INTRODUCTION

It is widely accepted that accumulation of mutations and chromosomal aberrations is the major causes of cancers.\(^1\) Mutations in KRAS are detected in approximately 30% of all human cancers.\(^2\) KRAS is involved in intracellular signal transduction, such as epidermal growth factor receptor (EGFR) signaling pathway.\(^3\) KRAS is activated abnormally when the KRAS gene is mutated.\(^4\) Point mutation rate of KRAS gene is 40%-50% in colorectal cancer.

An enzymatic on/off switch-mediated assay for KRAS hotspot point mutation detection of circulating tumor DNA

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Abstract
Background: To detect the mutations of KRAS gene in colorectal cancer patients and other cancer patients, it is of value to develop non-invasive, sensitive, specific, easy, and low-cost assays.

Methods: Templates harboring hotspot mutations of the KRAS gene were constructed, and primers were designed for evaluation of the specificity, and sensitivity of detection system consisted of exonuclease polymerase-mediated on/off switch; then, gel electrophoresis and real-time PCR were performed for verification. The assay was verified by testing the DNA pool of normal controls and circulating DNA (ctDNA) samples from 14 tumor patients, as compared to Sanger sequencing.

Results: A specific and sensitive assay consisted of exonuclease polymerase-mediated on/off switch, and multiplex real-time PCR method has been established. This assay could detect <100 copies of KRAS mutation in more than 10 million copies of wild-type KRAS gene fragments. This assay was applied to test KRAS gene mutations in three cases of fourteen ctDNA samples, and the results were consistent with Sanger sequencing. However, this PCR-based assay was more sensitive and easier to be interpreted.

Conclusion: This assay can detect the presence of KRAS hotspot mutations in clinical circulating tumor DNA samples. The assay has a potential to be used in early diagnosis of colorectal cancer as well as other types of cancer.

KEYWORDS
ctDNA, enzymatic on/off switch, KRAS mutations, multiplex PCR, mutation detection
The mutations are mainly located in codons 12 and 13 with approximate 80% occurrence in the 12th codon, 15% occurrence in the 13th codon, and 5% occurrence in codon 61 and others.\(^5\) The most majority of KRAS mutations identified are single nucleotide point mutations. The common patterns include G12D, G12A, G12R, G12C, G12S, G12V, and G13D. The mutation pattern of KRAS provides guidance of individualized treatment, and the KRAS wild-type colorectal cancer patients can benefit from treatment with anti-epidermal growth factor cetuximab.\(^6\,\,7\) KRAS mutation identification is strongly recommended by FDA to guide usage of cetuximab. Therefore, a sensitive, specific, and convenient method with detection capability of multi-mutations is highly desirable. In the past several decades, Sanger sequencing has been established for clinical diagnostics base on ctDNA samples. Here, we developed an assay of detecting KRAS hotspot mutations in ctDNA. Our assay provides a new approach for the detection of somatic mutations such as KRAS mutations. This mutation identification strategy is based on the molecular switch consisting of high-fidelity enzymes and the 3-terminal phosphorylation-modified primer. The mutation detection primers can only be extended when it perfectly matches the template. When primer and template are not perfectly matched, no or very low amount amplified products will be yielded, since high-fidelity enzymes cannot remove mismatched base due to its phosphorothioate modification. This assay can identify KRAS mutations in a normal background from an minimal amount ctDNA of peripheral blood. This assay is low-cost, fast, and high-throughput with 96-well or 384-well plates for thousands of samples per day. It may serve as a non-invasive and efficient diagnostic project for cancer patients or high-risk individuals.

\section{MATERIALS AND METHODS}

\subsection{The Statistical analysis of the database}

The gene mutation data and survival data of the colorectal cancer patients were downloaded from The Cancer Genome Atlas (TCGA) database. The overall survival rates between patients with KRAS mutations and patients without KRAS mutations were analyzed by Kaplan-Meier, and \(P < .05\) was considered statistically significant.

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|c|}
\hline
Assay & Specificity & Sensitivity & Multiplex & Time-saving & Result analysis \\
\hline
Multiplex PCR of on/off switch & Good & Good & Yes & Yes & Easy \\
\hline
Sanger sequence & Good & Ordinary & Yes & No & Difficult \\
\hline
HRM & Good & Good & No & Yes & Difficult \\
\hline
Allele-specific real-time PCR & Ordinary & Good & Yes & Yes & Easy \\
\hline
Isothermal-based optical sensor & Good & Good & No & Yes & Difficult \\
\hline
\end{tabular}
\caption{Comparison of the KRAS mutation detection assays}
\end{table}
2.2 | Preparation of KRAS hotspot mutation templates

Preparation of KRAS hotspot mutation templates was carried out by the method of overlapping extension PCR. Sixteen primers were listed in Table S1. These primers were designed according to the reference sequences of Gene ID 3845 in Genbank. The primers were designed to introduce hotspots mutations at codon 12 and codon 13 of KRAS gene. The primer-extended products were purified again and then subcloned into PGEM-T Easy Vector (Invitrogen). Subcloned products were sequenced for confirmation. The seven mutation templates of KRAS gene, including G12D, G12V, G12S, G12C, G12R, G12A, and G13D, are shown in Figure S1.

2.3 | The condition of the detection of KRAS mutations

The following nine mutation-specific primers were designed for mutation analysis (Table 2). The RKRAS is the common reverse primer. All primers were synthesized by Invitrogen, and the bold underlined nucleotide was phosphorothioate modified. A single PCR or multiplex PCR with proofreading polymerase-mediated on/off switch for KRAS mutations were detected in an ABI Prism 7500 system (Applied Biosystems). A 25 µL PCR detection system contained 2.5 µL of 10× Pfu buffer with MgSO4 (Fermentas), 1.2 µL of 20× Eva green (Biotium), 5n µmol/L of RKRAS primer, 5n µmol/L of FKRAS (n is depending on the number of primer), 0.5 U of Pfu, 0.4 µm of dNTP, 1 µl of template DNA, and ddH2O (to make up to 25 µl in total volume). After denature at 95°C for 5 minutes, primer extension was performed for 45 cycles with gradient PCR program, and the first stage was performed 10 cycles as denaturing at 95°C for 20 seconds, annealing at 68°C for 20 seconds with each cycle decreasing 1°C, and extension at 72°C for 30 seconds and the second stage 23 cycles, denaturing at 95°C for 20 seconds, annealing at 59°C and extension at 72°C for 30 seconds, and a final extension at 72°C for 10 minutes.

2.4 | PCR conditions for amplifying targets for KRAS mutations sequencing analysis

Taq polymerase-mediated regular PCR was used for sequencing of the DNA samples from the normal controls and cancer patients. The primers for amplifying the products for sequencing are listed in Table S1. This regular PCR was performed in a volume of 25 µL system containing 12.5 µL of 2× mix (Biomega), 10 µmol/L of primers, and about 40 ng of genomic DNA. After denature at 95°C for 5 minutes, primer extension was performed for 30 cycles with denaturing at 95°C for 20 seconds, annealing at 55°C for 20 seconds, extension at 72°C for 30 seconds, and with a final extension at 72°C for 10 minutes.

2.5 | Genomic DNA samples and ctDNA samples preparation

One pooled DNA sample contained two hundred normal controls was provided by the Nanhua University First Affiliated Hospital. Almost, the same amount of DNA in different final volume of each sample was mixed to form the gene pool after adjusted their concentrations of individual DNA samples.

Fourteen blood samples of tumor patients from the Second Affiliated Hospital of Soochow University were collected. Two mL of blood from the cubital vein was drawn to EDTA tubes and centrifuged at 12,000 g for 2 minutes, the ctDNA was extracted with the circulating DNA Extraction Kit (Omega), and extracted ctDNA was quantitatively measured by UV spectrometry.

TABLE 2 | The phosphorothioate-modified primers for detecting the seven hotspot mutations of KRAS gene

|       | FKRAS12-1 | FKRAS12-2 | FKRAS12-3 | FKRAS12-4 | FKRAS12-5 | FKRAS12-6 | FKRAS13 | F-12 | F-13 | RKRAS |
|-------|-----------|-----------|-----------|-----------|-----------|-----------|---------|------|------|-------|
| Primer| 5’-TAAACTTGTGGTAGTTGGAGCTG†ATC-3’ | 5’-TAAACTTGTGGTAGTTGGAGCTG†TC-3’ | 5’-TAAACTTGTGGTAGTTGGAGCTG†AGC-3’ | 5’-TAAACTTGTGGTAGTTGGAGCTG†TCG-3’ | 5’-TAAACTTGTGGTAGTTGGAGCTG†CGC-3’ | 5’-TAAACTTGTGGTAGTTGGAGCTG†TC-3’ | 5’-TTGTGGTAGTTGGAGCTGTG†ATCA-3’ | 5’-TAAACTTGTGGTAGTTGGAGCTG†TC-3’ | 5’-TTGTGGTAGTTGGAGCTGTG†CA-3’ | 5’-TCTGTATCAAAGAATGGTCCTG-3’ |

Note: Where “†” in place of phosphorothioate modified, base which is marked in bold and underlined meaning mutant base.

FIGURE 1 | The analysis of overall survival rate according to KRAS status in colorectal cancer patients. Colorectal cancer patients with KRAS mutations have significantly shorter survival time than patients with wild-type KRAS. (P < .0001)
3.1 | The prognosis of colorectal cancer patients with KRAS mutations was more poor compared with those without mutations

Taking advantage of The Cancer Genome Atlas (TCGA) database, the pattern of KRAS mutations in colorectal cancer patients was analyzed (Data S1, download from https://portal.gdc.cancer.gov/). A total of 103 KRAS mutations have been found in 263 cancer patients. The total survival rate of colorectal cancer patients harboring KRAS mutations was significantly lower than that of the patients without KRAS mutations (Figure 1). Therefore, it is of great clinical value of differentiating KRAS mutations to provide guidance of individualized treatments of colorectal cancer patients.

3.2 | A PCR-based assay of KRAS mutation detection was established

We established the detection assays of single or multiplex PCR system combination with high-fidelity enzyme "on/off" switch. As shown in Figure 2A, the multiplex PCR experimental design enables one of the codon 12 mutation primers to amplify the matched mutation templates. No observable products yielded from the mismatched primers. The seven mutant templates and the wild-type template were diluted to a final concentration ranging from $10^8$ to $10^2$ copies per PCR reaction (25 µL) to test the sensitivity and specificity of the assay. The specificity and sensitivity of the seven primer pairs were finally determined by electrophoresis, false-positive primer extension in most of the seven primer pairs was not produced until copy number of the mismatched template reached $10^7$, and few of them reached $10^6$. The sensitivity of the six mutation-specific primers for codon 12 of KRAS was at least $10^2$ copies, and the codon 13 of KRAS was as low as $10^3$ copies (Figure 2B). The codon 13 mutation primer can yield a dimer while the template concentration is low. The codon 12GCT and codon 13GAC were illustrated as representatives are shown in Figure 2B. Figure 2C shows that real-time PCR is more sensitive than conventional PCR. The sensitivity of this assay by real-time PCR for the KRAS seven hotpot mutations is at least 10 times higher than conventional PCR without compromising its specificity of this assay. Considering the high similarity in DNA sequence of primers to target against the 6 nucleotide variants at the mutation hotspot of codon 12 of KRAS gene, we developed multiplex PCR of codon 12. The sensitivity of all primers could reach at least $10^2$ copies, while false-positive amplification was not observed when the copy number of mismatched template was below $10^6$ copies. The final appropriate concentration of clinical samples in this assay is about between $10^2$ and $10^5$ copies, and this range is safe for clinical testing.

3.3 | KRAS mutations cannot be detected in healthy human DNA samples

This assay was further tested for its application in clinical samples. The efficient extension of KRAS mutation primers would not be observed in healthy human genomic DNA samples. As shown in Figure 3, we used one pooled DNA sample of 200 normal individuals for evaluating the specificity of our method.

FIGURE 2 The assay establishment for the KRAS mutation detection mediated by an enzymatic on/off switch. A, An illustration showing the multiplex PCR experimental design by on/off switch. B, Taking the 12GCT and 13GAC of KRAS as examples, the mutant template and Wt template were diluted in 10-fold serial and amplified by PCR with mutation-specific primer. The length of the PCR products was both 155 bp. C, Establishment of a multiplex PCR system with codon 12, as the melt curve and amplification plot shows, the detection limit reach to $10^2$ copies, and the specificity can reach to $10^6$. 
Mutation-specific primers failed to amplify products in these samples, while products were amplified by wild-type-specific detection primers. Codon 13 primers of KRAS could amplify a primer dimer, and we could distinguish the objective fragments from the dimer according to the Tm value. To further confirm this method, the PCR products of the KRAS gene were sent for sequencing and there were no point mutations in codon 12 and codon 13 of KRAS gene.

3.4 | Application of the current assay in KRAS hotspot mutation detection with cancer patients

CtDNA samples derived from fourteen tumor patients were tested with the newly developed KRAS hotspot mutation assay, two samples were detected carrying at least one type mutations of codon 12 and one sample was detected carrying codon 13 mutation according to the amplification curve and melt curve. The Sanger sequencing confirmed the KRAS gene mutations in the three cancer patients (Figure 4B): codon 12 mutations (GGT>AGT, GGT>GCT), codon 12 mutations (GGT>AGT, GGT>AGA), and mutation in codon 13 (GGC>GAC). Two of the three cancer patients carrying the KRAS gene mutations suffered from colorectal cancer surgery and adjuvant chemotherapy, and another case is lung cancer without chemotherapy. The other 11 samples could not be detected by our assay and had been confirmed by the sequencing. It indicates that the assay system we developed is highly sensitive and specific in mutation detection with clinical samples, especially for ctDNA samples.

**FIGURE 3** The current assay would not obtain false-positive signals of KRAS gene mutations in pooled DNA sample of health control. As the top melt curve and amplification plot shows, the pooled DNA sample could be amplified by the wild-type primers but not be amplified by the codon 12 mutation primers of KRAS. As the bottom melt curve and amplification plot shows, the DNA pool sample could be amplified by the KRAS wild-type primers but could not be amplified by the codon 13 mutation primers of KRAS.
The KRAS mutations are extremely rare in normal individuals, while they are presented in about 30% of cancer patients. High specificity and sensitivity of assays are crucial to avoid or minimize false-positive detection in normal DNA samples and false-negative results in patient samples. Sequencing is the most common technique to screen KRAS mutations, but it requires at least 10%-30% abundance of mutated templates.10,28 Screening the KRAS mutations by HRM had a sensitivity of 5%-6%.10,28 Hillary et al were able to detect KRAS mutations by allele-specific hybridization-induced aggregation (HIA) of oligonucleotide probe-conjugated microbeads with a sensitivity of 25%.29 Our multiplex real-time PCR method can detect KRAS mutation as few as 100 copies in at least $10^5$ wild-type counterparts. Currently, ctDNA concentrations vary from 0 to 1000 ng/mL blood, with the median concentration of ctDNA 790 ng/mL.30,31 In general, 1000 ng ctDNA includes approximately 150 000 copies of the genome31; accordingly to this, it is difficult to cause false-positive amplification in one PCR system by our assay.

In the present study, efficient primer extension was exclusively observed when the mutation-specific primers matched the mutation templates. One pooled DNA sample 200 normal individuals was used to test the newly developed assay, only wild-type primers but not mutation-specific primers was able to amplify the pooled DNA sample. For the 14 ctDNA samples of cancer patients, 3 of them generated PCR products when mutation-specific primers were used, and the presence of point mutations of KRAS in the 3 samples was further confirmed by DNA sequencing. It indicated the accuracy of the newly developed

![Melt Curve and Amplification Plot](image)

**FIGURE 4** The feasibility was tested for 7 hotspot mutations detecting of KRAS gene in cancer patients ctDNA samples. A, As the top melt curve and amplification plot shows, two of fourteen samples could be detected the codon 12 mutations of KRAS gene; as the bottom melt curve and amplification plot shows, one of fourteen samples could be detected the codon 13 mutation of KRAS gene. B, The sequencing of KRAS gene contained codon 12 and codon 13 of the three samples.
multiplex PCR system in genetic diagnostics. The high-fidelity DNA polymerase ensures the high sensitivity and specificity of the assay. This assay and similar assays have great potential to be widely applied in genetic diagnostics in clinic, especially for somatic mutations. In addition, multiplex PCR is time-efficient and less financial consuming.

5 | CONCLUSION

The present study has successfully established a non-invasive diagnostic method for KRAS mutation detection in circulating DNA of cancer patients. The allele-specific real-time PCR is mediated by mutation-sensitive “molecular switch” composed of high-fidelity DNA polymerase and phosphorylated-modified primers. We have demonstrated the success of using the assay in micro-mutation detection with ctDNA. This technology has a great potential in screening KRAS gene mutations thanks to its high specificity, sensitivity, low-cost, multiplex detection, and easy data interpretation.

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CONFLICT OF INTEREST

No competing financial interests exist.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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