, universal WTB-PCR resulted in much higher accuracy (i.e., 70%; 35/50) of detection of mutated samples, when compared with the percentage of *KRAS* mutants detected with traditional PCR (i.e., 30%; 15/50) (Table 3). According to the $2^{-\Delta\Delta Ct}$ mathematical model for relative quantification [26], we could obtain the standard curve presented in Fig. 4C. The ΔCq between *leptin* and KRA genes for these 15 samples suggested a mutant

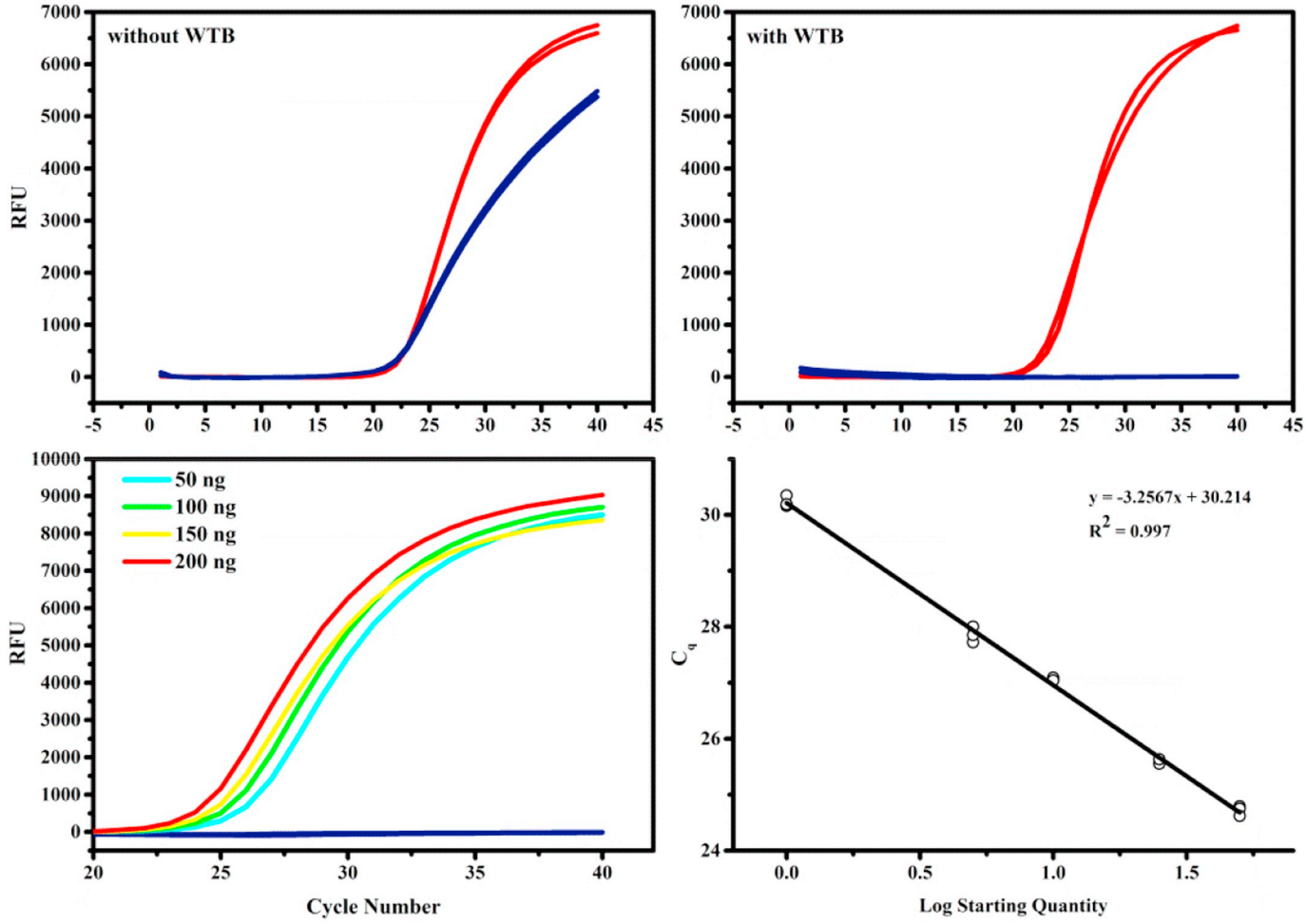

Fig. 3. Eliminating non-specific amplification introducing internal competitive reference.
Panels A to C show the amplification curves of universal WTB-PCR, in which the navy and red curves indicate the amplification of *KRAS* and *leptin* genes, respectively. Compared with PCR system without WTBs (i.e., panel A), the non-specific amplification of *KRAS* WT-alleles were eliminated in universal WTB-PCR (i.e., panel B), which were performed in 20 μL of reaction mixture having 50 ng of *KRAS* WT-gDNA, 500 nM of LSF and LSR targeting RIPC fragments (i.e., human *leptin* genes), and 500 nM of LSF and LSR targeting *KRAS* genes. Panel C further indicates that the non-specific amplification of *KRAS* WT-gDNA could be eliminated at amounts of up to 200 ng, as indicated. Panel D shows the standard curves of *leptin* genes in universal WTB-PCR, which were generated by plotting the average C_q values of *leptin* genes against the log starting quantity of gDNA (i.e., 1, 5, 10, 25 and 50 ng). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Optimization of oligonucleotides used in universal WTB-PCR.

Leptin	200 nM			500 nM		
KRAS	200 nM	500 nM	900 nM	200 nM	500 nM	900 nM
Maximum ΔC_q	14.36	18.43	18.36	18.6	18.44	18.26
Minimum ΔC_q	4.14	7.96	18.33	18.52	18.43	18.1
Average	9.25	13.2	18.35	18.56	18.44	18.18
SD[#]	7.23	7.4	0.02	0.06	0.01	0.11
CV[#]	78.13%	56.11%	0.12%	0.30%	0.04%	0.62%

Calculated from triplicate reaction mixtures. The ΔC_q values were calculated after 40 cycles. If there were no available C_q values, the 40 cycles were used to calculate the ΔC_q values in order to obtain the data.

percentage of > 20% (ranges: 20.36 to 80.84%); However, analysis of the 20 samples that were not analyzed using traditional PCR yielded a *KRAS* mutant percentage range from 18.24% to 0.03%. Interestingly, when compared with the preliminary WTB-PCR without RIPC fragments, the DNA sequencing results of the optimized universal WTB-PCR showed no artificial mutant nucleotides.

4. Discussion

The *KRAS* gene is the most commonly mutated gene in the RAS/RAF/MAPK pathway, and a mutation is present in approximately 35% to 45% of mCRC patients, making it one of the most commonly mutated genes in cancer. Codons 12 and 13 are two hotspots which account for about 95% of all *KRAS* mutations. Approximately 80% of mutations occur in codon 12 and 15% occur in codon 13 [3,27]. Available studies indicated that mCRC patients harboring *KRAS* mutations did not benefit from treatment with anti-*EGFR* monoclonal antibodies, e.g., Cetuximab and Panitumumab [28–30]. Because up to 90% of activating mutations in the *KRAS* gene are detected in codons 12 and 13 of *KRAS* [31], standard methods have been used to detect the mutations in these positions. However, because of intratumor heterogeneity, tumor cells containing mutated *KRAS* alleles frequently present as subclones in mCRC. Because of this, detection methods must be sensitive enough to detect mutations in tissue samples that contain an excess of WT-alleles and presence of mutant DNA as low as 0.01%. Clinical results of target gene mutation detection for individualized drug use show that the higher sensitivity of genetic mutation detection techniques can always screen individuals for appropriate treatment more effectively because

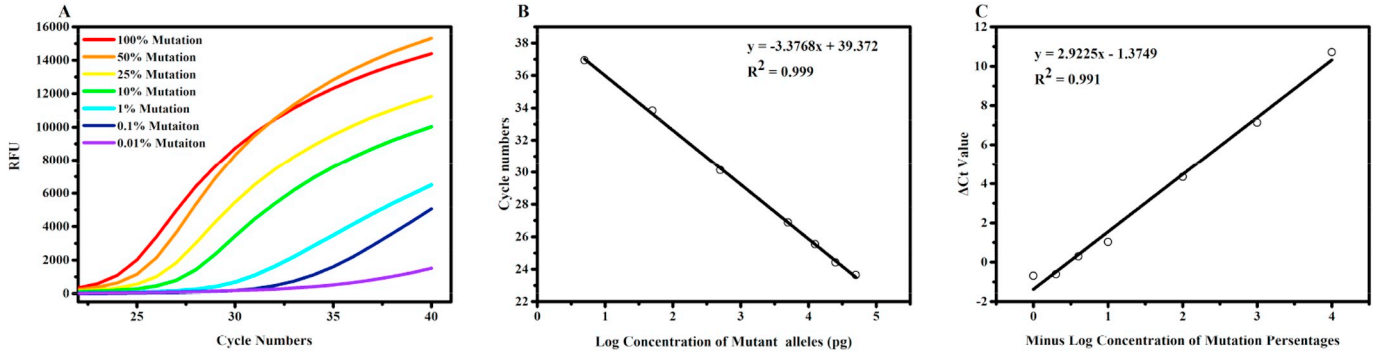

Fig. 4. Sensitivity and quantitative curves of universal WTB-PCR.
Panel A shows the amplification curves of *KRAS* MT-alleles at various mutant percentages as indicated (i.e., 0.01, 0.1, 1, 10, 25, 50, 100%) in universal WTB-PCR reaction mixtures, in which certain amounts (i.e., 5, 50, 500, 5000, 10,000, 25,000 and 50,000 pg) of cell line SW-480 gDNA (i.e., homozygous mutations at c.35G > C or p.G12 V of *KRAS* genes) were spiked into WT-gDNA to give a total of 50 ng of gDNA in 20 μL of reaction mixture. Panel B shows the standard curves generated by plotting the average C_q values from universal WTB-PCR against the log concentrations of *KRAS* MT-alleles from gDNA of cell line SW-480 (i.e., 5, 50, 500, 5000, 10,000, 25,000 and 50,000 pg). Panel C shows the standard curves generated by plotting the average ΔC_q values between *KRAS* and *leptin* genes against the minus log percentage concentrations of c.35G > C MT-alleles from cell line SW-480 (i.e., 0.01, 0.1, 1,10, 25, 50 and 100%) in 50 ng total of gDNA, which could be used to calculate the percentages of *KRAS* MT-alleles in clinical samples.

Table 3
Summary of *KRAS* detailed mutations in 50 clinical samples.

cDNA Position*	Protein Position#	Mutation Cases	
		Traditional PCR	universal WTB-PCR
c.34G > C	p.G12R	ND**	ND
c.34G > T	p.G12C	2	15
c.34G > A	p.G12S	ND	ND
c.35G > C	p.G12A	4	6
c.35G > T	p.G12V	ND	ND
c.35G > A	p.G12D	5	8
c.37G > C	p.G13R	ND	ND
c.37G > T	p.G13C	ND	ND
c.37G > A	p.G13S	ND	2
c.38G > C	p.G13A	ND	ND
c.38G > T	p.G13V	ND	ND
c.38G > A	p.G13D	4	4
Total		**15**	**35**
Percent		**30%**	**70%**

* "c." indicates cDNA. e.g., c.34G > C means nucleotide G mutated to C in position at 34 in cDNA.
# "p." indicates protein. e.g., p.G12A means glycine mutated to arginine in position at 12 in protein.
** ND indicates not detected.

even if there are a small number of drug-resistant mutant tumor cells, the predominant hyperplasia will lead to recurrence and metastasis. The higher the sensitivity of the mutation detection method, the more effective the detection of these rare cells or free DNA with drug-resistant mutations will be. This will help to avoid ineffective treatment. In a recent publication, results generated by Laurent-Puig, et al. suggested that mCRC patients harboring 1% *KRAS* mutated subclones (detected using ultra-high sensitivity pico-droplet dPCR, with 0.0005% sensitivity) could benefit from anti-*EGFR* treatment [32]. The reason for it may be that most of the tumor cells were *KRAS* WT-alleles that respond well to early-stage anti-*EGFR* antibody treatment. As a result, mCRC patients with low levels of *KRAS* mutated genes benefitted from anti-*EGFR* treatment compared with patients who have higher abundances of *KRAS* mutated genes. Even if there are a small number of drug-resistant mutant tumor cells, under the selective pressure of drugs, the predominant hyperplasia will lead to anti-*EGFR* treatment failure, recurrence and metastasis.

Compared with the above described methods, only one pair of primers coupled with one WTB probe was needed in WTB-PCR to specifically and sensitively amplify the multiple mutated alleles in excess WT-alleles which could eliminate the amplification of WT-alleles via complementary hybridizations with WT-alleles because the single base mismatches can potentially disrupt the duplex construction between WTB and mutant alleles, thereby selectively amplifying mutant alleles, and the amplified products could be directly used for DNA sequencing. Of the various methods used to detect low-abundance mutated genes, PCR mediated by WTB oligonucleotides such as peptide nucleic acid (PNA), locked nucleic acid (LNA), and LNA/DNA chimeras facilitate the discrimination of single base mismatches. WTB-PCR performs more optimally in the detection of mutants, selectively amplifying MT-alleles with sensitivity up to 0.01%.

The most common type of WTB-PCR system is that in which WTB oligonucleotides are located between the positions of LSF and LSR primers. This system is called elongation arrest [21,22]. Our team has been focused on it for several years and we have already developed detection methods [17,20,24,25]. The benefit of this system is that the allele-specific TaqMan (AST) probes could accurately discriminate the mutant bases. This has the further benefit of requiring no DNA sequencing of WTB-PCR amplicons. However, with this method, unmodified WTB could be hydrolyzed by DNA polymerase when primers were extended (Fig. 1A). Therefore, a WTB system is needed which has abilities to resist 5′ to 3′ exonuclease activities of *Taq* DNA polymerase used in WTB-PCR. Under these conditions, DNA polymerase is not required to prevent 5′ > 3′ exonuclease activity. In previously available WTB-PCR systems, PNA was one of the only available WTBs used in elongation arrest WTB-PCR. However, the PNA was too expensive to be acceptable in clinical applications.

In the present study, a novel type of WTB, i.e., WTBs having phosphorothioate (PS) modified based at their 5′-termini positions, were developed to resist the 5′ to 3′ exonuclease activity of *Taq* DNA polymerase. They were then used in the elongation arrest type of WTB-PCR to develop universal WTB-PCR (Fig. 1 C). This demonstrated that the exonucleolytic attack on the oligonucleotide can be efficiently prevented by the introduction of phosphorothioate bonds at the 3′-termini. PS-modified primers had been used in the single nucleotide polymorphism (SNP) assays [18,19], it is the first time it was used in WTB-PCR via modified WTBs to avoid hydrolysis by DNA polymerase. The results show that the antihydrolysis abilities of PS-modified WTBs were as high as those of PNA, but much less expensive in clinical applications. LNA/DNA oligonucleotide chimeras could enhance the ability of WTB, recognizing a single base mismatch to MT-alleles (e.g. c.35G > A), thus significantly decreasing the blocking affinity and allowing amplification of *KRAS* MT-alleles. Furthermore, the novel WTBs could also be modified by a hydrolysis block at the 5′-terminus to tolerate

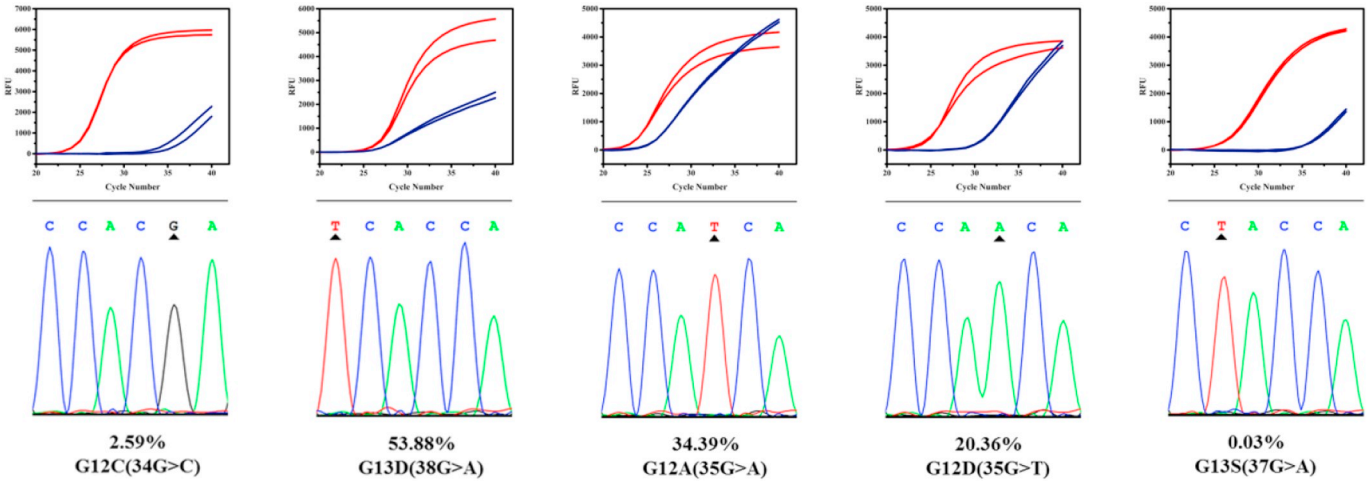

Fig. 5. Examples of results obtained from clinical samples.
The amplification curves of universal WTB-PCR (i.e., top panel) and their corresponding *KRAS* sequencing results (i.e., bottom panel), presented as examples of five clinical samples, in which detailed information about the MT-alleles and their corresponding percentages in clinical samples are indicated. The triangle arrows in the bottom panels indicate the positions of each mutant nucleotide in the sequencing chromatographs of *KRAS* genes.

5′ > 3′ exonuclease activity of DNA polymerase, including phosphorothioate-modified nucleotides, inverted nucleotide and other nucleic acid analogs the activity tolerating the force from DNA polymerase. Using LNA/DNA chimeras and 5′-terminus PS-modified WTBs, the universal WTB-PCR used in this study can simultaneously detect all 12 possible missense mutations at codons 12 and 13 of *KRAS* genes in a single closed tube. Based on the advantages associated with our WTB-PCR assay, similar strategies could be used to sensitively detect low-abundance mutations on other codons (e.g. codons 59, 6, 117 and 146) of *KRAS* and other virulence genes such as *NRAS* (e.g. codons 12, 13, 59, 6, 117 and 146), *BRAF* (e.g. codons 549, 596 and 600), and *PIK3CA* (e.g. exon 9 and 20).

In the previous study, we noticed that higher numbers of cycles resulted in false-positive results caused by artificial introduction of mutant nucleotides in amplicons. We hypothesize that these limitations are associated with the thermodynamic driving force of DNA polymerase. To satisfy the requirement for the thermodynamic driving force of DNA polymerase, RIPC fragments from human *leptin* genes were introduced in WTB-PCR, in a method called universal WTB-PCR. This approach was developed to eliminate non-specific amplification that frequently occurs in the WTB-PCR system and to monitor the input amount of sample genomic DNA (gDNA). The results showed that this universal WTB-PCR system could eliminate non-specific amplification. Moreover, the results further showed amplification efficiencies of *leptin* of 95.6% (R^2 = 0.999), which indicated that the system could be used to monitor the total amount of input DNA (Fig. 3D). Compared to other reaction systems, universal WTB-PCR showed up to 100% specificity and up to 0.01% selectivity and sensitivity. In conclusion, this strategy could solve the problem of artificial mutations caused by the low fidelity of Taq polymerase and over-abundance of thermal cycles.

The results of testing 50 clinical samples further confirmed the practicability of the method. The most frequent mutations of the 12 possible mutations at *KRAS* codons 12 and 13 are the 6 missense mutations associated with codon 12 (i.e., p.G12R, p.G12C, p.G12S, p.G12A, p.G12 V, and p.G12D) and the single mutation at codon 13 (i.e., p.G13D). In the present study, 70% (35/50) of the samples tested contained missense mutations and 94% (33/35) of these missense mutations were at the above positions, with only 12% (4/33) occurring in codon 13. Although commercially available kits targeting *KRAS* mutations have focused on these 7 common mutation sites, there is a need to further develop methods that target other mutation sites, such as the mutation site p.G13S at codon 13 that occur at a frequency of at least 6% (2/35). Our assay could detect all the mutation types present in clinical investigation, in order to provide better guidance in individualized therapy for tumor patients. Therefore, this novel system may be utilized in the clinical liquid biopsy screening for oncogenic mutations.

The major advantages associated with our universal WTB-PCR assay are that it facilitates detection of all 12 possible mutations of the *KRAS* gene at codons 12 and 13 in a single tube. The assay only requires approximately 60 min and requires no sequencing. Moreover, this assay only requires the use of a real-time PCR instrument, which is commonly available in most clinical laboratories, and requires no additional training for operating personnel. The assay product can be used to simultaneously sequence and analyze the internal control fragment. PS-modified WTB has more clinical application prospects because of the significant advantages of lower price and more stable performance. Further, we are developing the probe WTB-PCR assay using the mutant-specific TaqMan probes (MST) to monitor the amplification products and obtain mutation information directly, with no requirement for sequencing.

5. Conclusion

The introduction of RIPC (i.e., human *leptin* gene) into the WTB-PCR system to satisfy the thermodynamic driving force of thermophilic DNA polymerase prevented the occurrence of non-specific amplification in the WTB-PCR reaction system. Moreover, universal WTB-PCR provided the additional advantage of eliminating false-negative results caused by an inadequate amount of total DNA added. The universal WTB-PCR had the sensitivity to detect a single copy of the 12 possible human *KRAS* MT-alleles, with a selectivity of 0.01%. Therefore, these methods might serve as powerful and robust routine genotyping tools with clinical applications. In our laboratory, similar universal WTB-PCR assays targeting other oncogenic mutations in genes that play important roles in personalized treatments, such as *BRAF* and *EGFR* are being developed.

Acknowledgments

This study was partly supported by the Scientific Foundation of the Southwest Hospital (Nos. SWH2016YSCXZD-13 and SWH2016LHYS-01) and the National Nature Science Foundation of China (No. 81572066).

References

[1] L.A. Torre, et al., Global cancer statistics, 2012, CA Cancer J. Clin. 65 (2) (2015) 87–108.
[2] W. Chen, et al., Cancer statistics in China, 2015, CA Cancer J. Clin. 66 (2) (2016) 115–132.
[3] D. Cunningham, et al., Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer, N. Engl. J. Med. 351 (4) (2004) 337–345.
[4] C.S. Karapetis, et al., K-ras mutations and benefit from cetuximab in advanced colorectal cancer, N. Engl. J. Med. 359 (17) (2008) 1757–1765.
[5] A.A. Adjei, Blocking oncogenic Ras signaling for cancer therapy, J. Natl. Cancer Inst. 93 (14) (2001) 1062–1074.
[6] C.C. Maley, et al., Genetic clonal diversity predicts progression to esophageal adenocarcinoma, Nat. Genet. 38 (4) (2006) 468–473.
[7] T.D. Tlsty, L.M. Coussens, Tumor stroma and regulation of cancer development, Annu. Rev. Pathol. 1 (2006) 119–150.
[8] J.C. Fox, et al., The detection of K-ras mutations in colorectal cancer using the amplification-refractory mutation system, Br. J. Cancer 77 (8) (1998) 1267–1274.
[9] Y. Mitani, et al., Rapid SNP diagnostics using asymmetric isothermal amplification and a new mismatch-suppression technology, Nat. Methods 4 (3) (2007) 257–262.
[10] V. Taly, et al., Multiplex picodroplet digital PCR to detect *KRAS* mutations in circulating DNA from the plasma of colorectal cancer patients, Clin. Chem. 59 (12) (2013) 1722–1731.
[11] A. Lievre, et al., *KRAS* mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab, J. Clin. Oncol. 26 (3) (2008) 374–379.
[12] D. Dressman, et al., Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations, Proc. Natl. Acad. Sci. U. S. A. 100 (15) (2003) 8817–8822.
[13] A.R. Thierry, et al., Clinical validation of the detection of *KRAS* and *BRAF* mutations from circulating tumor DNA, Nat. Med. 20 (4) (2014) 430–435.
[14] M. Li, et al., BEAMing up for detection and quantification of rare sequence variants, Nat. Methods 3 (2) (2006) 95–97.
[15] F. Mouliere, et al., Multi-marker analysis of circulating cell-free DNA toward personalized medicine for colorectal cancer, Mol. Oncol. 8 (5) (2014) 927–941.
[16] A. Didelot, et al., Multiplex picoliter-droplet digital PCR for quantitative assessment of DNA integrity in clinical samples, Clin. Chem. 59 (5) (2013) 815–823.
[17] J.F. Huang, et al., Single-tubed wild-type blocking quantitative PCR detection assay for the sensitive detection of codon 12 and 13 *KRAS* mutations, PLoS ONE 10 (12) (2015) e0145698.
[18] J. Zhang, K. Li, On-off regulation of 3′ exonuclease excision to DNA polymerization by Exo+ polymerase, J. Biochem. Mol. Biol. 36 (6) (2003) 525–528.
[19] J. Zhang, K. Li, Single-base discrimination mediated by proofreading 3′ phosphorothioate-modified primers, Mol. Biotechnol. 25 (3) (2003) 223–228.
[20] Z. Yang, et al., Improved detection of *BRAF* V600E using allele-specific PCR coupled with external and internal controllers, Sci. Rep. 7 (1) (2017) 13817.
[21] H. Orum, et al., Single base pair mutation analysis by PNA directed PCR clamping, Nucleic Acids Res. 21 (23) (1993) 5332–5336.
[22] C. Thiede, et al., Simple and sensitive detection of mutations in the ras proto-oncogenes using PNA-mediated PCR clamping, Nucleic Acids Res. 24 (5) (1996) 983–984.
[23] S.C. Hamilton, J.W. Farchaus, M.C. Davis, DNA polymerases as engines for biotechnology, Biotechniques 31 (2) (2001) 370 (-6, 378-80, 382-3).
[24] D. Chen, et al., High-sensitivity PCR method for detecting *BRAF* V600E mutations in metastatic colorectal cancer using LNA/DNA chimeras to block wild-type alleles, Anal. Bioanal. Chem. 406 (9–10) (2014) 2477–2487.
[25] D. Chen, et al., Enhanced specificity of TPMT*2 genotyping using unidirectional wild-type and mutant allele-specific scorpion primers in a single tube, PLoS ONE 9 (4) (2014) e91824.
[26] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 29 (9) (2001) e45.
[27] D.J. Jonker, et al., Cetuximab for the treatment of colorectal cancer, N. Engl. J. Med. 357 (20) (2007) 2040–2048.
[28] W. De Roock, et al., *KRAS* wild-type state predicts survival and is associated to early radiological response in metastatic colorectal cancer treated with cetuximab, Ann. Oncol. 19 (3) (2008) 508–515.
[29] E. Van Cutsem, et al., Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor *KRAS* and *BRAF* mutation status, J. Clin. Oncol. 29 (15) (2011) 2011–2019.
[30] C. Bokemeyer, et al., Efficacy according to biomarker status of cetuximab plus FOLFOX-4 as first-line treatment for metastatic colorectal cancer: the OPUS study, Ann. Oncol. 22 (7) (2011) 1535–1546.
[31] S. Forbes, et al., COSMIC 2005, Br. J. Cancer 94 (2) (2006) 318–322.
[32] P. Laurent-Puig, et al., Clinical relevance of *KRAS*-mutated subclones detected with picodroplet digital PCR in advanced colorectal cancer treated with anti-*EGFR* therapy, Clin. Cancer Res. 21 (5) (2015) 1087–1097.