INTRODUCTION

K-ras gene mutations in codons 12 and 13 are one of the earliest events in colon carcinogenesis. DNA was extracted from 25 mg of tumour tissue (n = 70) that were taken from tumour mass and pairs with normal epithelial tissue distant from the tumour of colorectal cancer patients. Exon 1 and exon 2 of the K-ras gene were amplified. Hotspot mutations were detected using polymerase chain reaction-based single-strand conformation polymorphism method and confirmed by direct DNA sequencing analysis.

RESULTS

Mutations were identified in 14 out of the 70 (20%) colorectal carcinoma tissues. Single-base transition from GGT to GAT (glycine to aspartate) in codon 12 was detected in nine samples, while three samples presented with GGC to GAC transition in codon 13. Patients with large adenoma had a 12-fold higher likelihood of K-ras mutations (odds ratios [OR] 12.31; 95% confidence intervals [CI] 1.81–83.76). Tumours located at the left colon were more likely to present with K-ras mutations (OR 4.54; 95% CI 0.96–21.54).

CONCLUSION

Our study showed a high frequency of G to A transition of codon 12 mutation of the K-ras gene, with significant correlation with tumour size and tumour location.
Nevertheless, the presence and type of mutations have not been well studied for colon cancer patients in Asia, including the differences that may exist in mutation type among the different ethnic groups. Understanding the mutations that occur, especially in the early stages of colon cancer formation, has potential for screening as well as for contributing more information on tumourigenesis. Studies on the type of mutations for this gene in Asian colon cancer patients are scarce. With the increasing incidence of colon cancer, knowledge of the type of mutations may provide clues to its initiation and progression. Therefore, the aim of this study was to determine the type of mutation in codons 12, 13 and 61 of the K-ras gene in colon cancer.

**METHODS**

A total of 70 specimens of tumour tissue from the core with matched adjacent normal colon were obtained from colorectal cancer patients who underwent surgery in Universiti Kebangsaan Malaysia Medical Centre, Kuala Lumpur, Malaysia after informed consent was obtained. The study was approved by the Ethics and Research Committee of the Faculty of Medicine, Universiti Kebangsaan Malaysia. The fresh specimens were snap frozen in liquid nitrogen and stored in −80°C until used. Surgically removed tumours were histopathologically diagnosed, and appropriate areas of tumour and normal tissue were used for mutation analysis.

Tumours were histologically classified as moderately differentiated (n = 21) and well differentiated (n = 49) adenocarcinomas, and staged as Dukes’ B (n = 49) and Dukes’ C (n = 21). Most of the patients had left-sided tumours (n = 51), while the remaining (n = 19) tumours were located in the right colon.

Tissue genomic DNA was extracted from 25 mg of frozen colorectal tumour tissue (n = 70) and normal colonic tissue (n = 70) using QiAamp DNA Mini Kit (Qiagen, Hilden, Germany). Exons 1 and 2 of the K-ras gene encompassing codons 12, 13 and 61 were amplified. K-ras mutational hotspots were analysed using polymerase chain reaction-based single-strand conformation polymorphism (PCR-SSCP) method, and then confirmed by automated direct DNA sequencing analysis ABI Prism 3100 (Applied Biosystems, Foster City, CA, USA). Primers for exon 1 encompass codons 12 and 13, which amplify a 107 bp fragment. The sequences of the primers were sense: 5’-GAC TGA ATA TAA ACT TGT GGT AGT TGG ACC T-3’ and antisense: 5’-CTA TTG TTG GAT CAT ATT CGTCC-3’.(10) Primers for exon 2 amplify a 111 bp PCR product comprising codon 61 of the K-ras gene. The sequences of the primers were sense: 5’-TTC CTA CAG GAA GCA AGT AG-3’ and antisense: 5’-CAC AAA GAA AGC CCT CCC CA-3’.(10)

Hot start PCR was performed using the PTC-100 Programmable Thermal Controller (MJ Research,
by adding 2 μl sodium acetate (3M pH 4.6) diluted with 10 μl HIDI-formamide loading buffer before being denatured for two minutes and snap cooled on ice. Sequence analysis was performed using the ABI Prism 3100 sequencer. Data collection and image analysis were done using the software provided with ABI Prism 3100 DNA sequencing machine.

The association between each clinicopathological factor and the status of K-ras gene mutations in the tumour tissue was analysed using chi-square test. A p-value < 0.05 was considered to be statistically significant. The data was expressed as odd ratios and 95% confidence interval, as appropriate. All statistical analyses were performed with the Statistical Package for the Social Sciences software (SPSS Inc, Chicago, IL, USA). All p-values were estimated using a two-sided statistical test.

RESULTS

The 70 tumour samples were from 38 female and 32 male colorectal cancer patients. The average age of the patients was 62 (range 28–94) years (Table I). The patients represented three major ethnicities in Malaysia; the distribution of the patients was 59% Chinese (n = 41), 39% Malays (n = 27) and 3% Indians (n = 2). On histological analysis of the samples, 49 tumour samples were classified as Dukes’ B cancer stage and 21 as Dukes’ C cancer stage. Six tumours were located at the ascending colon, seven in the transverse colon and one in the descending colon. 17 cases arose from the rectosigmoid colon and 19 from the rectum. More than half of the study samples were well differentiated tumours (49/70), while 21 samples were moderately differentiated adenocarcinomas. The tumour sizes were 5—64 cm² (average 19.94 cm²).

K-ras gene mutations were successfully detected in the collected colorectal cancer specimens via PCR-SSCP analysis. Differences in mobility shift of the SSCP product suggest the

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Table II. Characteristics of colorectal cancer patients and clinicopathology features of tumours with K-ras gene mutation status.

<table>
<thead>
<tr>
<th>Case</th>
<th>Gender</th>
<th>Age (yrs)</th>
<th>Race</th>
<th>Tumour Site</th>
<th>Tumour Size (cm²)</th>
<th>Diff</th>
<th>Dukes' stages</th>
<th>K-ras gene mutations</th>
<th>Amino acid changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Female</td>
<td>64</td>
<td>Chinese</td>
<td>R</td>
<td>4.0</td>
<td>M</td>
<td>B</td>
<td>+ Codon 12: GGT to GAT</td>
<td>Glycine to aspartate</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>30</td>
<td>Malay</td>
<td>T</td>
<td>16.0</td>
<td>P</td>
<td>C</td>
<td>+ Codon 13: GGC to GAC</td>
<td>Glycine to aspartate</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>64</td>
<td>Malay</td>
<td>R</td>
<td>15.0</td>
<td>W</td>
<td>B</td>
<td>+ Codon 12: GGT to GAT</td>
<td>Glycine to aspartate</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>65</td>
<td>Chinese</td>
<td>S</td>
<td>51.0</td>
<td>W</td>
<td>B</td>
<td>+ Codon 13: GGC to GAC</td>
<td>Glycine to aspartate</td>
</tr>
<tr>
<td>5</td>
<td>Female</td>
<td>68</td>
<td>Chinese</td>
<td>S</td>
<td>20.0</td>
<td>W</td>
<td>C</td>
<td>+ Codon 12: GGT to GAT</td>
<td>Glycine to aspartate</td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>71</td>
<td>Malay</td>
<td>R</td>
<td>2.0</td>
<td>W</td>
<td>B</td>
<td>+ Codon 12: GGT to GAT</td>
<td>Glycine to aspartate</td>
</tr>
<tr>
<td>7</td>
<td>Male</td>
<td>56</td>
<td>Chinese</td>
<td>A</td>
<td>12.0</td>
<td>W</td>
<td>B</td>
<td>+ Codon 12: GGT to GAT</td>
<td>Glycine to aspartate</td>
</tr>
<tr>
<td>8</td>
<td>Female</td>
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<td>Chinese</td>
<td>A</td>
<td>20.0</td>
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<td>C</td>
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<td>Glycine to aspartate</td>
</tr>
<tr>
<td>9</td>
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<td>52</td>
<td>Chinese</td>
<td>A</td>
<td>36.0</td>
<td>W</td>
<td>B</td>
<td>+ Codon 12: GGT to GAT</td>
<td>Glycine to aspartate</td>
</tr>
<tr>
<td>10</td>
<td>Male</td>
<td>94</td>
<td>Chinese</td>
<td>D</td>
<td>39.5</td>
<td>W</td>
<td>B</td>
<td>+ Codon 12: GGT to GAT</td>
<td>Glycine to aspartate</td>
</tr>
<tr>
<td>11</td>
<td>Male</td>
<td>71</td>
<td>Chinese</td>
<td>S</td>
<td>66.0</td>
<td>W</td>
<td>B</td>
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<td>Glycine to aspartate</td>
</tr>
<tr>
<td>12</td>
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<td>65</td>
<td>Chinese</td>
<td>A</td>
<td>8.0</td>
<td>M</td>
<td>B</td>
<td>+ Codon 12: GGT to GAT</td>
<td>Glycine to aspartate</td>
</tr>
<tr>
<td>13</td>
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<td>67</td>
<td>Malay</td>
<td>A</td>
<td>44.0</td>
<td>M</td>
<td>B</td>
<td>+ Codon 12: GGT to GAT</td>
<td>Glycine to aspartate</td>
</tr>
<tr>
<td>14</td>
<td>Female</td>
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<td>Chinese</td>
<td>S</td>
<td>48.0</td>
<td>W</td>
<td>B</td>
<td>+ Codon 12: GGT to GAT</td>
<td>Glycine to aspartate</td>
</tr>
</tbody>
</table>

Diff: differentiation; SSCP: single-strand conformation polymorphism; A: ascending colon; T: transverse colon; D: descending colon; S: sigmoid; R: rectum; P: poorly differentiated; M: moderately differentiated; W: well differentiated

Massachusetts, MA, USA). Each PCR reaction contains 100 ng of genomic DNA, 5 μl of 1X reaction buffer, 20 μM dNTP mix, 1.5 mM MgCl₂, and 20 pmol primer in a final reaction volume of 50 μl. 0.5 unit Taq DNA polymerase (Promega Co, Madison, WI, USA) was added after the cycles began, with a denaturation step at 94°C for four minutes. It was then followed by 30 cycles of denaturation at 94°C for 75 seconds, annealing at 52°C for 90 seconds and elongation at 72°C for 120 seconds. A final elongation step at 72°C for three minutes completed the run. 5 μl of PCR products (20 ng) was mixed with 2 μl of Blue Orange loading dye and denatured at 95°C for 10 minutes. This was next chilled on ice for five minutes. SSCP analysis was then performed by allowing the DNA to migrate according to size on a 12.5% non-denaturing polyacrylamide gel electrophoresis at 150 volt, 200 mamp for two hours using a BioRad mini PROTEAN 3 electrophoresis set (Bio-Rad, Hercules, CA, USA). 1X TBE buffer was used as the running buffer. The gel was then stained with a DNA silver staining kit (Amersham Co, Uppsala, Sweden) to visualise the mobility shift of the PCR products.

The PCR products were electrophoresed on 2% agarose gel at 80 volt for one hour and purified using the QIAamp Gel Extraction kit (Qiagen, Hilden, Germany). Purified PCR products were used next as templates for sequencing analysis. Automated sequencing was performed using the Big Dye Terminator Kit V3.1 (Applied Biosystems, Foster City, CA, USA) using PTC-100 Programmable Thermal Controller. Reaction mixtures comprised 2 μl Big Dye Terminator, 2 μl buffer, 2 μl DNA template, 1 pmol of each primer and 2 μl sterile distilled water in a final reaction volume of 10 μl. Amplification included 25 cycles of denaturation at 95°C for ten seconds, annealing at 50°C for ten seconds and elongation at 60°C for four seconds. Cycle sequencing products were next purified

presence of mutations in the tumour samples, which were not detected in their respective normal colon tissues (Fig. 1). Out of the 70 tumour samples, 14 (20%) showed the presence of mutations, which was later confirmed by automated direct DNA sequencing technique (Fig. 2). The mutations were single-base substitutions, of which 11 (79%) cases were in codon 12 and three (21%) were located in codon 13. The codon 12 mutations showed a predominance of G to A transitions (9/11), which changed glycine to aspartic acid in p21ras protein (Fig. 3), while two other mutations (2/11) were G to T transversion (glycine to valine).

All the three cases in codon 13 also showed G to A transitions (Table II). All the G to A transitions on codon 12 were present in Chinese patients, except for one in a Malay patient, whereas G to A transitions on codon 13 were present in two Malay patients and one Chinese patient.

There was a significant correlation between K-ras gene mutations and tumour size ($p = 0.003$; OR 12.31 [1.81–83.76]). The average size of the tumour specimens with mutations was 36.47 cm$^2$, whereas those without mutation were considerably smaller, at an average size of 15.94 cm$^2$ (Table I). K-ras mutations also showed a significant correlation with tumour location ($p = 0.04$; OR 4.34 [0.96–21.54]), where 37% of tumours at the right colon present with K-ras mutations compared to those at the left colon (14%). Age appeared to also correlate with K-ras mutations, although not significantly. 80% of patients (8/11) who presented with mutations in codon 12 were above the average age of 62.5 (range 64–94) years, while the remaining two (20%) patients were 56 and 52 years old. Point mutations were only detected in tumour tissues and not in their respective normal tissues. None of the tumour samples exhibited mutations in codon 61 of the K-ras gene.

**DISCUSSION**

Studies have implicated K-ras gene mutations in the early stage of colorectal cancer. Different methods have been employed to detect K-ras mutations. In this study, we used the PCR-SSCP method, which has the advantage of being a simple yet sensitive, non-radioactive method to detect K-ras mutations. Silver staining detection was then used to visualise the DNA. PCR-SSCP is reported as a sensitive method that is able to detect a single-point mutation. Using this method, we were able to identify 14 K-ras single-point mutations in the tumour specimens, which were then confirmed by direct DNA sequencing.

The frequency of K-ras mutation was 20% (14/70), which is rather low compared to previous reports of 30%–80%. Nevertheless, the type of mutation observed in this study corresponds to the findings of previous studies, where the most common mutation detected was in codon 12 followed by codon 13, with none detected in codon 61. In addition, for codon 12, nine out of the 11 cases were transitions from GC to AT, while the remaining two were GC to TA transversions. The three cases with codon 13 mutations were GG to GA transitions.

A significant correlation between the presence of K-ras mutations and tumour size was seen in larger adenomas. Only one small adenoma in this study contained a mutation. Previous studies have reported a significant association of codon 13 K-ras mutation with tumour size ($>3$ cm, $p = 0.03$). These studies also reported the association of K-ras mutation with other clinical outcomes in terms of survival and lymph node involvement, and suggested that mutated exon 2 of K-ras represents a molecular lesion in the development of more aggressive disease. Previous studies have also suggested the possibility that K-ras oncogene might be useful as a potential predictor of metachronous...
adenomas\(^{(29)}\) and that \(K\)-ras mutations may be less common in small adenomas.\(^{(26)}\) This suggests that \(K\)-ras mutation is not only important as a tumour initiation factor but is also involved in the progression of aggressive tumour. Tortola et al.\(^{(29)}\) have shown that \(K\)-ras and \(p53\) gene mutations show significant correlation between tumour aggressiveness and survival rate of colorectal cancer patients. A study by Jen et al.\(^{(27)}\) found this mutation to be almost equally common in both non-dysplastic and dysplastic polyps.

An \textit{in vitro} study on transfecants of NIH3T3 cells by Guerrero et al.\(^{(29)}\) suggests that point mutation at codon 12 \(K\)-ras may increase aggressiveness not by altering the proliferative pathways but by differential regulation of the \(K\)-ras downstream pathways that lead to inhibition of apoptosis, enhanced loss of contact inhibition and increased predisposition to anchorage-independent growth, which offers a molecular explanation for the increased aggressiveness of tumours with \(K\)-ras codon 12 mutations observed in clinical setting.

Our result also showed a significant correlation between \(K\)-ras mutations and tumour location. \(K\)-ras mutations were more frequent in the left colon (37%) compared to the right colon (14%). The tumour at the right colon is three times more likely to present with \(K\)-ras mutations compared to the left colon. This finding is in agreement with that of Bleeker et al.\(^{(29)}\) who reported a higher frequency of \(K\)-ras mutation in right-sided tumours (38%) compared to left-sided ones (10%), although other studies have reported no difference in \(K\)-ras mutation at both tumour sites.\(^{(10)}\) Genetic differences between right and left tumours have been highlighted, where genetic evidence and phenotype as well as prognostic finding have provided insight into the differences of both tumour location. Differences in proximal and distal colorectal cancer suggest that each may arise through different pathogenetic mechanisms.\(^{(29)}\) Proximal tumours appear to represent a genetically more stable form of the disease and are usually related to the nucleotide instability pathway as microsatellite instability, whereas distal tumours show evidence of greater genetic instability, which are usually associated with specific chromosomal instability.\(^{(11,29,10)}\) These differences may be partially attributed to different embryological development and physiological circumstances.\(^{(28)}\)

Our results showed no significant correlation between \(K\)-ras mutations and histological tumour differentiation, although the mutations were more frequent in well-differentiated adenocarcinomas (10/14) compared to moderately differentiated ones (4/14). A study by Bazan et al.\(^{(30)}\) showed an association between \(K\)-ras mutations with mucinous histotype, which suggests that codon 12 \(K\)-ras mutations may have a role in the mucinous differentiation pathways. There was no significant correlation between Dukes’ tumour staging and \(K\)-ras gene mutation in our tumour specimens and no association with gender and ethnicities, although it was noted that the number of patients positive for the mutation was small. This result is consistent with that of other studies.\(^{(29)}\) \(K\)-ras gene mutations in our study also showed no correlations with survival, as reported by Bouzourene et al.\(^{(32)}\)

The results of our study showed no significant correlation between mutations of \(K\)-ras gene among the three different major ethnicities in Malaysia. 71% of the mutations (10/14) occurred in Chinese patients (\(n = 41\)) and 29% (4/14) in Malay patients (\(n = 27\)), whereas none of the two Indian patients showed any \(K\)-ras gene mutations. According to the Malaysia National Cancer Registry, in the year 2003, the Chinese population showed the highest incidence of colon and rectum cancers in Malaysia, with 451 (age-standardised rate [ASR]: 17.2) and 300 (ASR: 12.5) cases, respectively, followed by Malays with 201 (ASR: 3.4) and 242 (ASR: 6.7) cases, respectively.\(^{(30)}\)

In conclusion, this study showed that the frequency of \(K\)-ras gene mutations is low in Malaysian colorectal cancer patients. Patients with mutations most commonly had a G to A transition in codon 12. There is a significant correlation between \(K\)-ras mutation and tumour location. Tumour size provides evidence for the different genetic alteration in left and right colon tumourigenesis and for the importance of \(K\)-ras mutation in promoting tumour aggressiveness. Further studies into mutations of other genes such as \(p53\), \(c-myc\) and \(erb-b2\) involving a larger number of samples among the different ethnic groups are required.

REFERENCES

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The following are the judging criteria:

- The paper with the most potential impact on clinical practice
- Most rigorous study design/research methodologies
- Comprehensive data analysis and balanced discussion
- Data interpretation

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