Prevalence of KRAS, BRAF, PI3K and EGFR mutations among Asian patients with metastatic colorectal cancer

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Abstract. Mutations in oncogenes along the epidermal growth factor receptor (EGFR) signaling pathway have been implicated in the resistance to cetuximab in patients with metastatic colorectal cancer (mCRC). However, the relative significance of these mutations based on their frequencies of occurrence in the Singaporean population remains unclear. In the present study, the prevalence of Kirsten rat sarcoma viral oncogene homolog (*KRAS*), v-Raf murine sarcoma viral oncogene homolog B (*BRAF*), phosphoinositide 3-kinase (*PI3K*) and *EGFR* somatic mutations were determined among Singaporean patients with mCRC. DNA extracted from 45 pairs of surgically resected tumor and normal mucosa samples was subjected to direct sequencing or restriction fragment length polymorphism. Associations of the genetic mutations with various clinicopathological parameters were further explored. Mutations in either codon 12 or 13 of *KRAS* were confirmed as prominent phenomena among the included Singaporean mCRC patients, at a prevalence comparable with that of Caucasian and patients of other Asian ethnicities [33.3% (90% confidence interval, 21.8-44.9%)]. *KRAS* mutation was not associated with clinicopathological features, including age, gender and ethnicity of patients, or the tumor site, differentiation and mucinous status. Conversely, the prevalence of *BRAF* (0%), *PI3K* (2.2%) and *EGFR* (0%) mutations were low. The results of the present study indicate that *KRAS* mutations are prevalent among the studied population, and confirm the low prevalence of *BRAF*, *PI3K* and *EGFR* mutations. *KRAS* should be prioritized as an investigational gene for future studies of predictive biomarkers of cetuximab response among Singaporean patients with mCRC.

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Introduction

Over the past few decades, the incidence of colorectal cancer (CRC) has escalated rapidly in Asian countries (1). In Singapore, CRC is the most commonly diagnosed cancer, accounting for 17.6 and 13.9% of cancers in males and females, respectively (2). The relatively high incidence of this disease has prompted efforts by clinicians and scientists to enhance the therapeutic management of CRC. Cetuximab (Erbitux®) is a monoclonal antibody used widely in the targeted treatment of metastatic CRC (mCRC). It binds to the epidermal growth factor receptor (EGFR) and attenuates its downstream oncogenic signaling along the RAS/rapidly accelerated fibrosarcoma (RAF)/mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT axes, thereby inhibiting tumor growth and progression (3). However, resistance to cetuximab remains a relevant issue. Studies have indicated that up to 80% of patients may incur additional treatment costs and skin toxicity without deriving a beneficial response from the treatment (4-6). For example, in patients with chemotherapy-refractory colorectal cancer whose tumors express EGFR, 9% [95% confidence interval (CI), 3-19%] achieved a partial response. Toxicities such as an acne-like skin rash, predominantly on the face and upper torso, were experienced in 86% of the patients (6). In another study conducted on patients with irinotecan-refractory metastatic colorectal cancer, the rate of response to combination therapy of cetuximab plus irinotecan was 22.9%, while that of cetuximab monotherapy was 10.8% (5). The identification of predictive markers of cetuximab response is therefore pertinent to improving the cost-effectiveness of the treatment and optimizing the quality of life for patients.

Table I. Primers for polymerase chain reaction and gene sequencing.

Gene	Primer sequence	Annealing temperature, ˚C	Product, bp
KRAS			
Exon 2	Forward: 5'-GGTGGAGTATTTGATAGTGTATTAACC-3'		
	Reverse: 5'-AATGGTCCTGCACCAGTAATATG-3'	60	246
Exon 3	Forward: 5'-TCTTTGGAGCAGGAACAATG-3'		
	Reverse: 5'-TGCATGGCATTAGCAAAGAC-3'	55	402
BRAF			
Exon 11	Forward: 5'-TCCCTCTCAGGCATAAGGTAA-3'		
	Reverse: 5'-CGAACAGTGAATATTTCCTTTGAT-3'	55	313
Exon 15	Forward: 5'-TCATAATGCTTGCTCTGATAGGA-3'		
	Reverse: 5'-GGCCAAAAATTTAATCAGTGGA-3'	55	224
PI3K			
Exon 9	Forward: 5'-GGGAAAAATATGACAAAGAAAGC-3'		
	Reverse: 5'-CTGAGATCAGCCAAATTCAGTT-3'	55	250
Exon 20	Forward: 5'-TTTGCTCCAAACTGACCAA -3'		
	Reverse: 5'-TGGAATCCAGAGTGAGCTTTC -3'	55	349
EGFR			
Exon 18	Forward: 5'-GGCACTGCTTTCCAGCAT-3'		
	Reverse: 5'-CCCCACCAGACCATGAGA-3'	60	248
Exon 19	Forward: 5'-CCCAGTGTCCCTCACCTTC-3'		
	Reverse: 5'-CCACACAGCAAAGCAGAAAC-3'	60	239
Exon 21	Forward: 5'-TGATCTGTCCCTCACAGCAG-3'		
	Reverse: 5'-TCAGGAAAATGCTGGCTGAC-3'	60	231

Kirsten rat sarcoma viral oncogene homolog (*KRAS*), ensembl assession number ENSG00000133703; v-Raf murine sarcoma viral oncogene homolog B (*BRAF*), ensembl assession number ENSG00000157764; phosphoinositide 3-kinase (*PI3K*), ensembl assession number ENSG00000121879; epidermal growth factor receptor (*EGFR*), ensembl assession number ENSG00000146648.

In predicting the response of patients to anti-EGFR therapy, various genetic alterations along the EGFR pathway have emerged as promising markers. Landmark trials, including the multicenter CRYSTAL and OPUS studies, have revealed that activating mutations in Kirsten rat sarcoma viral oncogene homolog (*KRAS*), a critical regulatory protein along the RAS/RAF/MAPK axis, abrogate the therapeutic effect of cetuximab and serve as powerful negative predictors of its clinical efficacy (7-11). Therefore, major advisory bodies have promulgated restricting the administration of cetuximab to patients with mCRC and wild-type *KRAS* status (12,13). More recently, persuasive evidence has emerged for cetuximab resistance conferred by mutations in v-Raf murine sarcoma viral oncogene homolog B (*BRAF*) and *PI3K*, regulators of the RAS/RAF/MAPK and PI3K/AKT pathways respectively (14,15). Additionally, *EGFR* gene mutations, common features in non-small-cell lung cancer (NSCLC), have been linked to the efficacy of EGFR tyrosine kinase inhibitors including gefitinib (16-19). Given the similar mechanism of action of cetuximab and gefitinib, mutation at the *EGFR* tyrosine kinase domain could theoretically alter the sensitivity to cetuximab of mCRC.

While compelling data exists on the aforementioned mutations as potential predictive markers of cetuximab resistance in predominantly Caucasian patients with mCRC, the relevance and importance of these findings within specific populations in Asia depends upon the local prevalence of these genetic alterations. Despite widespread efforts to establish the prevalence of these mutations among Asian countries, including China and Japan (20-26), there is scarce data regarding their prevalence in Singapore, a country with ethnic diversity comprising Chinese, Malay and Indian individuals. As ethnicity and lifestyle may influence mutation patterns (1), it is important to investigate and establish the prevalence of these genetic mutations among patients with mCRC in Singapore. A thorough review of the literature to date was conducted by searching the following keywords on PubMed in June 2014: 'KRAS OR K-Ras OR BRAF OR B-Raf OR PI3KCA OR PI3K-CA OR PI3K OR PIK3CA OR PIK3-CA OR PIK3 OR EGFR' AND 'colorectal cancer OR rectal cancer OR colon cancer' AND 'metastatic' AND 'Singapore'. The search revealed only one relevant study, which assessed *KRAS* mutations in eight mCRC tumors in Singapore (27). Furthermore, the frequencies of other genetic mutations relevant to the chemoresistance of cetuximab (*BRAF*, *PI3K* and *EGFR*) were not analyzed.

In order to establish this information, the present study aimed to comprehensively profile the frequencies of mutations in the hotspot regions of *KRAS*, *BRAF*, *PI3K* and *EGFR* in Singaporean patients with mCRC. The associations between the gene mutations and various clinicopathological characteristics were further examined. The understanding of their prevalence will help prioritize

Table II. Mutational analysis methods for *KRAS*, *BRAF*, *PI3K* and *EGFR* genes.

Gene	Mutations	Analysis method
KRAS	Codon 12, 13 (Exon 2) Codon 61 (Exon 3)	Gene sequencing
BRAF	Codon 439, 459 (Exon 11) Codon 600, 601 (Exon 15)	Gene sequencing
PI3K	Codon 542, 545 (Exon 9) Codon 1043, 1047 (Exon 20)	Gene sequencing
EGFR	G719S (Exon 18)	RFLP
	L858R (Exon 21)	RFLP
	Deletions (Exon 19)	Gene sequencing

KRAS, Kirsten rat sarcoma viral oncogene homolog; *BRAF*, v-Raf murine sarcoma viral oncogene homolog B; *PI3K*, phosphoinositide 3-kinase; *EGFR*, epidermal growth factor receptor; RFLP, restriction fragment length polymorphism.

Table III. Clinicopathological characteristics of 45 patients with metastatic colorectal cancer.

Characteristic	Value
Age, years	
Mean	59
Range	30-83
Gender, n (%)	
Male	29 (64.4)
Female	16 (35.6)
Ethnicity, n (%)	
Chinese	34 (75.6)
Malay	7 (15.6)
Indian	4 (8.9)
Tumor site, n (%)	
Ascending colon	1 (2.2)
Hepatic flexure	1 (2.2)
Sigmoid colon	21 (46.7)
Rectosigmoid	6 (13.3)
Rectum	16 (35.6)
Tumor differentiation[a], n (%)	
Moderate	39 (86.7)
Poor	6 (13.3)

[a]All samples were moderately or poorly differentiated; no samples were well-differentiated.

investigational genes for future studies of predictive biomarkers of cetuximab response.

Materials and methods

Patients and tissue samples. Patients with mCRC (Dukes' Stage D) who underwent surgical tumor resection at the Singapore General Hospital (Singapore) between June 2010 and October 2012 were included in the current study. The inclusion criteria were as follows: i) Histologically confirmed mCRC; ii) availability of sufficient amounts of tissue samples from the primary lesions for mutational analyses; and iii) availability of clinical information.

Paired tumor and mucosal tissues were snap-frozen in liquid nitrogen, microdissected and stored at -80˚C until analysis. Careful microdissection ensured that ≥90% of the tumor specimen comprised cancer cells. Matched normal mucosa samples were obtained ≥5 cm from the edges of the tumor. Clinicopathological parameters, including the age, gender and ethnicity of the patients, tumor site, degree of histological differentiation and histological type (mucinous or non-mucinous) were recorded. The study was approved by the Institutional Review Board of the Singapore General Hospital (2010/041/B) and informed consent was obtained from all participants.

DNA extraction and polymerase chain reaction (PCR) amplification. Genomic DNA was extracted from tissue samples using the QIAmp DNA Mini kit (Qiagen, Alameda, CA, USA), according to the manufacturer's instructions, and subjected to PCR to amplify *KRAS* exons 2 and 3, *BRAF* exons 11 and 15, *PI3K* exons 9 and 20 and *EGFR* exons 18, 19 and 21. The primers used for PCR amplification were synthesized using First BASE Laboratories Sdn Bhd (Singapore) and are listed in Table I. These exons were selected for amplification as they encompass the mutational hotspots (Table II).

Each PCR reaction contained ~300 ng of genomic DNA, 2 μl each of forward and reverse primers (10 μM), 20 μl of 5 M betaine (Sigma-Aldrich, St. Louis, MO, USA), 5 μl of 2 mM deoxynucleotide triphosphates, 2 μl of 25 mM $MgSO_4$ and 1 μl of Novagen KOD Hot Start DNA polymerase (all from Merck Millipore, Tokyo, Japan) made up to a final volume of 50 μl. PCR cycling consisted of an initial denaturation at 94˚C for 2 min, 35 cycles of denaturation at 94˚C for 15 sec, primer annealing at 55 or 60˚C (as stated in Table I) for 30 sec and elongation at 68˚C for 1 min, followed by a final extension at 68˚C for 5 min. PCR products were then verified by 1.5% agarose gel electrophoresis (Sigma-Aldrich) and purified using a Multiscreen® $PCR_{\mu 96}$ plate (Merck Millipore, Carrigtwohill, Ireland) prior to either direct gene sequencing or restriction fragment length polymorphism (RFLP) analyses of the mutational hotspots (Table II) (28).

Gene sequencing. Purified PCR products of *KRAS* exons 2 and 3, *BRAF* exons 11 and 15, *PI3K* exons 9 and 20 and *EGFR* exon 19 were sequenced with BigDye® Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems Life Technologies, Foster City, CA, USA) as per the manufacturer's instructions, and purified and analyzed with a 3730 ABI capillary electrophoresis system (Applied Biosystems Life Technologies). All sequencing reactions were performed using forward primers as stated in Table I, with the exception of *PI3K* exons 9 and 20 in which 5'-GGGAAAAATATGACAAAGAAAGCTATA-3' and 5'-TTGCTCCAAACTGACCAAAC-3' were used, respectively. DNA of normal mucosae from each patient was also amplified and sequenced alongside matched tumor DNA samples to rule out the occurrence of non-somatic mutations or polymorphisms.

Table IV. Types of *KRAS* mutation detected in codons 12 and 13.

KRAS exon 2	Wild-type (amino acid)	Point mutation (amino acid)	Mutations, n (%)
Codon 12	GGT (G)	GAT (D)	7 (46.7)
	GGT (G)	GTT (V)	2 (13.3)
	GGT (G)	AGT (S)	1 (6.7)
	GGT (G)	GCT (A)	1 (6.7)
Codon 13	GGC (G)	GAC (D)	4 (26.7)

Amino acids: G, Glycine; D, Aspartic acid; V, Valine; S, Serine; A, Alanine. *KRAS*, Kirsten rat sarcoma viral oncogene homolog.

Table V. Associations between *KRAS* mutation and clinicopathological characteristics.

Characteristic	All (n=45)	*KRAS* wild-type (n=30)	*KRAS* mutant (n=15)	P-value
Age (mean ± SD), years[a]	59	56.6±10.2	64.5±8.9	0.013
Gender, n (%)[b]				
Male	29	16 (55.2)	13 (44.8)	0.028
Female	16	14 (87.5)	2 (12.5)	
Ethnicity, n (%)[c]				
Chinese	34	23 (67.6)	11 (32.4)	0.137
Malay	7	6 (85.7)	1 (14.3)	
Indian	4	1 (25.0)	3 (75.0)	
Tumor site, n (%)[b]				
Colon	29	22 (75.9)	7 (24.1)	0.078
Rectum	16	8 (50.0)	8 (50.0)	
Tumor differentiation, n (%)[c]				
Moderate	39	29 (74.4)	10 (25.6)	0.012
Poor	6	1 (16.7)	5 (83.3)	
Histological type, n (%)[c]				
Mucinous	6	2 (33.3)	4 (66.7)	0.157
Non-mucinous	39	28 (71.8)	11 (28.2)	

Obtained by [a]independent t-test, [b]χ^2 test or [c]Fisher's exact test. *KRAS*, Kirsten rat sarcoma viral oncogene homolog; SD, standard deviation.

RFLP analysis. The presence of G719S (*EGFR* exon 18) and L858R (*EGFR* exon 21) mutations were determined by RFLP analyses using restriction endonucleases *Dde*I and *Sau*96I (New England Biolabs, Inc., Singapore) (29), respectively. Purified PCR product (15 μl) was digested with 10 units of *Dde*I or *Sau*96I in a total volume of 20 μl at 37˚C for 2 h, and electrophoresed through a 2.5% agarose gel. Upon digestion by restriction enzyme *Dde*I, the wild-type allele of *EGFR* exon 18 produced fragments at 27 and 221 bp while the mutant G719S allele yielded fragments at 27, 92 and 129 bp. The SW48 human colorectal adenocarcinoma cell line (American Type Culture Collection, Manassas, VA, USA), which harbors a heterozygous G719S mutation (30), was run alongside as a positive control. Upon digestion by *Sau*96I, the wild-type allele of *EGFR* exon 21 yielded fragments at 55 and 176 bp, while the mutant L858R allele produced three fragments (55, 86 and 90 bp).

Statistical analysis. The normal approximation method was used to construct a 90% CI in estimating the prevalence of genetic mutation. This conservative CI was used due to the small sample size. Associations of genetic mutations with clinicopathological parameters, including gender, ethnicity, tumor location, tumor differentiation and histological type were explored using the χ^2 or Fisher's exact tests (SPSS version 16; SPSS Inc, Chicago, IL, USA). Associations with age were evaluated using an independent samples Student's t-test. A Bonferroni correction for multiple testing was performed by dividing the critical P-value (P=0.05) by the number of comparisons being made (n=6). Therefore, statistical significance was established at P<0.008.

Results

Patient characteristics. A total of 45 patients with mCRC, comprising the major ethnic groups in Singapore (34 Chinese, 7 Malay and 4 Indian patients) and reflecting their prevailing population distribution were enrolled into the study. Tumors were located predominantly in the sigmoid colon (46.7%),

Table VI. Frequencies of *KRAS*, *BRAF* and *PI3K* mutations in different populations of patients with mCRC.

			KRAS		*BRAF*		*PI3K*	
Study[a]	Location of population	n[b]	Codons/exons examined[c]	Prevalence of mutation	Codons/exons examined[c]	Prevalence of mutation	Codons/exons examined[c]	Prevalence of mutation
Current study	Singapore	45	Codons 12, 13 and 61	33.3% (15/45)[d] [90% CI, 21.8-44.9%]	Codons 439, 459, 600 and 601	0.0% (0/45)	Codons 542, 545, 1043 and 1047	2.2% (1/45)
Pang *et al* (27)	Singapore	8	Codons 12, 13 and 61	37.5% (3/8) in cytological specimens; 50.0% (4/8) in tumor[d]	Analysis conducted only on *KRAS* wild-type cases	-	-	-
Di Nicolantonio *et al* (31)	Italy/ Switzerland	113	Codons 12 and 13	30.1% (34/113)	Codon 600	9.7% (11/113)	-	-
Frattini *et al* (32)	Switzerland	27	Codons 12 and 13	37.0% (10/27)	-	-	-	-
Mollinari *et al* (33)	Italy/ Switzerland	111	Codons 12, 13 and 61	41.4% (46/111)	Codon 600	8.1% (9/111)	Codons 542, 545 and 1047	10.1% (11/109)
Lievre *et al* (34)	France	30	Codons 12 and 13	43.3% (13/30)	Exons 11 and 15	0.0% (0/30)	Exons 1, 2, 9 and 20	6.7% (2/30) (E542 K)
Gao *et al* (20)	China	59	Codons 12 and 13	18.6% (11/59)	Codon 600	8.5% (5/59)	-	-
Li *et al* (21)	China	190	Codons 12 and 13	31.1% (59/190)	-	-	-	-
Mao *et al* (22)	China	61	Codons 12 and 13	36.8% (21/57)	Codon 600	25.4% (15/59)[e]	Codons 542, 545 and 1047	8.2% (5/61)
Kato *et al* (23)	Japan	28	Codons 12 and 13	25.0% (7/28)	-	-	Codons 542, 545, 1043 and 1047	14.3% (4/28)
Kimura *et al* (24)	Japan	61	Codons 12 and 13	34.4% (21/61)	-	-	-	-
Ito *et al* (25)	Japan	242	Codons 12 and 13	43.8% (106/242)	Analysis conducted only on *KRAS* wild-type cases	-	Analysis conducted only on *KRAS* wild-type cases	-
Nakanishi *et al* (26)	Japan	34	Codons 12 and 13	50.0% (17/34)	Codon 600	8.8% (3/34)	-	-

[a]Studies involved unselected patients [i.e. no biased inclusion criteria (e.g. chemotherapy-refractory) or exclusion criteria (e.g. history of neoplasm)]. [b]Sample size of patients with mCRC. [c]Only data derived from direct sequencing was presented to eliminate technical variation that may obscure population differences. [d]All mutations were observed in either codon 12 or 13. [e]Authors noted that *KRAS* and *BRAF* mutations were not mutually exclusive. *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *BRAF*, v-Raf murine sarcoma viral oncogene homolog B; *PI3K*, phosphoinositide 3-kinase; mCRC, metastatic colorectal cancer; CI, confidence interval.

rectum (35.6%) and rectosigmoid region (13.3%) and were moderately or poorly differentiated. Table III summarizes the clinicopathological characteristics of the recruited patients.

KRAS mutational profiling. Tumor *KRAS* mutation was identified in 15 patients, equal to a prevalence of 33.3% (90% CI, 21.8-44.9%). In addition, 11 mutations (73.3%) were identified in codon 12, while 4 mutations occurred in codon 13 (26.7%). The types of gene mutations detected in *KRAS* are tabulated in Table IV. The most frequently observed mutation was a GGT>GAT transition (G12D). By contrast, no mutations were detected in codon 61 of exon 3. No normal mucosae exhibited any mutations, indicating that all tumor mutations were somatic in nature.

Correlation of KRAS gene mutations with clinicopathological characteristics. No statistically significant differences were identified in terms of age, gender, ethnicity, tumor site, tumor differentiation and mucinous status between patients with and without *KRAS* mutations (P>0.008; Table V).

PI3K mutational profiling. Of the 45 tumor samples, only one sample (2.2%) harbored a somatic mutation of the *PI3K* gene. The observed *PI3K* mutation was a heterozygous GAG>GCG transversion in codon 545 of exon 9 (E545A), and was identified in a sigmoid colonic tumor displaying *KRAS* wild-type, resected from a 30-year-old female patient of Chinese ethnicity (the youngest patient in the cohort).

BRAF and EGFR mutational profiling. No mutations (0/45 samples) were detected in codons 439, 459, 600 and 601 of the *BRAF* gene. Similarly, all samples exhibited wild-type status at codons 719 and 858 of the *EGFR* gene. No deletion mutations were observed at *EGFR* exon 19.

Discussion

Mutations in *KRAS*, *BRAF* and *PI3K*, encoding the key regulatory proteins downstream of EGFR, play vital roles in colorectal carcinogenesis and have been closely linked with clinical resistance to cetuximab (7-11,14-15). To further elucidate the importance of these genetic alterations in the context of Singaporean mCRC, their currently undefined local prevalence was characterized in the present study. The results revealed a substantial occurrence of *KRAS* mutations, the frequency of which (33.3%) resembled that in north Asian (e.g. Chinese and Japanese) and Caucasian populations of mCRC patients (20-50%) (20-26,31-34). For comparison, representative studies from Japan, China and Europe, in which direct sequencing of *KRAS* were conducted at similar codons, are summarized in Table VI. The substantial prevalence of *KRAS* mutations provides a strong basis for future investigations on its utility as a predictor of cetuximab efficacy in the Singaporean population. Notably, the observed mutations were located exclusively in codons 12 and 13 of exon 2, consistent with reports on its predominance (90%) in exon 2 and infrequent occurrence at codon 61 of exon 3 (35). It is also noteworthy that codons 12 and 13 of exon 2 encode for two adjacent glycine residues situated in close proximity to the catalytic site of *KRAS*. Mutations of these codons abolish the intrinsic guanosine triphosphatase activity of the KRAS protein (35), leading to its constitutive activation and tumor growth. Within exon 2, the distribution of mutations between codons 12 and 13 was also congruent with prior reports of patients with mCRC, in which ~70% of mutations occurred at codon 12 (20,32,33). In addition, G12D (GGT>GAT) was revealed to be the most prominent mutation type, in concordance with evidence from Chinese and Caucasian mCRC patients (21,22,32). Taken together, codons 12 and 13 represent potential subjects of interest for future Singapore-based studies investigating the role of *KRAS* mutation status in predicting treatment response.

A number of studies conducted in Caucasian and Asian CRC populations found no association between the prevalence of *KRAS* mutations and various clinicopathological parameters, including the gender and age of patients as well as tumor location, histological type and differentiation (23,26,36-38). Analogous findings were also evident among mCRC patients from Asian populations (39). Similarly, the various clinicopathological parameters of Singaporean patients with mCRC investigated in the present study did not correlate significantly with the occurrence of *KRAS* mutations. Such poor correlation between genotype and phenotype is not unexpected, as CRC is a heterogeneous disease defined by host genetic, environmental, nutritional and gut microbial factors (40).

The present study also revealed an extremely low prevalence of *BRAF* and *PI3K* mutations. Encoding a downstream effector of KRAS in the MAPK pathway, *BRAF* has also has been studied extensively with regard to CRC. The *BRAF* V600E mutation has been documented to occur at a lower rate (0-10%) than *KRAS* mutations in Caucasian and Asian mCRC patients (20,31,33,34) (Table VI). This observation was reflected in the present study, in which no *BRAF* mutations were detected. By contrast, the mutation rate of the gene encoding PI3K (2.2%), a regulator of PI3K/AKT signaling, appeared to be marginally lower compared with that of Chinese and Caucasian mCRC populations (~10%) (22,23,33,34) (Table VI). The low observed frequency of *PI3K* mutations may possibly be explained by environmental influences, such as diet and lifestyle, or a difference in hotspot codons in Singaporean patients.

As *EGFR* gene mutation has been a crucial determinant of the sensitivity of NSCLC to EGFR tyrosine kinase inhibitors, it was of interest to determine its mutation rate in patients with mCRC. In the present study, however, neither missense (G719S in exon 18 and L858R in exon 21) nor deletion mutations (in exon 19) were identified. Specifically, although the *EGFR* G719S mutation, an NSCLC-relevant somatic mutation, was previously discovered in the SW48 colon cancer cell line (30), the present data suggested this mutation was not clinically prevalent in the context of mCRC. The paucity of *EGFR* somatic mutations in Singaporean patients with mCRC was consistent with findings in their Caucasian counterparts (28). This highlights the presence of a different set of genetic alterations that drives the progression of mCRC compared with NSCLC. Considering that the *EGFR* activating gene mutation is responsible for the sensitivity of NSCLC to EGFR tyrosine kinase inhibitors, the non-existence of activating *EGFR* mutations may also explain the general lack of response towards anti-EGFR therapy in mCRC. Nevertheless, with regard to predicting the efficacy of cetuximab therapy, the present

analyses demonstrated collectively that *BRAF*, *PI3K* and *EGFR* mutations assume less significant roles, owing to their rarity of occurrence, compared with that of *KRAS* among Singaporean mCRC patients.

In conclusion, the frequencies of *KRAS*, *BRAF*, *PI3K* and *EGFR* mutations were determined in the Singaporean mCRC population, and *KRAS* mutations were confirmed to be prominent phenomena. The present study thereby lays the foundation for future investigations into predictive biomarkers of cetuximab response, and represents an important step towards personalized medicine for the local Singaporean mCRC population.

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