ORIGINAL ARTICLE

Evaluation of *ras* Mutations in Filipino Colorectal Patients

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ABSTRACT

Activating mutations in the *ras* proto-oncogene lead to increased, unregulated cellular proliferation. Point mutations in *ras* codons 12 and 13 are early events in 40-50% of colorectal cancer cases, and are associated with shortened patient survival. Procedures for mutation detection, which are used in patient diagnostic evaluation, have been developed for various populations, but not for Filipinos. This study aims to determine the incidence of *ras* mutations among Filipino colorectal cancer and non-cancer patients and to evaluate the usefulness of *ras* mutation detection in colorectal cancer diagnostics. Two rapid, cost-effective mutation detection methods are also evaluated. Restriction fragment length polymorphism (RFLP) detects mutations in codons 12 and 13 while single strand conformational polymorphism (SSCP) detects any mutation in the amplicons screened. PCR-amplified *ras* exon 1 from 21 colorectal cancer and 17 non-cancerous tissue samples were analyzed. DNA sequencing confirmed the presence of six substitution mutations: five (23.8%) in cancer samples and one (5.9%) in a non-cancer sample. RFLP detected all mutations, while SSCP failed to detect one, suggesting that RFLP is the better method for mutation screening. The incidence of *ras* mutations among Filipino colorectal cancer patients is lower than in other populations, suggesting that *ras* mutation detection is a highly sensitive but not specific diagnostic tool for colorectal cancer in Filipinos.

Key Words: ras, colorectal cancer, mutations

Introduction

The *ras* proto-oncogene encodes a 21-kD protein involved in G protein-mediated signal transduction. K-*ras* has guanosine triphosphate (GTP)-binding activity and participate, through a signaling cascade involving protein kinases, in transmitting mitogenic signals into the nucleus.[1,2] Activating mutations in the *ras* gene family, for example, in response to chemical carcinogens that produce base substitutions, result in constitutive activation of GTPase activity. These mutations commonly lead to increased and unregulated cellular proliferation and malignant transformation. Mutations of the K-*ras* oncogene were detected in about 70 – 95% of pancreatic cancers[3,4] and 30 – 50% of colorectal cancers.[5] The most common mutations associated with cancer alter the GTP-binding domain encoded by codons 12 and 13 in *ras* exon 1.[6,7,8]

Colorectal cancer is among the leading malignancies found in various populations, and is the second most frequent cause of cancer death in developed countries.[9,10] In the Philippines, it is ranked as the 7th-leading cause of cancer mortalities, with an incidence rate of approximately 10 per 100,000.[11] Activating point mutations in *ras* hotspot codons, such as codons 12 and 13, appear early in the colorectal neoplastic pathway and have been associated with tumor progression and shortened patient survival. Several studies, including a prospective study[12] and large meta-analyses[13,14] have shown that *ras* mutations are associated with poor prognosis in colorectal adenocarcinoma and that different gene mutations have different prognostic impact.[15] Diagnostic procedures for detection of *ras* mutations have been developed for various populations and cancer types. Methods used include mutation-specific oligonucleotide hybridization, polymerase chain reaction (PCR) with restriction fragment length polymorphism (RFLP) analysis, single-strand conformation polymorphism (SSCP) analysis, mutant allelic-specific amplification, or direct sequencing.[16]

Determination of the presence of *ras* mutations can be beneficial to the clinician making a diagnostic evaluation for colorectal cancer, as it is a cancer-specific molecular marker, compared to conventional cytological and immunohistochemical examination of blood samples, whose sensitivity and specificity for cancer cell detection are relatively low.[17,18] It is specifically a good potential marker for colorectal cancer because of the reported high frequency of *ras* mutations in this cancer type in various populations.[19,20,21,22]

Evaluation of *ras* mutations in colorectal tissue samples has not been previously done for Filipino populations. This study aims to determine the incidence of *ras* mutations among colorectal cancer and non-cancer patients and to evaluate the usefulness and accuracy of *ras* mutation detection in detecting colorectal cancer. The sensitivity and specificity of RFLP and SSCP in detecting *ras* mutations in colorectal tissue, compared to DNA sequencing, will also be assessed. RFLP employs restriction enzymes to cut DNA at specific sequences. RFLP is used in detecting

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ras mutations in codons 12 and 13.[23] However, it cannot detect mutations outside these mutational hotspots. SSCP utilizes the electrophoretic separation of single-stranded DNA based on subtle differences in sequence, often a single base pair, which results in a different secondary structure and a measurable difference in mobility through a gel.[24] It is capable of detecting random mutations in a particular amplicon.[25] In the Philippine setting, these two methods are probably the most practical to use since they are relatively cheap and easy to perform.

Methods

Tissue Source

All colorectal tissue samples were collected from the Philippine General Hospital (PGH), Jose R. Reyes Memorial Medical Center (JRRMMC), and Ospital ng Maynila (OM) from 2002 to 2004. The sample collection protocol was approved by the Institute's Ethical Review Committee. Colorectal tumor tissues were obtained from 21 colorectal cancer patients. Control colorectal tissues were obtained from 17 patients who had undergone resection because of nonmalignant diseases. Written informed consent was obtained from all patients included in the study. All tissues were stored at -80°C until further processing.

DNA Extraction and PCR amplification

Tissues weighing less than 50 mg were cut into small pieces and homogenized in PBS using a 1.5 mL homogenizer. The genomic DNA was extracted using the QIAmp DNA Mini Kit (Qiagen, Hilden, Germany). The relative purity and concentration of the extracted gDNA were determined using a UV-Vis spectrophotometer. R*as* exon 1 was PCR-amplified from genomic DNA using Takara Taq as described by Schimanski.[23] The primers used were: *Ras* A (forward):5'ACTGAATATAA ACTTGTGGTCCATGGAGCT-3' and *Ras* B (reverse): 5'-TTATCTGTATCAAAGAATGGTCCTGCACCA-3'. The *Ras* A primer contains a mismatch (underlined) while *Ras* B is of wild type sequence. The amplification profile was as follows: initial denaturation (5 minutes at 94°C), followed by 40 cycles of denaturation (30 seconds at 94°C), annealing (30 seconds at 55°C), and elongation (30 seconds at 72°C), and a final elongation step (5 minutes at 72°C). The products were visualized by ethidium bromide staining on a 3% gel.

RFLP Analysis

The mismatch incorporated by the *Ras* A primer introduces recognition sites in codon 12 for *BstXI* and in codon 13 for *XcmI* on wild type DNA. Each PCR product was digested with *BstXI* and *XcmI*. For *BstXI* digestion, 5 μL of the PCR product was digested with *BstXI* (3 U; Fermentas) in a 10 μL reaction mixture at 55°C for 5 hours. For *XcmI* digestion, 4 μL of the PCR product was digested with *XcmI* (4 U; New England Biolabs) in a 10 μL reaction mixture at 37°C for 6 hours. The digestion products were visualized by ethidium bromide staining on a 3% agarose gel.

SSCP Analysis

A 10 μL aliquot of the PCR product was denatured with formamide containing loading buffer at 95°C for 10 minutes and snap-chilled in ice for 5 minutes. The denatured samples were run in 12% polyacrylamide gel (29:1 acrylamide to bis-acrylamide ratio) for 1.5 hours at a temperature maintained between 15°C and 20°C. The gels were stained in ethidium bromide solution and viewed using a UV transilluminator.

DNA Sequencing

Twenty (20) microliters of PCR-amplified *ras* exon 1 from all case and control samples were sequenced using BigDye™ terminator cycling conditions. The sequencing products were purified using ethanol precipitation and run using ABI Automatic Sequencer 3730xl. All electropherograms were viewed and compared against the wild type *ras* reference sequence (Genbank accession number NM_021284) using the Sequencher 4.7 Software.

Results

K-*ras* mutations have long been established as early events in colorectal carcinogenesis. These are found mostly in the transition stage of a small benign adenoma into a larger, more aggressive villous adenoma.[20,26] In this study, the incidence of *ras* mutations among colorectal cancer and non-cancer Filipino patients was evaluated to determine the usefulness of *ras* mutations as biomarkers for colorectal cancer. *Ras* exon 1 was PCR amplified using mismatch primers from 38 colorectal tissue samples (21 cancerous and 17 non-cancerous tissue) (data not shown). All case and control samples were DNA sequenced to determine the presence of mutations (Fig. 1). Five of 21 cancer samples and one of six non-cancer samples showed substitution mutations in their DNA sequence. Of the five cancer samples, three mutations were found in codon 12: a G→A transition in the first base, a G→C transversion in the 2nd base, and a G→A transition in the 2nd base. Two mutations were found in codon 13; both were G→A transitions in the 2nd base. The mutation in the control sample was a G→T transversion in the first base of codon 1.

RFLP and SSCP were also evaluated for their effectiveness as mutation detection tools in comparison to DNA sequencing. In the RFLP analysis, codon 12 mutations were detected by *BstXI* digestion, and codon 13 mutations by *XcmI* digestion. Digestion with *BstXI* or *XcmI* generates a 138 bp or a 135 bp fragment, respectively, in wild type alleles (Figure 2). RFLP analysis found 13 samples with mutations. Six were confirmed by DNA sequencing to have true mutations, while the rest had wild-type sequence (Figure 4A). SSCP analysis detected 14 samples with abnormally migrating bands, suggesting the presence of mutations (Figure 3). Of these, five were confirmed to have true mutations using DNA sequencing, and the rest had wild-type sequence. One sample that was normal in SSCP was determined to contain a mutation using DNA sequencing (Figure 4B).

Discussion

This study is a preliminary evaluation of the presence of *ras* mutations in Filipino colorectal patients. In the sample population tested, the incidence of *ras* mutations among colorectal cancer patients is 5/21 (23.8%), which is a lower frequency than what has been reported for other populations, and 1/17 (5.8%) in non-colorectal patients. The crude odds ratio for *ras* mutations and colorectal cancer is 5 (confidence interval = 0.524 – 47.73).

RFLP appears to be the better method for mutation screening compared to SSCP. SSCP is a popular method for mutation detection because of its simplicity. However, it has less than 100% sensitivity, as exemplified in this study by its non-detection of one DNA sequencing-confirmed mutation. The sensitivity of SSCP may be improved by more sensitive visualization methods, for example, silver staining. However, these are more expensive and more difficult to perform. RFLP detected all codon 12 and codon 13 mutations. Although both screening methods give rise to a significant number of false positives, RFLP did not give any false negatives. Even though it cannot detect mutations outside codons 12 and 13, our preliminary results show that *ras* mutations are clustered in these codons, so RFLP can detect all potentially *ras* exon 1 mutation positive samples. Our results suggest that a screening method such as RFLP can narrow down a pool of samples to only those that have putative *ras* mutations. However, confirmation of mutation needs to be done by DNA sequencing to eliminate the possibility of false positives.

Studies have shown that evaluation of specific nucleotide changes, and corresponding amino acid changes, in *ras* may provide relevant information concerning the aggressive potential of the tumor and the clinical outcome of colorectal cancer patients.[12,27,28] For example, one group reported that G→A transitions in codon 13, leading to the amino acid substitution Gly13→Asp13, are significantly linked to a more biologically aggressive potential for tumors compared with any other K-*ras* mutations.[27] Samowitz et.al. observed that the same mutation was associated with reduced survival rate.[29] Codon 12 K-*ras* mutations may have a role in the mucinous differentiation pathway.[27] Ideally, therefore, direct DNA sequencing is the best way not only to detect mutations, but also to determine the exact type of mutation. However, DNA sequencing is expensive and requires specialized equipment. In the Philippine setting, a possible algorithm for rapid, cost-effective mutation screening is to test patients who have the potential to develop colorectal cancer metastases for *ras* mutations using RFLP. If positive, a second-tier analysis may be performed using direct DNA sequencing.

The detection of *ras* mutations in 1/17 (5.8%) non-cancer tissue samples is interesting. K-*ras* mutations have been detected in some patients with benign biliary strictures, such as chronic pancreatitis and gallstones; such mutations are considered to be of help in differentiating benign from malignant strictures.[16,30,31] Mutations have also been detected even in normal colonic mucosa from patients afflicted with colorectal tumors.[32] Most of the control samples used in the study were benign tumor tissues. Since *ras* is involved in tumor development rather than progression,[33] detection of *ras* mutation in non-cancer tissue may have potential for predicting eventual carcinogenesis, and intervention can thus be performed to prevent it.

In this study, only 23.8% of the colorectal cancer patients tested positive for mutations, although it should be noted that the study had a small sample size, and thus the observed frequency may not be totally reflective of the actual mutation frequency in the Filipino colorectal cancer population. Our results show that *ras* mutation detection

Table 1. Evaluation of ras mutation detection (using DNA sequencing) as a marker for colorectal cancer.

Parameter	Value (confidence interval)
Sensitivity	0.238 (0.106 – 0.45)
Specificity	0.941 (0.73 – 0.997)
Positive predictive value	0.833 (0.435 – 0.991)
Negative predictive value	0.500 (0.336 – 0.664)

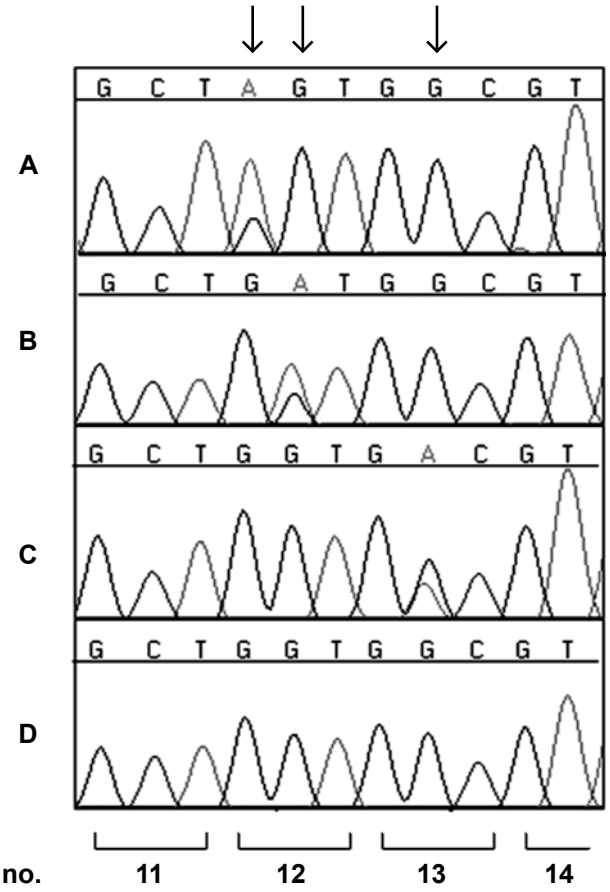

Figure 1. Direct DNA sequencing of colorectal tissue samples. A. G to A transition at the first base of codon 12. B. G to A transition at the second base of codon 12. C. G to A transition at the second base of codon 13; D. wild type sample.

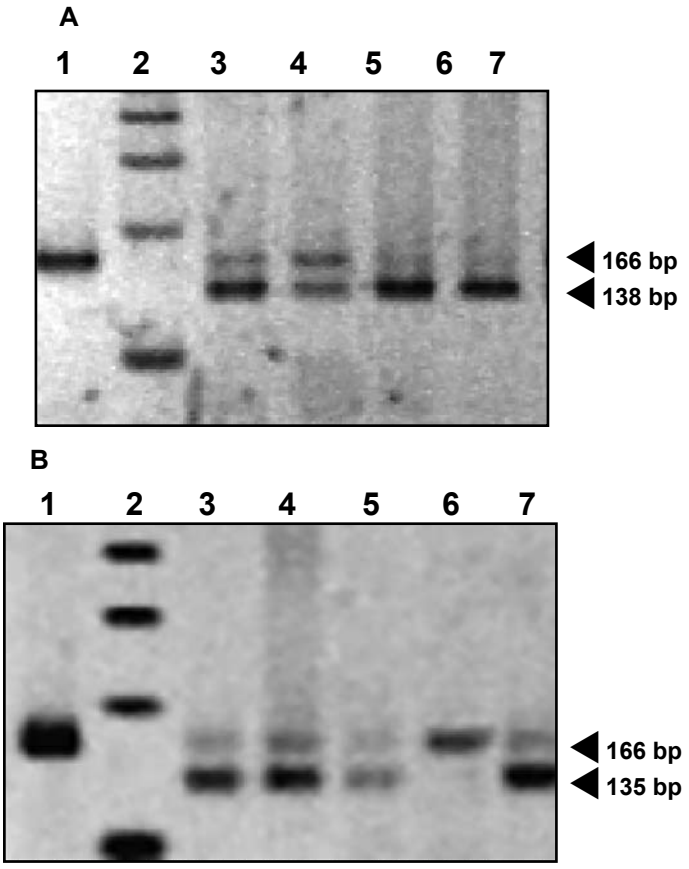

Figure 2.RFLP analysis for hotspot mutations. A. The 166 bp wild-type *ras* amplicons were digested with BstXI. Samples that remain uncut have codon 12 mutations that alter the recognition sequence for BstXI. Digested samples have wild-type sequence and are cut into a 138 bp fragment (indicated) and a 28 bp fragment (not visible). Lane 2 contains 100 bp molecular weight marker. Lane 1 contains the undigested PCR product, Lanes 3 and 4 contain mutant samples, and Lanes 5 and 6 contain wild-type samples. B. The 166 bp wild-type *ras* amplicons were digested with XcmI. Samples that remain uncut have codon 13 mutations that alter the recognition sequence for XcmI (Lane 6). Digested samples (Lanes 3, 4, 5, 7) have wild-type sequence and are cut into a 135 bp fragment (indicated) and a 31 bp fragment (not visible). Lane 2 contains a 100 bp molecular weight marker. Lane 1 contains an undigested PCR product.

is a highly sensitive but not specific diagnostic tool for colorectal cancer (Table 1). This is consistent with the suggestion that colorectal carcinogenesis can occur through several different pathways, not necessarily involving *ras*,[27] and that *ras* mutation screening is useful, but not sufficient, for general colorectal cancer screening.[12] Its usefulness may be in its predictive value for disease progression. Due to limitations in follow-up of the screened population, as well as limited information on the cancer stage of colorectal cancer patients screened, we are unable to make our own correlations between the presence and type of mutation, and the survival rates of these patients. Additional studies are suggested to determine the full correlation between the type of *ras* mutation present and the clinical outcome in the Filipino population.

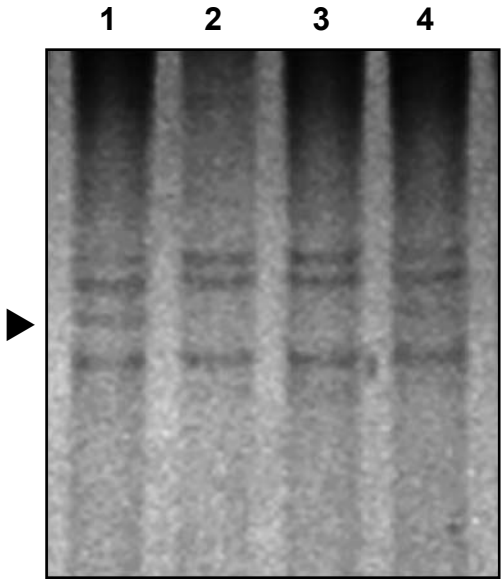

Figure 3. SSCP analysis of ras. The presence of an extra band in lanes 1 and 4 (indicated by wedge) differentiates the mutant from the wild-type (lanes 2 and 3).

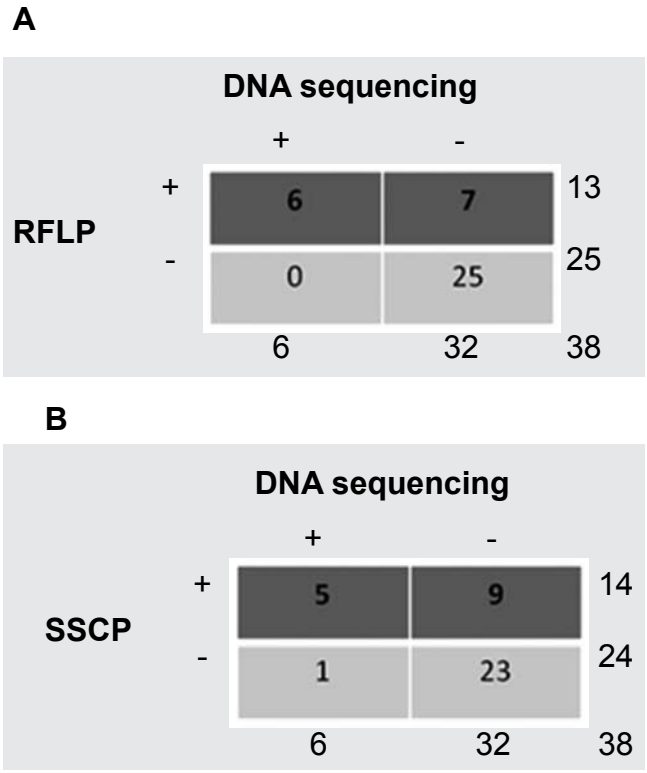

Figure 4. Assessment of RFLP (A) and SSCP (B) as screening methods for the detection of ras mutations. The effectiveness of the two methods in determining presence of *ras* mutations was assessed by comparing each to DNA sequencing.

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