Loss of heterozygosity of Kras2 gene on 12p12-13 in Chinese colon carcinoma patients

Jun Wan, Hong Li, Yuan Li, Mei-Ling Zhu, Po Zhao

INTRODUCTION

Clinical and experimental studies have shown that point mutation of Kras2 gene plays an important role in the development and progression of tumors [1-4]. However, it has been reported that wild type Kras2 gene has inhibitory effects on tumor growth and proliferation [5]. Inactivation of cancer suppressor gene is a frequently encountered early event in the development of tumors, leading to loss of heterozygosity (LOH) [6-10]. This study was to investigate the possible genetic variation of wild type Kras2 gene in the carcinogenesis of colon carcinoma by analyzing LOH in tumor and its adjacent tissues.

MATERIALS AND METHODS

Specimens
Ten specimens of primary carcinoma tissue, 10 specimens of adjacent tissue, and 10 specimens of normal tissue at the distal incision margin were taken respectively from patients with pathologically confirmed colon carcinoma before and after surgery at the Department of Surgery, General Hospital of the Chinese PLA from January to December 2003. The patients did not receive radiotherapy and chemotherapy before surgery.

DNA extraction from genome
Specimens of carcinoma and its adjacent tissues as well as normal tissue at the distal incision margin were suspended respectively in 50μL DNA lysate containing 50 mmol/L Tris- HCl, 1mmol/L EDTA, 0.1% Tween 20, 200 mg/L protease K, pH8.0, and digested overnight at 50°C. Protease K was denatured and inactivated at 95°C. Then the supernatant was centrifuged and stored at -20°C for use.

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Table 1 Primer sequences on 12p12-13 microsatellite markers

| Markers | Primer sequences | Length (bp) |
|---------|-----------------|-------------|
| D12S89  | D12S89-F: 5'-ATTAGAGCCGACGTGTTT-3' | 254-288     |
|         | D12S89-R: 5'-CCATATGGGACTGCGG-3' |             |
| D12x358 | D12x358-F: 5'-GGGGACAGGAAAACTTG-3' | 238-270     |
|         | D12x358-R: 5'-AAGACGCTTTTATTTTCTC-3' |             |
| D12x310 | D12x310-F: 5'-GAAGACTTGGTTAATC-3' | 243-253     |
|         | D12x310-R: 5'-TTTGAATCTCCAAATGGC-3' |             |
| D12S1057| D12S1057-F: 5'-GAAGACTTGGTTAATC-3' | 204-222     |
|         | D12S1057-R: 5'-TTTGAATCTCCAAATGGC-3' |             |
| D12s1592| D12s1592-F: 5'-AGGGTTCAAAAAGTGTGACG-3' | 215-261     |
|         | D12s1592-R: 5'-AGGGTTCAAAAAGTGTGACG-3' |             |
| D12S1596| D12S1596-F: 5'-CACTGTTCGCGGTAGGTT-3' | 280-302     |
|         | D12S1596-R: 5'-GCTGAGTGTGGTGGTCA-3' |             |
| D12S1035| D12S1035-F: 5'-CACTGTTCGCGGTAGGTT-3' | 234-276     |
|         | D12S1035-R: 5'-GCTGAGTGTGGTGGTCA-3' |             |
| D12S1606| D12S1606-F: 5'-CACTGTTCGCGGTAGGTT-3' | 119         |
|         | D12S1606-R: 5'-GCTGAGTGTGGTGGTCA-3' |             |
| D12s310 | D12s310-F: 5'-TCTTCTTGGTGGCTGG-3' | 243-253     |
|         | D12s310-R: 5'-TCTTCTTGGTGGCTGG-3' |             |
| D12s358 | D12s358-F: 5'-GGGGACAGGAAAACTTG-3' | 238-270     |
|         | D12s358-R: 5'-GGGGACAGGAAAACTTG-3' |             |
| D12S89  | D12S89-F: 5'-ATGTATGATGTTATGATGTT-3' | 254-288     |
|         | D12S89-R: 5'-ATGTATGATGTTATGATGTT-3' |             |
| D12S1591| D12S1591-F: 5'-CACTGTTCGCGGTAGGTT-3' | 280-302     |
|         | D12S1591-R: 5'-GCTGAGTGTGGTGGTCA-3' |             |

**Polymerase chain reaction (PCR)**

One μL 10xPCR buffer, 0.5 mmol/L magnesium ion, 0.2 mmol/L 4xDNTP, 0.4 μmol/L upstream primer, 0.4 μmol/L downstream primer, 1U Taq DNA polymerase/reaction and 0.5 μL DNA template were added into 10 μL PCR system at 94°C for 5 min. Fourteen PCR cycles of amplification were performed at 94°C for 30 s, at 55°C for 30 s, at 72°C for 30 s, followed by 16 cycles at 94°C for 30 s, at 55°C for 30 s, at 72°C for 30 s, and a final extension at 72°C for 5 min.

**Denaturing polyacrylamide gel electrophoresis**

PCR products (0.3μL) were added to the loading buffer containing GENESCAN-500 molecular weight as internal control and mixed with formamide, then denatured at 72°C for 2-3 min and electrophoresed by the AB1377 fluorescence sequencer. The standard sequencing PAGE 64-well denaturing polyacrylamide gel was used for electrophoresis. The electrophoresis was performed at the temperature higher than 42°C for 2.5-3 h. The electrophoresis channels were analyzed using GENESCAN version 3.1 Software. The types and intensity of fluorescence were collected for each channel, the size of amplified PCR products was determined using the molecular weight as internal control. Data of the type, intensity and molecular weight of the amplified microsatellite DNA fluorescence were obtained using GENOTYPE version 2.0 Software. The fluorescence intensity could indicate the amplified DNA within the linearity (relative fluorescence intensity was 200-300).

**LOH analysis**

Based on the principles of fluorescence labeling of primers, a fluorescence labeled primer group-matched sequence was linked to the 5' end of a specific primer, so that the PCR products were labeled with the fluorescence group in the process of PCR. The fluorescence labeled PCR products were electrophoresed using the AB 1377 fluorescence sequencer. Data collected by the electrophoresis were analyzed using the GENESCAN and GENOTYPE Softwares to obtain the peak and size of the map. Gene typing was performed. The peak was found in 2 allelic gene segments and compared to the normal value of the adjacent channels. The allelic ratio was calculated according to the following formula: allelic ratio=peak ratio of carcinoma tissue/peak ratio of normal tissue. LOH was considered when the allelic ratio was higher than 1.5 or lower than 0.67. Microsatellite instability (MSI) was considered when no abnormal peak point was found in DNA of carcinoma tissue compared to normal tissue.

**Statistical analysis**

The correlation between LOH and clinical and pathological parameters was evaluated by chi-square test. All statistical analyses were carried out by SPSS 10.0. P < 0.05 was considered statistically significant.

**RESULTS**

**Frequency of LOH in carcinoma adjacent tissue**

No LOH was detected in 70 (7/10) of adjacent tissue specimens (1, 2, 4-6, 8, 9) on all markers. LOH was detected in 30% (3/10) of adjacent tissue specimens (3, 7, 10) at least on one marker, 28.5% (2/7) of adjacent tissue specimens on D12S1034, 25% (1/4) on D12S1617, 14.29% (1/7) on D12S1596, 12.5% (1/8) on D12S89, and 0% on other markers (Table 2 and Table 3, Figure 1).

**Frequency of LOH in carcinoma tissue**

No LOH was detected in 40% (4/10) of primary colon carcinoma tissue specimens (1, 4-6) on all markers.
was detected in 60% (6/10) of colon carcinoma tissue specimens (2, 3, 7–10) at least on one marker, 42.86% (3/7) on D12S1034 and D12S1591, 33.33% (1/3) on D12S310, and 0% (0/4) on D12S1592 (Tables 2 and 3).

**Correlation between LOH on 12p12-13 and clinicopathological parameters**
Chi-square test was used to evaluate the correlation between LOH and clinicopathological parameters. The results showed that LOH did not correlate with age, sex, tumor size and lymph node metastasis (Table 4).

**DISCUSSION**
RASp21 consisting of Hras1, Nras and Kras2, is a GTP-coupled protein and can transfer signals from cell surface into cells. Its normal expression is necessary to maintain the normal physiological activities of cells. Activated Ras proto-oncogenes, especially Kras2, play an important role in the carcinogenesis of human and rodent tumors. Mutations of Kras2 gene have been found in tumor tissues of human organs, including bladder[11], breast[12], rectum[13], kidney[14], liver[15], lung[16], ovary[17], pancreas[18], stomach[19] and hematopoietic system[20]. In general, about 30% cancers display ras gene mutations, while the highest mutation rate is found in colonic and pancreatic cancer[21–23]. In samples of mutated ras gene, most mutations occur in Kras2 gene. Mutation and activation of ras gene usually occur at codons 12 and 13 or 61, leading to the transformation of proto-oncogene to oncogene[24]. This kind of activation can up-regulate the expression of ras/ErK signal channel in the absence of external stimuli and further increase the abnormality of associated signal channels, leading to malignant transformation of cells. Activated ras gene is usually considered as the dominant oncogene because of the existing expression of wild type ras and malignant transformation of activated ras[25]. However, is still controversial the effect of the dominant gene-ras is still controversial since wild type ras has been found in human and mouse pulmonary adenocarcinomas[26,27].

**In vivo and in vitro experiments**[28] have shown that tumors are found more frequently in normal mice with 2 wild type Kras2 copies than in those with LOH of one wild type Kras2 copy after they are treated with 2 carcinogens. The occurrence of tumor is 50-fold higher in mice with LOH of one wild type Kras2 copy than in normal mice with 2 wild type Kras2 copies. The tumor in the former group of mice is poorly-differentiated adenocarcinoma, while the tumor in the later group of mice is adenoma. Zhang et al[28] reported that wild type Kras2 gene can inhibit cell growth, formation of clones, and induce tumors in naked mice. In addition, LOH has

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**Table 2** Frequency of LOH on the 11 markers of 12p12-13 in colon carcinoma and its adjacent tissues

| Markers | LOH (%) | Signal (%) | Frequency of LOH (%) | Heterozygosity (%) | Tumor tissue |
|---------|---------|------------|----------------------|-------------------|--------------|
| D12S823 | 0       | 5          | 0                    | 50                | 20           |
| D12S1034| 2       | 7          | 28.57                | 70                | 42.88        |
| D12S1596| 1       | 4          | 25                   | 40                | 25           |
| D12S1591| 1       | 7          | 14.29                | 70                | 42.88        |
| D12S358 | 0       | 8          | 0                    | 80                | 25           |
| D12S310 | 0       | 3          | 0                    | 30                | 33.33        |
| D12S358 | 0       | 4          | 0                    | 40                | 0            |
| D12S89  | 1       | 8          | 12.5                 | 80                | 12.5         |
| D12S1617| 1       | 7          | 14.29                | 70                | 14.29        |
| D12S1037| 0       | 7          | 0                    | 70                | 28.57        |
| D12S1606| 0       | 6          | 0                    | 60                | 16.67        |

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**Table 3** Distribution of LOH on 12p12-13 in microsatellite-labeled primers

| Biomarker | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|-----------|---|---|---|---|---|---|---|---|---|----|
| D12S823   | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○  |
| D12S1034  | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○  |
| D12S1596  | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○  |
| D12S1591  | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○  |
| D12S358   | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○  |
| D12S310   | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○  |
| D12S1592  | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○  |
| D12S89    | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○  |
| D12S1617  | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○  |
| D12S1037  | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○  |
| D12S1606  | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○  |

○: Normal; ○: No signal; ●: LOH; A: adjacent tissue; T: cancer tissue

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been found in pulmonary adenocarcinoma induced by various chemical carcinogens. Point mutation of Kras2 gene is detected in 67-100% mice with LOH of wild type Kras 2 gene. These important findings will certainly query the established carcinogenesis of dominant Kras2 gene.

It was reported that the development and progression of colon carcinoma are a process involving multiple genes and factors, and characterized by its stages: normal mucosa → atypical hyperplasia including intestinal metaplasia → adenoma → adenocarcinoma [10]. Kras2 gene as a dominant oncogene due to its point mutation plays an important role in the progression of carcinoma, which is one of the reasons why the inhibitory effect of wild type kras2 gene on cancer is concealed. Since cancer suppressor gene can be inactivated by deleting mutation, we studied LOH of Kras 2 gene on 12p12-13 in primary colon carcinoma. These important findings will certainly query the established carcinogenesis of dominant Kras2 gene.

In conclusion, Kras2 gene can exert inhibitory effects on the proliferation of colon carcinoma cells. LOH on 12p12-13 does not correlate with the clinical and pathological parameters obtained from colon carcinoma.

### Table 4 Correlation between LOH on chromosome 12p12-13 and clinical pathologic factors

| Clinical character                  | LOH |  | Total | Ratio (%) | P   |
|------------------------------------|-----|---|-------|-----------|-----|
| Tissue class                       |     |   |       |           |     |
| Adjacent Tumor                     | 3   | 7 | 10    | 30        | 0.17753 |
| Tumor                              | 6   | 4 | 10    | 60        | 0.77816 |
| Age (yr)                           |     |   |       |           |     |
| ≤50                                | 4   | 3 | 7     | 70        | 0.77816 |
| >50                                | 2   | 1 | 3     | 30        | 0.57816 |
| Sex                                |     |   |       |           |     |
| Male                               | 5   | 2 | 7     | 70        | 0.77816 |
| Female                             | 1   | 2 | 3     | 30        | 0.57816 |
| Tumor size (cm)                    |     |   |       |           |     |
| <5                                 | 3   | 1 | 4     | 40        | 0.42919 |
| ≥5                                 | 3   | 3 | 6     | 60        | 0.57816 |
| Lymph node metastasis              |     |   |       |           |     |
| Yes                                | 4   | 2 | 6     | 60        | 0.59816 |
| No                                 | 2   | 2 | 4     | 40        | 0.59816 |

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