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Original Article

RAS mutation acts as a biomarker during colorectal cancer course in forming the lung metastasis

Xiaorong Lai[1*], Dongyang Yang[1*], Liyu Yan[2], Ying Li[1], Jianhua Liu[1], Weiwei Jiang[1], Dong Ma[1]

[1]Department of Internal Medicine of Gastrointestinal Tumor, Guangdong General Hospital Welfare Branch, Guangdong Academy of Medical Sciences, Guangzhou, China; [2]Department of Pathology, Guangdong General Hospital, Guangdong Academy of Medical Sciences, Guangzhou, China. [*]Equal contributors.

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Abstract: The metastasis is the most challenging issue and the most common cause of colorectal cancer (CRC) death in Chinese. In this study, we evaluated the association between the mutational status of KRAS, NRAS, BRAF and various clinicopathologic features, especially for the metastatic pattern in patients with CRC. The KRAS mutational status in exons 2, 3 and 4, NRAS in exons 2, 3 and 4, BRAF in exon 15 from formalin-fixed sections of primary tumors or related metastases were determined by sequencing analysis. We retrospectively evaluated data from 347 patients with CRC to analyze the relationship of KRAS, NRAS and BRAF mutational status with the clinicopathologic feature and the metastatic pattern. The results indicated that of the 347 patients enrolled, 148 (42.7%) had tumors with KRAS mutation, 10 (2.9%) had tumors with NRAS mutation and 16 (4.6%) had tumors with BRAF mutation. Female patients had higher tendency to develop tumors with a BRAF mutation than male patients (7.4% *vs.* 2.5%, *P*=0.038). Compare with no metastasis, KRAS mutations were significantly more frequent in multiple metastasis (P=0.017). Compared with patients free of metastasis, RAS mutational patients has a higher risk for lung metastasis (P=0.49, OR=2.83, 95% CI (1.005-8.013)) using the long-rank test. In conclusion, lung metastasis was more likely to develop during the CRC course in patients whose tumor had a RAS mutation than in those having no RAS mutation. This finding may have an impact on decision making for treatment and surveillance of metastatic disease.

Keywords: KRAS, NRAS, BRAF, metastasis, colorectal cancer

Introduction

Despite numerous advances in treatment for the colorectal cancer, metastasis remains the most challenging issue and the most common cause of colorectal cancer death [1]. Preclinical studies have suggested that mutant KRAS can constitutively promote tumors invasion and metastasis by activating downstream Raf/Erk/Map kinase and other signaling pathways [2, 3]. There are limited data evaluating whether the mutation profile affect the pattern of dissemination of metastasis in CRC patients. The few previous trials have suggested that KRAS mutations may affect the pattern of metastatic development [4, 5]. It is a practical need for predicting the subsequent metastases in patients with no evidence of organs involvement at the diagnosis. We aimed to determine the potential value of KRAS, NRAS and BRAF mutation as a predictive factor for development of metastasis.

Materials and methods

Patient population

Under approval of Cancer Centre Board of Guang Dong General Hospital, Guangdong Academy of Medical Sciences, we reviewed the patients (n=347) with cancers of the colon (n=214) or rectum (n=133) from May 2014 to June 2015 who received mutational analysis as part of their standard care. Clinical features of the patients and pathological profiles of the tumors were obtained from patient medical records. Among the 81 cases of metastatic colorectal cancers, 65 are single metastasis, 16 are multiple metastasis. The most common metastatic location is lung, liver, other location include

Table 1. Characteristics of the patients with KRAS, NRAS and BRAF mutations

	$KRAS^{mut}$	$KRAS^{WT}$	Ps	NAS^{mut}	NAS^{WT}	Ps	$BRAF^{mut}$	$BRAF^{WT}$	Ps
	PTS	PTS		PTS	PTS		PTS	PTS	
	N (%)	N (%)		N (%)	N (%)		N (%)	N (%)	
Age			0.913			0.108			0.127
≥ 65 years	63	87		7	143		10	140	
< 65 years	85	112		3	194		6	191	
Sex			0.062			0.526			0.038
Male	76	123		7	192		5	194	
Female	72	76		3	145		11	137	
Location			0.507			0.699			0.078
Right	68	104		5	167		12	160	
Left	19	23		2	40		2	40	
Rectum	61	72		3	130		2	131	
Differiation			0.503			0.923			0.013
Well	16	30		1	45		6	40	
Medium	131	168		9	290		10	289	
Poor	1	1		0	2		0	2	
Stage			0.293			0.381			0.679
I	16	34		1	49		3	47	
II	54	63		4	113		4	113	
III	47	68		5	110		7	108	
IV	31	34		0	15		2	63	

peritoneal cavity, Peritoneum, abdominal wall, ovary, bladder and distant lymph nodes.

Mutational analysis

Clinical tumor genotyping was performed on all 347 patients using nucleic acids extracted from diagnostic formalin-fixed, paraffin-embedded tumor tissue using QIAGEN system (QIAGEN, Dneasy Blood and Tissue Kit, Germany) according to the manufacturer's instructions. Mutational profiling simultaneously queried over previously described hotspot mutations across rats cancer genes, including Kirsten rat sarcoma viral oncogene homolog (KRAS); neuroblastoma RAS viral oncogene homolog (NRAS) and v-raf murine sarcoma viral oncogene homolog B (BRAF). This was performed using a custom-modified ABI 3500DX Sequencing System (Applied Biosystems/Life Technologies Corporation, Carlsbad, American), as previously described. First-generation sequencing was performed in both directions, and sequence analysis was performed using the Chromas software (Beckman Coulter), and the normal sequence was downloaded from Gene Bank. Microdissection was utilized under the guidance of a clinical pathologist as required to ensure >30% tumor cellularity.

Statistical analysis

The chi-square test was used to compare genotype frequency by age at diagnosis, sex, disease location, histology, stage at initial presentation, and number of metastasis. Patient characteristics and disease factors were summarized by descriptive statistics. The categorical parameters were compared by using the two-sided Pearson χ^2-test or Fisher exact test, as appropriate. The t-test was used to compare genotype frequency for continuous variables. SPSS software (version 16.0; SPSS, Chicago, IL, USA) was used for statistical analyses. A *P*-value < 0.05 was considered significant.

Results

Patient characteristics

Of the 347 patients enrolled in the present study, 57.3% (199) were male. The median age was 61.0 years (range, 23-92 years). One hundred and seventy-two cancers were from the proximal colon (cecum to transverse colon), 42 from the distal colon (descending colon to sigmoid colon) and 133 from the rectum. All 347 patients included in the study were diagnosed with either early stage (n=167) or advanced

Table 2. The relationship of metastatic sites with RAS mutation

Metastasis	Lung	Ps	Liver	Ps	Other	Ps	Multiple	Ps	Free
Kras		0.739		0.697		0.836		0.017	
Wild	6		19		16		4		154
Nutant	3		11		10		12		112
Nras		0.998		0.606		0.608		0.999	
Wild	9		30		26		16		256
Mutant	0		0		0		3		10
Braf		0.38		0.376		0.998		0.567	
Wild	8		30		25		15		253
Mutant	1		0		1		1		13

stage (n=180). CRC patients were categorized into three groups on the basis of KRAS, NRAS and BRAF mutations, and they were compared in terms of age, gender, colorectal tumor location, histological differentiation, UICC stage and number of metastasis.

KRAS mutation was identified in 148 of the tumors ($KRAS^{mut}$, 42.7%), and 199 tumors were KRAS wild type ($KRAS^{wt}$, 57.3%). NRAS mutation was identified in 10 of the tumors ($NRAS^{mut}$, 2.9%), and BRAF mutation was identified in 16 of the tumors ($BRAF^{mut}$, 4.6%). The groups were unbalanced according to location of primary tumor and patient's sex: female patients have more tendencies to develop tumors with a BRAF mutation than male patients (7.4% *vs.* 2.5%, P=0.038). Tumors with a BRAF mutation perhaps tended to occur more frequently in the right side of the colon than in the left colon or rectum (7.0% vs. 4.7%, P=0.078). Other patient and disease characteristics are shown in **Table 1**.

Impact of KRAS, NRAS and BRAF mutation status on metastatic characteristics of the colorectal cancer patients

All 347 CRC cases were examined for mutations in KRAS (exons 2, 3 and 4), NRAS (exons 2, 3 and 3) and BRAF (exon 15). We also observed the changes in the nucleotides and corresponding amino acids of mutation profiles. The distribution of mutations was consistent with those reported by previous studies. The most frequent mutation of KRAS is 2p.G12V (codon 12), occurring in 37 cases with the frequency 10.7%. The most frequent NRAS mutation is 3p.61 (codon 3), 3 cases with the frequency 0.9%, while the most frequent BRAF mutation is 15p.V600E (codon 15), occurring in 14 cases with the frequency 4.0%. Mapping associations between molecular markers revealed that 1 patient had both KRAS and NRAS mutations, whereas 1 patient had both KRAS and BRAF mutations. KRAS/NRAS mutation combinations were as follows: 4p.A146T/4p.116, KRAS/BRAF mutation combinations were as follows 2p.G12S/15p.V600E. In contrast, NRAS and BRAF mutations were mutually exclusive.

RAS mutation was detected in 51.0% (n=177) cases. Of the metastatic patients with RAS mutation included in the this studying, 4 cases (2.3%) were lung metastasis as the only site of metastasis, 11 cases (6.2%) were liver metastasis, 11 cases (6.2%) were other sites metastasis, 16 cases (9.0%) were multiple metastasis site, while 135 cases without metastasis were identified RAS mutation genes. Regarding the RAS status, there was significant difference of mutation frequency between the five groups ($P < 0.05$) (**Table 2**). Compare with no metastasis, KRAS mutations were significantly more frequent in multiple metastasis (P=0.017).

Analysis the association of RAS with metastatic patterns among CRC patients

There were no statistically significant differences for metastasis in age and primary location, sex and differentiation using the log-rank test. Compared with patients free of metastasis, RAS mutational patients had a higher risk for lung metastasis (P=0.49, RR=2.83, 95% CI 1.005-8.013), while patients with liver, other and multiple location metastasis was not proved to be correlated with RAS mutation (P> 0.05).

Discussion

Currently, the presence of KRAS mutation is regarded as the most important predictive biomarker for resistance to anti-epidermal growth factor receptor (EGFR) antibodies. Previously classified as KRAS 'wild-type' tumors based on practices testing only for mutations in codon 12 or 13 of KRAS, these less common atypical RAS mutations have been shown to negate the presumed benefit of anti-EGFR therapies for patients with RAS wild-type tumors [6-8].

Our institution has been performing the analysis of KRAS, NRAS and BRAF mutation on patients with CRC since 2014 May. This testing establishes a subset of patients for whom testing would assist in identifying targeted therapies for the individual. We retrospectively evaluated mutational signatures of KRAS, NRAS and BRAF for correlation with clinical characteristics, primary site, patient sex, age in CRC with advanced disease.

Few data are available that suggested RAS mutational profiles may influence the pattern of metastatic dissemination. It have been shown that KRAS mutational status has a prognostic role in specific surgical populations, for example, in predicting the risk of recurrence in localized CRC [9] and after hepatic or lung resection [4, 10-12] also identified differences in metastatic patterns according to KRAS status. Our study not only confirms the findings, but also extends this finding to demonstrate that, in CRC patients with multiple metastatic sites at diagnosis, KRAS may contribute to differences in the pattern of metastatic dissemination. This finding may need the validation of the future study, reiterating the potential clinical relevance of this mutation in considering patients for appropriate treatment.

Our study demonstrates that KRAS mutation was detected in 42.7%, NRAS mutation in 2.9% and BRAF mutation in 4.6% of CRC patients. This distribution of mutations of KRAS and BRAF was consistent with those reported by previous studies, the level of KRAS mutational is a little higher than the western, it maybe result from the difference of race. It has been reported that BRAF mutations have been associated with right-sided high-grade tumors in older patients. Our finding showed that tumors with a BRAF mutation occurred more frequently in the well-differentiated colon tumors and in female patients, validating the previous reports partially. Analyses of the clinical predictive factors for specific metastasis in patients revealed that clinical variables [gender, age (< 65 *vs.* ≥ 65 years), primary tumor sites (colon vs. rectum), histological grade, etc.] had no significance. Logistic regression analysis was carried out to improve that RAS status remained predictive for lung-only metastases [odds ratio (OR)=2.83]; 95% confidence interval (CI), 1.005-8.013; P=0.49 for liver-only metastasis, while no RAS mutational had not be confirmed to increase risk of developing liver-only, other location and multiple metastasis compared with colon cancer patients without metastasis. It is maybe related with small size of metastasis cases of our study, and need to be furthered in large scales.

To the best of our knowledge, with 347 CRC patients included, this is the largest retrospective series to analyze the role of KRAS, NRAS and BRAF mutational status in pattern of metastasis in Chinese patients. Although the mutational profiles of this research include KRAS, NRAS and BRAF, and the sample size is outstanding, our study has some limitations. First, it is a retrospective analysis of patients from a single institution and thus unrecognized biases might have influenced the external validity of this conclusion. Second, RAS mutational analysis does not incorporate potential discordance in RAS status between a primary tumor and its metastases, difference of the material obtained from needle biopsy or resected specimens. However, RAS mutations are thought to be highly concordant between primary and metastatic location during the progression of the disease [13], previous study showed biopsied specimens of primary tumor just has a trend for higher discordance rate than resected specimens without significance [12]. Third, although our study extended RAS testing including these critical codons of KRAS, NRAS and BRAF to improve the predictive efficacy for EGFR monoclonal antibody therapy, and more sensitive methods, such as current sequencing approaches for RAS mutation analysis are used in the investigational setting and not widely spread in the clinical practice. However, the significance of the additional low-allele frequency mutations revealed by the newer methodology is not clear and waits for further validation. For example, those with a codon 13 mutation are revealed to present as more aggressive disease with a higher rate of synchronous organ metastasis, compared with KRAS codon 12 mutation [14]. In our cohort, the relevance of the various mutations with clinic significance, especially the multiple metastases and the development of this disease, is not clear and needs to further validate.

These results support the conclusion that RAS status can be used to risk stratify patients for development of distant metastasis. In addition, multiple metastases had a higher rate of RAS mutation than primary tumor with no metastasis in colorectal cancer, suggesting a possibility

of specific RAS mutation in CRC patients. This observation needs to be further investigated in large studies.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Dong Ma, Department of Internal Medicine of Gastrointestinal Tumor, Guangdong General Hospital Welfare Branch, Guangdong Academy of Medical Sciences, Guangzhou 510120, China. E-mail: doc_madong@yeah.net

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