Singapore Med J 2012; 53(1) : 26

Predominance of G to A codon 12 mutation *K-ras* gene in Dukes' B colorectal cancer

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INTRODUCTION *K-ras* gene mutations in codons 12 and 13 are one of the earliest events in colon carcinogenesis.
METHODS 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.

Keywords: codon 12, colorectal cancer, Dukes' B, K-ras gene, mutation
Singapore Med J 2012; 53(1): 26–31

INTRODUCTION

The past few decades have seen a two- to four-fold increase in the incidence of colorectal cancer (CRC) in Asian countries such as China, Japan, Singapore and Malaysia.[1] This is attributed to an increasing affluence, leading to changes in dietary habits and lifestyle, despite major differences among the ethnic groups. The incidence of CRC is highest in the Chinese, followed by the Malays and Indians. A high incidence of CRC has also been reported in developed nations such as North America, Western Europe and Australia,[2] which is consistent with an affluent lifestyle – a diet that is high in red meat with frequent intake of alcohol and relatively low in fibre-rich, unrefined plant-based foods. The higher incidence of CRC in developed nations may also be attributed to lower levels of physical activity among the population. The molecular epidemiology of colon cancer in Asians has not been thoroughly studied in high-risk populations and among different ethnic groups in terms of its distribution.

The mutational events in early colorectal cancer tumourigenesis include mutations in *adenomatous polyposis coli* and hypermethylation, followed by *K-ras* mutations. Deletion in colon cancer and mutations in the *p53* gene occur later in CRC tumourigenesis, although the exact order may vary.[3] *K-ras*, located on the short arm of chromosome 12 at position 12p12.1, is an oncogene that is implicated in the carcinogenesis of colorectal cancer. The *K-ras* gene product is a guanosine triphosphate/guanosine diphosphate (GTP/GDP)-binding protein with a molecular weight of approximately 21 KDa. This protein is localised to the inner side of cell membranes. It acts as an 'on-off' switch that regulates GTP-signal transductions, controlling cell growth, differentiation and survival of the cell.[4]

K-ras mutations in positions 12, 13 and 61 result in GDP-resistant mutants locked into active GTP-bound states. The mutants remain 'on' and induce uncontrollable cellular proliferation and tumour formation.[5] Somatic mutational activation of *K-ras* genes is a frequent phenomenon occurring in various human cancers.[6,7] In CRC, this mutation may have potential as a biomarker, both in early diagnosis and susceptibility assessment, and has been reported to be 30%–80% depending on the population studied.[8,9] Most of the mutations (70%–80%) that have been reported in the West were found in codon 12 of the gene, with variations in the type of mutation.[10] The differences in type of mutation are still unclear, and no definite pattern as to their origin and predisposing factor has been observed.[1]

Previous studies have reported 27.4% and 38.0% of *K-ras* mutations in CRC patients from the UK and Switzerland, respectively.[10,11] Tortola et al[12] have shown that *K-ras* mutations were present in 41% (54/132) of colorectal patients, with 52% (22/42) of positive *K-ras* mutation in codon 12 with G to A transition in colorectal cancer patients from Spain. Other studies have found the presence of 25.0%–41.3% (57/112) of *K-ras* mutations, with G to T transversion followed by 31.9%–32% with G to A transition, and only 7% with G to C transition in colorectal patients from the USA.[13,14] Oliveira et al[15] reported of *K-ras* mutations in 32% (272/854) of sporadic CRC, with a spectrum of 63% with G to A transition on codon 12 and 29% with G to T transversion.

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Table I. Comparison of *K-ras* gene mutation frequency between clinicopathology features of colorectal cancer patients.

	No. (%)			p-value χ^2 test	Crude OR (95% CI)	Adjusted OR[b] (95% CI)
	Total	*K-ras* mutations +ve	*K-ras* mutations −ve			
All patients	70 (100)	14 (20)	56 (80)			
Age (yrs)				0.36[a]		
Average age	61.94	64.5	61.3			
< 60	23 (33)	3 (13)	20 (87)		1.00	
> 60	47 (67)	11 (23)	36 (77)		2.04 (0.51–8.12)	2.07 (0.33–12.91)
Gender				0.38		
Male	32 (46)	8 (25)	24 (75)		1.00	
Female	38 (54)	6 (16)	32 (84)		1.78 (0.54–5.81)	1.63 (0.38–6.89)
Race/ethnicity				0.55		
Malay	27(39)	4 (15)	23(85)		1.00	
Chinese	41(59)	10(24)	31(76)		1.59 (0.44–5.73)	2.03 (0.37–11.00)
Indian	2 (3)	0(0)	2(100)			
Site				0.04[a]		
Left colon	51 (73)	7 (14)	44 (86)		1.00	
Right colon	19 (27)	7 (37)	12 (63)		3.67 (1.07–12.3)	4.54 (0.96–21.54)
Stage				1.00[a]		
Dukes' B	49 (70)	49 (70)	39 (80)		1.00	
Dukes' C	21 (30)	21 (30)	17 (81)		0.92 (0.25–3.34)	1.08 (0.20–5.78)
Differentiation				1.00[a]		
Moderate	21 (30)	4 (19)	17 (81)		1.00	
Well-differentiated	49 (70)	10 (20)	39 (80)		1.09 (0.29–3.96)	0.63 (0.11–3.71)
Tumour size (cm^2)				0.003		
Average size	19.94	36.47	15.94			
< 15	31 (44)	3 (10)	28 (90)		1.00	
15–34	29 (41)	5 (17)	24 (83)		1.87 (0.41–8.69)	1.30 (0.20–8.27)
> 35	10 (14)	6 (60)	4 (40)		13.50 (2.37–76.82)	12.31 (1.81–83.76)

[a]Fisher's exact test compares the differences in proportion between *K-ras* mutations +ve and *K-ras* mutations −ve.
[b]Nagelkerker R Square = 0.33
OR: odds ratio; CI: confidence interval

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 tissues 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'.[16] 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'.[17]

Hot start PCR was performed using the PTC-100 Programmable Thermal Controller (MJ Research,

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

Case	Gender	Age (yrs)	Race	Tumour			Dukes' stages	*K-ras* gene mutations		Amino acid changes
				Site	Size (cm^2)	Diff		SSCP	DNA Seq	
1	Female	64	Chinese	R	4.0	M	B	+	Codon 12 : GGT to GAT	Glycine to aspartate
2	Male	30	Malay	T	16.0	P	C	+	Codon 13 : GGC to GAC	Glycine to aspartate
3	Female	64	Malay	R	15.0	W	B	+	Codon 12 : GGT to GAT	Glycine to aspartate
4	Male	65	Chinese	S	51.0	W	B	+	Codon 12 : GGT to GAT	Glycine to aspartate
5	Female	68	Chinese	S	20.0	W	C	+	Codon 13 : GGC to GAC	Glycine to aspartate
6	Male	71	Malay	R	2.0	W	B	+	Codon 12 : GGT to GAT	Glycine to aspartate
7	Male	56	Chinese	A	15.0	W	B	+	Codon 12 : GGT to GAT	Glycine to aspartate
8	Female	76	Chinese	A	20.0	W	C	+	Codon 12 : GGT to GTT	Glycine to valine
9	Female	52	Chinese	A	36.0	W	C	+	Codon 12 : GGT to GAT	Glycine to aspartate
10	Male	94	Chinese	D	39.5	W	B	+	Codon 12 : GGT to GAT	Glycine to aspartate
11	Male	71	Chinese	S	66.0	W	B	+	Codon 12 : GGT to GAT	Glycine to aspartate
12	Male	65	Chinese	A	8.0	M	B	+	Codon 12 : GGT to GTT	Glycine to valine
13	Male	67	Malay	A	64.0	M	B	+	Codon 13 : GGC to GAC	Glycine to aspartate
14	Female	61	Chinese	S	48.0	W	B	+	Codon 12 : GGT to GAT	Glycine to aspartate

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 $MgCl_2$, and 20 pmol primer in a final reaction volume of 50µl. 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 ten 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 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 cm^2 (average 19.94 cm^2).

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

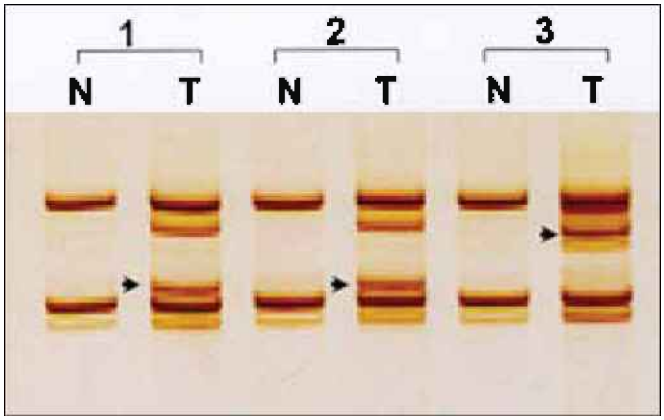

Fig. 1 Polymerase chain reaction-based single-strand conformation polymorphism analysis of K-ras gene in colorectal cancer tissue. Tumour tissue (T) from samples 1, 2 and 3 show an additional band (arrow), indicating the presence of mutations. No such band was detected in their respective normal (N) samples.

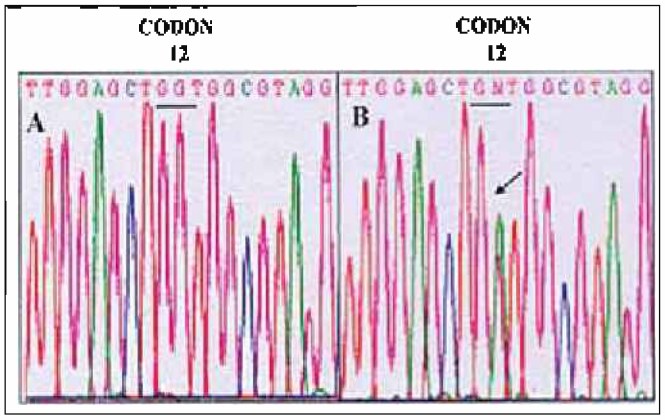

Fig. 2 DNA sequencing analysis of K-ras gene for colorectal samples. (A) Sequence analysis exon 1 K-ras gene normal tissues sample with wild type DNA sequence. (B) Sample tumour shows the presence of interfering peak (arrow) at codon 12, indicating that the mutation occurred in the tumour tissues with transition GC to AT at codon 12.

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 4.54 [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.

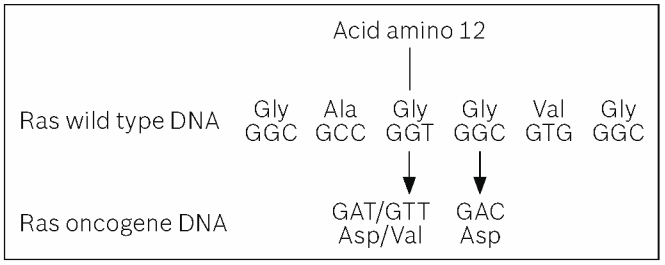

Fig. 3 Point mutations in K-ras gene with transition G to A. Amino acids also change from glycine to aspartic acids. Transversion G to T with changes of amino acid glycine to valine. These K-ras mutations impair conversion of GTP to GDP+P.

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.[16,17] 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.[18] PCR-SSCP is reported as a sensitive method that is able to detect a single point mutation.[18] 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%.[19,20] 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.[21-23] 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).[13,24] 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.[24] Previous studies have also suggested the possibility that *K-ras* oncogene might be useful as a potential predictor of metachronous

adenomas[25] 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[12] 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 *in vitro* study on transfectants of NIH3T3 cells by Guerrero et al[28] 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,[19] 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.[11,29,30] These differences may be partially attributed to different embryological development and physiological circumstances.[30]

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[31] 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.[32] *K-ras* gene mutations in our study also showed no correlations with survival, as reported by Bouzourene et al.[33]

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, with the lowest incidence for Indians with 30 (ASR: 3.4) and 38 (ASR: 6.8) cases, respectively.[34]

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 aberration 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.

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