Mutational Analyses of *K-ras* Exon 2 in Thailand Colorectal Cancer Tissue Samples

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ABSTRACT

Objective: The main purpose of this study was to elucidate the genotype of *K-ras* gene in Thailand colorectal cancer tissue samples, especially in exon 2, which has never before been reported.

Methods: 106 patients samples in formalin fixed paraffin embedded tissue blocks were investigated in this study. DNA was extracted and PCR was performed by using primers specific for the *K-ras* gene at exon 2. Direct sequencing was performed in a Genetic Analyser ABI3130 with specific software.

Results: The mutation of *K-ras* exon 2 gene in Thailand colorectal cancer samples accounted for 37.7% of the total. The most common mutation found in this series was the G→A transition which accounted for 70%.

Conclusion: The incidence of *K-ras* exon 2 mutation in Thailand colorectal cancer samples was remarkedly similar to previous reports.

Keywords: Mutation, *K-ras*, exon 2, Thailand and colorectal cancer

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Colorectal cancer is one of the most common cancers and causes of cancer-related mortality worldwide. In Thailand, the incidence of this cancer as reported from the Ministry of Public Health in 2007 is 8.8 for males and 7.6 for females which is the third in frequency of malignant diseases.[1] From the annual statistical report 2007 at Siriraj Hospital, colorectal cancer is the third leading cause of malignancies accounting for 8.26% with the incidence rate in males and females accounting for 9.02% and 7.58% respectively.[2] The 5 year survival rate in patients with colorectal cancer though has improved from 22% to 47%, although the overall survival rate remains uncertain.[3]

Based on previous reports, approximately 30-50% in colorectal tumor samples exhibited *K-ras* mutation which most often occurred at a point mutation in codon 12,13 in exon 2.[4,5] In context, *K-ras* signaling pathways are activated from extracellular growth factor receptors and their ligands which regulate cell progression through cell cycle and proliferation.[6,7] *K-ras* gene encodes p21 proteins which are small guanine-nucleotide binding proteins that act as switches in several signal transduction pathways. The mutations of *K-ras* gene increase the oncogenic properties of Ras proteins by inhibiting the GTPase activity. Additionally, several studies demonstrated that the oncogenic mutant *K-ras* increased the GTP-bound state and resulted in loss of control of cell proliferation and survival, and eventually the promotion of metastasis.[8]

Recent treatment in advanced colorectal cancer with targeted therapy, especially cetuximab, has been reported to provide significant benefit in tumors bearing only wild type *K-ras*. The mutation status of *K-ras* gene had no influence on overall survival among patients treated with cetuximab and best supportive care alone.[9-12] Moreover, some collaborative studies also demonstrated that the significant presence of *K-ras* mutation in the primary tumor was important for cancer progression.[13]

The main purpose of this study was to elucidate the genotype of *K-ras* gene in Thailand colorectal cancer tissue samples, especially in exon 2, which is markedly essential in both drug influence and prediction of survival. Mutant detection for *K-ras* exon 2 was analyzed by using direct sequencing together with specific software which is the gold standard technique.

MATERIALS AND METHODS

Tissue samples

Samples in formalin fixed paraffin embedded tissues blocks were obtained from patients diagnosed with colorectal cancer sent for *K-ras* testing at Siriraj Hospital.

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All tissue blocks were re-examined using histopathological type by pathologist, Dr. Paisal Parichatikanond, Faculty of Medicine Siriraj Hospital, Mahidol University. Most tissues sites were from the colorectum except one from a lung metastatic site and another one from a liver metastatic site.

DNA extraction

For each sample, the serial sections of 20 µm thickness for 30 pieces were obtained with a standard microtome and disposable blades. DNA was extracted by using a QIAamp DNA mini kit (Qiagen) following the manufacturers protocol. In brief, wax was removed from tissue blocks with xylene followed by centrifugation at 6,000 g for 10 minutes. After this initial step, pellets were resuspended in absolute ethanol for 5 minutes and centrifuged. Paraffin-free tissues were air dried for 15 minutes at room temperature and subjected to the DNA extraction procedures described by the kit. The concentration and purity of obtained DNA was measured with a Smartspect (BIO-RAD, USA).

Polymerase chain reaction and gel extraction

All PCR reactions were performed on a MyCycler (BIO-RAD, USA). The sequences of PCR primers for *K-ras* exon 2 were as follows: forward primer 5'TTTATTATAAGG CCTGCTGAA-3',5"-GCCTGCT GAAAATGACTGAA-3', and 5'-GTGTATTAACCTTATGTGTGAC-3' and reverse primer 5'-TTAGCT GTATCGTCAAGGCA-3', 5"-AGA ATGGTCCTGCACCAGTA-3' and 5'-CTATTGTTGGAT-CATATTCG TC-3'. PCR was performed by mixing 10 mM of dNTP 1.5 µl,10x Pfx amplification buffer 10 µl, Pfx DNA polymerase 0.4 µl, 20 pmol/ µl of each primer 1.5 µl, 50 mM of $MgSO_4$ 1 µl, 200 ng of DNA template, adding up to a final volume of 50 µl with water. Cycling parameters were: Denaturation for 15 seconds at 94°C, annealing for 30 seconds at 60°C, and extension for 30 seconds at 68°C, for 35 cycles. The PCR products were then run on 1.5% agarose gel and stained with ethidium bromide to check for the amplicon at size 84 bps. Gel was then cut and the PCR product was eluted and purified by using a QIAquick Gel extraction mini kit (Qiagen) following the manufacturers instructions.

Direct sequencing method

For direct sequencing, PCR product purified from gel was further added to with Big Dye Terminator V3.1. The mixture was composed of sequencing buffer 2 µl, Big Dye Terminator 4 µl, 0.8 pmol/ µl of primer 4 µl, purified template (50-300 ng), and then water was added up to a final volume of 20 µl. The reaction was performed on a MyCycler (BIO-RAD, USA). Cycling parameters were: Denaturation for 10 seconds at 96°C, annealing for 5 seconds at 50°C, and extension for 4 minutes at 60°C, for 25 cycles. Then the product was precipitated by ethanol/ sodium acetate precipitation with 95% ethanol 62.5 µl, 3M NaOAc (pH 5.0) 3 µl in a final volume of 80 µl. Hi-Di Formamide was added to the precipitated product before analyzing the sequences in a Genetic Analyser ABI3130 with specific software.

Statistical analysis

The patients characteristics such as age and sex, and *K-ras* mutational type were retrospectively identified and analyzed using descriptive analysis. The retrospective research was performed after the approval from "The Siriraj Institutional Review Board (SIRB)."

RESULTS

Patients characterizations

A total of 106 patient samples diagnosed as colorectal cancer based on histopathological section were enrolled in this retrospective study. The number of male patients was 57 and the number of female patients was 49 with a male to female ratio equal to 1.16:1. The age of patients ranged from 21 to 89 years. The mean age was 60.6 years. The mean age of male patients was 61.5 years, whereas the mean age of females was 59.58 years.

K-ras mutations in tumor samples

For sequence analysis of all 106 samples, 40 samples showed mutated *K-ras* status accounting for 37.7% as shown in Table 1. Twenty-nine out of 40 tumor samples were mutated in codon 12 and 11 samples were mutated in codon 13 accounting for 72.5% and 27.5%, respectively. Only one sample was mutated in both codon 12 and 13. The most frequent mutation detected in codon 12 is AGT. For codon 13, the most frequent mutation is GAC, accounting for 20%. DNA sequencing of *K-ras* gene at codon 12 with GGT→AGT was demonstrated in Fig 1 and a wild type pattern was shown in Fig 2. Additionally, in our experience, the failure for PCR or direct sequencing to detect *K-ras* exon 2 in this study was about 30-40%.

DISCUSSION

This is the first report that elucidated the genotype of *K-ras* mutations in Thailand colorectal cancer patients by using direct sequencing. The detection of mutation by direct sequencing has been applied by clinical laboratories for decades. Although it is considered as a gold standard,

TABLE 1. Spectrums of *K-ras* mutation detected at Siriraj Hospital.

Condon number	Mutation type	Number of case	Amino acid substitute	Histologic type
12. GGT (glycine)	G**A**T	2	Aspartate	
12	G**T**T	7	Valine	
12	**A**GT	16	Serine	
12	**T**GT	2	Cysteine	
12	GC**T**	1	Alanine	
12	**AGG**	1	Arginine	Adenocarcinoma
13. GGC (glycine)	GG**A**	1	Glycine	
13	GA**C**	8	Aspartate	
13	GT**C**	1	Valine	
13	GG**T**	1	Glycine	

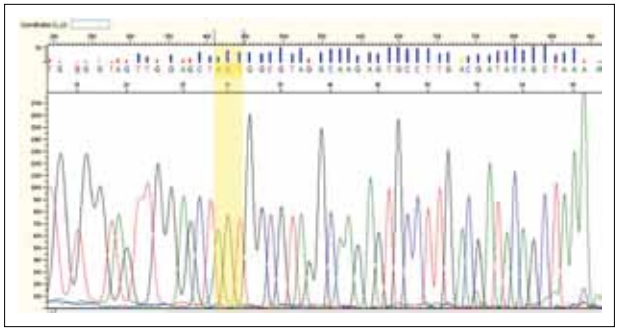

Fig 1. DNA sequencing of *K-ras* mutation at codon 12 with GGT→AGT transition.

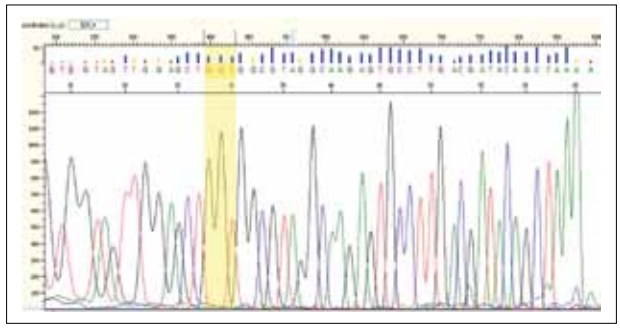

Fig 2. DNA sequencing of *K-ras* gene at codon 12 and 13 showed *K-ras* wild type.

it is usually a time consuming and not always a successful process.[14,15] Comparing direct sequencing to restriction fragment length polymorphism (RFLP), RFLP provides a more easy way, but it does not reveal the true extent of genetic diversity.[16]

Based on these data, *K-ras* mutations, which were detected by using direct sequencing, were presented in Thailand colorectal cancer tissue samples accounting for 37.7%. This incidence of mutation was remarkably similar to previous reports from other countries.[14] As mentioned earlier, most *K-ras* mutations were reported to occur at codon 12, 13 and less frequently found at codon 61.[15] The ratio of codon 12 to 13 mutations in our series was 2.6:1 which was not different from another report.[16] Actually, codon 12 and 13 are GGT and GGC, respectively, and, both are glycine amino acid which is one of the amino acids with a simple structure.[17]

The distribution of specific nucleotides changes at codon 12 and 13 of *K-ras* gene in our series is summarized in Table 1. At codon 12, we found 7 types of *K-ras* mutations and the most frequent type detected in codon 12 in our series was G →A (AGT) in the first nucleotide, which was serine, instead of glycine amino acid accounting for 40%. This frequency was different from the Taiwan report in which the second nucleotide mutation was the most common type.[18] However, the highest frequency of overall mutation from that report was the same as ours. In addition, the second common type of mutation at codon 12 was in the second nucleotide which was G→T (GTT). The amino acid substituted in this position was valine, accounting for 17.5%.

For codon 13, five types of *K-ras* mutation were detected in our paraffin embedded tissues and the most common mutation was G→A (GAC) in the second nucleotide resulting in aspartate substitution which was similar to a previous report.[18] Interestingly, only one case in our series had a mutation in both codon 12 and 13 which accounted for only 0.94%. Based on our data, the most frequent overall *K-ras* mutation was G→A transition accounting for 70%, followed by G→T transition accounting for 25%. When compared to previous data, the high incidence of *K-ras* gene in exon 2 at codon 12 and 13 from G→A transition mutation in Thailand colorectal cancer is similar to frequencies previously reported.[19] This observation of G→A transition in *K-ras* gene might be the effect of several common environmental carcinogens, especially alkylating agents.[20]

Recently, *K-ras* wild type colorectal cancer patients in metastatic stage have been considered to gain benefit from treatment with cetuximab which is the monoclonal antibody against epidermal growth factor.[21] From previous observations, the clinical signs of disease, histopathological parameters, anatomical location of tumors, age and gender of patients does not necessarily coincide with *K-ras* mutations occurrence.[22,23] However, structural changes in amino acids might modify carcinoma aggression, especially at codon 12 valine for glycine (G12V) and codon 12 aspartate for glycine (G12D). Both of these codon 12 mutations were less commonly found in our series.[8] Notably, the phenotypic consequence of *K-ras* gene Glycine→Glycine substitution should be evaluated for clinical significance, especially in the context of response to cetuximab.[24]

In conclusion, by using direct sequencing, the incidence of *K-ras* mutation and frequency of mutation type in Thailand colorectal cancer was found to be the same as reported before in several series. Therefore, ethnicity might not affect the incidence of *K-ras* mutation.

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