Wild-type blocking pcr coupled with internal competitive amplified fragment improved the detection of rare mutation of *KRAS*

JIA PENG[1,2*], KUN WEI[1*], XIANG ZHAO[1], KE YANG[1], HUAN WANG[1], YANG ZHANG[1], MEI GUO[1], JING HE[1], HAIYAN WU[1], YONGCHUAN LI[1], NA ZHAO[1], QING HUANG[1] and WEILING FU[1]

[1]Department of Laboratory Medicine, Southwest Hospital, The Third Military Medical University, Chongqing 400038; [2]Department of Laboratory Medicine, 324th Hospital of PLA, Chongqing 400020, P.R. China

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Abstract. Mutant KRAS proto-oncogene GTPase (*KRAS*) serves an important role in predicting the development, diagnosis, treatment and efficacy of targeted drug therapies for colorectal cancer. To improve the detection efficacy of trace amount of mutant *KRAS*, the locked nucleic acid-based method was modified in the present study. Internal competitive amplification fragments were used to improve the inhibition of wild-type *KRAS* with a wild-type blocking (WTB) probe and specifically amplify the trace amounts of mutant *KRAS*. The modified method, quantitative clamp-based polymerase chain reaction technology using WTB coupled with internal competitive reference to enhance the amplification specificity, named WIRE-PCR, completely blocked the amplification of wild-type *KRAS* in 50-150 ng DNA templates. The added internal competitive amplified fragments were amplified together with the target gene, which were used to reduce base mismatch due to the high number of cycles in PCR and quantify the total amount of DNA. The results demonstrated that WIRE-PCR facilitated the detection of mutated alleles at a single molecular level. In the colorectal biopsies from 50 patients with suspected colorectal cancer, 18 cases (36%) contained mutant *KRAS*, and the amount of mutant DNA accounted for 18.6-64.2% of the total DNA. WIRE-PCR is a simple, rapid and low-cost quantitative analysis method for the detection of trace amounts of the mutant *KRAS*.

Correspondence to: Professor Weiling Fu or Professor Qing Huang, Department of Laboratory Medicine, Southwest Hospital, The Third Military Medical University, 30 Gaotanyan Street, Chongqing 400038, P.R. China
E-mail: weiling_fu@163.com
E-mail: drq.huang@gmail.com

*Contributed equally

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Introduction

Colorectal carcinoma (CRC) is one of the highest incident malignant tumors in the world. The five-year survival rate is less than 10%, and 50-60% of the CRC patients eventually progress to metastatic colorectal carcinoma (mCRC) (1,2). Although FOLFIRI, FLOFLOX, and other chemotherapy alone or in combination with anti-VGFR monoclonal antibody (e.g., Bevacizumab) can improve the prognosis of patients with mCRC, who may produce drug resistance, application of epidermal growth factor receptor (EGFR) monoclonal antibody (e.g., Panitumumab) at the moment is still effective (3,4). As a member of ErbB transmembrane tyrosine kinase receptor family, EGFR activates MEK/ERK, PI3K/AKT, and STAT signaling pathways to induce cell proliferation, dedifferentiation and blocking of apoptosis (5). Anti-EGFR monoclonal antibodies, mainly include Cetuximab and Panitumumab, have been used in the clinical therapy of mCRC (6). *KRAS* is a downstream EGFR-signaling pathway-core component of MAPK pathway. Previous studies have shown that its *KRAS* mutation leads to failure of Erbitux, Panini, or other monoclonal antibody therapy due to ineffective inhibition of EGFR signaling pathway using those monoclonal antibodies, indicating that mutant *KRAS* is critical for EGFR monoclonal and targeted antibody therapy (7). The European Medicines Agency requires clinicians to detect *KRAS* mutation in the patients before administrating monoclonal antitumor drug (8).

A variety of detection methods have been available for the screening of mutant *KRAS*, including sequencing, single-stranded confirmation polymorphism (SSCP), AS-PCR, TaqMan probe PCR, beads, emulsion, amplification, and magnetics (BEAMing), LigAmp assay, and clamping-based PCR (9,10). Clamping-based PCR is the most sensitive method for the detection of low abundance mutations by selectively adding the wild-type amplified nucleic acid into the reaction system to block the amplification of wild-type gene (11,12). The related techniques have been successfully applied to detect the trace amount of gene mutation in some tumors (12,13). However, the application of clamping-based PCR also has a problem: Susceptible to the interference of DNA polymerase in the reaction system, which may result

in base mismatch in PCR with high cycle number (14-16). To solve this problem, the present study modified the existing clamping-based PCR by adding internal competitive amplified fragments to enhance the inhibition of wild-type *KRAS* via locked nucleic acid (LNA) probe and established a method for detecting the trace amount of mutant *KRAS* in colorectal neoplasms. The method was applied to detect mutant *KRAS* from the colorectal biopsies of 50 patients with suspected colorectal cancer, followed by DNA sequencing and pathological analysis to validate the test results. The present study provided a reference for effectively predicting the therapeutic outcomes of colorectal cancer patients through our methods on mutant *KRAS* detection.

Materials and methods

Patients and DNA extraction from colorectal biopsies. The present study recruited Han Chinese patients from the outpatient and inpatient clinics of the Southwest Hospital (Chongqing City, China). Our study protocol was approved by the Ethics Committee of the Southwest Hospital. All patients or their guardians signed the informed consents before participating in the present study.

Fresh colorectal biopsies obtained during colonoscopy from the patients with suspected colorectal cancer were washed in PCR and subsequently placed in new Eppendorf tubes, followed by DNA extraction using Tissue DNA kit (Catalogue no. 536-050, Gene Tech Biotechnology Co., Ltd., Shanghai, China) with reference to the manufacturer's instructions and DNA quantification using Nanodrop to obtain mean value of total DNA of each sample from the triplicated measurements.

Amplification of mutant KRAS using WIRE-PCR. As shown in Table I, the PCR system in the present study contained 500 nM primer set (SW-329/330) and 100 nM florescent probe (SW-1294) for the detection of internal reference gene; 500 nM primer set (SW-1595/1596) and 250 nM fluorescent probe (SW-1438) for the detection of *KRAS*; and 500 nM LNA probe (SW-144). The PCR conditions were 50˚C for 2 min and 60 cycles of 95˚C for 2 min, 95˚C for 15 sec, and 60˚C for 1 min. The 2X SuperMix-UDG was used as the enzyme for the PCR system. To determine the sensitivity of WIRE-PCR system, a concentration gradient of mutant template was prepared by mixing increasing concentrations of plasmid (10^5, 10^4, 10^3, 10^2, 10^1, 10^0, copies/μl) from the previously constructed containing mutated single nucleotides (c34G>C;G12R.1R) with human WT-gDNA in total 50 ng. The reaction conditions were 50˚C for 2 min and 60 cycles of 95˚C for 2 min, 95˚C for 15 sec, and 60˚C for 1 min in total 20 μl.

Table I. Sequences of oligonucleotides used in the present study.

Oligo ID	Oligo sequences (5'-3')
SW-329	CAGTCTCCTCCAAACAGAAAGTCA
SW-330	GTCCATCTTGGATAAGGTCAGGA
SW-1294	(Texas Red) CGGTTTGGACTTCATTCCTGG GCTCC (BHQ2)
SW-1595	TTTATTATAAGGCCTGCTGAAAATGAC
SW-1596	CGTCAAGGCACTCTTGCCTAC
SW-1438	(VIC) ACTACCACAAGTTTATATTC (MGB)
SW-144	TACGCCACCAGCT

[1]SW-329 and SW-330 were the primer sets of the internal reference *LEPTIN*; SW-1294 was the fluorescent probe of internal reference *LEPTIN*; SW-1595 and SW-1596 were the primer sets of the *KRAS* gene; and SW-1438 was the fluorescent probe of KRAS. The underlined letters in SW-144 indicate the LNA.

Results

Establishment and optimization of WIRE-PCR. Given the importance of the annealing temperature in PCR system, we optimized the best anneal temperature for the primer sets used in the present study. For example, anneal temperatures for the amplification of *KRAS* gene using SW-1595/1596 primers were set as 60-68˚C to amplify the PCR products at eight gradient temperatures. The results of agarose gel electrophoresis separating the PCR products of different annealing temperatures, and the results showed the optimal temperature 60˚C, with good amplification efficacy and the corresponding annealing, which was suggested to be the optimal annealing temperature for the reaction system.

The mean CT values ± standard deviation in the internal reference gene, *LEPTIN*-involved amplification reaction using 500 and 250 nM *KRAS* primer set were 25.6±0.23 and 26.52±0.36, respectively. The small CT value of the reaction system using 500 nM *KRAS* primer set showed good reproducibility, and thus we used 500 nM *KRAS* primer set to optimize the *KRAS* amplification. Subsequent test using different concentrations of fluorescent probe of the internal reference gene (i.e., 50, 100, and 200 nM) showed that the wild-type blocking (WTB) probe in our WIRE-PCR using different concentrations of fluorescent probe of the internal reference gene could effectively block the amplification of wild-type *KRAS*. With reference to the results of *LEPTIN* and *KRAS* amplifications, the mean CT values of *LEPTIN* amplification group and *KRAS* amplification group were relatively large when using 200 nM fluorescent probe of the internal reference gene (Table II). Therefore, 50 nM and 100 nM fluorescence probe for the internal reference gene were considered to be the optimal concentrations in the system. Application of 100 nM fluorescent probe of the internal reference gene better enhanced the fluorescence intensity of the *KRAS* amplification than the other two concentrations of the probe. In addition, the fluorescence signal was stable. Therefore, a final concentration of 100 nM fluorescent probe of the internal reference gene was selected to optimize the PCR reaction, which contained 500 nM primer set (SW-329/330) and 100 nM florescent probe (SW-1294) for the detection of internal reference gene, *LEPTIN*; and 500 nM primer set (SW-1595/1596) and 250 nM fluorescent probe (SW-1438) for the detection of *KRAS*.

Blocking effect of different concentrations of wild-type template in the reaction system. The concentrations of most DNA samples extracted from the clinical biopsies ranged from 50 to 150 ng. Evaluation if the WTB concentration used in

Table II. Comparison of the mean CT values of the *LEPTIN* and *KRAS* amplification groups.

Mean CT value ± (SD)	Fluorescent probe 50 nM	Fluorescent probe 100 nM	Fluorescent probe 200 nM
LEPTIN (with WTB)	24.4±0.24	24.33±0.68	26.15±0.15
LEPTIN (without WTB)	23.75±0.46	23.81±0.76	25.62±0.27
KRAS (with WTB)	NA	NA	NA
KRAS (without WTB)	25.55±0.23	25.68±0.55	26.22±0.21

Table III. Blocking effects of different concentrations of wild-type template in the constructed wire PCR system.

Mean CT value ± (SD)	Template 50 ng/μl	Template 100 ng/μl	Template 150 ng/μl	Template 200 ng/μl
LEPTIN (with WTB)	24.46±0.63	23.75±0.20	24.21±0.25	23.54±0.2
LEPTIN (without WTB)	24.07±0.08	23.41±0.11	24.86±0.15	23.21±0.13
KRAS (with WTB)	NA	NA	NA	36.78±0.61
KRAS (without WTB)	24.16±0.90	23.49±0.60	22.75±0.11	22.64±0.35

the reaction system could effectively block the amplification of different amounts of DNA templates was necessary at the early experimental stage. Different concentrations of DNA template (i.e., 50, 100, 150, and 200 ng/μl) used in the *KRAS* amplification group imparted an effective blocking effect on 50-200 ng of the wild-type template under the WTB reaction. Non-specific amplification of *KRAS* was found in the system using 200 ng/μl template and near 40 cycles (with the mean CT value of 36.78±0.61), indicating that application of 500 nM WTB probe did not completely block the amplification of wild-type gene when using 200 ng wild-type template (Table III and Fig. 1A and B). No significant difference of the mean CT values of *LEPTIN* amplification was found before and after adding WTB (25.39±0.21 and 25.37±0.04, respectively $P>0.05$), suggesting that WTB had no significant effect on *LEPTIN* amplification (Fig. 1C and D). The results with and without WTB when added *LEPTIN* showed the internal amplified fragment were amplified together with the target gene, which were used to reduce base mismatch due to high number of cycles in PCR and enhanced the specificity (Fig. 1E and F).

The sensitivity capabilities of the WIRE-PCR system. According to the blocking efficiency curves in Fig. 2, we used the concentration gradient (10^5, 10^4, 10^3, 10^2, 10^1, 10^0, copies/μl) plasmid (c34G>C;G12R.1R) mixed with WT-gDNA in total 50 ng as template to assessment the sensitivity. The result shown in Fig. 4 indicated that in the WIRE-PCR system, different concentration result in different C_q values even at the level of single base pair of Plasmid. However, a previous study showed that traditional PCR always lead to consistent C_q values despite the differences in the mutated template concentrations associated with the *KRAS* MT-alleles because of both WT-alleles and MT-alleles could be equivalently amplified (17). We used the C_q values associated with traditional PCR to indicate the total quantity of input DNA in previous research. When WIRE-PCR utilized, the C_q values increased with the quantity of *KRAS* mutation template, indicating that the amplification of *KRAS* WT-alleles was efficiently inhibited. And we added internal competitive amplified gene, not only can it amplified together with the target gene, but can used to reduce base mismatch due to high number of cycles in PCRs and quantify the total amount of DNA. The result showed the high blocking efficiency and indicated that the WIRE-PCR promotes the detection of *KRAS* MT-alleles with high sensitivity even at single base pair level (Fig. 2).

Based on the amplification curves in Fig. 2, we concluded that the WIRE-PCR was capable of detecting a single copy of *KRAS* MT-allele in high presence of WT-alleles with an amplification efficiency of 93.8% and R^2=0.998 (Fig. 3). The linear association suggest the amount of MT-alleles in a given sample could be quantified by the real-time PCR standard curve.

The race amount of mutant KRAS in the clinical biopsies. In the present study, WIRE-PCR was used to detect the trace amount of mutant *KRAS* in 50 colorectal biopsies collected during colonoscopy and find 18 positive cases, indicating that approximately 36% of the colorectal biopsies from the 50 patients had trace amount of mutant *KRAS* (18). Fig. 4A shows a colorectal biopsy that harbored a mutant *KRAS* detected by our early constructed system. The green dashed line shows continued amplification of mutant *KRAS* after adding LNA, indicating that gene mutation in *KRAS* occurred. Fig. 4B shows no specific gene amplification after adding LNA, suggesting no mutant *KRAS* is contained in that particular sample. Moreover, the red lines in Fig. 4A indicated that the amplification of internal competitive fragment was not affected before and after the addition of LNA, indicating a good reproducibility of our experimental results. To further confirm the mutation in 18 colorectal biopsies, we sent the PCR products of these 18 cases with positive *KRAS* mutation for sequencing a biotechnology company in Shanghai, China. The results showed that 18 specimens contained *KRAS* gene mutation. As shown in Fig. 4C and D, the green peak demonstrates the G to A mutation, which was consistent with the immunohistochemical analysis

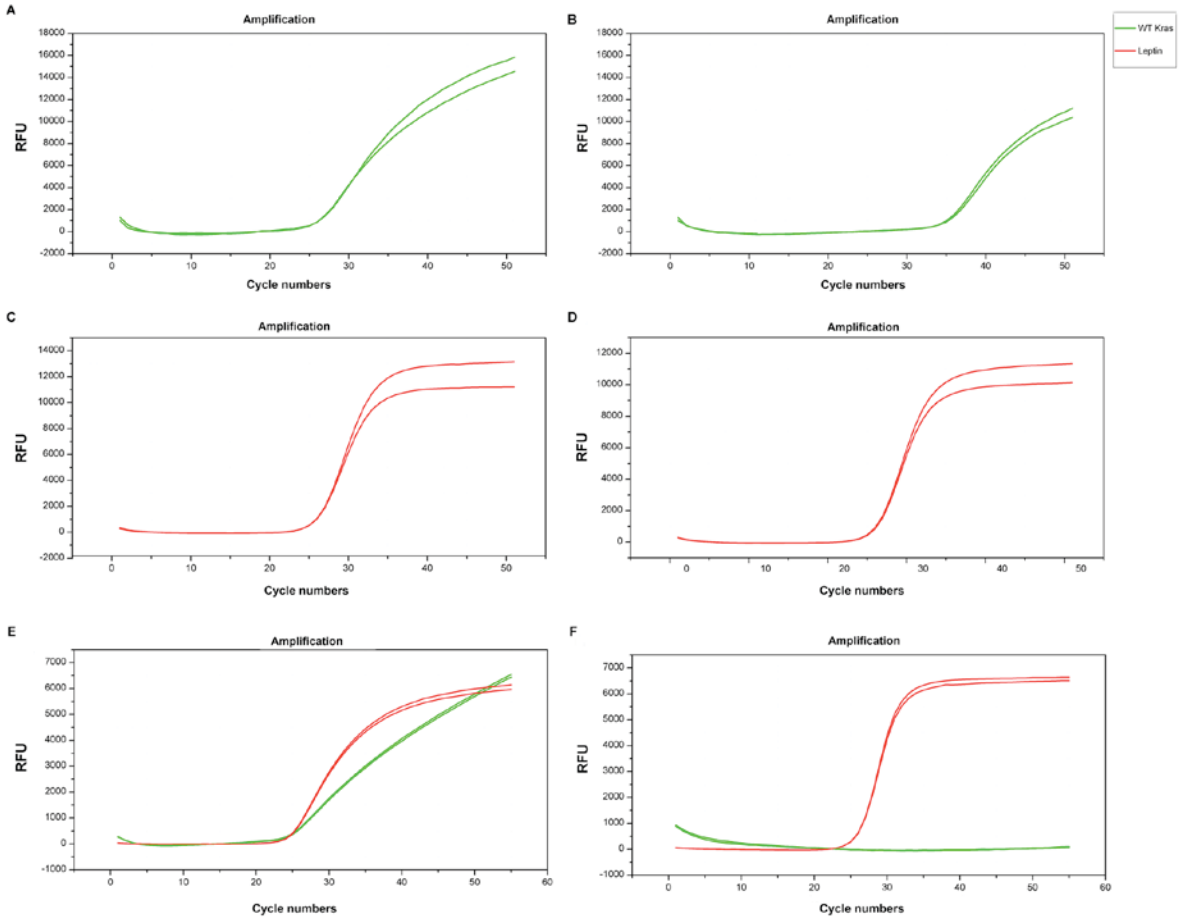

Figure 1. Blocking effects of the constructed WIRE-PCR system on wild-type template. Amplification curves of *LEPTIN* and *KRAS*. Green line represents *KRAS* and red represents *LEPTIN*. (A) Wild-type *KRAS* without WTB LNA probe. (B) Wild-type with WTB LNA probe. (C and D) *LEPTIN* without and with WTB LNA probe. (E and F) WIRE-PCR system without and with WTB LNA probe. Addition of WTB LNA resulted in *LEPTIN* amplification only (red line) but a complete blockage of wild-type *KRAS* amplification. NTC were not found in amplification curves. WTB, wild-type blocking; LNA, locked nucleic acid.

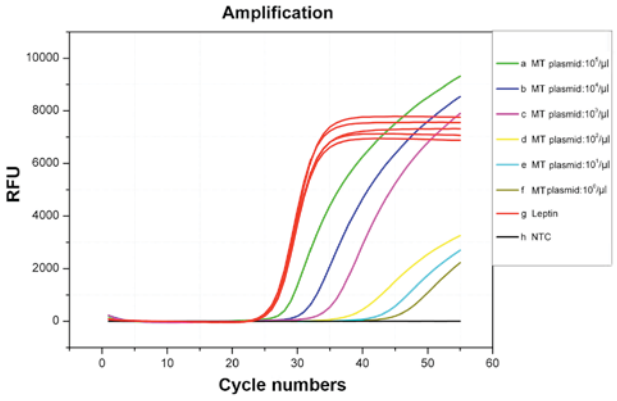

Figure 2. Sensitivity of real-time WIRE-PCR with internal competitive amplified fragment. Curves a to f show the amplification curves of real-time PCR with the WTB probe. Specified concentrations of template (MT-*KRAS* plasmid mixed with human WT-gDNA) indicated in each curve. *LEPTIN* gene as internal competitive amplified fragment in curve g. The amplification completed in a 20 μl reaction mixture. *KRAS* Plasmid (c34G>C;G12R.1R) were diluted in different concentrations (10^5, 10^4, 10^3, 10^2, 10^1, 10^0, copies/μl) spiked into samples containing WT-gDNA. WTB, wild-type blocking.

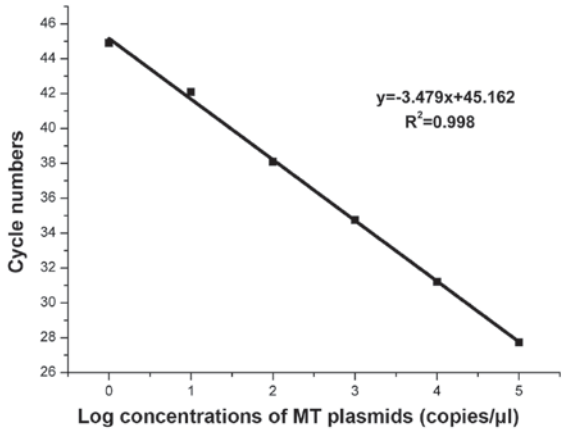

Figure 3. Quantitative curves of real-time WIRE-PCR. Standard curve was generated by plotting the average C_q values from real-time PCR against the log concentrations of mutant *KRAS* plasmid. The amplification efficiency of real-time WTB-PCR was 93.8% (slope, -3.479;R^2=0.998). WTB, wild-type blocking.

of the corresponding specimens under 20x magnification of light microscopy. Calculation of the changes of CT values of *KRAS* amplification group before and after the addition of LNA as well as the CT values of the internal competitive amplified fragment, *LEPTIN*, helped assess the *KRAS* mutation rate in each positive sample. Among the 18 biopsies with positive mutations, the *KRAS* mutation rate ranged from 18.6

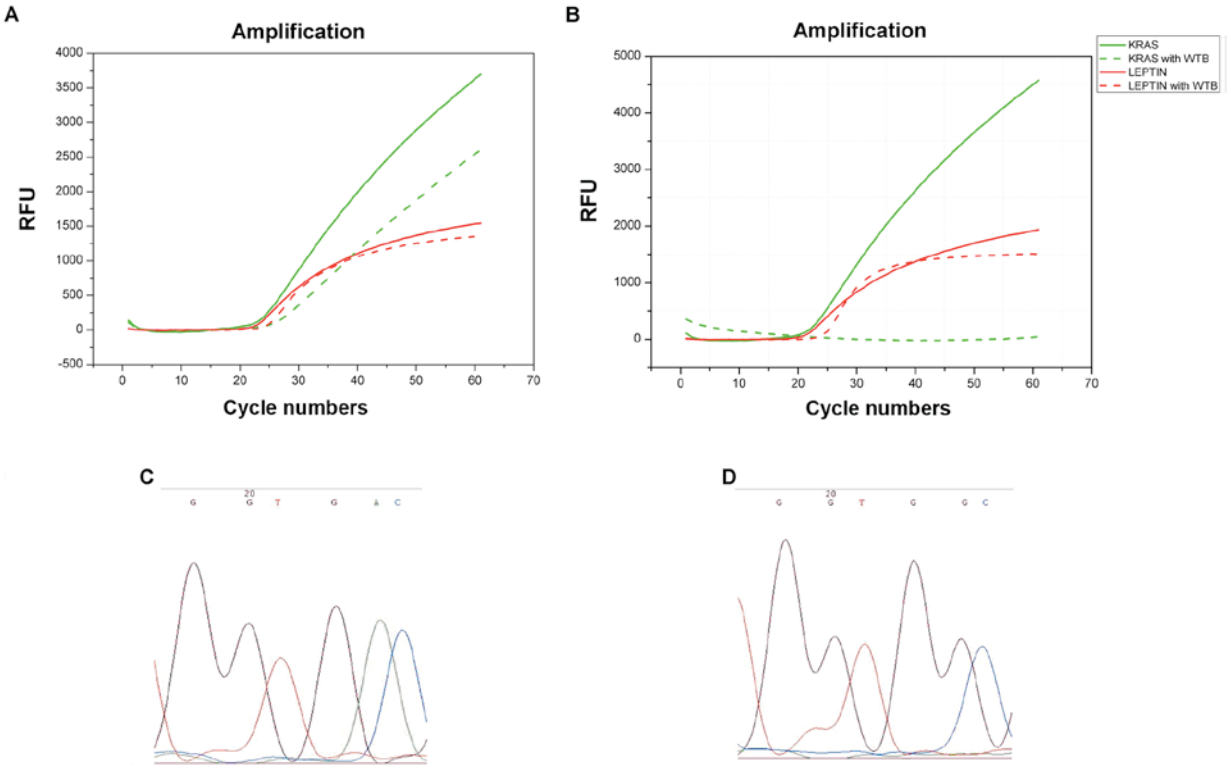

Figure 4. Detection of the trace amount of mutant *KRAS* in the clinical biopsies of colonoscopy. Panels (A and C) show the detection and amplification of trace amount of mutant *KRAS*. Panels (B and D) indicate no *KRAS* mutation. Green and red lines represents KRAS and internal amplified fragment (*LEPTIN*) respectively, Dash lines indicate WTB LNA probe were added in the reaction. NTC were not found in amplification curves. WTB, wild-type blocking; LNA, locked nucleic acid.

to 64.2%. In the present study, the constructed WIRE-PCR effectively detected the trace amount of mutant *KRAS* in the clinical biopsies.

Discussion

Different proportions of *KRAS* mutation have been discovered in a variety of human malignancies, such as malignant melanoma, lung cancer, colorectal cancer, and thyroid cancer. The *KRAS* mutation rate in the patients with rectal cancer is approximately 40% that included the point mutations in codons 12, 13, 15, 18, 61, 117, and 146 of exon 2, in which, the point mutations in codons 12 and 13 of exon 2 were common, accounting for approximately 40% (19,20). Mutant *KRAS* has become one of the markers affecting the prognosis of the colorectal cancer patients, and detection of mutant *KRAS* is particularly important (21,22). In a large number of detection assays for gene mutation, clamp-based PCR has given us good inspiration. Application of peptide nucleic acid (PNA) or LNA forms LNA/DNA chimeras, which closely bind with wild-type template and prevent the amplification of the wild-type template based on the high affinity binding between LNA/PNA and DNA (23). In clinical practice, LNA has been used as a substitute for PNA. According to the high affinity binding between LNA and DNA, LNA probe has been used to inhibit the wild-type PCR amplification (16). The principle is that the design of upstream and downstream primers is outside the LNA/DNA chimeras. In this reaction system, the polymerase lacking 5' to 3' end exonuclease activity ensured that LNA/DNA chimera probes were not hydrolyzed in the reaction system and LNA/DNA chimera probes were used for the inhibition of wild-type gene (24). To target the design of complementary oligonucleotide of sense strand *KRAS*, the present study added WTB probe into the reaction system. The added WTB probe sequence was partially overlapped with the wild-type template. *KRAS* and WTB probes competitively bound to the wild-type template in the same reaction system. Binding efficiency between LNA of the WTB probe and template was higher, occupying the base complementary binding region of primers and templates, thereby interfering the binding between primers and wild-type templates and inhibiting the amplification of the wild-type gene (11). In the 20 μl PCR system, the addition of polymerase and bases is often superfluous and necessary for common PCR amplification reactions, However, non-targeted mutant gene amplification occurred between LNA/DNA chimeras and template binding region in the WTB-involved PCR leads to false positive results. With increasing number of PCR cycles, the products of non-targeted mutant gene amplification are continuously increased, which seriously affects the reading in the detection of trace amount of the single-point mutant gene.

In our previous study, we found that the KRAS of the wild-type samples also amplified under the WTB reaction when PCR system near high cycles. Under the thermodynamic driving force of DNA polymerase, the single base terminal mismatch between primers and template could easily trigger the non-specific amplification of an input DNA having opposite genotype (e.g., WT genotype) (25,26). Moreover, weak-destabilization effects of terminal mismatches could

further promote non-specific amplification (27). Although stringent reaction conditions can be used to dramatically reduce or eliminate non-specific amplification, boptimization is time-consuming, and sometimes unsuccessful. Internal reference gene added as internal competitive amplified fragments in the reaction system consumed any excess DNA polymerase and free base fragments, thereby enhancing the blocking effect of WTB probe on wild-type *KRAS* template and improving the detection efficiency for trace amount of *KRAS* gene. In addition, since the internal competitive amplified fragment had no complementary binding site with WTB probe, WTB probe exclusively bound to the complementary template region but not the internal competitive amplified fragment, which did not affect the amplification of the internal competitive amplified fragment. Addition of the primer sets of internal reference gene, *LEPTIN*, massively produce the internal competitive amplified fragments, which helped consuming any excess DNA polymerase and free base fragment and reducing the likelihood of false mismatch, thereby increasing the blocking effect of LNA probe on the wild-type gene. Because this was the same reaction system amplifying the same template, this method was able to quantify the total amount of DNA template and reduce contamination by simplifying the steps of the reaction. The present study analyzed the CT values and fluorescence intensity obtained from the amplification to optimize the WIRE-PCR system by adjusting the final concentrations of primers of the internal reference gene and its probe, the primer for *KRAS* gene and its probe, and the LNA in the reaction system as follows: 500 nM of the primer sets and 100 nM of the fluorescent probe of the internal reference gene; 500 nM of the primer sets and 250 nM of the fluorescent probe of the *KRAS* gene; and 500 nM of the LNA probe. Subsequent evaluation of the blocking effect on the common DNA quantity in the clinical sample under the optimized reaction system showed that when the wild-type templates ranged from 50 ng to 150 ng, the WTB probe effectively blocked the amplification of the wild-type template in the reaction system. Subsequent detection of the trace amount of mutant *KRAS* in the 50 colorectal biopsies of the patients with suspected colorectal cancer showed that 36% of the specimens had mutant *KRAS*, with the mutation rate ranging from 18.6 to 64.2%.

In summary, the constructed internal competitive amplified fragment improved the detection of trace amount of mutant *KRAS* by WTB in a real-time fluorescence-based quantitative detection assay. Series optimization in primer concentrations, fluorescent probe concentrations, and LNA concentration effectively block the wild-type DNA templates of the specimens used in the PCR system, which in turn, effectively enriched the mutant gene. The resulted showed the sensitivity is as low as single base pair level and completely inhabited WT-alleles of *KRAS*. Among the 50 colorectal biopsies collected during colonoscopy, the mutation rate of detected trace amount of mutant *KRAS* was 36%. The wire PCR in the present study was highly sensitive and specific, easily operated and inexpensive method compared to the direct sequencing approach. It could be extensively used to monitor gene mutation in clinical practice and provide references for tumor monitoring and individualized drug therapies.

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