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• GASTRIC CANCER •

Studies on microsatellite instability in p16 gene and expression of hMSH2 mRNA in human gastric cancer tissues

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Abstract

AIM: To detect the loss of heterozygosity (LOH) frequency of microsatellite sites D9s171, D9s1604 of p16 gene and expression of hMSH2 mRNA in various differentiated types of gastric cancer, adjacent cancer tissues and normal gastric mucosa.

METHODS: LOH was detected by polymerase chain reaction (PCR)-denaturing polyacrylamide gel electrophoresis-silver staining. The expression of hMSH2 mRNA was examined with in situ hybridization.

RESULTS: The frequency rate of LOH was significantly higher in gastric cancers than that in adjacent cancer tissues (P=0.032). No significant difference was noted among various differentiated types and various clinical stages of gastric cancers. The significantly reduced expression of hMSH2 mRNA positive signal cells exhibited in gastric cancers, in comparison with that in the adjacent cancer tissues and normal gastric mucosa, respectively (P=0.001). No significant difference was noted among various clinical stages of gastric cancers (P>0.05). The difference of positive signal cells in poorly differentiated cancers and those in well and moderately differentiated cancers were significant (P<0.001).

CONCLUSION: The frequencies of LOH in two microsatellite sites, D9s171 and D9s1604, in p16 genome were associated with development of gastric cancer and no significant correlation was demonstrated between the LOH frequency and the cell differentiated types of tumor cells or clinical stages. There was a positive relationship between the expression of hMSH2 mRNA and the differentiated types of gastric cancer.

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INTRODUCTION

Microsatellite instability (MI) occurs frequently adjacent to the loci of tumor suppressor genes[1]. The defects of mismatch repair (MMR) gene are closely related to the occurrence of MI and abnormality of genes[2, 3]. Expression of p16 gene was significantly reduced in gastric cancer[4-12] and was associated with the progression and metastasis[13, 14]. The relationship between the MI of p16 gene in the gastric cancer and adjacent cancer tissue and the abnormal expression of MMR gene has rarely been reported. In this paper, two microsatellite loci, D9s171 and D9s1604 located at the upstream of p16 gene, were selected to study the loss of heterozygosity (LOH) of 9p21-22 region in gastric cancer tissues. The expression of hMSH2 mRNA in gastric cancer, adjacent cancer and normal gastric tissues was detected by *in situ* hybridization with hMSH2 oligonucleotide probe.

MATERIALS AND METHODS

Specimens

All the specimens were collected from the First and Second Affiliated Hospital of Medical College of Zhengzhou University and the People Hospital of Henan Province.

Specimens used to extract DNA: Specimens of gastric cancer tissue, adjacent cancer tissue and normal gastric mucosa were from each of 20 patients with gastric cancer. Of 20 patients, there were 4 cases with well differentiated and 16 moderately and poorly differentiated gastric cancer tissue. All specimens were used for isolation of DNA.

Specimens used *in situ* hybridization: gastric cancer specimens in 27 cases (including 20 cases gastric cancer specimens, with no history of radio- or chemotherapy preoperatively), adjacent cancer tissue specimens in 10 cases and normal gastric tissue specimens in 19 cases were used *in situ* hybridization. All the specimens were diagnosed pathologically (well differentiated in 5 cases, moderately 9 and poorly differentiated cancer 13). According to the PTNM of International Alliance of Anticancer in 1987, the specimens were divided into 4 clinical stages, the number in stage I, II, III and IV was 5, 10, 9 and 3, respectively.

Detection of microsatellite instability

The tissue DNA was extracted by routine phenol-chloroform method. The primers were synthesized by Shanghai Cell Biology Research Institute of China Scientific Institute and purified with PAGE. The sequence of primer was as follows, D9s171: 5' AGCTAAGTGAACCTCATCTCTGTCT3', 5' ACCCTAGCACTGATGGTATAGTCT3', and the length of amplified fragment was 159-177bp;D9s1604: 5' CCTGGGTCTCCAATTTGTCA3', 5' AGCACATGACACTGTGTGTG3', and the length of amplified fragment was 172-196bp. The annealing temperature of PCR was 60 ℃ and 50 ℃, respectively. The PCR products were electrophoresized on 80 g/L denatured polyacrylamide gel under constant voltage of 30 V/cm. The gel was stained with silver staining after electrophoresis.

In situ hybridization

Digoxigenin-labeled hMSH2 oligonucleotide probe and BCIP/NBT staining system were used to demonstrate the expression

of hMSN2 mRNA. Control consisted of specimens pretreated with 0.05 g/L RNase A at 37 ℃ for 30 min, and specimens hybridized with hybridization buffer without probe.

Analysis of results

Compared with that of normal gastric mucosa removed from the same case, if the band of the identical allele disappeared or its intensity reduced over 50 %, the result was defined as LOH positive. Specimens *in situ* hybridization observed under microscope, the cells with cytoplasm containing bluish violet granules were determined as positive signal cells. Five fields in each specimen was checked randomly.

Statistic analysis

Data of electrophoretic specimens were analyzed using Fisher' s exact test of probabilities with SPSS 10.0 statistic software. Correlative analysis were decided by using paired χ^2 test for numerical samples and $P<0.05$ was considered as significant difference. *In situ* hybridization specimens were treated using one way analysis of variance and $P<0.05$ was considered as different significantly.

RESULTS

Results of LOH at D9s171 and D9s 1604 of p16 gene in gastric cancer and adjacent cancer tissues (Figure 1 and 2)

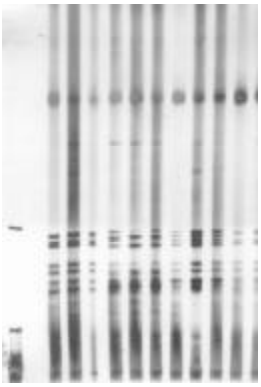

Figure 1 LOH of D9s171 in gastric cancer. Left 1: Marker Left 6: LOH(+).

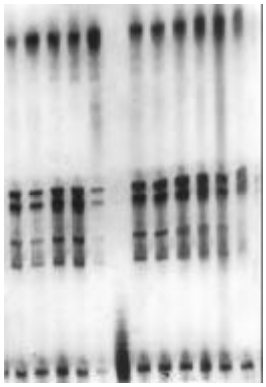

Figure 2 LOH of D9s1604 in gastric cancer. Left 6: LOH(+).

The number of LOH at D9s171 and D9s1604 in cancer tissues in 20 cases was 3 and 10, respectively, and that in adjacent cancer tissues was 2 and 4, respectively. There was no significant difference between the ratio of LOH at the two microsatellite loci. However, the combined ratio of LOH at the two microsatellite loci in gastric cancer tissues was obviously higher than that in adjacent cancer tissue ($P<0.05$) (Table 1).

Table 1 LOH at D9s171 and D9s 1604 in gastric cancer and adjacent cancer tissue

	LOH(+)	LOH(-)	Total
Gastric cancer tissue	13	7	20
Adjacent cancer tissue	6	14	20
Total	19	21	40

[a]$P<0.05$ *vs* adjacent cancer tissue.

Relationship between the ratio of LOH and the differentiated type and clinical stage of gastric cancer

The difference of incidence of LOH at D9s1604 in well differentiated adenocarcinoma (1/4) and that in moderately and poorly differentiated cancer (10/16) was not significant ($P>0.05$). The proportion of LOH in early stage and progressive stage of cancer was 50 % (4/8) and 58.2 (7/12), respectively.

Relationship between the LOH occurred at D9s171 and at D9s1604 in gastric cancer and adjacent cancer tissues

The incidence of LOH at D9s171 and D9s1604 were showed in Table 2. Analysis by paired χ^2 test for numerical sample showed that an intrinsic relation exhibits between them.

Table 2 Relation between LOH occurred at D9s171 and at D9s1604 in gastric cancer

D9s1604	D9s171		Total
	+	-	
+	4	10	14
-	1	25	26
Total	5	35	40

+: LOH (+); -: LOH (-); [a]$P<0.05$ *vs* LOH (-).

Expression of hMSH2 mRNA in normal gastric mucosa, gastric cancer and adjacent cancer tissues

The *in situ* hybridization positive signals of hMSH2 mRNA appeared as bluish violet granules distributed in the cytoplasm. No positive signals were found in nucleus. There were round or irregular granules in positive cells in normal gastric tissues. The number of positive signal cells increased from the superficial to deep layer of mucosa. A few positive signal cells scattered in the submucosa and no positive signal cells in muscular layer (Figure 3). Expression of hMSH2 mRNA in gastric cancer and adjacent cancer tissues was significantly decreased than that in normal gastric tissues (Table 3). The positive signal cells mainly scattered in the deep layer of mucosa. No positive signal cells were found in the submucosa and muscular layer (Figure 4, 5).

Table 3 hMSH2 mRNA in the normal gastric mucosa, gastric cancer and adjacent cancer tissue ($\bar{x}\pm s$) (*In situ* hybridization)

	n	No. of positive cases (ratio)	No. of positive cells in each scope
Normal gastric mucosa	19	13(68.4 %)	175.8±26.4[a]
Adjacent cancer tissue	10	6(60 %)	99.7±16.8[b]
Gastric cancer tissue	27	20(74.1%)	42.1±25.9[c]

[a]$P<0.01$ *vs* adjacent cancer tissue; [b]$P<0.01$ *vs* gastric cancer tissue.

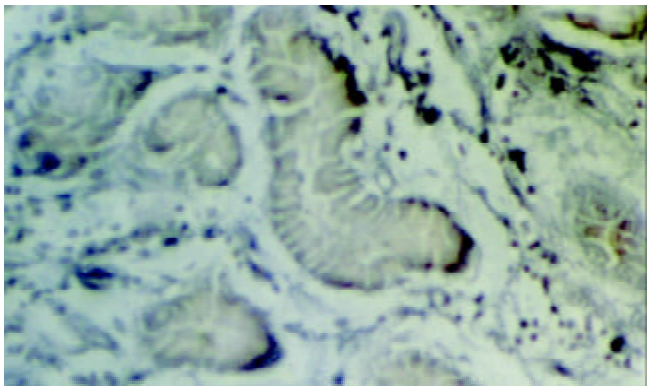

Figure 3 hMSH2 in normal gastric mucosa (×1000).

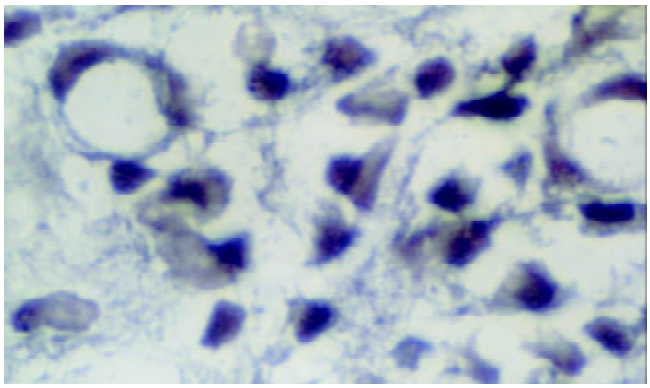

Figure 4 hMSH2 in adjacent gastric cancer tissue (×1000).

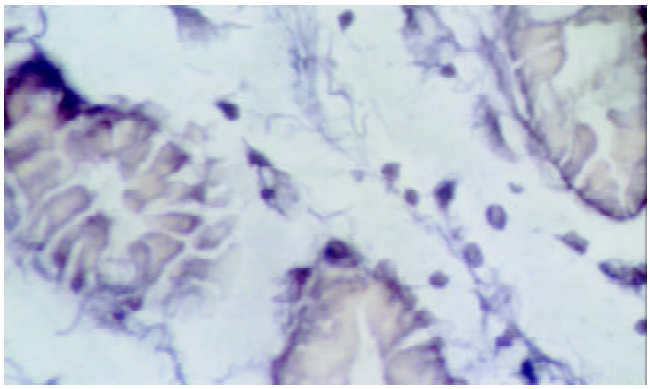

Figure 5 hMSH2 in gastric cancer tissue (×1000).

Expression of hMSH2 mRNA in various clinical stage of gastric cancer

Compared with that in the normal gastric tissue, the expression of hMSH2 mRNA in gastric cancer tissues was reduced significantly. However, there was no obvious difference in the number of positive cells among the various clinical stages of gastric cancer (P>0.05) (Table 4).

Table 4 hMSH2 mRNA in various clinical stage gastric cancer (*In situ* hybridization)

Clinical stage	*n*	No. of positive cases	No. of positive cells	F value	P value
I	5	4	62.8±25.4	2.495	0.097
II	10	8	46.3±24.4		
III	9	6	32.3±22.3		
IV	3	2	13.5±3.5		

Expression of hMSH2 mRNA in various differentiated types of gastric cancer

Compared with that in the normal gastric tissue, the expression of hMSH2 mRNA in gastric cancer tissue was decreased significantly (P>0.05). The number of positive signal cells differed among various differentiated types of gastric cancer. In poorly differentiated cancer tissue, the positive signal cells scattered in the middle and lower parts of mucosa, and the number of positive signal cells was smallest (Figure 6). There was a significant difference between the number of positive signal cells in the poorly differentiated gastric cancer tissue and that in the moderately and well differentiated gastric cancer tissue (Figure 7, 8).

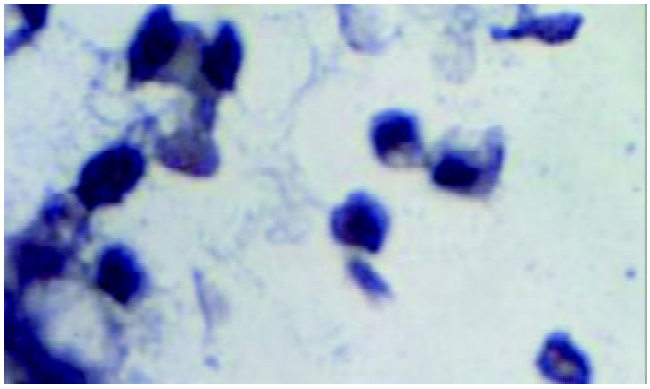

Figure 6 hMSH2 in poorly differentiated gastric cancer (×1000).

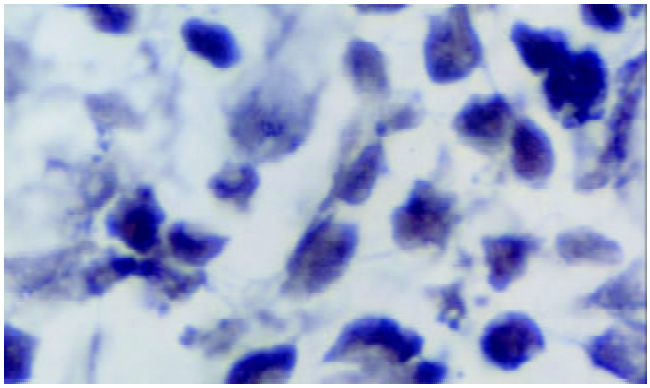

Figure 7 hMSH2 in moderately differentiated gastric cancer (×1000).

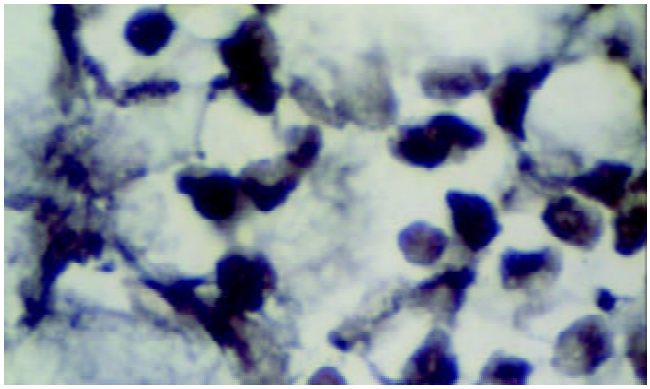

Figure 8 hMSH2 in well differentiated gastric cancer (×1000).

Negative reaction was showed in situ hybridization assay in control specimen.

DISCUSSION

Microsatellite instability of p16 gene

Microsatellite DNA is a genome-wide simple repeat sequence. Its normal length is shorter than 350bp, and the number of repeating is less than 60. The number of repeat unit of MI varied with individuals or tissues even in same body[15]. MI varied in different kinds of cancer[16-19]. In the gastric cancer tissues, MI was a frequent event, and the average frequency of MI was reported in different papers to be 33.9 %, 32.1 % and 25 %, respectively[20-22]. The results showed that the LOH frequency of D9s171 and D9s1604 microsatellite loci, located at upstream of p16 gene, were 15 % and 50 %, respectively. The LOH frequency in well differentiated gastric cancer tissue was lower than that in the moderately and poorly differentiated gastric cancer tissue without significant difference (P>0.05). The relation of MI and the differentiation degree of gastric cancer has been not known clearly[23,24].

It was showed that MI occurred at early stage of malignancy and gradually caused the formation of cancer[25,26]. The results in this study showed that the LOH of microsatellite loci exhibited 50 % frequency at the early stage of gastric cancer, which suggested that the alteration of these loci might activate specific oncogenes and deactivate tumor suppressor genes, therefore, cause the development and progress of cancer. The alteration of microsatellite DNA normally appears as LOH. If LOH occurs frequently at the same locus of one chromosome in the tumor, the site of occurring LOH usually is the location of tumor suppressor gene[27]. Through the analysis of the loci adjacent to p16 gene, small losses (<200bp) of p16 gene could be found in many kinds of cancer, which might be one of deactivation mechanisms of p16 gene. In this paper, two microsatellite loci were selected to demonstrate MI, D9s171 located at the site between the upstream of p16 gene and an adjacent tumor suppressor gene p15, D9s1604 located at the site between the exon 1α and exon 1β of p16 gene. LOH was detected at both D9s171 and D9s1604, and correlation between the occurrence of MI at the D9s171 and at the D9s1604 was existed, which suggested that occurrence of MI at the two loci might be related molecular event.

Expression of hMSH2 mRNA

Mismatch repair gene superfamily belongs to housekeeping genes, and is able to correct unmatched or mismatched bases in the process of DNA replication and DNA damage repairing, and control the accuracy of replication and recombination. Up to date, 6 human mismatch repair genes have been found, including 3 homologous of bacterial MutS (hMSH2, hMSH6 and hMSH3) and 3 homologous of bacterial MutL (hMSH1, hPMS1 and hPMS2). Loss of hMSH2 protein existed in the colonic and other cancer[28-33] and genetic alterations in hMSH2 was observed in gastric cancer cell line[34]. Loss of hMSH2 protein in the cancer tissue indicated that hMSH2 peptide or its coding mRNA was at an instable state. The number of the positive signal cells of hMSH2 mRNA in normal gastric mucosa, in adjacent cancer tissue, and in gastric cancer tissue was 175.8±26.4, 99.7±16.8 and 42.1±25.9, respectively. The number of positive signal cells was decreased significantly in adjacent cancer and gastric cancer tissue (P<0.01) No significant difference was found in the number of the positive signal cells of hMSH2 mRNA at various clinical stages in gastric cancer. A correlation might exist between the instability of gene expression and the development of gastric cancer. Tumor development is a multi-step process of somatic cell mutation and colonial amplification. With the hMSH2 (or other mismatch repair genes) mutation and the cell proliferation, the instability of genome occurred, and then the mutator acted selectively at the mutated site, caused enlargement of genome instability in deepness and wideness, the accumulation of oncogene mutation was accelerated and caused the formation of tumor finally[35].

Expression of hMSH2 protein in 115 bladder cancers was studied with immunohistochemistry[36] and showed that low expression of hMSH2 protein exhibited in 25 % cases and complete loss in 2 cases. A closely correlation existed between the decrease of hMSH2 mRNA and the recurrence of poorly differentiated cancer. The results in this work showed that expression of hMSH2 mRNA significantly decreased in gastric cancer tissues, especially in moderately and poorly differentiated cancer (P<0.05). The results suggested that low expression of hMSH2 mRNA in poorly differentiated cancer might be related to the metastasis and prognosis of cancer. The lower the gastric cancer was differentiated, the more unstable the gene expression was. As the ratio of DNA mismatch increased, the instability of genome enhanced, tumor became more invasive and the prognosis got worse.

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