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• GASTRIC CANCER •

Methylation and mutation analysis of p16 gene in gastric cancer

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Abstract

AIM: To study methylation, frequencies of homozygous deletion and mutation of p16 gene in gastric carcinoma.

METHODS: The methylation pattern in exon 1 and exon 2 of p16 gene was studied with polymerase chain reaction (PCR), using methylation sensitive restriction endonuclease HpaII and methylation insensitive restriction endonuclease MspI. PCR technique was used to detect homozygous deletions of exon 1 and exon 2 of p16 gene and single strand conformation polymorphism (SSCP) technique was used to detect the mutation of the gene.

RESULTS: Hypermethylation changes in exon 1 and exon 2 of p16 gene were observed in 25 % and 45 % of 20 gastric cancer tissues, respectively, while no methylation abnormality was found in normal tissues. The homozygous deletion frequency of exon 1 and exon 2 of p16 gene in 20 gastric cancer tissues was 20 % and 10 %, respectively. No mutation was found in exon 1 of p16 gene, while abnormal single strands were found in 2 (10 %) cases in exon 2 as detected by SSCP.

CONCLUSION: The results suggest that hypermethylation and abnormality of p16 gene may play a key role in the progress of gastric cancer. Hypermethylation of exon 2 of p16 gene may have effects on the carcinogenesis of gastric mucosa and may be a later event.

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INTRODUCTION

DNA methylation abnormality could influence gene transcription directly, and it could also cause the abnormal gene expression through the C→T mutation induced by deamination of 5' methyl cytosine (5 mC)[1]. In the eukaryote, 5 mC mainly appears in the CpG sequence of genome, and it is the site in which p16 gene mutation occurr frequently[2]. Highly frequent homozygous deletion, mutation and abnormal methylation of p16 gene exhibit in many kinds of carcinoma[3-9]. In China, studies on p16 gene methylation abnormality and deletion, mutation in gastric carcinoma have scarcely been reported. In this paper, restriction endonuclease-polymerase chain reaction (PCR) and single strand conformation polymorphism (SSCP) techniques were used to detect the CpG methylation and mutation in exon 1 and 2 of p16 gene. The biological siginificance of p16 gene and its methylation abnormality in the development and progress of gastric carcinoma were discussed.

MATERIALS AND METHODS

Specimens

20 specimens of gastric carcinoma and their corresponding adjacent normal-appearing gastric tissue were collected from the First and Second Affiliated Hospital of Medical College of Zhengzhou University and frozen in liquid nitrogen in 30 min. All the specimens were pathologically diagnosed and without radio or chemical therapy before operation.

Analysis of methylation

Tissue DNA was extracted by normal phenol-chloroform method. DNA samples were treated with HpaII and MspI. Primers were synthesized by Shanghai Cell Biology Research Institute of China Scientific Institute and purified with PAGE. The primers of p16 exon 1 (E1): 5'-GAA GAA AGA GGA GGG GCT G-3'; 5'-GCG CTA CCT GAT TCC AAT TC-3'; the primers of exon 2 (E2): 5'-CAC AAG CTT CCT TTC CGT CAT G-3', 5'-TCT GAG CTT TGG AAG CTC TCA GG -3'. The length of amplified fragments was 336bp and 424bp respectively. The parameter of PCR cycle was: 92 ℃ 60 s, 60 ℃ (renaturing temperature of E2 was 58.5 ℃) 60 s, 71 ℃ 90 s. After 24 cycles, the reaction system was thermal retarded at 71 ℃ for 10 min. 8 μl of PCR products were electrophoresized on 20 g/L agarose gel. After the electrophoresis, the gel was visualized under ultraviolet and photographed.

PCR-SSCP

The primers were the same as mentioned above. The parameter of PCR cycle was: 91.5 ℃ 60 s, 61.5 ℃ (E1) or 59.5 ℃ (E2) 60 s, 70.5 ℃ 90 s. After 30 cycles, the reaction system was thermal retarded at 70.5 ℃ for 10 min. PCR products were electrophoresized on 20 g/L agarose gel and stained with ethidium bromide. SSCP was taken on the 80 g/L undenatured polyacrylamide gel. After denaturing at 95 ℃ for 5 min, the samples were ice bathed immediately for 5 to 10 min and electrophorized under constant voltage 160 V for 4-6 h. After electrophoresis the gel was removed and silver stained.

Statistic analysis

Data were analyzed using Fisher's exact test of probabilities with SPSS 10.0 statistic software.

RESULTS

Methylation analysis of DNA

HpaII is a methylation sensitive restriction endonuclease, when methylation occurrs at the second C in the CCGG target sequence, HpaII can not recognize the target site. However,

Msp I is isoenzyme of HpaII, and can recognize the target site whether or not methylation occurs at the second C in the CCGG target sequence. The exon 1 and 2 of p16 gene include 2 and 4 5' -CCGG-3' sites. If methylation occurs, HpaII can not identify the target sequence, the specific patterns would appear after PCR products are electrophoresed (336bp or 424bp) (Figure 1). If no specific bands were amplified by PCR, then no methylation alteration at second C in 5' -CCGG-3' sequence is indicated (Figure 2).

Figure 1 Methylation analysis of p16 gene exon 1 (abnormality). 1: DNA marker; 2: negative control; 3-5 carcinoma tissue; (3: without enzyme treatment, 4: Hpa II treatment, 5: Msp I treatment); 6-8 normal tissue (6: without enzyme treatment, 7: Hpa II treatment, 8: MspI treatment).

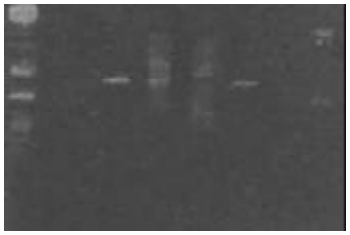

Figure 2 Methylation analysis of p16 gene exon 1 (no abnormality). 1: DNA marker; 2: negative control; 3-5 carcinoma tissue (3: without enzyme treatment, 4: Hpa II treatment, 5: MspI treatment); 6-8 normal tissue (6: without enzyme treatment, 7: Hpa II treatment, 8: MspI treatment).

Figure 3 PCR of p16 gene exon 1 of gastric carcinoma tissue. 1: Marker; 3: negative control; 2-8: 336bp of E1 PCR.

Figure 4 PCR of p16 gene exon 2 of gastric carcinoma tissue. 1: Marker; 2-8: 424bp of E2 PCR; 3: deletion.

Homozygous deletion analysis

After agarose electrophoresis of PCR products, if no amplified products were found at the sites corresponding to 336bp or 424bp, then homozygous deletion of E1 or E2 could be determined. In gastric carcinoma tissues, 4 cases (20 %) of E1 deletion and 2 cases (10 %) of E2 deletion were found. The 6 cases with homozygous deletion included 1 with well differentiated and 5 with moderately or poorly differentiated gastric carcinoma tissues (Figure 3 and 4).

PCR-SSCP analysis

Mobility shift is defined when abnormal bands appear or the position of bands alter. No abnormal alteration was found at E1 of p16 gene (Figure 5). At E2, abnormal single strand of mobility shift exhibited in 2 (10 %) cases, in 1 of which (IIIa stage, poorly differentiated adenocarcinoma) mobility shift occurred in both carcinoma and adjacent carcinoma tissues (Figure 6).

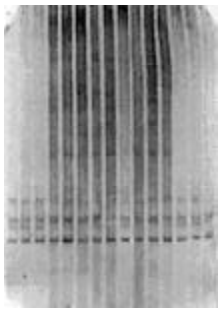

Figure 5 PCR-SSCP of p16 gene exon 1 (no abnormality).

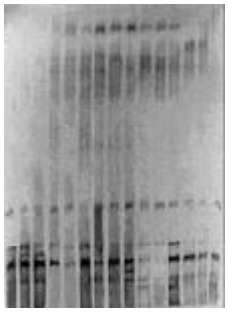

Figure 6 PCR-SSCP of p16 gene exon 1. Abnormal single strand occurred in 2, 3 lane (adjacent carcinoma and carcinoma tissue) and 9 lane (carcinoma tissue).

DISCUSSION

Methylation of p16 gene

In the process of multistage canceration, abnormality of gene expression may be controlled by genetic mechanism and epigenetic mechanism. Epigenetic mechanism is indicated by methylation alteration at 5 mC which cause gene expression abnormality without change in the DNA sequence and product of gene expression, and is a key mechanism causing genomic instability and canceration. Hypermethylation at CpG induces abnormality of DNA conformation stability which may influence the binding of specific protein and DNA regulating sequence, and cause gene silence. Inability to transcribe the tumor suppressor genes resulted in dysfunction of the genes and induced the development of carcinoma[10, 11].

Regional hypermethylation plays an important role in the alteration of gene expression in human carcinoma and in the progression of carcinoma. In the present paper, methylation at CpG in exons 1 and 2 of p16 gene in gastric carcinoma tissues was detected by treatment of methylation sensitive restriction endonuclease combined with PCR technique. The results showed that abnormal methylation was present in 5 and 9 of 20 cancer tissues, respectively, but no abnormality was found in corresponding adjacent normal gastric mucosa, suggesting

an association between methylation of p16 gene and gastric carcinogenesis. Homozygous deletion of p16 gene occurred, to some extent, in many kinds of human carcinoma cells. However, gene mutation rarely occurred and the frequency of homozygous deletion was low in primary carcinoma. It is interesting that in some human carcinoma without site mutation or homozygous deletion, for example, in the pulmonary cancer cell line in oat cell type, the frequency of remethylation at p16 CpG island is 78 % resulting in the loss of p16 gene transcription activity. The same phenomena exists in mammary, prostate, gastric and colon carcinomas, especially in the colon carcinoma with the frequency of methylation being high as 92 %. In the cells of colon carcinoma without homozygous deletion, methylation occurr at both alleles of p16 gene, and is related to its entire deactivation[1]. Based on the fact that the alteration of methylation of p16 gene and other genes occur in many kinds of carcinomas lacking of mutation and deletion, methylation might be a key mechanism of deactivation of tumor suppressor genes in primary carcinoma. Expression of p16 gene in gastric carcinoma is decreased significantly[12-22]. However, the frequency of mutation and deletion of p16 gene is low, suggesting that abnormal methylation might be a key mechanism in alteration of the gene expression in gastric carcinoma.

The results in the present study showed that abnormal methylation mainly appeared in poorly differentiated gastric carcinoma. Two cases with metrhylation in both exons were poorly differentiated and progressive gastric carcinoma. Hypermethylation of exon 2 mainly exhibited in the cases of late stage of gastric carcinoma, suggesting that hypermethylation of exon 2 is related to the differentiated degree and the clinical progression of gastric carcinoma, and thus might be a late event. Kampster *et al*[23]. reported in their study on methylation of p16 gene in esophagus carcinoma that alteration of methylation at exon 2 was obviously related to clinical stage and progression of carcinoma, and a correlation existed between hypermethylation of exon 1 and no gene expression. Yi *et al*[24] reported that methylation of p16 gene in colorectal cancer was obviously related to the Duke' s stage. Methylation of p16 gene was increased gradually with the progression of carcinoma, and could induce detectable alteration and consequence to late stage which may be related to clinical stage of gastric carcinoma.

Deletion and mutation of p16 gene

Deletion and mutation of p16 gene are also important mechanisms responsible for the dysfunction of tumor suppressor genes. Abnormality in 9p21-22 of chromosome has been reported in many kinds of carcinoma cells, and p16 gene is an important gene located in this region. By analysis of the sites adjacent to p16 gene, simultaneous mini-deletions (<200bp) of p16 allele were found in many carcinomas, and homozygous deletion of p16 gene has been testified in many kinds of primary carcinoma[25-27]. Lu *et al*[28]. detected that the deletion of E1 of p16 gene in 16.4 % of gastric carcinoma tissues, Wu *et al*[29]. reported a rate of 10 % (6/60). Different deletion rates of p16 gene in gastric carcinoma were reported by the other investigators[30-32]. In the present study, deletion of E1 and E2 was detected in 20 % and 10 %, respectively, of 20 cases gastric carcinoma cases, but amplified products appeared in corresponding normal gastric mucosa tissues.

Mutation of p16 gene mainly includes nonsense, missense, and frame shift mutation. The frequency of mutation is significantly lower than deletion with 70-90 % being present on E2[2]. Mutation of p16 gene in gastric carcinoma is rare, but the frequency is much higher than the natural mutation (10-6~10-4) of general genes, thus, it is conceivable that mutation of p16 gene might be involved in the development and progression of gastric carcinoma. In the present study, mutation of p16 gene E2 was detected in 2 cases of gastric carcinoma tissues, and no E1 mutation was found. Both the gastric carcinoma cases with mutation were progressive gastric carcinoma. One of them exhibited mobility shift in both carcinoma and adjacent carcinoma tissues, and belonged to IIIa stage and poorly differentiated adenocarcinoma, suggesting that p16 gene mutation might be a late event in the process of gastric carcinoma. It has been reported that the mutation site of p16 gene is the same as that of p53 gene, i.e. at CpG. It is believed that mutation is induced by nucleotide methylation[2]. It is suggested that mutation of p16 gene might be the consequence of the DNA genomic insatability, and gradually causes the canceration.

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