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Development of a Multiplex Allele-Specific Real-time Polymerase Chain Reaction Assay for detection of KRAS gene mutations in Thai colorectal cancer tissues

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KEYWORDS
Multiplex Allele-Specific Real-time PCR Assay; KRAS gene mutations; Colorectal cancer tissues.

ABSTRACT
Mutation analysis of KRAS is necessary before starting treatment with monoclonal anti-EGFR antibodies for effective and appropriate treatment for individual patients. The objective of this study is to develop a Multiplex Allele-Specific Real-time PCR assay for analysis of the mutational status of KRAS codons 12 and 13, including 7 types of KRAS mutations (G12D, G12A, G12R, G12C, G12S, G12V, and G13D). 160 of FFPE colorectal cancer tissues were collected from Department of Medical Services, Institute of Pathology. DNA was isolated from the FFPE tissue using AS-primers specific for mutant DNA. Moreover, the MAS-Real-time PCR analysis of samples showed good concordance (K=0.837, 95% CI 0.740-0.933) with pyrosequencing. In addition, the MAS-Real-time assay has a sensitivity of 78.26% and specificity of 100%. Our developed MAS-Real-time PCR can be applied for detection of KRAS gene mutations in FFPE tissues which is a reliable, rapid, cost-effective method and not requiring advanced instruments.

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Introduction

Colorectal cancer (CRC) is the most prevalent cancer worldwide and ranks as the third leading cause of cancer-related deaths[1]. In Thailand, CRC is the third most common cancer among men and the fifth most common among women[2]. Mutations in the KRAS gene lead to the activation of the RAS protein, promoting increased cell division, inhibition of apoptosis, induction of angiogenesis, and enhanced metastasis[3]. Monoclonal antibodies like cetuximab and panitumumab have been developed for cancer treatment, targeting the EGFR[4]. These antibodies aim to inhibit EGFR tyrosine kinase activation and downstream signaling[5]. However, these treatments are effective only for CRC with a wild-type KRAS proto-oncogene, with no response in cases with KRAS mutations[6]. Therefore, detecting KRAS gene mutations is crucial for tailoring personalized therapeutic strategies for patients[4].

Various molecular methods have been developed to detect KRAS mutations, including direct sequencing[6], pyrosequencing[7,8], co-amplification at lower denaturation temperature PCR[9], and digital PCR[10]. Additionally, commercial molecular kits are available for KRAS mutation detection, such as the cobas® KRAS Mutation Test[11], therascreen® KRAS RGQ PCR Kit[12], and KRAS PyroMark Q96 V2.0 Kit[13]. However, these methods require specialized equipment and expertise, making them costly for cancer patient prognosis and diagnosis. In contrast, Multiplex Allele-Specific Polymerase Chain Reaction (MAS-PCR) is an economical, reliable method for detecting known mutations and single-nucleotide polymorphisms[14]. Real-time PCR methods for SNP detection are increasingly important for genotyping variations in genomes[15]. MAS-Real-time PCR uses primers with allele-specific 3' termini that specifically target mutated DNA templates[14,16].

In this study, we developed a MAS-Real-time PCR assay to analyze mutations in KRAS codons 12 and 13. The most frequent single nucleotide point mutations in the KRAS gene are found in codons 12 and 13, accounting for 80 to 82% of the mutations[17-19]. The most frequent point mutations in codons 12 and 13, G12D, G12A, G12R, G12C, G12S, G12V, and G13D[8,19,20], were found to be present in formalin-fixed, paraffin-embedded tissue samples from 160 patients with colorectal cancer. The MAS-Real-time PCR method for FFPE samples was devised due to the efficient preservation of cellular, architectural, and morphological details in formalin-fixed paraffin-embedded (FFPE) tissues, which can be conveniently stored at room temperature for long durations, thus streamlining handling and storage processes[7,11]. However, FFPE processing can degrade DNA extraction efficacy and quality, posing challenges to conducting precise molecular analyses and potentially impacting the accuracy of KRAS analysis results. Furthermore, in the future, researchers plan to develop MAS-Real-time PCR for analyzing KRAS mutations from cfDNA samples. This is because there is a growing preference for detecting cancer gene mutations from cfDNA samples obtained through liquid biopsy[9,10], rather than from solid tissue or FFPE DNA samples. Pyrosequencing, a well-established and sensitive method, served as the reference for evaluating the sensitivity of the MAS-Real-time PCR assay in detecting KRAS mutant alleles.

Materials and methods

Clinical samples

Formalin-fixed, paraffin-embedded colorectal adenocarcinomas from 160 patients with CRC were collected from the Institute of Pathology, Ministry of Public Health, Bangkok, Thailand. The study was approved by the Ethics Committee of the Institute of Pathology (IOP-KM-R64-002). The pathologist reexamined the histomorphology of all samples to review and mark the adenocarcinoma areas of the hematoxylin and eosin stained slides.

DNA extraction of FFPE tissues

Each tissue sample was manually micro-dissected from areas containing only cancerous cells within paraffin-embedded blocks. Ten µm thick ribbon sections were put in a microcentrifuge tube. Paraffin was removed from the tissue sections with xylene and rehydration with 100% ethanol, and samples were air-dried. DNA was extracted from FFPE tissues and purified using a QIAamp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA quantity was determined by NanoDrop spectrophotometry (NanoDrop Technologies, Wilmington, DE).

PCR amplification and pyrosequencing

Pyrosequencing for analysis of a KRAS gene fragment spanning codons 12 and 13 was performed as previously described by Ogino et al[8]. Reactions and PCR conditions were performed as previously described by Seekhuntod et al[19]. PCR products were confirmed by 8% polyacrylamide gel electrophoresis, and gels were stained with SYBR Green I Nucleic Acid Gel Stain (1:400, Lonza, USA). PCR products in 30 µl were mixed with 3 µl streptavidin-conjugated Sepharose beads (Amersham Biosciences AB, Sweden), 40 µl binding buffer, and 17 µl distilled water. The mixture was shaken at 1400 rpm for 10 min. The biotinylated PCR products were captured using a vacuum prep tool. Single-stranded DNA purification involved washing the vacuum prep tool with 70% ethanol for 5 s, denaturation solution for 5 s, and washing buffer for 10 s. Biotinylated single-stranded DNA was introduced into a 96-well microtiter plate, which contained 40 µl of a 0.4 µM solution of sequencing PF1-primer (5'-TGTGGTAGTTG-GAGCTG-3') for analyzing positions 35 and 38 in the nucleotide sequence, along with PF2-primer (5'-TGTGGTAGTTGGAGCT-3') for analyzing position 34[8]. Afterward, the plate was incubated at 80°C for 2 minutes, followed by a 5-minute cooling period to room temperature, and then loaded onto the PyroMark Q96 ID system (Qiagen, Germany), as shown in supplementary figure S1.

DNA cloning

Genomic DNA from eight clinical samples containing KRAS wild-type DNA and seven KRAS codon 12 and 13 point mutations (G12D, G12A, G12R, G12C, G12S, G12V, and G13D) underwent PCR amplification using universal KRAS primers (KRAS-codon 12/13-F and KRAS-codon 12/13-R) were performed as previously described by Seekhuntod et al[19]. The resulting 259-bp PCR products were cloned into the psc-A-amp/kan vector, transformed into competent Escherichia coli cells using a Strataclone PCR cloning kit from Agilent Technologies (USA). Transformed bacteria were plated on selective LB-agar plates with ampicillin and X-Gal and incubated at 37°C overnight. White colonies were randomly selected and cultured in LB medium overnight. Plasmids were extracted using the Wizard® genomic DNA purification kit from Geneaid (Taipei, Taiwan) and screened for the insert fragment through PCR. Positive PCR products were sequenced by the Bioneer Corporation in Daejeon, Republic of Korea.

Primer and Probe Design

Allele-specific (AS) primers were custom-designed for seven distinct mutations, each tailored to the specific mutation. A mutation-unspecific segment was used as a reference amplicon. The 3′ terminal base of each AS primer was selected according to its corresponding mutation. Amplification reactions involved the primary KRAS forward primer and five AS primers (G12R-F, G12C-F, G12D-F, G12A-F, and G13D-F), which shared one common antisense KRAS reverse primer. Additionally, reactions with two AS primers (G12S-R and G12V-R) shared a common sense KRAS forward primer[19]. All primers were synthesized and provided by BioDesign Co., Ltd. (BioDesign, Pathumthani, Thailand). Probes were employed to detect target amplification. Reference and allele-specific PCRs used the same probe, with opposing PCR primers. These probes were procured from Applied Biosystems, Foster City, CA. Probes for KRAS PCR quantification were labeled

with 6-fluorescein at the 5' end, with a black hole quencher™ domain at the 3' end. An exogenous internal control PCR product, a 100-base-long segment in TBXAS1 exon9, was co-amplified in both reference and allele-specific PCRs. A probe was used for internal control PCR detection, labeled with a Texas Red-fluorophore at the 5' end and featuring a black hole quencher™ domain at the 3' end. All primer and probe sequences are listed in supplementary table S1.

Multiplex Allele-Specific Real-time PCR (MAS-Real-time PCR) Assay

To perform the MAS-Real-time PCR reaction for detecting KRAS gene mutations at codons 12 and 13, this assay was designed with primers and probes specific to gene NM_004985.4, which have different PCR product sizes as shown in supplementary table S1. All reactions were carried out in 1-8 tubes, each designated for a specific mutation type: wild-type, G12S, G12R, G12C, G12D, G12A, G12V, and G13D, respectively. In each tube, the final volume was 20 µl, consisting of 10 µl of 2X KAPA probe fast qPCR master mix, 0.5 µl each of 10 µM Oligonucleotide primer Internal control-F and Internal control-R, 0.25 µl of 10 µM Oligonucleotide probe Internal control-P, and the specific AS-primer as indicated in supplementary table S2. Additionally, 2 µl of DNA template with a concentration of 20-50 ng/µl was added, and the final volume was adjusted with nuclease-free water to 20 µl. The protocol included an initial activation step at 95°C for 5 minutes, followed by an amplification step consisting of 10 cycles at 95°C for 30 seconds, 64°C for 45 seconds, and 72°C for 30 seconds. Subsequently, there were 30 cycles of amplification at 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. Data collection was performed during the annealing phase, with measurements in the FAM channel for KRAS gene detection. The reactions were analyzed using a Bio-RAD CFX96 real-time PCR machine for qualitative detection, with the analysis based on the amplification curve characteristics above the threshold line. The criteria for result interpretation were as follows: a ct value of < 25 in the FAM channel indicated a positive result (mutation detected), while a ct value of ≥ 25 indicated a negative result (no mutation detected). In the Texas Red channel, a ct value of < 20 indicated a valid reaction. The DNA samples used in this assay were of high quality, and the wild-type tube along with the positive 113-bp as an internal control. Wild-type DNA at any of the seven positions prevents allele-specific amplification resulting in a corresponding missing amplification curve, as shown in figure 1.

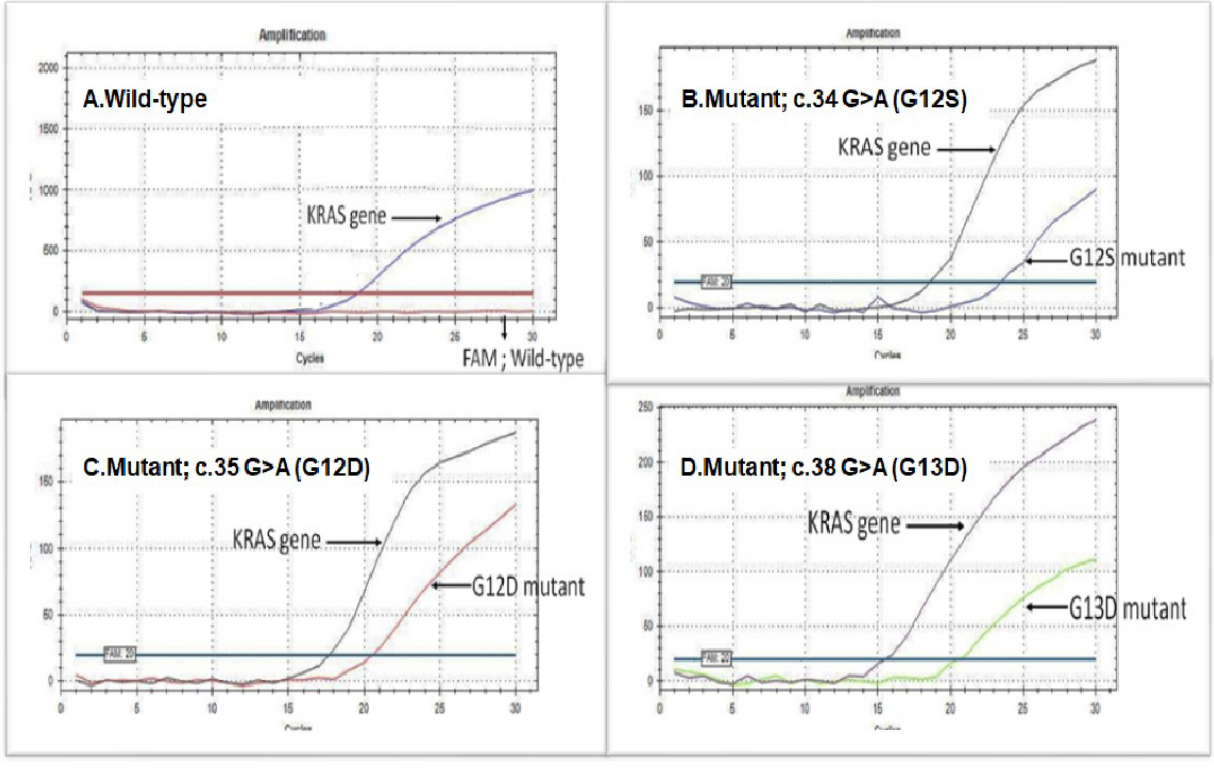

Figure 1 Genomic DNA from FFPE tissues were used for MAS-Real-time PCR assay.

(A) Wild-type nucleotide 35 by the KRAS-G12D-F primer. In red curves indicated wild-type DNA.

(B) c.34G>A (codon 12 AGT) mutation by the KRAS-G12S-R primer. In blue curves indicated G12S mutant DNA.

(C) c.35G>A (codon 12 GAT) mutation by the KRAS-G12D-F primer. In red curves indicated G12D mutant DNA.

(D) c.38G>A (codon 13 GAC) mutation by the KRAS-G13D-F primer. In green curves indicated G13D mutant DNA.

Sensitivity of MAS-Real-time PCR Assay

The eight plasmid clones containing KRAS wild-type sequences and seven plasmid clones with various KRAS mutations (G12D, G12A, G12R, G12C, G12S, G12V, or G13D) were isolated. Each mutated plasmid DNA was combined with wild-type plasmid DNA to create a total of 100 ng. In order to assess precision and reproducibility, we gradually reduced the proportion of mutant plasmid DNA, resulting in decreasing ratios of mutant to wild-type DNA at levels of 100%, 50%, 25%, 10%, 5%, 2%, 1%, and 0.1%. Precision and reproducibility were assessed through four repeated runs, with analysis conducted within the range of the lowest detection limit as determined by MAS-Real-time PCR.

Statistical analysis

The results obtained from both MAS-Real-time PCR and pyrosequencing were subject to comparison for 160 formalin-fixed, paraffin-embedded specimens. The significance of this evaluation was determined through Kappa statistics. A Kappa value greater than 0.81 was considered noteworthy, signifying that both methods produced highly accurate results. Additionally, we explored variances in categorical

variables such as age, gender, histologic grade, and tumor location among patients with KRAS mutations, employing a chi-square test. All statistical analyses were conducted as two-sided tests, with a significance threshold of *p*-value < 0.05. The analysis was carried out using SPSS software (version 19).

Results

Pyrosequencing analysis of KRAS gene mutations in CRC clinical samples

In the initial phase of the study, 160 clinical samples were analyzed through pyrosequencing, specifically targeting six distinct point mutations within codon 12 (G12S, G12R, G12C, G12D, G12A, and G12V), as well as one point mutation within codon 13 (G13D) of the KRAS gene. These mutations were selected due to their frequent occurrence in patients with colorectal cancer. Among the 160 tissue specimens, the sequencing results indicated that 46 cases (28.75%) displayed a mutation in either codon 12 or 13 of the KRAS gene, while 114 cases (71.25%) were categorized as KRAS wild type (as shown in Table 2). Of the 46 cases with a KRAS mutation, 40 (86.96%) had a mutation in codon 12. The most commonly observed codon 12 mutation was G12D, accounting for 36.96%, followed by G12V at 30.43%. The less common codon 12 mutations included G12S, G12A, G12C, and G12R, with frequencies of 8.70%, 4.35%, 4.35%, and 2.17%, respectively. The sole mutation detected in codon 13 was G13D, representing 13.04% of cases.

MAS-Real-time PCR analysis of KRAS gene mutations in CRC clinical samples

The MAS-Real-time PCR assay, performed on the 160 CRC tissue specimens to detect mutations in KRAS codon 12 and codon 13, produced results that closely corresponded with those obtained from pyrosequencing. As indicated in table 2, the MAS-Real-time PCR assay identified 36 cases (22.5%) with KRAS mutations in codons 12 and 13, while 124 cases (77.5%) were classified as KRAS wild type. More specifically, among these, 32 samples (88.89%) displayed mutations in codon 12, with the predominant mutation pattern being G12D at 36.11%, followed by G12V at 33.33%. The less common mutation patterns in codon 12 were G12S and G12R, accounting for 11.11% and 8.33%, respectively. In codon 13, only one mutation pattern, G13D, was observed in 11.11% of cases. Among the 114 FFPE DNA samples initially identified as wild-type KRAS by pyrosequencing assay, 46 samples were found to contain mutations. Within this subgroup, 27 cases (58.70%) demonstrated nucleotide transitions from G to A, followed by 16 cases (34.78%) showing nucleotide transversions from G>T, and 3 cases (6.52%) with nucleotide transversions from G>C at codon 12. The predominant mutation type observed in codon 12 was G12D (GAT), resulting in the amino acid change from glycine to asparagine, occurring in 17 cases (36.96%) among the total 46 samples, as detailed in table 1.

Table 1 KRAS mutational status in 160 CRC patients

KRAS status	Type of mutations Amino acid change	Pyrosequencing Number of mutations (%)	MAS-Real-time PCR Number of mutations (%)
Wild-type	-	114/160 (71.25%)	124/160 (77.50%)
Mutant	All Codon 12 and 13	46/160 (28.75%)	36/160 (22.50%)
Mutated Codon 12	All Codon 12	40/46 (86.96%)	32/36 (88.89%)
G12D	GGT>G<u>A</u>T Gly→Arg	17/46 (36.96%)	13/36 (36.11%)
G12V	GGT>G<u>T</u>A Gly→Val	14/46 (30.43%)	12/36 (33.33%)
G12A	GGT>G<u>C</u>T Gly→Ala	2/46 (4.35%)	0/36 ND
G12S	GGT><u>A</u>GT Gly→Ser	4/46 (8.70%)	4/36 (11.11%)
G12C	GGT><u>T</u>GT Gly→Cys	2/46 (4.35%)	0/36 ND
G12R	GGT><u>C</u>GT Gly→Arg	1/46 (2.17%)	3/36 (8.33%)
Mutated Codon 13	All Codon 13	6/46 (13.04%)	4/36 (11.11%)
G13D	GGC>G<u>A</u>C Gly→Asp	6/46 (13.04%)	4/36 (11.11%)

Note: Underlined bases represent the substitutions in the respective codon. ND denotes not detected.

Comparison of MAS-Real-time PCR and Pyrosequencing assays

The results obtained from the 160 FFPE samples were subjected to an agreement analysis, comparing the outcomes of the MAS-Real-time PCR with those from the pyrosequencing method (refer to Table 2). Among these samples, 150 out of 160 displayed matching results, indicating a high level of accuracy for both methods (p-value < 0.05), and there was no statistically significant difference between the two assays (K = 0.837, 95%CI = 0.740 to 0.933). The positive agreement was 100%, while the negative agreement was 91.94%.

Table 2 Pairwise comparison and agreement analyses between MAS-Real-time PCR and pyrosequencing assays

		Pyrosequncing		Total (%)
		Positive (%)	Negative (%)	
MAS-Real-time-PCR	Positive (%)	36 (22.50%)	0	36 (22.50%)
	Negative (%)	10 (6.25%)	114 (71.25%)	124 (77.50%)
	Total (%)	46 (28.75%)	114 (71.25%)	160 (100.00%)

Note: Kappa= 0.837, 95% confidence interval: From 0.740 to 0.933, Positive agreement: 100%, Negative agreement: 91.94%.

Examining the Association Between Patient Characteristics and Mutations in KRAS Codon 12 and 13

The median age of the patients was 64 years, with an age range spanning from 30 to 87 years. The majority of patients fell within the age group of 60 to 79 years, accounting for 60% of the total. The male-to-female ratio was 1.25 to 1. In terms of histological characteristics, the most prevalent type was moderately differentiated, comprising 68.12% of cases (109 out of 160), and the majority of cases (88.12%) were primary colorectal tumors (as detailed in Table 3). An investigation was conducted to explore potential correlations between patients' demographic attributes and the presence of detected KRAS mutations, as depicted in table 3. Among the patients with KRAS-mutated carcinomas, no statistically significant differences were identified in relation to age, gender, histologic grade, or tumor site.

Table 3 Correlation between KRAS mutation and patients' characteristics of 160 colorectal carcinomas

Characteristics	N (%)	WT KRAS N (%)	MT KRAS N (%)	*p*-value
Total patients	160			
Median age: (range)	64 years (30-87)			
20-39 years	5 (3.12%)	4 (80.00%)	1 (20.00%)	0.916
40-59 years	51 (31.88%)	36 (70.59%)	15 (29.41%)	
60-79 years	96 (60.00%)	69 (71.88%)	27 (28.12%)	
80-90 years	8 (5.00%)	5 (62.50%)	3 (37.50%)	
Gender:				
Male	89 (55.63%)	68 (76.40%)	21 (23.60%)	0.107
Female	71 (44.37%)	46 (64.79%)	25 (35.21%)	
Histologic grade:				
Well differentiated	34 (21.25%)	19 (55.88%)	15 (44.12%)	0.066
Moderate- differentiated	109 (68.12%)	81 (74.31%)	28 (25.69%)	
Poorly differentiated	17 (10.63%)	14 (82.35%)	3 (17.65%)	
Site:				
Colorectal primary	141 (88.12%)	99 (70.21%)	42 (29.79%)	
Metastasis	19 (11.88%)	15 (78.95%)	4 (21.05%)	0.430

The Sensitivity, Accuracy, and Consistency of KRAS Mutation Detection Using MAS-Real-time PCR

To assess the sensitivity of the MAS-Real-time PCR assay, plasmid DNA from each of the seven KRAS mutant clones was diluted in separated amplification reactions alongside plasmid DNA from a wild-type KRAS clone. The objective was to gradually decrease the proportion of mutant DNA to achieve lower ratios of mutant to wild-type DNA. The MAS-Real-time PCR assay successfully detected mutant alleles, reaching a sensitivity as low as 5% for the G12R, G12D, G12A, G12V, and G13D mutants, and 20% for the G12S and G12C mutants. An illustrative example of the MAS-Real-time PCR assay's lowest limit of detection can be seen in figure 2. To evaluate the precision and reproducibility of our MAS-Real-time PCR assay, we quantified KRAS mutations within DNA mixtures consisting of each of the seven mutant KRAS DNA samples and wild-type DNA at various ratios (1%, 5%, 10%, 15%, 20%, 30%, 50% and 100%) in four repeated runs. The results consistently demonstrated precision and reproducibility, consistently detecting the same lowest quantity of KRAS mutant alleles in all repeated runs.

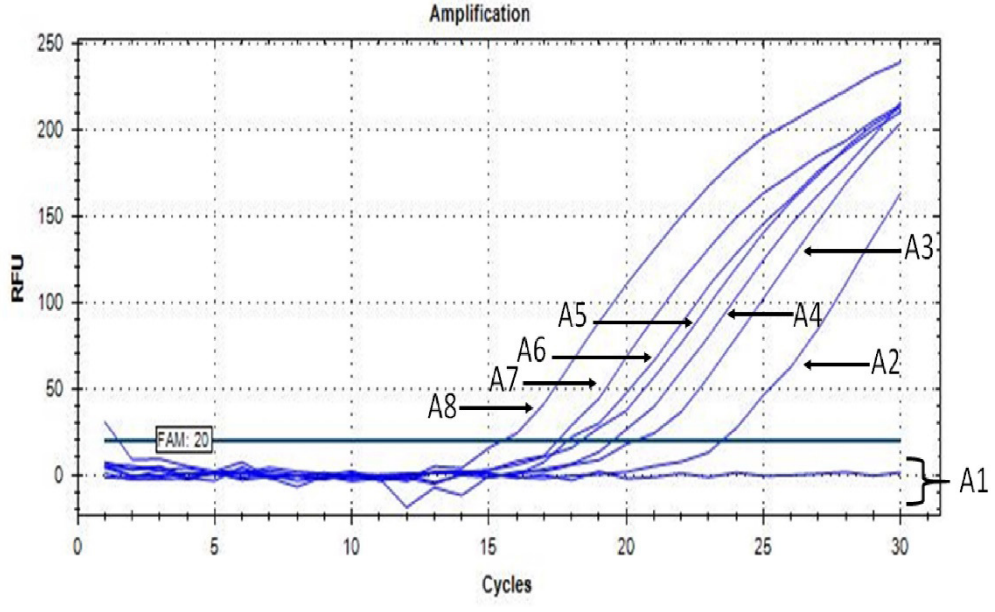

Figure 2 Sensitivity of MAS-Real-time PCR assay for identifying KRAS gene mutations.
Note: A representative amplification curve is shown. Dilutions of G12D mutant plasmid and wildtype plasmid DNA (from 1%, 5%, 10%, 15%, 20%, 30%, 50% and 100% mutated alleles). Designations A1-A8 represent DNA samples that yield positive results for the G12D mutation across varying mutation allele frequencies of 1%, 5%, 10%, 15%, 20%, 30%, 50%, and 100%, respectively.

Discussion

Colorectal cancer (CRC) ranks as the third most prevalent cancer globally and is a primary contributor to cancer-related fatalities[21]. Within Thailand, there has been a notable rise in the occurrence of CRC in recent times. Numerous studies have explored the link between KRAS mutations and colorectal cancer (CRC)[20]. These mutations in CRC patients are associated with resistance to anti-EGFR treatments like cetuximab or panitumumab[4]. Predicting therapeutic responses accurately is crucial to avoid unnecessary treatments and focus on more individualized and effective therapies. Several methods and

commercial molecular kits are available for detecting KRAS mutations[6,22,23], each with its own set of challenges. For instance, although direct sequencing is commonly used, it has low sensitivity and requires a substantial percentage (10%-30%) of mutated alleles in a wild-type background[22,24]. Pyrosequencing is accurate and feasible, with superior sensitivity (approximately 5% mutant allele)[8,22], but it involves expensive equipment and consumables, making it cost-prohibitive in developing countries. Commercial molecular kits offer advantages like high sensitivity (detection limit around 1% to < 5%), speed, easy data interpretation, and detection of various KRAS mutation positions. However, they also require costly instruments, expensive reagents, and have a relatively high cost per sample[25]. Hence, there is a pressing need to develop an accurate, simple, and cost-effective method for detecting KRAS mutations associated with CRC that can be deployed in developing countries.

In this research, we have effectively created a MAS-Real-time PCR assay that is both highly sensitive and specific, focusing on the seven most prevalent mutations (G12S, G12R, G12C, G12D, G12A, G12V, and G13D) in codons 12 and 13 of the KRAS gene. Additionally, the use of probe-based real-time PCR methods provides advantages in preventing interference between samples and/or environmental contaminants during experimental procedures.

Our findings demonstrated a high level of agreement between the MAS-Real-time PCR assay and pyrosequencing (K=0.837). However, in 10 cases, there were discrepancies in the results, particularly concerning mutations in codon 12 and codon 13. These mutations were detectable through pyrosequencing and Sanger direct sequencing but not with the MAS-Real-time PCR assay. Specifically, G12D was identified in three samples, G12V in two samples, G12A in two samples, G12C in two samples, and G13D in one sample. The inability of the MAS-Real-time PCR to detect mutations in all 10 samples may be attributed to issues such as biased amplification, primer interactions, and DNA damage resulting from the formalin fixation process during long-term storage[26,27]. This damage can lead to compromised DNA quality[28] and cross-linking with proteins, hindering the success of the MAS-PCR reaction[29,30].

Interestingly, our MAS-Real-time PCR assay exhibited a strikingly high analytical sensitivity for detection, successfully identifying approximately 5% mutant alleles in DNA mixing experiments. These experiments utilized genomic DNA isolated from plasmid cloned DNA for the G12R, G12D, G12A, G12V, and G13D mutants. Furthermore, the sensitivity of the MAS-Real-time PCR assay we developed surpasses that reported for direct sequencing and HRM (ranging from 5% to 20%), and is on par with that reported for pyrosequencing and commercial molecular kits (ranging from 1% to 5%)[8,24].

In this study, the occurrence of mutations in the KRAS gene at codons 12 and 13 was investigated in colorectal cancer patients in Thailand. A total of 160 tissue samples from colorectal cancer patients, embedded in paraffin and examined at the Institute of Pathology, Department of Medical Sciences, Ministry of Public Health, were analyzed. The findings revealed an overall mutation rate of 28.75% (46/160), with the mutation frequency ranging from 20% to 50%, closely aligned with previous research reports[19]. Mutations at codon 12 accounted for 86.96% (40/46), while those at codon 13 were found in 13.04% (6/46) of cases. The mutation rate at codon 12 ranged from 70% to 90%, while the rate at codon 13 ranged from 10% to 30%, consistent with earlier studies[18]. The most prevalent mutation types were G12D, G12V, and G13D, resembling findings from prior studies[7]. The highest mutation pattern involved the substitution of the base sequence Glycine (G) with Aspartic (A), accounting for 58.70% (27/46), in accordance with previous research[7,17].

General characteristics of colorectal cancer patients, including age, gender, histopathological features of tissue samples, and the location of cancerous masses, were also examined. It was observed that there was no significant difference in the occurrence of mutations based on age or the absence of mutations. However, some studies have reported an association between KRAS gene mutations and gender, with higher occurrences in females compared to males[6], which contradicts the findings of Poehlmann and colleagues, who reported higher occurrences in males[7].

The MAS-Real-time PCR method has a sensitivity comparable to that of direct sequencing and HRM (high-resolution melting) methods, which can detect mutant alleles in the range of 5% to 20%[8,25]. MAS-Real-time PCR is a rapid assay that can be completed in under 1.5 hours (excluding DNA isolation). It is cost-effective, with an approximate cost of $10 per test. Utilizing only a Real-time PCR instrument, the assay offers benefits in avoiding interference between samples and/or environmental contaminants during experimental procedures. Additionally, it does not demand a high level of technical expertise and specialized equipment.

Conclusion

In conclusion, we developed a MAS-Real-time PCR assay for detection of the seven most common mutations in codons 12 and 13 of the KRAS gene. MAS-Real-time PCR assay is a DNA-based protocol that was easy to perform, being rapid, cost-effective, highly sensitive and highly specific. An assay with these characteristics is important for analysis of clinical samples, such as FFPE tissues, in particular to assist clinicians in predicting the clinical course of monoclonal anti-EGFR antibody treatment of mCRC patients.

Take home messages

The MAS-Real-time PCR assay, developed here, demonstrates high sensitivity and specificity for detecting seven KRAS gene mutations (G12S, G12R, G12C, G12D, G12A, G12V, and G13D) in codons 12 and 13.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary

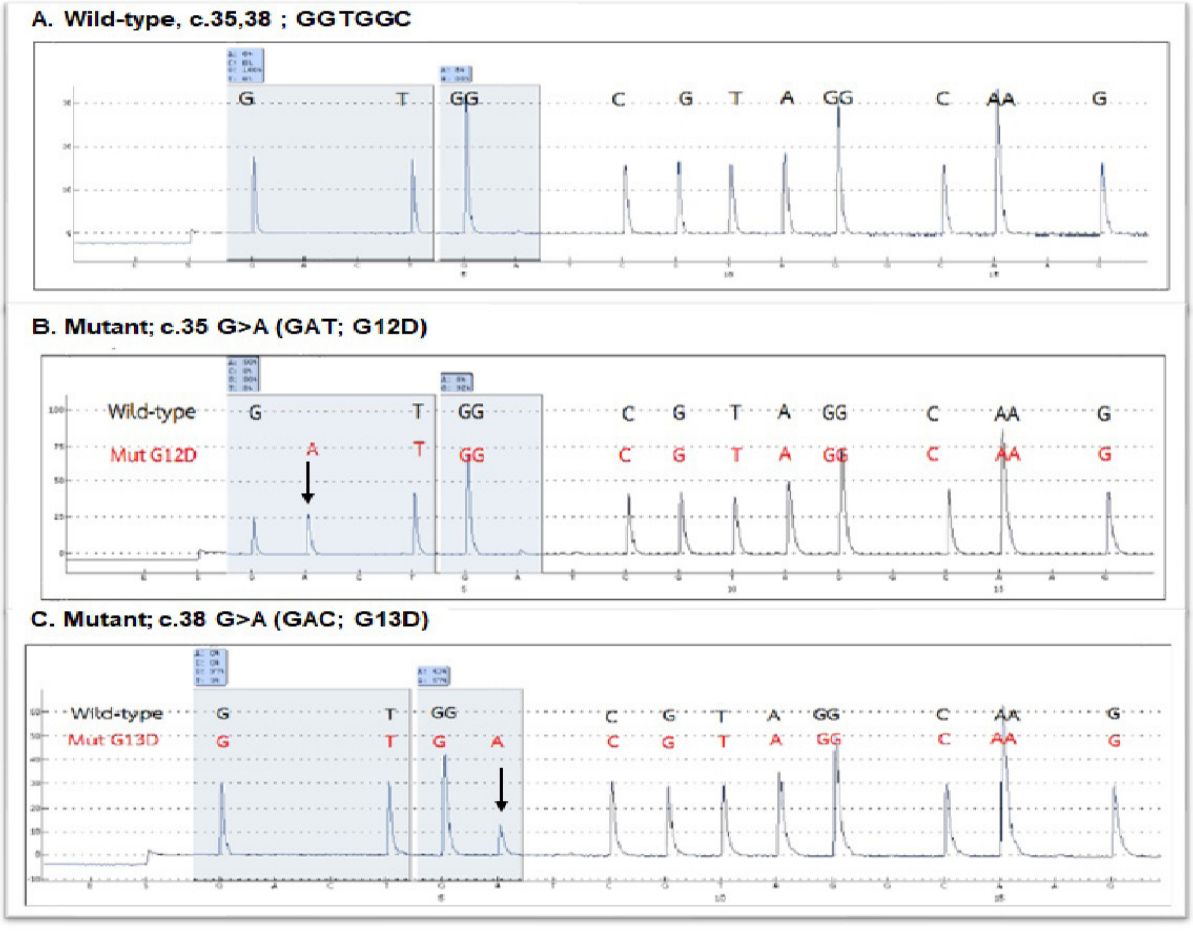

Figure S1 Pyrograms of pyrosequencing assay for identifying KRAS gene mutations.

(A) Wild-type nucleotide 35 and 38 by the KRAS-PF1 primer.

(B) c.35G>A (codon 12 GAT) mutation by the KRAS-PF1 primer.

(C) c.38G>A (codon 13 GAC) mutation by the KRAS-PF1 primer.

Note: Arrows indicate the presence of mutant alleles.

Table S1 Primers and Probe used in MAS-Real-time PCR for detecting the most common mutations in codons 12 and 13 of KRAS gene

Primer	Sequence	Sequence length (bp)	Product length (bp)
KRAS-F	5'-GGCCTGCTGAAAATGACTGAA-3'	21	113 bp
KRAS-R	5'-GGATCATATTCGTCCACAAAATG-3'	23	113 bp
KRAS-Probe	5'-FAM-TGTGGTAGTTGGAGCTGGTG-BHQ1-3'	20	-
KRAS-G12S-R	5'-CACTCTTGCCTACGCCACT-3'	19	64 bp
KRAS-G12R-F	5'-TTGTGGTAGTTGGAGCTC-3'	18	85 bp
KRAS-G12C-F	5'-CTGAATATAAACTTGTGGTAGTTGGAGCTT-3'	30	97 bp
KRAS-G12D-F	5'-ATAAACTTGTGGTAGTTGGAGCTGA-3'	25	91 bp
KRAS-G12A-F	5'-GTGGTAGTTGGAGCTGC-3'	17	83 bp
KRAS-G12V-R	5'-AAGGCACTCTTGCCTACGCCAA-3'	22	68 bp
KRAS-G13D-F	5'-AAACTTGTGGTAGTTGGAGCTGGTGA-3'	26	89 bp
Internal control-F	5'-GCCCGACATTCTGCAAGTCC-3'	20	100 bp
Internal control-R	5'-GGTGTTGCCGGGAAGGGTT-3'	19	100 bp
Internal control-Probe	5'-Texas Red-CTCCTCTACTGGGTGCAAGC-BHQ1-3'	20	-

Table S2 Details of adjusting the volume of AS-primer in the MAS-Real-time PCR reaction for KRAS gene mutations

G12S tube			G12R tube			G12C tube			G12D tube		
Primer name	**Conc.**	**Vol.**	**Primer name**	**Conc.**	**Vol.**	**Primer name**	**Conc.**	**Vol.**	**Primer name**	**Conc.**	**Vol.**
G12S-R	10 μM	0.1 μl	G12R-F	10 μM	0.65 μl	G12C-F	10 μM	0.25 μl	G12D-F	10 μM	0.15 μl
KRAS-F	10 μM	0.1 μl	KRAS-R	10 μM	0.5 μl	KRAS-R	10 μM	0.25 μl	KRAS-R	10 μM	0.4 μl
KRAS-P	10 μM	0.1 μl	KRAS-P	10 μM	0.5 μl	KRAS-P	10 μM	0.25 μl	KRAS-P	10 μM	0.2 μl
G12A tube			**G12V tube**			**G13D tube**			Wild-type tube		
Primer name	**Conc.**	**Vol.**	**Primer name**	**Conc.**	**Vol.**	**Primer name**	**Conc.**	**Vol.**	**Primer name**	**Conc.**	**Vol.**
G12A-F	10 μM	0.4 μl	G12V-R	10 μM	0.09 μl	G13D-F	10 μM	0.2 μl	KRAS-F	10 μM	0.15 μl
KRAS-R	10 μM	0.4 μl	KRAS-F	10 μM	0.09 μl	KRAS-R	10 μM	0.2 μl	KRAS-R	10 μM	0.15 μl
KRAS-P	10 μM	0.4 μl	KRAS-P	10 μM	0.09 μl	KRAS-P	10 μM	0.2 μl	KRAS-P	10 μM	0.15 μl