RESEARCH ARTICLE

Somatic Mutations of K-Ras and BRAF in Thai Colorectal Cancer and their Prognostic Value

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Abstract

Background: The study aimed to determine the incidence of K-ras and BRAF mutations in colorectal cancers (CRCs) in Thai patients and evaluate association with clinicopathological parameters including treatment outcomes in terms of event free survival (EFS). Materials and Methods: Two-hundred colorectal cancer specimens were collected for studies of K-Ras codon 12, 13 and 61, and BRAF codon 600 by polymerase chain reaction and direct nucleotide sequencing. Results: The overall incidence of K-Ras mutations in our patients was 23%. K-ras mutation frequencies in CRC stages (AJCC) I, II, III and IV were 6.7%, 16.1%, 23.3% and 26.6%, respectively (p-value>0.05). The three most common mutation forms were G12D, G12V and G13D. K-Ras mutation status was associated with poorer EFS in stage I-III CRCs (p-value 0.03). Conclusions: The study found a lower mutation frequency of K-Ras and BRAF compared to reports involving other ethnic groups. However, K-Ras mutations did have a negative prognostic value in early-stage CRCs.

Keywords: Colorectal cancer - K-Ras - BRAF - prognostic significance

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Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide (Jemal et al., 2010). The incidence of CRC in developing countries, including Thailand, has been increasing in recent years (Sriplung et al., 2006; Khuhaprema and Srivatanakul, 2008). Although surgical removal of the primary tumor is the mainstay treatment for localized CRC, other modalities including chemotherapy, external irradiation and molecular targeted therapy provide adjuvant and palliative roles in selected groups of patients. Recently, epidermal growth factor receptor (EGFR) targeting has become an integral part of progression control in advanced stage disease (Siena et al., 2009; Yen et al., 2010).

Somatic mutations within codons 12 and 13 of K-Ras have been reported in around 25-40% of CRCs, with the highest incidence in metastatic disease (Brink et al., 2003; Wu et al., 2005; Jeon et al., 2008; Neumann et al., 2009; Kummalue et al., 2011; Licara et al., 2011; Shen et al., 2011; Jakovjevic et al., 2012). Incidence and spectrum of K-Ras mutations in CRC vary according to ethnicity. The mutation status is an important predictive marker for EGFR-targeted therapy, as advanced CRCs harboring a mutated form of K-Ras seem to have a poorer response to the therapy than those with a wild-type sequence. For those reasons, it is necessary to check for K-Ras mutation status before initiation of treatment. The incidence of K-Ras mutations in Thai CRC has been reported at 37.7% (Kummalue et al., 2011). As well as K-Ras, a mutation of BRAF, which is another gene in the EGFR signaling system, has been reported as a potential predictor of targeted therapy response (Di Nicolantonio et al., 2008).

Our study aimed to explore the incidence and spectrum of K-Ras and BRAF mutations in Thai patients with CRCs and to evaluate the prognostic value of the K-Ras mutation in terms of survival outcome.

Materials and Methods

The Research Ethics Committee of the Faculty of Medicine, Prince of Songkla University, approved collection of the specimens and access to patients' electronic medical records.

CRC specimens

Two types of specimen were used in this study; snap-frozen tumor tissue from 133 CRC patients (65 females and 68 males) and formalin-fixed paraffin embedded (FFPE) tumor tissue from 67 CRC patients who developed distant metastasis. The snap-frozen tumor tissue samples were taken immediately after the surgical operation from the colonic specimen by the surgeons. The FFPE specimens were retrieved from archival tissue from

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the Department of Pathology or paraffin blocks from other institutes that were submitted to check for K-Ras mutations before targeted therapy. As most of the FFPE specimens were submitted from other institutes, and we had no history of those cases, they were not included in the subsequent clinic-pathological association analysis.

K-Ras and BRAF mutations screening by PCR and direct nucleotide sequencing

DNA was extracted from each specimen with a QIAamp DNA mini kit (Qiagen, Germany), following the manufacturer's protocol. After extraction, the quantity of DNA was measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific, DE). Mutation studies were performed using polymerase chain reaction and direct nucleotide sequencing. The study covered codons 12, 13 and 61of K-Ras and codon 600 of BRAF (the primer sequence used in this study will be provided upon request). Sequencing was performed by capillary electrophoresis on an ABI3130 automated sequencer (Applied Biosystem, Inc.). All sequences obtained were aligned with previously published nucleotide sequence NG_007524.1 (K-Ras) and NG_007873.2 (BRAF).

Clinical management of colorectal cancers

Before their surgical treatment, all patients had routine pre-operative investigations including a chest-x-ray, blood chemistry including serum albumin and carcinoembryonic antigens (CEA), and an evaluation for liver metastasis by ultrasonography and/or computerized tomography. Primary tumor staging followed the sixth edition of the TNM staging system of the American Joint Committee on Cancer (AJCC). Adjuvant chemotherapy was given to stage III colonic cancer patients, and adjuvant chemo-radiation was given to stages II and III rectal cancer cases.

All patients were scheduled for evaluation at 1-month intervals during the first year after surgery, every 3 months during the second year, and every 6 months thereafter.

Statistical analysis

Survival data used hospital-based data from the electronic hospital information system and/or death registry data on the database of the institutional Cancer Registry Unit, as of September 2012. Treatment outcomes included overall survival (OS) and event-free survival (EFS) with metastasis, local recurrence, and death set as sensors for the EFS analysis. Continuous demographic data are presented as mean and range if not stated otherwise. Associations between K-Ras mutation status and other parameters were evaluated by Chi-square test. Univariate survival analysis used the Log-rank test. Statistical significance was considered at a P-value<0.05. All analysis was done using the Stata version 6.0 program (Stata Corporation, TX).

Results

Demographic data of the patients is shown in Table 1. There were no statistically significant differences in age, sex or tumor location between cases with and without mutations.

Incidence and distribution of K-Ras and BRAF mutations in Thai CRC

In the study frozen tissue, the incidence of K-Ras mutations increased with AJCC tumor stage; 6.7% in stage I, 16.1% in stage II, 23.3% in stage III and 33.3% in stage IV (mCRCs) (Table 1). The incidence of K-Ras mutations in the FFPE specimen from stage IV patients was 17/67 (25.3%). Overall, considering both sampling methods, the K-Ras mutation frequency in mCRCs was 26.6%. There were no statistically significant differences between the incidence of mutations when considering sex, site of tumor or histological differentiation. The incidence of mutation tended to increase with tumor stage, however,

Table 1. Mutation Frequency of K-Ras, According to Clinicopathological Parameters

Characteristics	Total	K-Ras wildtype	K-Ras mutated
All	133	104 -78.20%	29 -21.80%
Age (years)			(p-value 0.6)
Median (range)	61 (25-87)	61 (26-87)	60 (25-86)
Gender			(p-value 0.94)
male	68	53 (78.60%)	15 (21.30%)
female	65	51 (77.80%)	14 (22.20%)
Tumor location			(p-value 0.86)
proximal colon	19	15 (79.00%)	4 (21.10%)
distal colon	36	27 (75.00%)	9 (25.00%)
rectum	78	62 (79.50%)	16 (20.50%)
Tumor differentiation			(p-value 0.8)
well	63	50 (78.50%)	13 (21.50%)
moderate	53	42 (79.30%)	11 (20.80%)
poor	14	10 (71.40%)	4 (28.60%)
AJCC stage			(p-value 0.19)
stage I	15	14 (93.30%)	1 (6.70%)
stage II	31	26 (83.90%)	5 (16.10%)
stage III	60	46 (76.70%)	14 (23.30%)
stage IV	27	18 (66.70%)	9 (33.30%)
pT			(p-value 0.17)
pT1-2	20	18 (90.00%)	2 (10.00%)
pT3-4	113	86 (76.10%)	27 (23.90%)
pN			(p-value 0.39)
pN0	56	47 (83.90%)	9 (16.10%)
pN1	73	54 (74.00%)	19 (26.30%)
pN2	4	3 (75.00%)	1 (25.00%)
Metastasis			(p-value 0.14)
present	28	19 (81.00%)	9 (19.10%)
absent	105	85 (67.90%)	20 (23.90%)
Pericolic fat invasion			(p-value 0.07)
present	31	28 (90.30%)	3 (9.70%)
absent	101	76 (75.30%)	26 (24.80%)
Lymph-vascular invasion			(p-value 0.813)
present	39	30 (76.90%)	9 (23.10%)
absent	71	56 (78.90%)	15 (21.10%)
CEA (ng/ml)	269.2	253.2	326.5 (p-value 0.8)
Two-year EFS	74.70%	77.70%	61.90%(p-value 0.03)
Two-year OS	79.20%	82.70%	65.70%(p-value 0.08)

Table 2. Distribution of Mutation Pattern and their Percentages Compared to All K-Ras Mutations

Codon	Nucleotide change	Amino acid change	Number of cases	% of all K-Ras mutated cases
Codon 12	GGT12GAT	G12D	26	56.50%
	GGT12GTT	G12V	8	18.60%
	GGT12GCT	G12A	1	2.20%
Codon 13	GGC13GAC	G13D	9	19.60%
Codon 61	CAA61CAC	E61H	1	2.20%
	CAA61CAT	E61H	1	2.20%

Table 3. Selected Reports of K-Ras Mutations Studies in Colorectal Cancers and the Distribution of Mutation Patterns

Author (year)	Country	n (cases)	%mutated	Percentage of each mutation type					
				G12D	G12V	G12S	G12C	G12A	G13D
Brink M (2003)	the Netherland	737	37%	27%	25%	6%	6%	6%	21%
Wu CM (2005)	Taiwan	181	27%	44%	23%	4%	6%	6%	15%
Poehlmann (2007)	Germany	65	29%	63%	27%	-	-	-	12%
Jeon CH (2008)	South Korea	78	30%	44%	22%	13%	-	-	17%
Neumann J (2009)	Germany	1,018	39%	36%	22%	7%	8%	6%	20%
Licar A (2011)	Slovenia	215	46%	38%	22%	3%	9%	7%	19%
Shen H (2011)	China	118	35%	39%	20%	5%	-	5%	29%
Kummalue T (2011)	central Thailand	106	38%	5%	18%	40%	5%	3%	20%
Jakovljevic K (2012)	Serbia	190	35%	44%	21%	5%	8%	11%	11%
Current study	southern Thailand	200	23%	57%	19%	-	-	2%	20%

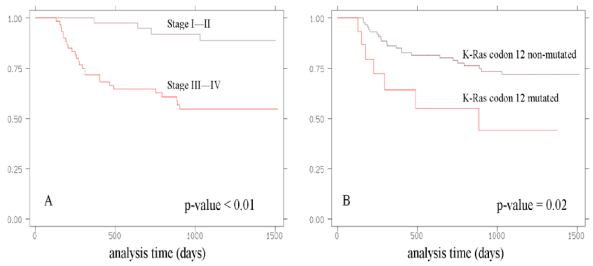

Figure 1. Kaplan-Meier Curves Showing Event Free Survival (EFS). A) stage groups and B) mutation status of K-RAS codon 12

the correlation was not statistically significant.

The distribution of K-Ras mutation is displayed in Table 2. Among the 5 types of mutation detected in this study, G-A transitions leading to a substitution of glycine by asparagine at codon 12 (G12D) was the most common (26 cases, 57% of all mutations), followed by G-T transversions leading to a substitution of glutamine at the codon 12 by valine (G12V) (8 cases, 19%). Mutations of codon 13 were detected in 9 cases (20%) and codon 61 mutations were found in only 2 cases (5%). Two mutations were detected in BRAF; one deletion mutation involving codon 599-600 and one single base substitution at codon 595 (TTT595TTG), which lead to a substitution of phenylalanine by leucine (F595L).

Association between K-Ras mutations and other clinicopathological parameters

The mean follow-up period was 41.8 months (range 16-52 months). The two-year OS and EFS rates were 79.2% and 74.7%, respectively. Survival analysis by log-rank test found that tumor stages 3-4 (p-value <0.01) and pericolic fat invasion (p-value 0.04) were the pathological parameters that were significantly associated with poorer EFS. K-Ras mutations were significantly associated with EFS status at two years, however, the association did not hold significance when a log-rank test was applied and longer follow-up data were taken into account. When each individual codon was analyzed against EFS, only codon 12 mutations were associated with a significant survival impact (p-value 0.02). (Figure 1)

On subgroup analysis, K-Ras mutations were associated with poorer EFS in stage I-III CRC (p-value 0.03). The two-year EFS rates in stage I-III CRCs with and without K-Ras mutations were 64.8% and 81.8%, respectively.

Discussion

Determination of K-Ras and BRAF mutation status has been suggested to be included in the clinical algorithm in CRCs (Lamy et al., 2011; Heideman et al., 2012; Tan and Du, 2012). Mutation status of K-Ras helps predict the response to EGFR targeted therapy, which is becoming an integral part of progress control for advanced disease (Yen et al., 2010). BRAF can be used as a molecular marker to exclude Lynch syndrome from microsatellite-instability CRCs (Domingo et al., 2005; Sinicrope and Sargent, 2012), and may define a worse-prognosis group. Previous studies have shown that mutations of K-Ras in CRC are confined to a hotspot region spanning codons 12 and 13 on exon 2, with an incidence rate ranging from 27-46% (Table 3). The overall mutation rate of 23% in our patients was rather low when compared to other studies, and thus the K-Ras mutation might not be a good diagnostic marker in our population. On the other hand, the relatively high mutation rate in stage IV disease suggests that determination of the mutation status as a predictive marker for targeted therapy can not be excluded. It has been suggested that as well as K-Ras and BRAF, mutations of PIK3CA and expression PTEN should also be screened before targeted therapy in mCRCs (Tan et al., 2012).

The prognostic value of K-Ras mutations has been demonstrated in case-control studies (Bazan et al., 2005; Conlin et al., 2005; Poehlmann et al., 2007). In our study, although the association between K-Ras mutation status and EFS was limited to stage I-III, we found that mutations at codon 12 of K-Ras were significantly associated with poorer EFS in all stages. This finding is consistent with some previous studies that found a prognostic value of codon 12 mutations in CRC (Font et al., 2001; Poehlmann et al., 2007; Winder et al., 2009; El-serafi, 2010). The prognostic value of this specific codon might be explained in a functional study which proved that malignant transformation of NIH3T3 cells by codon 12-mutated K-Ras had a more aggressive phenotype when compared to codon13-mutated K-Ras (Guerrero et al., 2000). The incidence of BRAF mutations in our patients was relatively low, compared to other reports (Tol et al., 2009; Mao et al. 2011). Interestingly, we found no cases

with mutation in BRAF hotspot codon 600, but found 2 mutations involving the nearby codon 595, suggesting that direct nucleotide sequencing might be a preferable molecular method than spot analysis that may miss a mutation in codons surrounding V600.

In summary, this study explored the incidence of KRAS and BRAF mutations in Thai patients with colorectal cancer and found a lower mutation incidence compared to previous studies in other populations. In addition, the study demonstrated a potential prognostic value of K-Ras mutation in terms of EFS in CRCs stages I-III.

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