Relationship between inactivation of p16 gene and gastric carcinoma

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Abstract
AIM: To investigate the relationship between inactivation of p16 gene and gastric carcinoma, and the mechanism of inactivation of p16 gene in gastric carcinogenesis.

METHODS: 40 fresh tumor tissue specimens were taken from primary gastric cancer patients. Expression of P16 protein was detected by immunohistochemical method. Deletion and point mutation of p16 gene were analyzed by polymerase chain reaction (PCR) and DNA sequencing, respectively.

RESULTS: The frequency of loss of P16 protein expression in the gastric cancer tissue, adjacent nontumor tissue, and distal normal tissue was 77.5 % (31/40), 55.0 % (22/40), and 17.5 % (7/40), respectively (P=0.005). Homozygous deletion of exon 1 and exon 3 was observed in two and three cases, respectively, giving an overall frequency of homozygous deletion of 12.5 %. All five cases had diffuse type gastric carcinoma. No p16 gene point mutation was detected.

CONCLUSION: These findings suggest a close correlation between inactivation of p16 gene and gastric carcinoma. Further investigations are needed to testify the mechanism of inactivation of p16 gene in gastric carcinogenesis.

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INTRODUCTION
Gastric carcinoma (GC) is common in China[1-19], From 1991 to 2000, 18,029 inpatients with malignant tumor had been treated in the Affiliated Yijishan Hospital of Wannan Medical College. 2,859 cases were GC sufferers, which accounted for 16 % of the total inpatients and ranked the first in malignant tumor. The statistical data showed that GC was one of the commonest tumors in South Anhui province. Therefore, it would have clinical significance to explore the pathogenesis of gastric carcinogenesis.

Early in 1994, Kamb et al[20] and Norobi et al[21] reported their studies on p16 gene simultaneously but independently. p16 gene is located in chromosome 9p21, consisting of 2 introns and 3 exons. Exon 1 contains a region of 126 bp, while exon 2 contains a region of 307 bp, and exon 3 contains a region of 11 bp. In recent years, studies have revealed homozygous deletion and mutation of p16 gene, predominantly in exon 2, in various malignant tumor, suggesting that p16 gene is a multiple tumor suppressor 1 (MTS1)[22-25].

The mechanism of p16 gene in gastric carcinogenesis remains unidentified. Some studies have suggested that p16 gene alteration in GC presents itself in different mechanism from other tumors. The alteration is infrequent in exon 2, but exists predominantly in exon 1 and 3[26-30]. In this study, using polymerase chain reaction (PCR), DNA sequencing analysis and an immunohistochemical method, the fresh tumor specimens of 40 GC patients were examined for homozygous deletion, point mutation of p16 gene and expression of P16 protein to verify the relationship between p16 gene alteration and GC.

MATERIALS AND METHODS
Gastric carcinoma specimens
Under sterile conditions, fresh tumor specimens and their adjacent non-tumor tissue (≤3 cm away from the tumor) and distal (≥5 cm away from the tumor) normal appearing tissues were obtained in the course of surgery from 40 patients with primary GC at the Affiliated Yijishan Hospital of Wannan Medical College. None of the patients had received either chemotherapy or radiotherapy prior to surgery. All of the primary tumors were pathologically confirmed to be GC cases, among which there were 26 males and 14 females. The age ranged from 31 to 68 (mean 55.4) years old. 14 cases had intestinal type and 26 cases diffuse type. The age ranged from 31 to 68 (mean 55.4) years old. 14 cases had intestinal type and 26 cases diffuse type. The age ranged from 31 to 68 (mean 55.4) years old. 14 cases had intestinal type and 26 cases diffuse type.

DNA extraction
Approximately 50 mg tissue of GC was abraded with routine methods and digested with proteinase K. DNA was extracted by means of the routine phenol-chloroform method. The purity and concentration of extracted DNA were detected with ultraviolet spectrophotometer (Daojin Company, UV-2201 type). The extracted DNA was stored at 4 °C until use.

Polymerase chain reaction
The three exons of p16 gene were amplified by employing 3 pairs of primers. The primers 1 and 3 were synthesized by Shanghai Boya Company, and the primer 2 by Saer Biotechnology Company of Shanghai Cell Biology Institution. The sequence of primers and the length of PCR products were listed in Table 1.

PCR was performed in 50 μL reaction volume containing 0.2 μg DNA template, 2 U Taq DNA polymerase (Shanghai Sangon Biotechnology Company of Shanghai Cell Biology Institution). The purity and concentration of extracted DNA were detected with ultraviolet spectrophotometer (Daojin Company, UV-2201 type). The extracted DNA was stored at 4 °C until use.

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Company), 10 pmol/L each pair of primers, 0.2 mmol/L dNTP, 1.5 mmol/L MgCl₂, 5 µL 10×buffer. PCR reaction was carried out in a thermal cycle machine (Perkin-Elmer, 480 type) for an initial 5 min denaturation step at 98 °C. After Taq DNA polymerase was added, the thermal cycle followed. The reaction conditions consisted of denaturation at 94 °C for 45 s, annealing at 58 °C for 50 s for exon 1, 56 °C for 60 s for exon 2, 52 °C for 50 s for exon 3, extension at 72 °C for 60 s, on completion of 35 cycles, at last extension at 72 °C for 5 min. 10 µL of PCR product was loaded into the gel, and a 100 bp DNA ladder was used as a marker. The PCR products were electrophoresed at voltage of 100 V on 2 % agarose gel for 30 min and visualized under UV illumination using an ethidium bromide stain. The results were photographed by a digital camera.

Table 1 The primer sequence of the p16 gene

| Exon | Sequence of the primer | Product (bp) | Temperature for annealing (°C) |
|------|------------------------|-------------|--------------------------------|
| 1S   | 5'TCTCGGAGAGGGGGAGAGCAG 3' | 280         | 58                             |
| 1A   | 5'GCCGATCCCTGATATCCATTC 3' |             |                                |
| 2S   | 5'TTCCTTTCCGTCATGCCGG 3' | 394         | 56                             |
| 2A   | 5'GTACAAATTCTCAGATCATCAGTCCTC 3' |             |                                |
| 3S   | 5'GGATGTTCCACACATCTTTG 3' | 189         | 52                             |
| 3A   | 5'ATGAAAACTACGAGAGGCCC 3' |             |                                |

S: sense; A: antisense.

DNA sequencing analysis

The GC specimens that produced amplified product were reamplified by PCR. PCR was performed in 100 µL reaction volume. The products were purified using Spin PCRapid Purification kit (Promage Company), and then together with PCR primers were sequenced (dideoxy chain termination) by an ABI Prism 377 DNA sequencer (Perkin-Elmer Company) in Shanghai Sangon Company.

Immunohistochemistry

The sections were deparaffined, boiled and retrieved in citric acid buffer for 10 min. Then, the immunohistochemical streptavidin-peroxidase (SP) method was used according to the specification of SP kit. The known positive sections were used as a positive control. Phosphatic buffer solution (PBS) replacing the primary antibody was used as a negative control in every experiment. Mouse-anti-human P16 (ZJ11) and SP kit were products of Fuzhou Maixin Biotechnology Company.

RESULTS

Deletion and mutation of p16 gene

Of 40 GC specimens in PCR products, exon 1 and exon 3 were not detected in 2 cases and 3 cases, respectively. But all of specimens were detected the PCR product of exon 2. These findings indicate that homozygous deletion of p16 gene exon 1 and exon 3 exist in GC (Figure 1, 2, 3). The frequency of deletion of p16 gene was 12.5 % (5/40). All five cases with p16 gene deletion had poorly differentiated were all diffuse type GC, with staging of PT³N³M₀, as assessed by pathological section analysis.

Amongst the 35 GC cases in whom p16 deletion was not detected, 10 cases were randomly selected for DNA sequencing. No point mutation was detected in these GC specimens when compared with the normal p16 gene cDNA sequence[37].

Table 2 P16 protein expression at GC

| Histological types               | n  | Positive | Negative | The frequency of P16 protein expression loss (%) |
|----------------------------------|----|----------|----------|-----------------------------------------------|
| Gastric carcinoma                | 40 | 9        | 31       | 77.5                                          |
| Adjacent nontumor tissue (≤3 cm) | 40 | 18       | 22       | 55.0                                          |
| Distal normal tissue (≥5 cm)     | 40 | 33       | 7        | 17.5                                          |
In this study, homozygous deletion of exon 1 and exon 3 of p16 gene and carcinogenesis of various tumors has been verified. It is suggested that alteration of p16 gene is associated with the differentiation degree and metastasis of GC. In contrast, Wu et al. reported that deletion of p16 gene was frequently encountered in the intestinal type, with a lower frequency in the diffuse type. Furthermore, He et al. reported that there was homozygous deletion of p16 gene exon 2 in GC, with a frequency of 20.0%. Our finding does not tally with above observations. We randomly selected 10 of the 35 GC cases without p16 gene deletion for DNA sequencing, no point mutation was detected. The results reveal that point mutation of p16 gene is rare in gastric carcinogenesis. It coincides with previous findings.

In human primary GC, deletion and point mutation of p16 gene are infrequent, but loss of expression of P16 protein is common. Some studies have found that the frequency of loss of P16 protein expression ranges from 52% to 90%. Our results agree with these studies. We found that the frequency of loss of expression of P16 protein was 77.5%. It had statistically significant difference with adjacent non-tumor tissue (55.0%) and distal normal appearing tissue (17.5%) (P<0.005). These results suggest that inactivation of p16 gene is strongly associated with gastric carcinogenesis, however, deletion and point mutation of p16 gene are not the major mechanism of p16 inactivation in GC.

The methylation-specific PCR (MSP) method established by Herman et al. in 1996 has significantly promoted the research on the interrelationship between hypermethylation and gene silencing. Subsequent studies have revealed high frequency of hypermethylation in p16 gene 5’ promoter regions in various tumors, with frequency ranging from 60% to 89%. Inactivation of p16 gene correlates with hypermethylation of 5’ promoter regions CpG island in GC. Some studies have found that hypermethylation in the promoter regions CpG island is an important mechanism for p16 gene inactivation in GC, whereas deletion and point mutation of the gene are rarely seen. The CpG island hypermethylation occurred early in multistep gastric carcinogenesis tends to accumulate along the process. P16 gene with methylated promotor regions would reexpress P16 protein when it is treated with 5-aza-2’-deoxycytidine which demethylates or when the inducers of hypermethylation are eliminated. The effect mechanism of p16 gene is to inhibit cell cycle directly, and it is easy to perform gene targeting or protein modification because p16 gene is small. Therefore, it is needed to further investigate the relationship between p16 gene inactivation and gastric carcinogenesis, and the mechanism of p16 gene inactivation, since it is of significant clinical implications in early diagnosis, therapy and prognosis.

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