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Original Article

The mutations of K-ras and BRAF gene in colorectal carcinoma and its clinical significance

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Abstract: Objectives: To assess the mutation of BARF and K-ras genes of colorectal carcinoma patients, and investigate their correlation with clinical pathological parameters. Methods: The PCR and direct sequencing were applied to detect mutations in the K-ras (codons 12, 13 and 61) and BRAF genes (codon 600). Besides, it was analyzed that correlation between two gene mutations and clinical pathological parameters. Results: K-ras mutations were found in 75 cases, and mutation rate was 34.4%. Among these, the codon 12 was 62 cases (28.4%), in which there were 18 cases (8.3%) GGT→GAT (G12A); 2 cases (0.92%) GGT→GCT (G12A); 17 cases (7.80%) GGT→GTT (G12V); 8 cases (3.7%) GGT→TGT (G12C); 17 cases (7.80%) GGT→AGT (G12S). The codon 13 was 13 cases that was GGC→GAC (G13A) (6.0%). The frequency of K-ras gene mutation in cases with lymph node metastasis (50%) and liver metastasis (55.3%) were significantly higher than those in cases without nodal (24.6%) or hepatic involvement (30%) (P<0.05). The frequency of K-ras gene with stage of I+II, III, IV were 19.2% (21/109), 50.8% (30/59), 48% (24/50), respectively. With the higher TNM stage, the chance of K-ras gene mutation increased (P<0.05). Only 4 cases (1.8%) were detected with BRAF gene mutation, and all of them had a wild-type K-ras gene. Conclusions: K-ras gene mutation is correlated to lymph node metastasis, liver metastasis and tumor stage, while BRAF gene mutation is rare. Detection K-ras and BRAF genes mutations may be useful in guiding treatment of CRC.

Keywords: K-ras gene, BRAF gene, gene mutation, colorectal carcinoma (CRC)

Introduction

Colorectal cancer (CRC) is one of the most common malignancies in the world and it is a disease that can be easily cured either by surgery or by endoscopic excision when diagnosed at an early stage [1]. It has been generally accepted that a majority of CRC develop through a well-defined adenoma-carcinoma sequence in which multiple genetic changes are involved in this pathway that is known as chromosomal instability pathway. K-ras gene is known to play an important role along this pathway in transitioning from early to intermediate adenomas [2]. However, increasing evidence accumulates that a subset of CRC arises via the hyperplastic polyp-serrated adenoma -carcinoma sequence that is associated closely with microsatellite instability positive colorectal carcinomas [3-5]. These polyps are usually large and/or multiple and/or located in the proximal colon [6, 7]. As we all known, the K-ras and BRAF gene were all located in epidermal growth factor receptor (EGFR) signal pathway, and this pathway included RAS-RAF-MEK-ERK-MAPK [8] and PI3K-PTEN-AKT pathway [9]. Both BRAF and K-ras are proto-oncogenes that interact in tandem in the RAS-RAF-MEK-ERK-MAP kinase signaling pathway, which plays an important role in the control of cell differentiation, proliferation, survival and apoptosis [10].

Following the further studies of tumor signal pathway mechanism and the application of molecular targeted agents, it was found that K-ras gene status was closely related with treatment of CRC. But, recently, some studies found that CRC patients without K-ras gene mutation obtained the most benefits from EGFR inhibitors, while there was no significant effect of EGFR inhibitors to patients with mutation K-ras gene [11, 12]. So, the K-ras gene was the

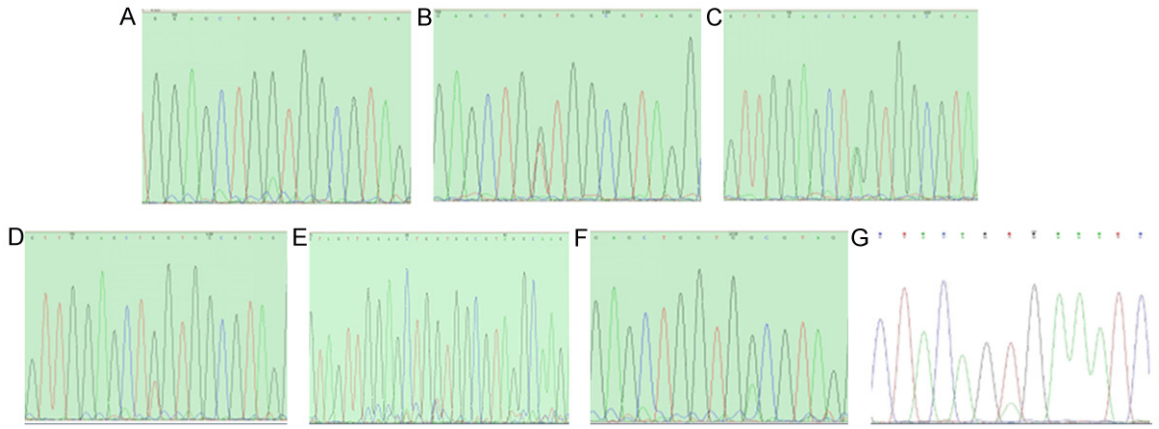

Figure 1. The mutation of K-ras gene codon 12 and 13 as well as BRAF gene. A. K-ras gene codon 12 GGT→GAT; B. K-ras gene codon 12 GGT→GTT; C. K-ras gene codon 12 GGT→AGT; D. K-ras gene codon 12 GGT→TGT; E. K-ras gene codon 12 GGT→GCT; F. K-ras gene codon 13 GGC→GAC; G. BRAF gene codon 600 site GTG→GAG.

molecular marker of treatment with molecular targeted agents to anti-EGFR mono-antibody. While there was few studies on the relationship between K-ras gene status and prognosis of patients with CRC, and its predictive value was controversial. A study found that patients with G12V mutation showed worse clinical manifestations [13], and they had lower total survival rate than ones with other mutation genotype [14]. A large sample study showed that the K-ras gene codon 13 G→A mutation had close relationship with survival stages of CRC patients [15].

To better predict the sensitivity of CRC patients to anti-EGFR targeted regents, recently, the studies on the K-ras gene were all combined with other genes [16, 17], in which the BRAF attracted more attentions. K-ras and BRAF gene all played key regulated roles in the Ras/MAPK signal pathway. Their mutations could contribute to the uncontrolled proliferation, which were one of the key targets in clinical tumor treatments at present. The multi-mutation of K-ras and BRAF gene might cause various signal pathway regulated function and further influence the selection of targeted treatment of tumor cells. Therefore, studies on the clinical pathological relationship between K-ras and BRAF gene mutation features could provide theoretical basis for the regulated effects of K-ras and BRAF gene mutation to tumor cell signal pathway, and provide references for selection of new targets of treatment of CRC.

Materials and methods

Subjects

We recruited 218 patients who underwent colonoscopy for various reasons at the the first affiliated hospital of Nanchang University from June 2013 to March 2015. All patients were diagnosed as CRC by endoscopic biopsies, and they received radical resection without chemotherapy before operation. All samples were fixed with 10% neutral formalin. Patients were classified according to the TNM classification of WHO in 2000, in which included 125 males and 93 females aged 21 to 89 years old, median age was 52; right colon cancer 84 cases, left colon cancer 69 cases, rectal cancer 65 cases; lymph node metastasis 80 cases, non-lymph node metastasis 138 cases; liver metastasis 38 cases, non-liver metastasis 180 cases; lung metastasis 32 cases, non-lung metastasis 186 cases; in TNM classification, phase I 35 cases, phase II 74 cases, phase III 59 cases, phase IV 50 cases.

Methods

Extraction of tumors DNA: The 5 to 10 paraffin-embedded tissues sections were treated with dewaxing and Benzene removal. And then the tumor tissues were scraped into EP tubes, and then the protease K and protein precipitation liquid were added to precipitate protein and extract DNA.

PCR: Amplification reaction program was as following: initial denaturation for 7 min at 94°C;

Table 1. Relationship between K-ras gene status and clinical pathological parameters (cases/%)

Clinical pathological parameters	Cases	K-ras gene mutation	χ^2	*P* value
Gender				
Male	125	41 (32.8)	0.394	>0.05
Female	93	34 (36.6)		
Age (year)				
<40	25	5 (20)	2.798	>0.05
≥40 and ≤60	131	45 (34.4)		
>60	62	25 (40.3)		
Tumor type				
Ulcerative type	163	59 (36.2)	5.905	>0.05
Infiltrating type	20	9 (45)		
Eminence type	35	6 (17.1)		
Tumor size (cm)				
<5	86	23 (26.7)	4.937	>0.05
≥5 and ≤10	99	42 (42.4)		
>10	33	10 (30.3)		
Tumor site				
Right colon	84	28 (33.3)	1.275	>0.05
Left colon	69	27 (39.1)		
Rectal	65	20 (30.8)		
Differentiation degree				
High-medium degree	118	41 (34.7)	5.464	>0.05
Low degree	100	33 (33)		
Lymph Node Metastasis				
Yes	80	40 (50)	14.447	>0.05
No	138	34 (24.6)		
Liver Metastasis				
Yes	38	21 (55.3)	7.789	<0.05
No	180	54 (30)		
Lung Metastasis				
Yes	32	13 (40.6)	0.946	>0.05
No	186	61 (32.8)		
TNM stage				
I+II	109	21 (19.3)	22.924	<0.05
III	59	30 (50.8)		
IV	50	24 (48)		

35 cycles, denaturation for 45 s at 94°C, annealing for 45 s at 62°C and extension 45 s at 72°C; then extension 7 min at 72°C. The PCR products were taken out and stored at 4°C.

DNA sequencing: The K-ras and BRAF gene mutations were detected by DNA directly sequencing using Sequencing machine (ABI37-30XL), sequencing regents were Big Dye, and all samples were sequenced using bi-directional sequencing. The known K-ras and BRAF gene mutation peaks of CRC were as the positive control, and the DNA Star software was used to analyze gene sequences.

Statistical analysis

Data was analyzed using the χ^2-test. All tests were performed two-tailed with a confidence interval (CI) of 95%. Statistical analysis was performed by using SPSS version 20.0 software. A *P* value <0.05 was considered statistically significant.

Results

Mutation of K-ras

In 218 cases CRC tissues, K-ras gene mutation was 75, and mutation rate was 34.4%. Among these, the codon 12 was 62 cases (28.4%), in which there were 18 cases (8.3%) GGT→GAT (G12A); 2 cases (0.92%) GGT→GCT (G12A); 17 cases (7.80%) GGT→GTT (G12V); 8 cases (3.7%) GGT→TGT (G12C); 17 cases (7.80%) GGT→AGT (G12S). The codon 13 was 13 cases that was GGC→GAC (G13A) (6.0%). There was no case with codon 12 and 13 mutation, and the condon 61 was found no mutation (**Figure 1A-F**; **Table 1**).

Relationship between mutation of K-ras and clinicopathological parameters

After χ^2 test, results showed that the frequency of K-ras gene mutation in cases with lymph node metastasis (50%) and liver metastasis (55.3%) were significantly higher than those in cases without nodal (24.6%) or hepatic involvement (30%) (*P*<0.05). The frequency of K-ras gene with stage of I+II, III, IV were 19.2% (21/109), 50.8% (30/59), 48% (24/50), respectively. With the higher TNM stage, the chance of K-ras gene mutation increased (P<0.05). Differences among gender, age, tumor morphology, whether liver or lung metastasis as well as codon 12 and 13 mutation rates had no statistical signifi-

Table 2. Mutation type and frequencies of K-ras gene codon 12 and 13

Codon	Wild	Point mutation	Case
Codon 12	GGT (Gly)	GAT (Asp)	18
	GGT (Gly)	GTT (Val)	17
	GGT (Gly)	AGT (Ser)	17
	GGT (Gly)	TGT (Cys)	8
	GGT (Gly)	GCT (Ala)	2
Codon 13	GGC (Gly)	GAC (Asp)	13

cances (P>0.05). Results were shown in **Table 1**.

Mutation of BRAF and its relationship with clinicopathological parameters

In 218 cancer tissues, there were only 4 cases (1.8%) detected with BRAF gene mutation, and mutations were all GTG→GAG. These included 2 cases females and 2 cases males, who were aged 72, 43, 45 and 57 years old separately; 1 case was right colon cancer, 2 cases were left colon cancer and 1 case was rectal cancer; 1 case with lymph node, liver and lung metastasis, another 3 cases were all with lymph node metastasis; 3 cases were phase III, 1 case was phase IV; and all of them had a wild-type K-ras gene (**Tables 2**, **3** and **Figure 1A-G**).

Discussions

The K-ras gene mutation status was detected in 218 cases CRC in this study, we found that 75 cases were K-ras mutation, and the mutation rate was 34.4%, in which the codon 12 and 13 was 28.4% and 6.0% separately. Foreign research revealed that K-ras mutation rate was about 30-50% in CRC, and mutation sites mostly focused on the condon 12 and 13. Our results were close to before study results [18]. In 75 mutation cases of condon 12, there were 35 cases G→A, which was the most common mutation site. The second site was G→T (25 cases). There were 6 kinds of mutation type: Gly12Asp (18 cases), Gly12Val (17 cases), Gly12Ser (17 cases), Gly12Ala (2 cases) and Gly13Asp (13 cases), in which mutation rate of Gly12Asp site was the highest.

But it had already been confirmed that K-ras gene mutation played an important role in the CRC [19]. Report suggested that the K-ras mutation could be detected in adenoma tissues. There was no a final conclusion that K-ras gene mutation had relationship with clinical pathological characters. For example, Gao F [20] et al found that female patients were easier to obtain K-ras gene mutation by detecting 48 cases Chinese CRC patients. Zlobec [21] et al indicated that K-ras gene mutation had no relationship with some parameters such as age, gender, tumor size and site as well as TNM classification and so on. Naguib [22] et al reported that K-ras gene mutation was related to Dukes' stage and microsatellite instability. Mannan [23] et al detected K-ras gene mutation status in 88 cases CRC tissues and found that K-ras gene mutation had significant correlation with lymph node metastasis and tumor stage. Our results showed that K-ras gene mutation rate of lymph node and liver metastasis were 50% and 55.3% separately, and significant higher than no-lymph node and liver metastasis (24.6% and 30%). And with the increased of TNM classification stage, the K-ras gene mutation rate was also improved. These results all indicated that CRC with K-ras gene mutation was easier to be lymph node and liver metastasis, and that was related with high tumor stage. Detecting K-ras gene status and analyzing its mutation and type could provide references for predict lymph node and liver metastasis as well as tumor development. But it needed further confirmed its mutation types and expressed changes brought by these mutations. For example, the cell signal pathway active mechanism contributed by gene mutation and how to participate the lymph node and liver metastasis and so on, which was our next studies direction.

K-ras/BRAF/ERK signal pathway plays a critical role in the incidence of CRC. A foreign study [24] revealed that BRAF gene mutation rate was about 12% to 15.6% in CRC, and the BRFA gene mutation was related with tumor differentiation and microsatellite instability. Richman [25] et al held that BRAF gene mutation had relationship with prognosis. Nieolantonio [26] et al reported that 112 cases patients with mCRC showed drug resistance and survival situation was related with BRAF gene status after treatment of panitumumab or cetuximab. Our results showed that, there was only 4 cases BRAF gene mutation (1.8%) in 218 cases. Therefore, it was unclear that BRAF mutation had relationship with clinical pathological character. Hsieh [27] et al found that BRAF mutation rate was 1.1% in 180 cases CRC samples

Table 3. Clinical pathological materials of 4 cases with BRAF gene mutation

Case	Age (year)	Gender	Tumor size (cm)	Tumor type	Tumor site	Differentiation degree	Invasive depth	Lymph Node Metastasis	Liver metastasis	Lung metastasis	TNM stage	K-ras mutation
1	72	Female	6×5	Ulcerative type	Rectal	Low	Out of outer membrane	Yes	Yes	Yes	IV	No
2	43	Female	3×3.4	Eminence type	Left colon	High-medium	Muscular layer	Yes	No	No	III	No
3	45	Male	6.7×7	Eminence type	Left colon	Medium	Total layer	Yes	No	No	III	No
4	57	Male	12×13	Eminence type	Right colon	Low	Out of size film	Yes	No	No	III	No

from Chinese people in Taiwan; Shen [28] et al also found that BRAF I5 (V600E) mutation rate was 1.7% in 118 cases CRC; Liao [29] et al found 3 cases BRAF gene mutation (4.9%) in 61 cases Chinese people; Li [30] et al found 14 cases (7.0%) BRAFI5 (V600E) in 200 cases CRC patients, which were lower than data from west countries. The reasons of this difference might be related with races, living environments as well as specificity and sensitivity of detection methods.

Siu [31] et al reported that they found no K-ras gene mutation in tumors with BRAF gene mutation. Calistri [32] et al found that P53, BRAF and K-ras gene mutation rates were 35%, 4% and 30% separately by DNA sequencing analysis of 100 cases CRC, while there were rare cases simultaneously with these gene mutations. In this study, 4 cases with BRAF gene mutation were all wild genotype of K-ras gene, which proved that there were some kinds of mechanism in the development of CRC. Some researchers applied RNAi for CRC cell lines with BRAF or K-ras mutation to inhibit BRAF gene, they found that CRC cells with BRAF mutation showed significant growth inhibition and increased apoptosis, while there was no above change in CRC cells with K-ras mutation. Besides, the expression of phosphated ERK1/2 and cyclin D1 significantly decreased in CRC cells with BRAF mutation than K-ras mutation after inhibition of BRAF. So, patients with BRAF mutation were better to treat than with K-ras gene mutation.

It was reported that BRAF could influence the treatment of anti-EGFR. Nieolantonio [26] et al found 11 cases BRAF mutation in 79 cases with wild genotype K-ras, while there was no patients response for panitumumab or cetuximab who were with BRAF mutation, and there was no patient with BRAF mutation who response for panitumumab or cetuximab. Obviously, wild BRAF gene was also necessary to the effectiveness of panitumumab or cetuximab. Therefore, it was important significance that K-ras and BRAF genes were simultaneously detected in the patients with CRC, and provided our further research direction.

Disclosure of conflict of interest

None.

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