Original Article
K-ras genetic mutation and influencing factor analysis for Han and Uygur nationality colorectal cancer patients

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Abstract: To investigate the K-ras genetic mutation status in colorectal cancer patients, compare the difference of K-ras genetic mutation rate in Han and Uygur nationality and analyze the influencing factor. 91 cases (52 cases of Han nationality and 39 cases of Uygur nationality) of colorectal biopsy or surgical ablation pathology specimen from the first affiliated hospital of Xinjiang Medical University during January, 2010 to March, 2013 were collected to detect the 12th and 13th code mutation status of K-ras gene exon 2 with pyrosequencing method and compare the difference of K-ras gene mutation rate between Han and Uygur nationality patients. Single factor analysis and multiple factor logistic regression analysis were utilized to analyze the influencing factor for K-ras genetic mutation. 33 cases of patients with K-ras genetic mutation were found from the 91 cases colorectal cancer patients and the total mutation rate was 36.3%. Among them, 24 cases (72.7%) were found with mutation only in the 12th code, 9 cases (27.3%) were found with mutation only in the 13th code and no one case was found with mutation in both the two codes. Mutation rate of the 12th code in the Uygur nationality was significantly higher than that in the Han nationality (P<0.05), but there were no significant difference in the comparison of the total mutation rate and the 13th code mutation rate between the two groups (P>0.05). There were no associativity (P>0.05) between the K-ras genetic mutation and sex, age, smoking history, drinking history, tumor location, macropathology type, differentiation level, staging, invasive depth, lymph nodes transferring and metastasis in colorectal cancer patients (P>0.05). K-ras genetic mutation rate is high in colorectal cancer patients. The mutation rate of 12th code in Uygur nationality is higher than that in Han nationality. There is no significant associativity between K-ras genetic mutation rate and patients' clinical pathology characteristic.

Keywords: Colorectal cancer, Han nationality, Uygur nationality, gene, mutation

Introduction

Colorectal cancer is a clinical frequent malignant tumor. More than 1.2 million new patients are suffered from colorectal cancer and more than 0.6 million people died of the disease every year all over the world. Data from America national cancer institute has showed that there were 143460 new colorectal cancer patients, 51690 people were died of this disease and the morbidity and mortality are in the third palace among malignant tumors in 2012 in America [1]. In China, The morbidity and mortality of colorectal cancer increase year by year and that in advanced stage is with higher incidence rate. Recently years, the morbidity of colorectal cancer increases quickly and has become one of the 5 most frequent malignant tumors. The 5-year survival rate of this disease

is about 60% and no significant improvement in the treatment effectiveness is acquired within 30 years [2]. K-ras genetic mutation in colorectal cancer is more frequent than mutation of other genes in ras family. Colorectal cancer is also one of the tumors in which the K-ras genetic mutation is mostly studied and applied in clinical detection. K-ras genetic mutation frequency in colorectal cancer is 14%-50% [1-3]. The activation of this gene plays important roles in the generation and development of colorectal cancer and has considerable reference value for molecule targeted therapy of the disease. In some research, the change has also been regarded as an unfavorable prognosis for the colorectal patients [3, 4].

The Uygur nationality in Xinjiang keep different genetic background compared with other

Table 1. Han and Uyghur Clinical characteristic of patients with colorectal cancer

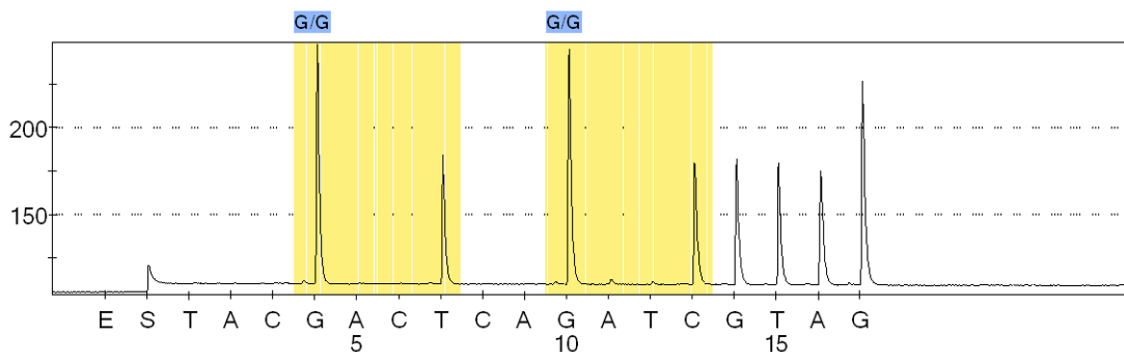| Group	cases	Sex		Age [M (P_{25}, P_{75}) years old]	Smoking history		Drinking history	
		male	female		no	yes	no	yes
Han nationality	52	28 (53.8)	24 (46.2)	57 (50, 65)	35 (67.3)	17 (32.7)	40 (76.9)	12 (23.1)
Uygur nationality	39	26 (66.7)	13 (33.3)	56 (45, 62)	32 (82.1)	7 (17.9)	31 (79.5)	8 (20.5)
X^2 (u) value		1.158		885*	2.495		0.085	
P value		0.314		0.300	0.114		0.770	

Figure 1. K-ras gene wild-type standard test patterns. Note: Detection results of the two sites were all G/G, suggesting that the K-ras gene was wild type. Results of G/A, G/C or G/T suggesting that the K-ras gene had mutated.
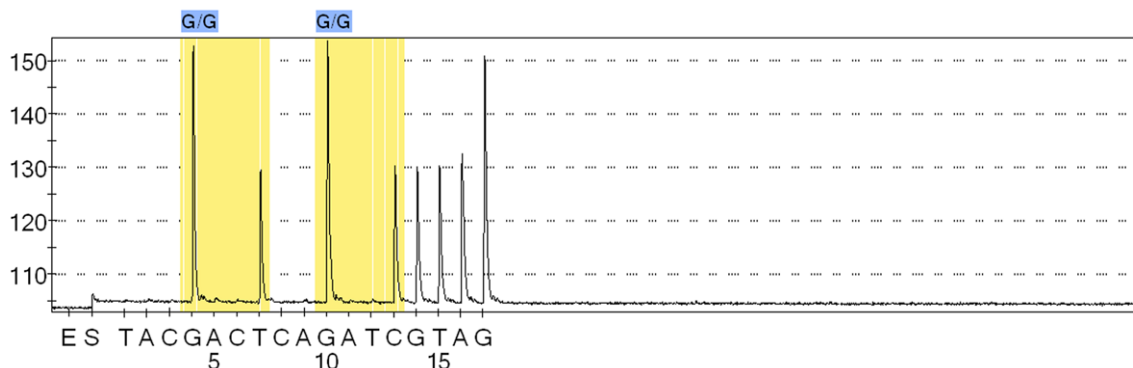

Figure 2. The samples tested wild-type K-ras gene report.

nationalities in addition to the difference of social culture and living habits. K-ras genetic mutations are different in colorectal cancer of different races all over the world. A few studies have revealed the mutation status of K-ras gene and its associativity with the generation and development of colorectal cancer in Uygur nationality in Xinjiang. Previous studies have showed that detecting the 12th and 13th code mutation in K-ras gene exon 2 of colorectal cancer patients with pyrosequencing method is sensitive and accurate and could be utilized for the detection of tumor genetic mutation in clinical individualized treatment [5]. This study detect the 12th and 13th code mutation status in K-ras gene exon 2 in colorectal cancer patients paraffin specimen from Han and Uygur nationality with pyrosequencing method to analyze the clinical pathology significance of K-ras genetic mutation.

Materials and methods

Inclusion and exclusion criteria

Inclusion criteria: a. Patients with ages range from 18 to 80 years old, b. patients who were diagnosed as colorectal cancer by enteroscope
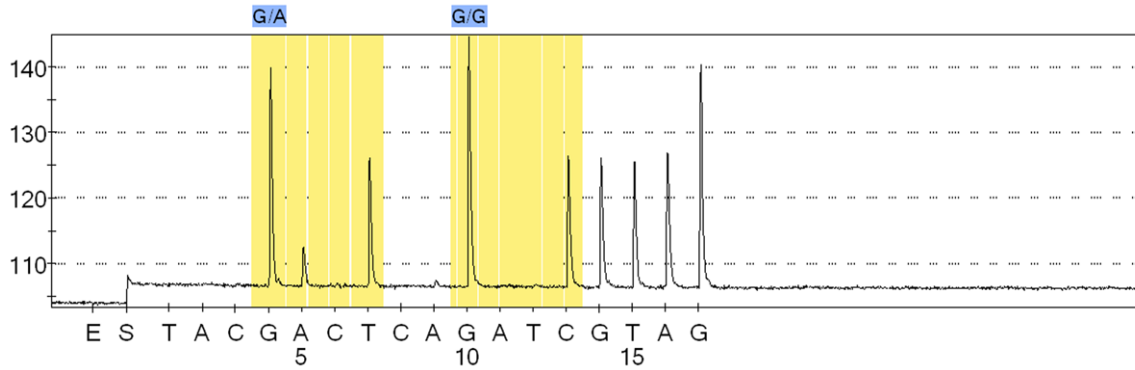
Figure 3. The samples tested mutant K-ras gene mutation report (codon 12 GAT mutation).
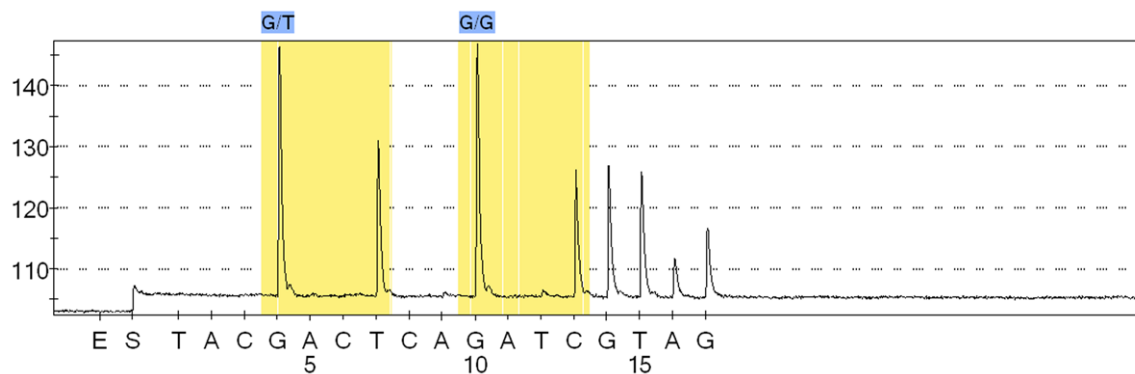

Figure 4. The samples tested mutant K-ras gene mutation report (codon 12 GTT mutation).
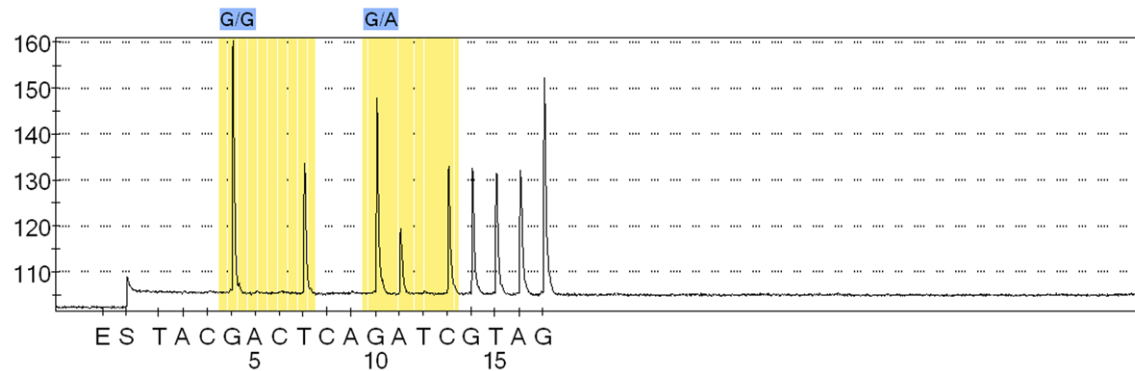

Figure 5. The samples tested mutant K-ras gene mutation report (codon 13 GAC mutation).

pathologic diagnosis, c. patients who were finally diagnosed as colorectal cancer by post-operative pathology, d. patients with complete clinical data, patients who signed informed consent to participate this study.

Exclusion criteria: a. Patients who were under 18 and over 80 years old, b. patients who had treated with radiotherapy, chemotherapy and molecular targeted drug. c. patients with critical illness or severe hypertension, diabetes mellitus, heart and cerebral vessels, d. patients with metastatic tumor in colon and rectum. e. patients who refused to participate the research.

Clinical data

91 cases of colorectal cancer biopsy or surgical ablation pathology specimen were collected from the first affiliated hospital of Xinjiang Medical University during January, 2010 to

Table 2. Han and Uyghur Clinical characteristic of patients with colorectal cancer

Group	Tumor location			Macropathology type			Differentiation level			Staging			
	colon	rectum	ulcer type	protrude type	infiltrating type	well differentiated	moderately differentiated	poorly differentiated	I stage	II stage	III stage	IV stage
Han nationality	27 (51.9)	25 (48.1)	27 (55.1)	21 (42.9)	1 (2.0)	7 (14.3)	28 (57.1)	14 (28.6)	9 (18.4)	10 (20.4)	19 (38.8)	11 (22.4)
Uygur nationality	22 (56.4)	17 (43.6)	23 (62.2)	11 (29.7)	3 (8.1)	4 (10.8)	22 (59.5)	11 (29.7)	6 (16.2)	11 (29.7)	18 (48.6)	2 (5.4)
X^2 (u) value	0.181			2.826			0.228			5.858			
P value	0.671			0.239			0.867			0.119			

Table 3. Han and Uyghur Clinical characteristic of patients with colorectal cancer

Group	Invasion depth (T)			lymph nodes transferring (N)			Metastasis (M)	
	T2	T3	T4	N0	N1	N2	M0	M1
Han nationality	12 (24.5)	20 (40.8)	17 (34.7)	23 (46.9)	18 (36.7)	8 (16.3)	36 (73.5)	13 (26.5)
Uygur nationality	8 (21.6)	18 (48.6)	11 (29.7)	18 (48.6)	11 (29.7)	8 (21.6)	34 (91.9)	3 (8.1)
X^2 (u) value	0.527			0.637			3.586	
P value	0.768			0.727			0.058	

Table 4. K-ras gene mutation in colorectal cancer tissues between two nationalities

Group	Cases	Total mutation	the 12th code mutation	the 13th code mutation
Han nationality	52	15 (28.8)	9 (17.3)	6 (11.5)
Uygur nationality	39	18 (46.2)	15 (38.5)	3 (7.7)
X^2 (u) value		2.888	5.136	0.370
P value		0.089	0.023	0.543

March, 2013, including 52 cases of Han nationality and 39 cases of Uygur nationality, or 5 cases of biopsy or 86 cases of surgical specimen. Patients were not treated with any chemoradiation, molecular targeted drug and so on when we obtained pathology specimen from them. We collected related clinical and pathology data (macropathology type, differentiation level and staging in 5 cases of biopsy were not identified) and there were no significant difference in sex, age, smoking history, drinking history, tumor location, macropathology type, differentiation level, staging, invasive depth, lymph nodes transferring and metastasis between the two groups (P>0.05, **Tables 1**-3).

Methods

Pyrosequencing method was utilized to detect the 12th and 13th code mutation status of K-ras gene exon 2 in paraffin section of both Han and Uygur nationality colorectal cancer patients.

Collection of paraffin embedding tissue sample: Cut each specimen into 8 pieces of 5 μm thick paraffin sections and make sure each section containing more than 70% tumor tissue. All specimens were stored at room temperature for less than 3 years and diagnosed as tumor tissue.

Extraction of genome DNA in paraffin section tissue: Kits were purchased from gene technology (Shanghai) company. We selected appropriate amount of sections according to tissue size

and immerged them into xylene for 1 min and then 95% ethanol solution for 1 min, scraped tissues into 1.5 ml centrifuge tube and added 30-50 μl 0.01% SDS lysate with 50 μg/ml protease K. (5 μl protease K in 50 μl lysate) before blending them, put them into water bath to react for 1.5-2 hours at 56°C and then 10 min at 100°C, centrifuged them for 10 min at 10 000×g and obtained the supernatant as PCR template.

Primer design: Primers for K-ras gene fragment amplification were synthesized by gene technology (Shanghai) company. The upper stream primer 5'-biotin-TGACTGAATATAAACTTGTGG-TAGTTG-3' (biotin labeling for 5' end) and the downstream primer 5'-TCGTCCACAAAATGAT-TCTGAA-3' were synthesized for a 91 bp product and primer 5'-GCACTCTTGCCTACG-3' was synthesized for sequencing.

PCR amplification: PCR amplification was carried out according to the PCR amplification kit [purchased from gene technology (Shanghai) company]. The reaction condition was set up as follows: holding at 37°C for 5 min, initial denaturation at 95°C for 3 min, with 50 cycle of denaturation at 95°C for 10 s, annealing at 56°C for 20 s and extension at 72°C for 30 s and end with a final extension at 72°C for 5 min. The PCR product was detected by Agarose electrophoretic analysis to validate the PCR amplification efficiency.

Pyrosequencing: Single stranded DNA was purified from PCR reaction solution utilizing single stranded purification devices in PyroMark ID type pyrophosphoric acid sequenator (Sweden Biotage AB company). Sequencing reaction plate was placed in the sequenator for sequencing. Data analysis (SNP analysis was the preference) was proceeded utilizing the analysis software in pyrophosphoric acid sequenator. Sites

Table 5. The relationship of Colorectal cancer tissues K-ras gene mutation and clinicopathological features

	Cases	Total mutation (%)	X^2 value	*P* value
Sex			2.302	0.129
male	54	23 (42.6)		
female	37	10 (27.0)		
Age (years old)			0.599	0.439
<60	49	16 (32.7)		
≥60	42	17 (40.5)		
Smoking history			1.292	0.256
No	67	22 (32.8)		
Yes	24	11 (45.8)		
Drinking history			2.093	0.148
No	71	23 (32.4)		
Yes	20	10 (50.0)		
Tumor location			0.010	0.920
Colon	49	18 (36.7)		
Rectum	42	15 (35.7)		
macropathology type			0.270	0.847
Ulcer type	50	19 (38.0)		
protrude type	32	12 (37.5)		
infiltrating type	4	1 (25.0)		
differentiation level			5.564	0.062
well differentiated	11	1 (9.1)		
moderately differentiated	50	22 (44.0)		
poorly differentiated	25	9 (36.0)		
Staging			3.877	0.275
I stage	15	7 (46.7)		
II stage	21	9 (42.9)		
III stage	37	14 (37.8)		
IV stage	13	2 (15.4)		
Invasion depth (T)			1.351	0.509
T2	20	8 (40.0)		
T3	38	16 (42.1)		
T4	28	8 (28.6)		
lymph nodes transferring (N)			0.653	0.721
N0	41	17 (41.5)		
N1	29	10 (34.5)		
N2	16	5 (31.3)		
Metastasis (M)			2.867	0.090
M0	70	29 (41.4)		
M1	16	3 (18.8)		

with heterozygote need further gene frequency analysis (**Figures 1**-**5**).

Statistic methods

We utilized SPSS 19.0 software to proceed statistic treatment. The ages that did not coincide with normal distribution were represented by median (upper quartile, lower quartile). Rank sum test was utilized for group comparison. X^2 test and Fisher precise test were utilized for enumeration data. Univariate and multivariate logistic regression analysis were utilized for K-ras genetic mutation influencing factors of colorectal cancer patients ($\alpha=0.05$).

Results

Comparison of K-ras genetic mutation status in two groups of colorectal cancer patients

33 cases (36.26%) of colorectal cancer patients were mutant. Among them, 24 cases (72.7%) were found with mutation only in the 12th code, 9 cases (27.3%) were found with mutation only in the 13th code and no one case was found with mutation in both the two codes. Mutation rate of the 12th code in the Uygur nationality was significantly higher than that in the Han nationality (P<0.05), but there were no significant difference in the comparison of the total mutation rate and the 13th code mutation rate between the two groups (P>0.05, **Table 4**).

Influencing factor analysis for K-ras genetic mutation in colorectal cancer

Univariate logistic regression results showed that there were no significant difference between the K-ras genetic mutation and sex, age, smoking history, drinking history, tumor location, macropathology type, differentiation level, staging, invasive depth, lymph nodes transferring and metastasis in colorectal cancer patients (P>0.05, **Table 5**).

Taking sex, age, smoking history, drinking history, tumor location, macropathology type, differentiation level, staging, invasive depth, lymph nodes transferring and metastasis in

Table 6. Assignment of influencing factors for colorectal cancer tissues K-ras gene mutation and clinicopathological features

Factor	assignment
Sex	Male=0, female=1
Age (years old)	initial data
smoking	Nonsmoking=0, smoking=1
drinking	Nondrinking=0, drinking=1
Tumor location	Rectum=0, colon=1
macropathology type	Ulcer type=1, protrude type=2, infiltrating type=3
differentiation level	well differentiated=1, moderately differentiated=2, poorly differentiated=3
Staging	I stage=1, II stage=2, III stage=3, IV stage=4
Invasion depth (T)	T2=1, T3=2, T4=3
lymph nodes transferring (N)	N0=1, N1=2, N2=3
Metastasis (M)	M0=0, M1=1

Table 7. The multivariate logistic regression analysis results of Colorectal cancer tissues K-ras gene mutation and clinicopathological features

variables	β	SE	Wald X^2 value	df	P value	OR value	95% CI
Sex	-0.746	0.600	1.543	1	0.214	0.474	(0.146, 1.539)
Age	-0.028	0.038	0.553	1	0.457	0.972	(0.902, 1.048)
Smoking	-0.965	1.435	0.452	1	0.501	0.381	(0.023, 6.340)
Drinking	1.860	1.648	1.274	1	0.259	6.426	(0.254, 162.482)
Tumor position	0.016	0.572	0.001	1	0.978	1.016	(0.331, 3.116)
macropathology type							
Ulcer type	-0.019	1.357	0.000	1	0.989	0.981	(0.902, 1.048)
protrude type	-0.427	1.397	0.093	1	0.760	0.653	(0.125, 3.022)
differentiation level							
well differentiated	3.012	1.336	5.081	1	0.024	20.328	(1.481, 278.915)
moderately differentiated	0.585	0.699	0.702	1	0.402	1.796	(0.457, 7.062)
Staging							
I stage	-1.662	2.559	0.422	1	0.516	0.190	(0.001, 28.563)
II stage	-1.294	2.256	0.329	1	0.566	0.274	(0.003, 22.843)
III stage	-0.552	1.802	0.094	1	0.759	0.576	(0.017, 19.675)
Invasion depth (T)							
T2	-0.662	1.427	0.215	1	0.643	0.516	(0.031, 8.456)
T3	-0.668	0.626	1.139	1	0.286	0.513	(0.150, 1.748)
lymph nodes transferring (N)							
N0	0.134	1.396	0.009	1	0.923	1.144	(0.074, 17.649)
N1	-0.742	0.866	0.733	1	0.392	0.476	(0.087, 2.601)
Metastasis (M)	-0.876	1.488	0.347	1	0.556	0.416	(0.023, 7.692)

colorectal cancer patients as independent variable (**Table 6**), multivariate logistic regression results showed that there were no regression association between the K-ras genetic mutation and the above index (P>0.05, **Table 7**).

Discussion

The generation and development mechanism of colorectal cancer are still not clear to us by now. Colorectal cancer generation is a complex process with multiple genes (including oncogene, tumor suppressor gene, mismatch repair gene and several modifying gene) and multi procedure changes. Oncogenes related with colorectal cancer are ras gene and c-myc gene. Among them the K-ras genetic mutation has closely relationship with colorectal generation, anti epidermal growth factor receptor expression and targeted drug treatment. K-ras is an

important member of ras proto-oncogene family (including K-ras, N-ras and H-ras) and associated with tumor (especially human colorectal) generation, development, migration, diffusion and angiogenesis. The frequent mutational sites of K-ras gene (including mutant and wild type) are the 12th and 13th codon on exon 2 and the 61th codon on exon 3. There are differences in genetic mutation of colorectal cancer patients in different nationality all around the world. K-ras genetic mutation rate is 30%-50% in American [6], 45%-48% in European [7-8, 21], 23.0%-35.4% in Thailand, India, Republic of Korea and Japan [10-14] and 14.3%-33.57% in China (lower than that in European and America but similar with that in the East Asian) [15-21]. In this study, the total K-ras mutation rate is 36.3%, including the 12th codon mutation (72.7%) and the 13th codon mutation (27.3%), which are similar to most research results [6, 10, 13-21]. The total K-ras mutation rate in Uygur nationality is 46.2% and that in Han nationality is 28.8%. The 12th codon mutation rate in Uygur nationality (38.5%) is higher than that in Han nationality (17.3%), but there is no significant difference in total mutation rate and the 13th codon mutation rate between the two nationalities. The K-ras genetic mutation in Han nationality is similar with that in the East Asian countries and that in Uygur nationality is similar with that in European countries. However, the results from Dapeng Fu et al [22] are that K-ras genetic mutation rate in 144 cases of Han and Uygur nationality are 20.41% (20/98) and 26.09% (12/46) respectively. Difference of genetic mutation rate in Uygur nationality between our results and the results from Dapeng Fu may be associated with sample source and size. We also find that there are no associativity between the K-ras genetic mutation and sex, age, smoking history, drinking history, tumor location, macropathology type and differentiation level in colorectal cancer patients, which are similar to most reports at home and abroad [17]. However, results from Wei Liu et al [16] suggest that the K-ras genetic mutation rate are higher in females over 60 years old and that from Wei Wu et al [18] suggest that there are differences in primary position, lymph node transferring and metastasis between the 12th codon mutation and the 13th codon mutation. In our study, there are no associativity between K-ras genetic mutation rate and staging, invasion depth, lymph node transferring and metastasis, most reports at

home and abroad also show that there is no associativity between K-ras genetic mutation rate and staging, but Wenhui Wu et al [20] believe the associativity between that and lymph node transferring, liver transferring and TNM staging.

Wild type K-ras is a prediction index for satisfactory curative effect of molecule targeted therapy combined with chemotherapy. Identifying the K-ras gene status before targeted therapy is helpful to select suitable patients and obtain the best curative effect [23-25]. Many studies suggest that the 12th and 13th codon mutation in K-ras coding region exon 2 prognosticate resistance to EGFR-targeted antibody therapy [25, 26]. So panel in NCCN intensively recommend that tumor tissue (primary tumor or metastatic lesion) gene analysis should be utilized for all the colorectal cancer patients when they are diagnosed as IV stage metastatic carcinoma in the newest guide in 2013 because determining the K-ras genetic status as early as possible will provide reference for the selection of the following treatment protocols.

In this study, the K-ras genetic mutation rate of colorectal cancer patients in Uygur nationality is 46.2% and that in Han nationality is 28.8%. The 12th codon mutation rate in Uygur nationality is higher than that in Han nationality. There are no associativity between K-ras genetic mutation rate and sex, age, smoking history, drinking history, tumor location, macropathology type, differentiation level, staging, invasive depth, lymph nodes transferring and metastasis in colorectal cancer patients. The study provide the K-ras genetic mutation rate information of Uygur nationality colorectal cancer patients, but its associativity with prognosis need to be further studied. Treatment of EGFR-targeted antibody drugs based on K-ras mutation status will benefit vast colorectal cancer patients. The clinical pathology parameter of K-ras genetic mutation in this study are different with that in other reports, which is probably because of the difference in detection methods, sample source and size. The differences in mutation rate between the two nationalities need to be further confirmed by vast clinical studies.

Disclosure of conflict of interest

None.

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