Analysis of *KRAS* Mutations in Cases of Metastatic Colorectal Cancer at a Single Institution in Tochigi, Japan

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Key Words

Metastatic colorectal cancer · *KRAS* · Carcinogen · Mutational analysis

Abstract

Objective: Colorectal cancer patients bearing wild-type *KRAS* benefit from anti-epidermal growth factor receptor (EGFR) antibody treatment. Since clinical studies showed the efficacy of anti-EGFR antibody treatment for metastatic colorectal cancer (mCRC), we analyzed *KRAS* mutations in mCRC to gain insight into the association between these mutations and clinicopathological characteristics. **Methods:** *KRAS* mutations were analyzed in 109 tissue samples of mCRC using amplification refractory mutation system-Scorpion (ARMS/S) assay (68 samples) and direct sequencing (41 samples). **Results:** In the ARMS/S assay, 36.5 and 7.4% of mCRCs harbored mutations at codons 12 and 13, respectively. In direct sequencing, corresponding values were 24.4 and 19.5%. Overall, 37.6% (codon 12/13, 25.7/11.9%) of mCRCs harbored *KRAS* mutations. No significant differences were found between *KRAS* mutations and clinicopathological variables. Among mCRC patients <65 years of age, the incidence of *KRAS* mutations at codon 13 was significantly higher in female than male patients ($p = 0.035$). **Conclusion:** The incidence of *KRAS* mutations in mCRC was similar to that of non-mCRC as previously

reported. *KRAS* codon 13 mutations might be associated with younger female patients with mCRC, but further investigation is necessary to clarify the association between this type of mutation and metastatic potential in female CRC patients.

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Introduction

The incidence of colorectal cancer (CRC) has recently increased remarkably in Japan with a reported incidence of 69,901 tumors located in the colon and 37,003 in the rectum in 2006 [1]. In addition, a shift in the distribution of CRCs towards the right-sided colon has occurred in Japan [2], and major determinants of this shift may be related to the increasing proportion of elderly people and/or changes in dietary habits [2, 3]. In addition, the incidence of right-sided colon cancer in Japan has increased in female patients [2]. Of note, right-sided colon cancer is more likely to be detected at an advanced stage as compared to left-sided colon cancers [4].

For the treatment of metastatic CRC (mCRC), strategies including conventional chemotherapy and molecu-

N.S. and S.T. contributed equally to this work.

larly targeted therapy are still needed. For cases of mCRC, anti-epidermal growth factor receptor (EGFR) antibody (cetuximab, etc.), a molecularly targeted agent, has recently been developed as an optional treatment [5, 6]. This new agent leads to cancer inhibition via cell cycle arrest and apoptosis, as well as to anti-angiogenic effects associated with the functions of the downstream effectors of the EGFR signaling cascade [6]. KRAS protein is an important downstream component of the EGFR signaling cascade [5], and its mutation leads to loss of proper regulation of this signaling pathway [7]. Clinical studies have shown that the *KRAS* mutation is an established predictive biomarker of resistance to cetuximab in patients with mCRC [8–10]. Since patients with a tumor bearing wild-type *KRAS* benefit from cetuximab, mutational analysis of this gene has become an essential molecular test for patients with mCRC. In this setting, a mutation-specific real-time polymerase chain reaction (PCR)-based technique combining amplification refractory mutation system (ARMS) and a unique bi-functional fluorescent primer/probe molecule (Scorpion) has been introduced and recommended for *KRAS* mutational analysis [11, 12].

In this study, we analyzed *KRAS* mutations in 109 cases of mCRC at a single institution in Japan to gain insight into the association between these mutations and clinicopathological characteristics, and to facilitate the development of a better molecular targeting therapy.

Materials and Methods

Patients and Tissue Samples

We obtained 109 tissue samples of synchronous or metachronous mCRC by surgical resection ($n = 91$) or biopsy ($n = 18$) from 109 patients at the Dokkyo Medical University School of Medicine, Tochigi, Japan, between 2007 and 2012. The 109 patients comprised 63 males and 46 females, with a mean age of 63.5 years (range 20–82). The tumor location in the colon was classified as right side (cecum, appendix, ascending colon, and transverse colon) or left side (descending colon, sigmoid colon, and rectum). The histological classification was adopted as its predominant histological component. For example, for a tumor comprising mainly of well-differentiated adenocarcinoma with a partial moderately-differentiated adenocarcinoma component, a diagnosis of well-differentiated type was made. Depth of tumor invasion and tumor staging were assessed according to the UICC/TNM classification.

Informed consent was obtained from all patients. To adhere to the ethics prescriptions, a linkable anonymizing method was used to ensure the study was conducted in a blinded manner. Samples used in this study were surgical or biopsy materials obtained for diagnosis or treatment and not for research purposes. Participation in the present study did not increase medical disadvantage or risk for patients, and data were used strictly for analysis of information as part of the therapeutic intervention.

Analysis of KRAS Mutations

DNA Extraction

Genomic DNA was extracted from five 10- μ m-thick formalin-fixed paraffin-embedded tissue sections, in which tumor cells occupied >70% using the DNA FFPE Tissue Kit (Qiagen, Valencia, Calif., USA). The concentration and purity of the extracted DNA was determined by spectrophotometry.

ARMS-Scorpion Assay

For the ARMS-Scorpion (ARMS/S) assay, *KRAS* mutations were detected using the *KRAS* Mutation Test Kit (TheraScreen, DxS-Qiagen, Manchester, UK) in accordance with the manufacturer's instructions. Purified genomic DNA was applied for a set of 7 known *KRAS* codon 12 or 13 mutation-detecting reactions or a control reaction. Reactions were conducted in a LightCycler II RT-PCR instrument (Roche Diagnostics, Tokyo, Japan) and analyzed using LightCycler Adapt software v1.1 (Roche Diagnostics) [11, 12].

Direct Sequencing

Mutational analyses for *KRAS* were performed using genomic DNA by PCR followed by direct sequencing. The primer sequences were as follows: forward: 5'-GTGTGACATGTTCTAATATAGTCA-3', and reverse: 5'-GAATGGTCCTGCACCAGTAA-3' with minor modification. The BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif., USA) and ABI 3130xl Genetic Analyzer (Applied Biosystems) were used according to the manufacturer's instructions. Analyses of the DNA sequences were performed with the Sequence Scanner Software (Applied Biosystems).

Statistical Analysis

All statistical analyses were carried out using StatView for Windows version 5.0 (SAS Institute Inc., Cary, N.C., USA). Categorical analysis of variables was performed using either the χ^2 test (with Yates' correction) or Fisher's exact test, as appropriate. A p value <0.05 was considered statistically significant.

Results

In this study, *KRAS* mutations were analyzed using the ARMS/S assay for 68 cases and direct sequencing for 41 cases. The concordance of both methods was also confirmed in some cases. In the ARMS/S assay, 36.5% (18/68 cases) of mCRCs harbored mutations at codon 12, and 7.4% (5/68) had mutations at codon 13. In direct sequencing, 24.4% (10/41) harbored mutations at codon 12, and 19.5% (8/41) had mutations at codon 13. No significant differences were found in the incidence of *KRAS* mutations between the two methods. In total, 25.7% (28/109) of mCRCs harbored mutations at codon 12, and 11.9% (13/109) had mutations at codon 13 [overall, 37.6% (41/109) mutations at codons 12 and 13]. There were 14 of 28 (50.0%) tumors with mutations from GGT (Gly) to GAT (Asp), 9 of 28 (32.1%) tumors with mutations from

Table 1. Associations of clinicopathological variables with mutational status of *KRAS* in mCRC

Variable	Mutation at codon 12			Mutation at codon 13		
	positive (n = 28)	negative (n = 81)	p value	positive (n = 13)	negative (n = 96)	p value
Sex						
Male	19	44	0.269	5	58	0.148
Female	9	37		8	38	
Age						
<65 years	14	38	0.829	7	45	0.770
≥65 years	14	43		6	51	
Location						
Right side	11	20	0.152	5	26	0.513
Left side	17	61		8	70	
Tumor						
Well 4	1	1	0.251	1	14	0.813
Moderate	17	50		10	57	
Poor	1	13		1	13	
Mucinous	3	3		1	5	
Others (adenosquamous)	1	1		0	2	
n.a.	2	3		0	5	
Depth of tumor invasion						
pT2	1	1	0.738	0	2	0.802
pT3	12	38		6	44	
pT4	10	31		6	35	
n.a.	5	11		1	15	
Lymph node metastasis						
Positive	17	53	0.718	7	63	0.152
Negative	5	16		5	16	
n.a.	6	12		1	17	
TNM stage						
II	1	8	0.559	2	7	0.717
III	8	20		3	25	
IV	17	46		8	55	
n.a.	2	7		0	9	

n.a. = Not available.

GGT (Gly) to GTT (Val), 3 of 28 (10.5%) tumors with mutations from GGT (Gly) to AGT (Ser), 1 of 28 (3.6%) tumors with mutations from GGT (Gly) to GCT (Ala), and 1 of 28 (3.6%) tumors with mutations from GGT (Gly) to TGT (Cys) at codon 12. In addition, all 13 tumors showed mutations from GGC (Gly) to GAC (Asp) at codon 13.

No significant differences were found in mCRC patients with mutational status of *KRAS* codon 12 or 13 with regard to clinicopathological variables (table 1). Among mCRC patients under 65 years of age, the incidence of *KRAS* mutations at codon 13 was significantly higher in females (26.1%, 6/23) than males (3.6%, 1/29; $p = 0.035$; table 2). There were no significant differences in tumor

Table 2. Associations of sex and age with mutational status of *KRAS* in mCRC

Variable	Mutation at codon 12		Mutation at codon 13	
	positive (n = 28)	negative (n = 81)	positive (n = 13)	negative (n = 96)
Male <65 years	10	19	1	28*
Male ≥65 years	9	25	4	30
Female <65 years	4	19	6	17*
Female ≥65 years	5	18	2	21

* $p = 0.035$.

location among the younger (<65 years) female patients with mCRC harboring a *KRAS* mutation at codon 13 (data not shown).

Discussion

In this study, the *KRAS* mutation rate in patients with mCRC was 37.6% (codon 12/13, 25.7/11.9%), which was similar to previous studies showing that *KRAS* was mutated in about 30–40% (codon 12/13, 25.5/14.5%) of mCRCs [5, 6, 9, 10]. Similar to mCRC, *KRAS* mutations have been reported to occur in 36–41% of non-mCRCs [8, 13]. With regard to subsites of *KRAS* mutations, mutations at codon 12 (approximately 70–80%) were predominant as opposed to those at codon 13 [7, 14], in line with our results that mutations at codons 12 and 13 were present in 68.4 and 31.6%, respectively. Furthermore, in the current study, *KRAS* codon 13 mutations were detected more frequently in female than in male patients who were <65 years of age. This fact is supported by several lines of evidence suggesting that female sex hormones, via their effects on bile acid production, bowel transit time, colonic bacteria and production of volatile fatty acids, are related to colorectal mutagens [13, 15]. By contrast, *KRAS* codon 12 G→C transversions were mainly observed in male patients with tumors in the distal colon and in female patients with tumors in the proximal colon [16]. In a study of mCRC patients who were administered cetuximab, patients with *KRAS* codon 13 mutation had a longer survival than those with other types of *KRAS* mutations [10]. In line with this, *KRAS* codon 13 mutations exhibited weaker in vitro transforming activity than codon 12 mutations, in association with an alteration of the predisposition to apoptosis [17].

Several reports have suggested that artificial transition at codon 13 of *KRAS* has occurred in formalin-fixed material [18–20]. In our study, transitions were detected only at codon 13, whereas transversions and transitions were found at codon 12. Our previous study on pyrosequencing for *KRAS* has shown that codon 13 G→T transversions were revealed at similar levels to *KRAS* codon 12 G→T transversions in the DNA mixtures extracted from a cultured cancer cell line known to contain a *KRAS* codon 12 G→T transversion and a cultured cancer cell line known to contain no *KRAS* mutations [18].

In conclusion, the incidence of *KRAS* mutations in mCRC was similar to that of non-mCRC as previously reported. *KRAS* codon 13 mutations might be associated with younger female patients with mCRC. However, large multicenter studies are needed to assess the clinical effect and the biological behavior of *KRAS* codon 13 mutations in mCRC in order to develop better therapeutic options for female patients.

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