

# Overexpression of wild-type p21Ras plays a prominent role in colorectal cancer

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**Abstract.** Colorectal cancer (CRC) is the most common gastrointestinal type of cancer. The overexpression of Ras proteins, particularly p21Ras, are involved in the development of CRC. However, the subtypes of the p21Ras proteins that are overexpressed and the mutation status remain unknown restricting the development of therapeutic antibodies targeting p21Ras proteins. The present study aimed to investigate the mutation status of *ras* genes associated with Ras proteins that are overexpressed in CRC and explore whether or not wild-type p21Ras could be a target for CRC therapy. p21Ras expression was examined immunohistochemically in normal colorectal epithelium, benign lesions and malignant colorectal tumor tissues by monoclonal antibody (Mab) KGH-R1 which is able to react with three types of p21Ras proteins: H-p21Ras, N-p21Ras and K-p21Ras. Then, the expression levels of p21Ras subtypes were determined in CRC by a specific Mab for each p21Ras subtype. Mutation status of *ras* genes in p21Ras-overexpressing CRC was detected by DNA sequencing. There was rare p21Ras expression in normal colorectal epithelium but a high level of p21Ras expression in CRC, with a significant increase from normal colorectal epithelium to inflammatory polyps, low-grade intraepithelial neoplasia, high-grade intraepithelial neoplasia and invasive colorectal adenocarcinoma, respectively. Overexpression of K-p21Ras was found in all CRC tissues tested, overexpression of N-p21Ras was found in 85.7% of the CRC tissues, while H-p21Ras expression was not found in any CRC tissue. DNA sequencing showed that there were no *K-ras* mutations in 60% of the K-p21Ras-overexpressing CRC, while 40% of the CRC tissues harbored *K-ras* mutations. *N-ras* mutations were not found in any N-p21Ras-overexpressing CRC. Our findings

indicate that overexpression of wild-type p21Ras may play a prominent role in the development of CRC in addition to *ras* mutations and could be a promising target for CRC therapy.

## Introduction

Colorectal cancer (CRC) is the most common gastrointestinal malignancy, and is one of the leading causes of cancer-related deaths worldwide. Approximately 50,000 individuals die of CRC each year. The estimated number of deaths are 26,020 and 23,170 for men and women, respectively, in 2016 (1).

Surgery, chemotherapy and irradiation remain the most common treatments of this devastating disease. Yet, the therapeutic effect of these traditional treatments is not as effective as expected (2). Therefore, improvement in the therapeutic approach is imperative for more efficacious treatment of CRC. Targeted therapy is a new treatment for human cancers and is a developing future trend. Several targeted drugs have been used clinically for CRC therapy, such as cetuximab (3) and panitumumab (4), monoclonal antibodies which target the epidermal growth factor receptor (EGFR). EGFR is overexpressed in 60-70% of CRC cases, and these patients could benefit from EGFR-targeted therapy (5,6). Unfortunately, cetuximab is ineffective for patients without EGFR overexpression or with EGFR extracellular domain mutations. Thus, it is necessary to identify new targets of therapy in EGFR signaling pathways.

The *Ras* gene, which locates at the downstream of EGFR in the RAS/RAF/MAPK pathway plays a major role in the development of CRC (7). *K-ras* mutation is an early event in colorectal tumorigenesis (8-10), and occurs in 30-60% of all CRC cases (11-18). However, *N-ras* mutations are rare in CRC (19). Glarakis *et al* found that the incidence of *N-ras* mutations is 1.3% in CRC (1/76) (20). *H-ras* mutations are far less common. From COSMIC database, among 765 colon adenocarcinomas only 1% were found to harbor *H-ras* mutation (21). Mutant p21Ras resulting from mutations of the *ras* gene abolish GAP-induced GTP hydrolysis of Ras proteins, leading to constitutive activation of *ras* (GTP-bound active form). Such activating mutations result in constitutive signaling, and thereby cause an increase in proliferation and in malignant transformation (22).

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As known, most oncogenes play a carcinogenic role by gene amplification and the overexpression of wild-type proteins (23,24). Ras proteins, p21Ras, are overexpressed in 29-76% of CRC (25), but the subtype of p21Ras proteins that are overexpressed in CRC and mutation status remain unknown, limiting the development of therapeutic antibodies targeting the *ras* gene. Thus, the present study was performed to investigate the subtypes of p21Ras proteins and mutation status in CRC by immunohistochemistry and direct sequence analysis to explore whether or not wild-type p21Ras could be a target for CRC therapy.

## Materials and methods

**Samples.** All samples were collected from archives at the Kunming General Hospital between April 2009 and May 2015. This study was approved by the Ethics Committee of Kunming General Hospital. Written informed consent was provided by all patients. A total of 378 samples were used, including 45 cases of normal colorectal tissues (with a 5-cm distance from the tumor margin), 73 cases of colorectal inflammatory polyps, 48 cases of colorectal low-grade intraepithelial neoplasia, 83 cases of colorectal high-grade intraepithelial neoplasia and 129 cases of colorectal cancers. All of the samples were formalin-fixed and paraffin embedded (FFPE).

Among the 129 CRC patients, 85 were males and 44 were females with an average age of 58.06 years (range 30-93 years). Microscopically, 48 were poorly differentiated, 58 were moderately differentiated, and 13 were well differentiated. Seventy-eight patients presented with regional lymph node metastasis. All of the patients receive no radiation therapy or neoadjuvant chemotherapy prior to surgery.

**Antibodies.** Monoclonal antibody KGH-R1 which is able to react with all three subtypes of p21Ras, H-p21Ras, N-p21Ras and K-p21Ras, was prepared in our laboratory (26). Monoclonal antibody 60309-1-Ig to K-p21Ras was purchased from ProteinTech Group (Chicago, IL, USA), and monoclonal antibody sc-31 to N-p21Ras and monoclonal antibody sc-29 to H-p21Ras were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

**Tissue microarray (TMA) construction.** FFPE colorectal tissue samples were selected for TMA construction (Boyikang, Beijing, China). Briefly, the areas containing cancer tissues were annotated on hematoxylin and eosin (H&E) slides and identified by two pathologists. Four-millimeter cores were removed from the selected area (region of interest) with a needle punch. These 4-mm donor cores were subsequently embedded in previously arranged recipient paraffin blocks through a precisely spaced 36-hole array pattern. Core positions in the recipient paraffin block were noted on a TMA map. After paraffin was intenerated and cooling, the recipient blocks were cut using a microtome and used for immunohistochemistry.

**Immunohistochemistry.** Sections (4- $\mu$ m) from the TMA containing CRC and normal tissues were collected on glass slides coated with adhesive polylysine. The sections were incubated in an oven at 60°C for 3 h, then deparaffinized in xylene,

hydrated in descending concentrations of ethanol (2 x 100% for 3 min, 95% for 3 min and 85% for 3 min) and washed in double-distilled water three times. Subsequently, the slides were subjected to specific epitope unmasking by an autoclave (1600 W, 2 min) in citrate acid buffer (0.01 M pH 6.0), and then exposed to 3% H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidase reactivity for 10 min, followed by washing in distilled water. To avoid unspecific staining, 10% BSA was used to block the sections for 40 min at 37°C. Then the sections were incubated in the p21Ras monoclonal antibody at 4°C overnight. Controls were obtained by incubating serial sections with the blocking solution but incubated in phosphate-buffered solutions (PBS, pH 7.2) instead of the primary antibodies, and then washed in 0.01 mol/l PBS. The sections were then sequentially exposed to horseradish peroxidase secondary antibody (ZSGB-BIO, Beijing, China) for 30 min, and washed with PBS three times. To visualize the sections, diaminobenzidine as a chromogen was applied for 5-7 min and hematoxylin counterstain for 1 min. Finally, all slides were dehydrated and mounted.

The expression of p21Ras was evaluated by the percentage of positive cells and histological score (Hscore) (27). Briefly, at least 300 cells were counted in every component on every slide. The staining patterns were graded as membranous or cytoplasmic. Protein immunoreactivity was scored according to the intensity of staining, which was graded on an arbitrary scale ranging from 0 to 3: 0, negative (no stained cells); 1, low (primrose yellow cells); 2, medium (yellow cells); and 3, high expression (tawny cells). A mean percentage of positive tumor cells was determined in at least 3 areas at x40 magnifications and ranged from 0 to 100%.

**DNA extraction.** Tumor areas in the FFPE tissue blocks were circled by two experienced pathologists. FFPE serial sections (10- $\mu$ m) were used for DNA extraction by means of the QIAamp DNA FFPE tissue kit according to the QIAamp DNA FFPE tissue handbook. The purity of the extracted DNA was tested using an ultraviolet spectrophotometer.

**PCR amplification reaction.** Primer 5.0 and oligo softwares were used to design primers for *K-ras*, *N-ras* and *H-ras* amplification. Primer sequences were as follows *K-ras* exon 2 sense, 5'-TTA TTATAAGGCCTGCTG-3' and antisense, 5'-TGTATCAAAGA ATGGTCC-3'; *K-ras* exon 3 sense, 5'-GTGTTTCTCCCTTC TCAG-3' and antisense, 5'-GGCATTAGCAAAGACTCA-3'; *K-ras* exon 4 sense, 5'-TGTTACTAATGACTGTGCTA-3' and antisense, 5'-TAACAGTTATGATTTTGC-3'; *K-ras* exon 5 sense, 5'-ACATGGCTTTCCAGTAA-3' and antisense, 5'-GTTGCC ACCTTGTTACCT-3'; *N-ras* exon 2 sense, 5'-AATTAACCCTG ATTACTGG-3' and antisense, 5'-TAAAGATGATCCGAC AAG-3'; *N-ras* exon 3 sense, 5'-TAACCTTGGCAATAGCAT-3' and antisense, 5'-TAACCTCATTTCCTCCATA-3'; *N-ras* exon 4 sense, 5'-CATGACCCACTGTACCCA-3' and antisense, 5'-TTG CACAAATGCTGAAAG-3'; *N-ras* exon 5 sense, 5'-GAGATA CAAATGCAAGAG-3' and antisense, 5'-AAACACCAGCACT CCT-3'; *H-ras* exon 2 sense, 5'-AGACCCTGTAGGAGGACCC-3' and antisense, 5'-CTGCTGGCACCTGGAC-3'; *H-ras* exon 3 sense, 5'-CACGGAAGTCCCTGAGGGG-3' and antisense, 5'-GCCTGGCCCCACCTGTG-3'; *H-ras* exon 4 sense, 5'-CTC TCGCTTTCCACCTCT-3' and antisense, 5'-AGCTGTGGG

Table I. p21Ras expression in benign lesions and malignant tumors of colorectal cancer.

Tissue	n	Positive	Percentage of positive tissues (%)	Intensity	Percentage of positive cells (%)	Hscore
Normal epithelium	45	0	0	0.0123	1.23±2.98	1.23±2.98
Inflammatory polyps	73	7	9.59	0.0741	7.06±15.64	7.41±16.74
Low-grade intraepithelial neoplasia	48	31	64.58	0.6654	53.33±42.36	66.54±59.91
High-grade intraepithelial neoplasia	83	50	60.24	0.942	57.28±48.73	94.2±87.46
Colorectal carcinoma	94	61	64.89	1.161	63.11±47.11	116.14±103.30

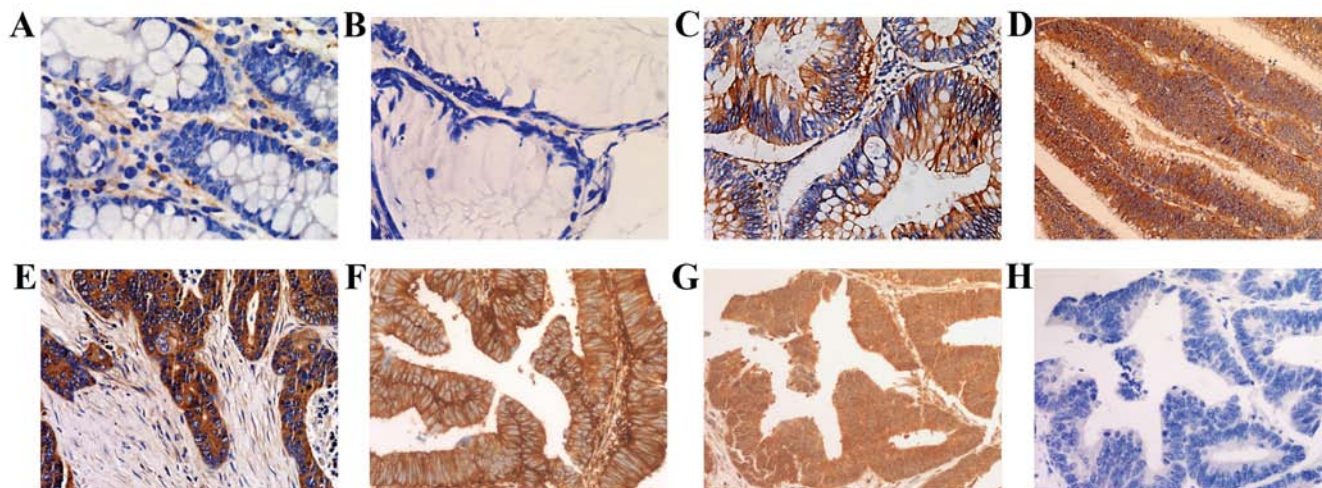


Figure 1. Expression of total p21Ras and p21Ras subtypes was detected by immunohistochemistry. (A-E) Total p21Ras expression detected by Mab KGHR-1. (A) Negative staining in NE; (B) negative staining in IP; (C) strong staining in LIN; (D) strong staining in HIN; (E) strong staining in CRC. (F-H) Expression of p21Ras subtypes in CRC. (F) Strong staining of K-p21Ras; (G) strong staining of N-p21Ras; (H) no staining of H-p21Ras. NE, normal epithelium; IP, inflammatory polyps; LIN, low-grade intraepithelial neoplasia; HIN, high-grade intraepithelial neoplasia; CRC, colorectal cancer.

TGGAGA-3'; *H-ras* exon 5 sense, 5'-GGCAGGCGGCCAC AGG-3' and antisense, 5'-ATCCGGTGGGCGTGGC-3'. Human *K-ras*, *N-ras* and *H-ras* gene sequences were obtained from GeneBank AF493917, AF493919 and AF493916, respectively. Exon 1 is an untranslated region (UTR). The amplification was performed in a final volume of 25  $\mu$ l containing 2.5  $\mu$ l 10X PCR buffer, 2  $\mu$ l dNTP mixture, 1.0  $\mu$ l *Taq* enzyme, 1.0  $\mu$ l forward primer (10  $\mu$ mol/l), 1.0  $\mu$ l reverse primer (10  $\mu$ mol/l), and at least 800 ng DNA. PCR reaction conditions were as follows: initial denaturation at 95°C for 4 min, 30 cycles at 95°C for 30 sec, suited annealing temperature for 30 sec, amplification at 72°C for 30 sec; and a final extension at 72°C for 10 min. The PCR amplification products were separated by 1% agarose gel electrophoresis (AGE) for 30 min and imaged using the Syngene imaging system (Synoptics Ltd., Cambridge UK).

**DNA sequence analysis.** Twenty microliters of PCR products were sent to the Beijing Genomics Institute (BGI) for sequencing. The sequencing was conducted in both directions (forward and reverse), and then analyzed by Align X (Invitrogen, Carlsbad, CA, USA) and Chromas (Technelysium Pty Ltd., Queensland, Australia) softwares.

**Statistical analysis.** The statistical analysis was performed using the SPSS software package, standard version 22.0 (SPSS

Inc., Chicago, IL, USA). Data are expressed as mean  $\pm$  SD. Statistical significance was determined by the Student's t-test. A value of  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Expression of p21Ras.** p21Ras expression was detected in none of the normal colorectal epithelium (0/45), in 9.59% of the inflammatory polyps (7/73), in 64.58% of the low-grade intraepithelial neoplasia samples (31/48), in 60.24% of the high-grade intraepithelial neoplasia samples (50/83) and 64.89% of invasive colorectal carcinoma samples (61/94) (Fig. 1 and Table I). The expression products were localized in the cytoplasm and cell membrane.

We used the percentage of positive cells and Hscore to evaluate the expression level of p21Ras. In normal colorectal epithelium, inflammatory polyps, low-grade intraepithelial neoplasia, high-grade intraepithelial neoplasia and invasive colorectal carcinoma samples, the mean percentages of positive cells were 1.23±2.98, 7.06±15.64, 53.33±42.36, 57.28±48.73 and 63.11±47.11%, and the mean Hscores were 1.23±2.98, 7.41±16.74, 66.54±59.91, 94.20±87.46 and 116.14±103.30, respectively (Fig. 2 and Table I), which indicated a gradual increase from normal colorectal epithelium to CRC.

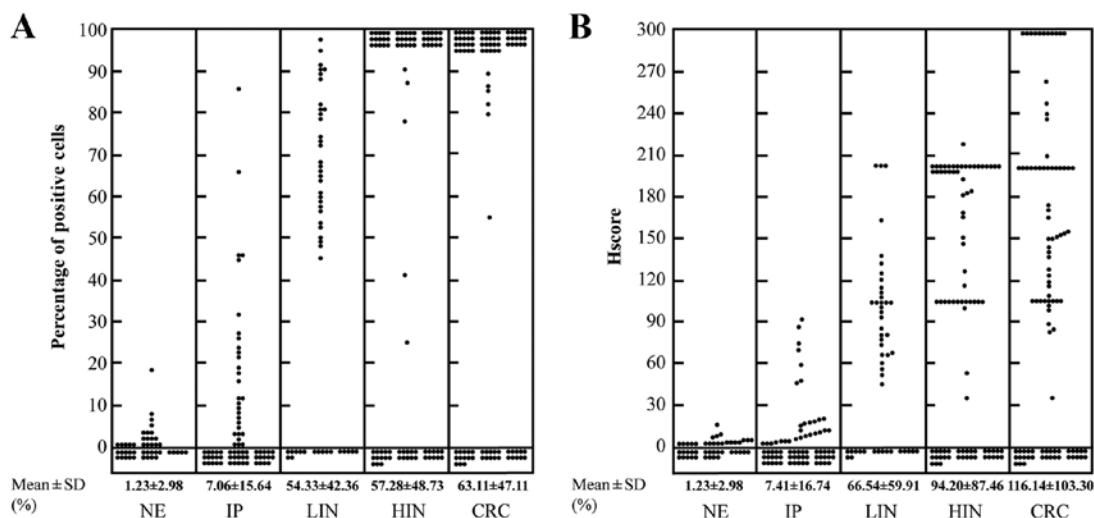


Figure 2. The percentage of positive cells and Hscore of p21Ras expression in the different colorectal lesions. (A) The percentage of positive cells of p21Ras distributed in normal, hyperplastic and malignant lesions of colorectal tissue. (B) The Hscore of p21Ras expression for each sample in hyperplastic and malignant lesions of colorectal tissue. Hscore, histological score. NE, normal epithelium; IP, inflammatory polyps; LIN, low-grade intraepithelial neoplasia; HIN, high-grade intraepithelial neoplasia; CRC, colorectal cancer.

Table II. Correlation between p21Ras expression and the clinicopathologic features of the invasive colorectal adenocarcinoma patients.

Clinicopathologic features	Cases (N=94)	Hscore	P-value	Percentage of positive cells (%)	P-value
Gender			>0.05		>0.05
Male	64	114.29±103.76		63.33±46.78	
Female	30	120.10±103.97		62.63±48.62	
Age (years)			>0.05		>0.05
≤50	28	105.07±104.43		55.95±49.55	
<50	66	120.84±103.26		66.14±46.09	
Histologic type			<0.05		<0.05
Non-mucinous adenocarcinoma	84	121.43±101.70		66.40±46.20	
Mucinous adenocarcinoma	10	35.4±66.99		25.40±42.77	
Differentiation			<0.01		<0.01
Well	13	40.92±73.16		28.77±45.08	
Moderate	34	102.78±90.99		63.75±47.95	
Poor	47	182.08±91.68		87.47±31.42	
Invasive depth			>0.05		>0.05
Superficial muscle	7	83.71±83.61		57.14±53.45	
Deep muscle	17	103.65±108.56		57.59±49.91	
Full thickness	70	122.42±104.20		65.04±46.37	
Tumor size (cm)			>0.05		>0.05
<2	12	72.17±93.12		45.96±48.44	
2-5	59	123.94±101.51		67.81±45.85	
>5	22	119.09±111.27		60.00±49.36	
Lymph node metastasis			<0.05		<0.05
-	63	131.21±104.57		68.97±45.88	
+	31	85.52±95.06		51.19±48.12	

Correlation between p21Ras expression and clinicopathologic variables. A total of 94 CRC cases were adopted here.

There was a statistical correlation between histologic type and Hscores, and also lymph node metastasis and Hscores

Table III. p21Ras expression and *ras* mutations in CRC.

Patients	<i>K-ras</i>			<i>N-ras</i>		<i>H-ras</i>		
	Expression	Exon	Mutation	Expression	Mutation	Expression	Exon	Mutation
201503220	+	Exon 4	c.436G>A→p.A146T	-	-	-	Exon 2	c.81T>C
201503213	+	-	-	-	-	-	Exon 2	c.81T>C
201503102	+	-	-	-	-	-	-	-
201502928	+	Exon 4	c.436G>A→p.A146T	+	-	-	-	-
201502903	+	-	-	+	-	-	Exon 2	c.81T>C
201502056	+	Exon 2	c.35G>A→p.G12D	+	-	-	-	-
201501338	+	-	-	+	-	-	Exon 2	c.81T>C
201501304	+	-	-	+	-	-	-	-
201500934	+	-	-	+	-	-	Exon 2	c.81T>C
201500667	+	-	-	+	-	-	-	-
201412412	+	Exon 2	c.38G>A→p.G13D	+	-	-	-	-
201410010	+	-	-	+	-	-	-	-
201409737	+	Exon 2	c.38G>A→p.G13D	+	-	-	-	-
201408315	+	Exon 2	c.35G>C→p.G12A	+	-	-	Exon 2	c.81T>C
201407762	+	-	-	+	-	-	-	-
201406231	+	Exon 4 Exon 5	c.436G>A→p.A146T c.526>T→p.E176Stop	-	-	-	-	-
201405694	+	-	-	+	-	-	Exon 2	c.81T>C
201405647	+	Exon 2	c.35G>A→p.G12D	+	-	-	Exon 2	c.81T>C
201503581	+	-	-	+	-	-	-	-
201503452	+	-	-	-	-	-	-	-
201502977	+	Exon 5	c.467T>C→p.F156S	+	-	-	-	-
201502204	+	-	-	+	-	-	-	-
201501593	+	Exon 2	c.35G>T→p.G12V	+	-	-	Exon 2	c.81T>C
201501337	+	-	-	+	-	-	-	-
201501079	+	-	-	+	-	-	-	-
201407828	+	Exon 2	c.38G>A→p.G13D	+	-	-	-	-
201407425	+	-	-	+	-	-	Exon 2	c.81T>C
201407236	+	Exon 2	c.35G>A→p.G12D	+	-	-	-	-
201405983	+	Exon 2	c.38G>A→p.G13D	+	-	-	Exon 2	c.81T>C
201405149	+	-	-	+	-	-	-	-
201404719	+	-	-	+	-	-	-	-
201404718	+	Exon 2	c.38G>A→p.G13D	+	-	-	Exon 2	c.81T>C
201404238	+	-	-	+	-	-	-	-
201401791	+	-	-	+	-	-	-	-
201401610	+	-	-	+	-	-	Exon 2	c.81T>C

( $P < 0.05$ ). However, no correlation was observed between Hscore and the other patient clinicopathologic parameters ( $P > 0.05$ ), which suggests that the expression of p21Ras and indices such as gender, age, invasive depth are independent events. Furthermore, the same results were found between the percentage of positive cells and the clinicopathologic variables (Table II).

**Expression of p21Ras subtypes in CRC.** Three Mabs, each of which is able to recognize one of the p21Ras subtypes were used to detect the expression of the three p21Ras subtypes by immunohistochemistry (Fig. 1). It was demonstrated

that K-p21Ras was expressed in all 35 CRC, N-p21Ras was expressed in 30/35 of CRC samples, and H-p21Ras was not expressed in all of the CRCs tested (Table III). Notably, over-expression of both K-p21Ras and N-p21Ras were detected in 30 cases (Table III). Analysis of the immunohistochemical staining of K-p21Ras, N-p21Ras and H-p21Ras was also evaluated according to the percentage of positive cells and Hscore, which were  $92.08 \pm 10.98$ ,  $77.00 \pm 33.21$ , 0% and  $180.08 \pm 50.81$ ,  $154.04 \pm 92.26$ , 0, respectively.

**Ras mutation status in CRC.** All 12 exons of *K-ras*, *N-ras* and *H-ras* in 35 of the CRC cases were amplified successfully

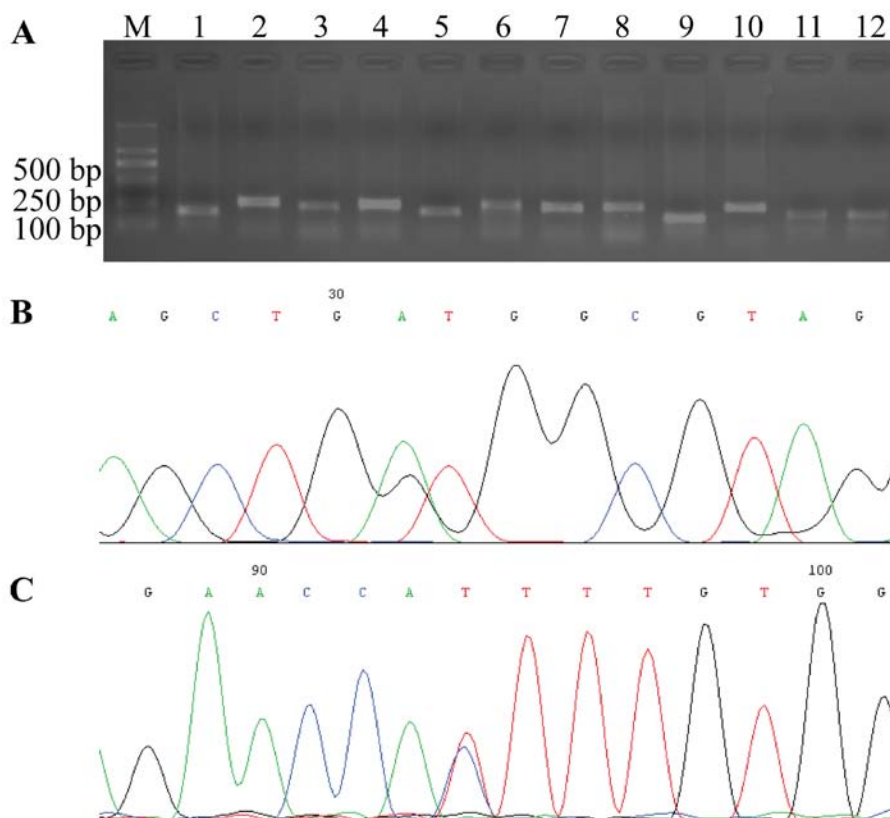


Figure 3. Mutation status of *ras* was detected by direct sequencing. (A) Representative 1% agarose gel revealing 12 exon amplicons in CRC. *K-ras* exon 2-5 (lane 1-4), *N-ras* exon 2-5 (lane 5-8), *H-ras* exon 2-5 (lane 9-12). (B) *K-ras* mutation in CRC (c.35G>A). (C) *H-ras* mutation in CRC (c.81C>T). CRC, colorectal cancer.

by designed primers. Each PCR product was confirmed by agarose gel electrophoresis with expected sizes of 185, 274, 255, 263, 217, 339, 255, 286, 187, 276, 207 and 215 bp, respectively (Fig. 3).

Among the 35 cases of CRC with K-p21Ras overexpression, *K-ras* mutations were detected in 40% of the cases (14/35), however, *K-ras* mutations were not detected in the other 60% of CRC cases which indicated that the overexpression of p21Ras in these 60% CRC were wild-type. *K-ras* mutation was present in codon 12 (5 cases), 13 (5 cases), 146 (3 cases), 156 (1 case) and 176 (1 case). The most frequent *K-ras* mutation was transition of base G/A (5/14, 35.7%) in codon 13, which resulted in the substitution of glycine with aspartate. *N-ras* mutations were not found in all 30 of the N-p21Ras-overexpressing CRC cases (Table III). In CRC without N-p21Ras expression no *N-ras* mutation was detected, and in CRC without H-p21Ras expression only a *H-ras* nonsense mutation at codon 27 was found (Fig. 3).

## Discussion

Amplification of oncogenes and protein overexpression have been identified in various solid tumors. Overexpression of the human epidermal growth factor receptor 2 (HER2) gene occurs in 15-25% of human breast cancers (28). EGFR is overexpressed in 40-60% of non-small cell lung cancer cases (23). Overexpression is considered to be the main activation mechanisms of oncogenes, and oncogene proteins could be potential targets for cancer therapy. Trastuzumab (29),

pertuzumab (30) and lapatinib (31) targeting HER2 protein have been approved as standard care for inhibiting HER2 activity in the treatment of HER2-positive breast cancer. Cetuximab (3,32), panitumumab (4,33) and nimotuzomab (34) targeting EGFR protein have been used to treat human cancers with EGFR overexpression, such as CRC and non-small cell lung cancer.

*Ras* gene protein p21Ras was found to be overexpressed in most human tumors, including CRC (35), bladder cancer (36), breast cancer (37,38), stomach adenocarcinomas (39), thyroid cancer (40) and laryngeal cancer (41). However, no targeted drugs that target against p21Ras directly have been exploited. Recently, we prepared a novel anti-p21Ras Mab, KGH-R1, which can recognize and react with three type of p21Ras, including H-p21Ras, N-p21Ras and K-p21Ras, and the single chain antibody derived from this Mab could regress p21Ras-overexpressing tumors *in vitro* and *in vivo* (26,42). In this study, the Mab was employed to examine p21Ras expression in normal colorectal epithelium, inflammatory polyps, low-grade intraepithelial neoplasia, high-grade intraepithelial neoplasia and invasive CRC. The results showed that there was almost no p21Ras expression in normal colorectal mucosa, but high level expression of p21Ras in CRC and colorectal intraepithelial neoplasia. Together with the reported data (15,43-46), we confirmed that p21Ras overexpression is an important event in colorectal carcinogenesis and plays a major role in the development of CRC.

Subsequently, we evaluated expression of p21Ras subtypes by immunohistochemistry using anti-K-ras, anti-N-ras or anti-H-ras Mab, and found that K-p21Ras was expressed in all of

the tested CRC tissues, which was significantly higher than the results of Elsabah and Adel (42.3%) (47). The different frequency of Ras expression probably resulted from region variations, and the difference in sample sources (48,49). Additionally, we found that N-p21Ras was expressed in 85.7% of the CRC cases, but H-p21Ras was not expressed in any tested CRC case. Our data indicated that K-p21Ras and N-p21Ras are deeply involved in CRC development.

Furthermore, DNA sequencing was used to reveal the mutation status of the overexpressed p21Ras, and found that 60% of K-p21Ras-overexpressing CRC samples did not harbor *K-ras* mutation. *N-ras* mutation was not found in any of the N-p21Ras-overexpressing CRCs. Thus, overexpression of the wild-type p21Ras may be another important mechanism in CRC development, and the therapeutic antibodies targeting wild-type p21Ras may have better prospect for the therapy of CRC. To date, few studies have reported the overexpression of wild-type p21Ras in cancers. To the best of our knowledge, this is the first time to reveal wild-type p21Ras expression in CRC. The mechanism involved in the induction of tumorigenesis by the overexpression of wild-type p21Ras remains unclear. Zheng *et al* reported that overexpression of the wild-type N-p21Ras induces IL-8 by binding and activating the cytoplasmic pool of JAK2. IL-8 then acts on tumor cells and promotes the progression of cancer (50). In addition, we speculated that overexpression of the wild-type p21Ras leads to the excessive GTP-bound active form that cannot be completely hydrolyzed, and finally stimulates persistent cell proliferation and tumorigenesis. However, on the other hand, Spandidos and Wilkie reported that after rat 208F cells (a derivative of Rat-1 cells) were transfected with T24 mutant *H-ras* (51) or the mutant *N-ras* (52), the expression level of normal *H-ras1* gene was elevated, leading to suppression of the transformed and tumorigenic phenotypes induced by mutant *ras* genes. Thus, wild-type H-p21Ras plays a complex role in the development of cancers, and further studies are needed to clarify the mechanisms of wild-type p21Ras overexpression in cancer development.

In conclusion, we detected the expression level of p21Ras in benign and malignant CRC, as well as the p21Ras subtypes and mutation status of the *ras* gene in CRC. We conclude that the overexpression of wild-type p21Ras, especially wild-type K-p21Ras and N-p21Ras play a prominent role in the development of CRC. This also implies that wild-type p21Ras is a promising target for CRC therapy and it is feasible to develop the antibody drugs against wild-type p21Ras.

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