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Detection of *KRAS* codon 12 and 13 mutations by mutant-enriched PCR assay

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ABSTRACT

Background: The identification of *KRAS* mutations before the administration of anti-epidermal growth factor receptor (EGFR) therapy of metastatic colorectal cancer (mCRC) has become important. The aim of the present study was to develop a novel technology that can increase detection sensitivity for *KRAS* mutations.
Methods: DNAs were extracted from colorectal cancer tissues and formalin-fixed, paraffin-embedded (FFPE) colorectal cancer samples. Mutant-enriched PCR assay utilizes the exceptionally thermostable endonucleases, PspGI for codon 12 and PhoI for codon 13, for specific amplifying *KRAS* mutations from mixed samples. The amplified PCR products were subjected to single-base primer extension or sequencing. Digital PCR was used to evaluate some of the results.
Results: We compared the results with that from direct sequencing. In the FFPE samples, thirteen discordant samples were found. We showed that the mutant-enriched PCR assay can identify the codons 12 and 13 mutation in a mixed population of mutant and wild type DNA sequences at 1:1000 and 1:400, respectively. The sensitivity of this method is lower than the digital PCR.
Conclusions: We developed a rapid and highly sensitive method to detect codons 12 and 13 mutations of the *KRAS* gene. This method is a powerful tool for finding low-abundance variations in genomic DNA.

1. Introduction

Colorectal cancer (CRC) is one of the most common causes of cancer death in the world. There are over one million of individuals diagnosed every year worldwide [1,2]. In recent years, the numbers of CRC incidence have increased considerably in the Asian population. Similarly, CRC is the third leading cause of cancer deaths in both men and women in the Taiwanese population. Recently, significant improvements have been achieved including patient survival and prognosis by applying new therapies. Anti-EGFR-targeted therapies with monoclonal antibodies, such as cetuximab and panitumumab, are a successful strategy for the treatment of metastatic colorectal cancer (mCRC) or after the failure of conventional chemotherapy. These factors have been developed to inhibit ligands binding the extracellular domain of EGFR and then block the downstream intracellular signaling pathways. *KRAS* is a downstream molecule in the EGFR signal transduction pathway and more evidence shows that the patients with *KRAS* mutations do not benefit from the addition of cetuximab or panitumumab to standard chemotherapy [3–7]. Therefore, *KRAS* mutation testing should be performed in all individuals from advanced CRC refractory to first-line regimens to identify which patient's tumors will not answer to the expensive monoclonal antibody inhibitors of EGFR. Guidelines from the National Comprehensive Cancer Network have been changed, stating that anti-EGFR monoclonal antibody therapies are to be restricted to patients with wild-type *KRAS* tumors [8].

Several commonly used methodologies are available for detecting the mutational status of the *KRAS* gene. These methods including Sanger sequencing [9], pyrosequencing [10], amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) [11], high resolution melting (HRM) [12], real-time PCR [13], peptide nucleic acid (PNA) clamp polymerase chain reaction (PCR) assay [14], wild-type blocking PCR (WTB-PCR) [15], pyrophosphorolysis-activated polymerization allele-specific amplification (PAP-ASA) [16], next generation sequencing (NGS) [17], co-amplification at lower denaturation temperature PCR (COLD-PCR) [18], or digital PCR [19]. All of the methods have their own advantages and disadvantages. In this study, we used a mutant-enriched PCR assay to examine the presence of *KRAS* codon 12 and 13 mutations of patients with colorectal cancer using frozen stored tissues and formalin-fixed, paraffin-embedded (FFPE) tissues.

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2. Materials and methods

2.1. Source and extraction of genomic DNA

2.1.1. Tissue specimens

Resected primary colorectal cancers were obtained from 106 patients in the Changhua Christian Hospital, and these tissues were stored in liquid nitrogen before DNA extraction. Extraction of DNA was performed as described earlier [20]. The concentration of DNA was determined at 260 nm using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE). This study was approved by the Institutional Review Board of the Changhua Christian Hospital. Moreover, we have checked the *K-RAS* mutations in these tissue specimens using direct sequencing and single-base primer extension, and the results have been published [21], and we used these samples to develop the mutant-enriched PCR assay.

2.1.2. FFPE tissues

The specimens consisted of 83 FFPE colorectal adenocarcinomas submitted for clinical *KRAS* mutation analysis. All samples were tested for mutant-enriched PCR assay. FFPE samples were deparaffinized and air dried, subsequently, DNA was isolated using the proteinase K and QIAamp® micro DNA extraction kit (QIAGEN, Maryland, MD, USA) according to the manufacturer's instructions. The clinic-pathological characteristics of these patients are presented in the Table 1.

2.2. Mutant-enriched PCR assay for screening mutation of KRAS codon 12 mutations

The mutant-enriched PCR is a one-step PCR with restriction digestion to eliminate wild-type genes selectively, thus enriching the mutated genes. This assay was performed as described previously with some modifications [22]. Briefly, to detect the codon 12 mutations, the sequences of primers for PCR amplification are as follows: 5′-ACTTGTGG TAGTTGGACCT-3′ (forward primer) and 5′-TAACTTGAAACCCAAGGT AC-3′ (reverse primer). The forward primer harbors one mismatched base (G to C) to introduce a new CCWGG sequence after PCR amplification of wild-type alleles. The thermostable restriction enzyme PspGI was used to digest the CCWGG sequence in the amplicon of the wild-type. In contrast, codon 12 1st base and 2nd base mutant alleles were not digested because of the base substitution of G-to-A nucleotide, G-to-T nucleotide and G-to-C nucleotide at fourth and fifth base of CCWGG, resulting in the enriched of mutant alleles. The unusually thermostable restriction enzyme PspGI (activity half-life of 2 h at 95 °C) [23] resists deactivation during thermal cycling of the reaction is the key of mutant-enriched PCR assay. PCR amplification of 0.1 μg DNA with 2.5 U of Pro Taq Plus DNA polymerase (Protech Technology Enterprise, Taipei, Taiwan) and 0.5 U of PspGI (New England Biolabs, Ipswich, MA, USA) in the presence of 200 μM dNTPs, 0.2 μM primers, and 1× reaction buffer was carried out in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The reaction mixture contained a final concentration of 10 mM Tris–HCl, 50 mM KCl, 0.01% gelatin, 1.5 mM $MgCl_2$ and 0.1% Triton X-100. The PCR program consisted of 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 1 min at 56 °C, and 1 min at 72 °C. There was a final amplification step of 7 min at 72 °C. The PCR products were semi-quantified on a 2.5% agarose gel in 0.5× Tris-borate-EDTA (TBE) and visualized by staining with ethidium bromide (EtBr). The nucleotide of *KRAS* codon 12 was examined by single-base primer extension reaction or direct sequencing.

Table 1
Patients' characteristics.

Characteristics	No.	Percentage
Total patients	83	
Age		
Mean	49	
Gender		
Male	51	61
Female	32	39
Grade		
Well	2	2
Moderate	77	93
Poor	4	5
Location		
Colon	45	54
Rectum	28	34
Sigmoid	10	12
No. of EGFR-targeted therapy (Cetuximab)		
Yes	48	58
No	35	42
Response to EGFR-targeted therapy		
Responders	22	27
Nonresponders	61	73

2.3. Mutant-enriched PCR assay for screening mutation of KRAS codon 13 mutations

To detect the codon 13 mutations, the sequences of primers for PCR amplification are as follows: 5′-GTTCTAATATAGTCACATTTTCATTATTTT TATTATAAAGC-3′ (forward primer) and 5′-GTCAAGGCACTCTTGC CTAGG -3′ (reverse primer). The forward primer harbors one mismatched base (G to A) to prevent the restriction enzyme PhoI cut. The reverse primer harbors one mismatched base (C to G) to introduce a new GGCC sequence after PCR amplification of wild-type alleles. The restriction enzyme PhoI was used to digest the GGCC sequence in the amplicon of the wild-type. In contrast, codon 13 1st base and 2nd base mutant alleles were not digested because of the base substitution of G-to-A nucleotide, G-to-T nucleotide and G-to-C nucleotide at first or second base of GGCC, resulting in the enriched of mutant alleles. PCR amplification of 0.1 μg DNA with 2.5 U of Pro Taq Plus DNA polymerase (Protech Technology Enterprise, Taipei, Taiwan) and 0.1 U of PhoI (New England Biolabs, Ipswich, MA, USA) in the presence of 200 μM dNTPs, 0.2 μM primers, and 1× reaction buffer was carried out in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems). The reaction mixture contained a final concentration of 10 mM Tris–HCl, 50 mM KCl, 0.01% gelatin, 1.5 mM $MgCl_2$ and 0.1% Triton X-100. The PCR program consisted of 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 1 min at 56 °C, and 1 min at 72 °C. There was a final amplification step of 7 min at 72 °C. The PCR products were semi-quantified on a 2.5% agarose gel in 0.5× TBE and visualized by staining with EtBr. The nucleotide of *KRAS* codon 13 was examined by single-base primer extension reaction or direct sequencing.

2.4. Single-base primer extension reaction

A single-base primer extension reaction was done using ABI Prism SNaPshot Multiplex kit (Applied Biosystems). The two probes for either codon 12 1st base and codon 12 2nd base or codon 13 1st base and codon 13 2nd base were added to the tube containing 1.5 μl of purified PCR products, as well as 4 μl of SNaPshot Ready Reaction premix containing AmpliTaq® DNA polymerase and fluorescently labeled ddNTPs. The sequences of the probes used for mutation analysis have been previously described [21]. Each 10 μl mixture was subjected to 25 single-base extension cycles consisting of a denaturing step at 96 °C for 10 s and primer annealing and extension at 55 °C for 35 s. After cycle extension, unincorporated fluorescent ddNTPs were incubated with 1 μl of shrimp alkaline phosphatase (United States Biochemical, Cleveland, OH, USA) at 37 °C for 1 h, followed by enzyme deactivation at 75 °C for 15 min. The primer extension reaction products were resolved by automated capillary electrophoresis on a capillary electrophoresis platform. Briefly, 14 μl of Hi-Di™ Formamide (Applied Biosystems) and 0.28 μl of GeneScan ™-120LIZ® Size Standard (Applied Biosystems)

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