Genic expression and promotor methylation of hMSH2 in esophageal cancer tissues☆

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Abstract

Objective. To detect hSMH2 gene expression and test aberrant methylation of hSMH2 gene promotor in esophageal cancer tissues. *Methods.* The *in situ* hybridization (ISH) and PCR were to detect hMSH2 mRNA expression and hMSH2 promotor methylation in 32 cases of esophageal cancer tissue. *Results.* The 46.88% esophageal cancer tissue was hMSH2 mRNA positive, lower than the normal tissue (84.38%). The correlation analysis revealed that the hMSH2 had weak correlation with age and sex of esophageal cancer patients, the size and location of tumor, pathologic type, tissue-profiling, lymph node metastasis, and tumor infiltration depth. The occurrence rate of hMSH2 promotor methylation was 32.4% in the total 32 cases. No methylation was found in normal esophageal tissue. The positive expression rate of methylation in elder patients (age ≥ 70) was much higher than that in younger patients (age < 70); in addition, hMSH2 promotor methylation rate for patients with pathology level III – IV was much bigger than that with level I – II. *Conclusion*. hMSH2 gene was inactivated at the early stage of esophageal cancer. The hMSH2 gene inactivation may due to methylation of promotor, and hMSH2 gene promotor methylation is related to patient's age and tumor pathology type. [Life Science Journal. 2008; 5(4): 23 – 27] (ISSN: 1097 – 8135).

Keywords: esophageal cancer; hMSH2; *in situ* hybridization; methylation

1 Introduction

Oncogene, antioncogene and DNA repair gene are the most important genes in the onset and development of carcinoma[1]. People have done researches on the effect of DNA mismatch repair (MMR) gene in hereditary nonpolyposis colorectal cancer (HNPCC) recently. Loss and malfunction of MMR gene also exist in many other types of carcinoma. hMSH2 was the firstly detected MMR gene. The inactivation of hMSH2 has strong relationship with carcinogenesis. hMSH2 gene was inactivated by gene loss, mutation, and methylation. Currently, research regarding MMR on methylation are mainly in the field of intestinal cancer, ovarian cancer, pharyngeal cancer, and lung cancer. China is a country with the highest morbidity and mortality of esophageal carcinoma in the world. Each year, 300 thousand people develop esophageal carcinoma, while more than half of them are Chinese. Linxian (in Henan Province) and the neighbor counties such as Huixian, Anyang, etc. are the areas with high esophageal cancer incidence and mortality in the world[2]. In this research, the expression of hMSH2 mRNA in esophageal cancer tissues and normal tissues was detected by *in situ* hybridization (ISH), and the pathological data of patients were analyzed. The hMSH2 gene promotor methylation in esophageal cancer tissues was detected by the methylation specific PCR, and its relationship with the incidence and development of esophageal cancer was discussed.

2 Materials and Methods

2.1 Subjects

Thirty-two cases of esophageal cancer patients were all diagnosed with esophageal carcinoma and admitted in the First Afflited Hospital of Zhengzhou University between October 2002 and May 2003. Nineteen patients were

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males and thirteen were females. The age ranged from 50 to 78 years old, with mean age was 62.8 ± 7.4 years old. None of them ever took radiotherapy, chemotherapy before operation. All histological samples removed from the surgeries were diagnosed and verified by more than two pathologists. Among the samples: twenty-five cases were squamous cell carcinoma, five cases adenocarcinoma, and two cases adeno-squamous cell carcionma. Histologically, twenty-two neoplastic lesions were stage I – II, and ten were stage III – IV. Twenty-three of them were squamous cell carcinoma (SQ), and nine were adenocarcinoma (AD). As for the location, four cases were found in the upper esophagus, sixteen were in the middle esophagus, and three were in the lower esophagus. Seventeen samples were less than 5 cm, and the rest fifteen were larger than 5 cm. One sample of tumor was confined to esophageal mucosa, eighteen were infiltrated to esophageal muscularis, and the rest thirteen cases infiltrated to all esophageal layers. All samples were resected from surgery, and divided into two parts: one part was paraffin-embedded, and the other was stored in liquid nitrogen to extract DNA.

2.2 Methods

2.2.1 ISH. ISH reagent was purchased from Wuhan Bo-Shi-De Biotech Limited Ltd (China). We followed the user's guide provided by manufacture[3] protocol.

2.2.2 DNA extraction and modification. Genomic DNA was extracted by proteinase-K digestion and phenol/chloroform extraction, followed by ethanol precipitation. DNA was modified using CpGenome DNA modification kit (Intergen, USA).

2.2.3 PCR. The specific primers of hMSH2 gene methylation and normal were as follows: For the hMSH2 unmethylation: (sense) 5'-GGTTGTTGTGGTTG-GATGTTGTTT-3', (antisense) 5'-CAACTACAA-CATCTCCTTCAACTACACCA-3', the target sequence to be amplified was 151 bp in length; For the hMSH2 methylation: (sense) 5'-TCGTGGTCGGACGTCGTTC-3', (antisense) 5'-CAACGTCTCCTTCGACTACACCG-3' with the target sequence to be amplified was 150 bp in length. Total volume was 50 μl: 10 × PCR buffer (Mg^{2+} plus) (Takara, Japan) 5 μl , 2.5 mmol/L dNTP (Takara) 4 μl , 20 μmol/L Primer pair (Sangon, China) 1 μl, modified template 2 μl, 5 U/μl Taq DNA polymerase (Takara) 0.5 μl. Circle parameter: 95 ºC for 5 minutes for denaturalization; then 36 cycles: 95 ºC for 50 seconds, 58 ºC normal/56 ºC methylation for 50 seconds, and 72 ºC for 50 seconds; at last prolongation, 72 ºC for 5 minutes.

2.3 Result verification

After ISH, samples were observed under light microscope. Positive signal was appeared as brown granules in cytoplasm, and colorless cytoplasm was negative. PCR product was judged by 2% agarose gel electrophoresis.

2.4 Statistical analysis

Comparison between different groups was conducted by two-sample *t* test. The Pearson χ^2 test and Fisher's exact test were also used ($P = 0.05$).

3 Results

3.1 The expression of hMSH2 mRNA in esophageal carcinoma tissue and normal tissue

hMSH2 was mainly expressed in glandular cells of the mucosa, but not in muscle tissue. The expression of hMSH2 mRNA in esophageal cancer tissue and the normal tissues was demonstrated in Table 1. And hMSH2 mRNA expression in different pathological types of esophageal cancer was listed in Table 2.

3.2 hMSH2 promotor methylation

Methylation results were judged by agarose gel electrophoresis. Of 32 carcinoma samples, 34.38% were methylated, but no methylation was found in normal esophageal tissues (Table 1).

The measurement of methylation of hMSH2 promotor in different types of esophageal cancer showed that hMSH2 promotor methylation was associated with age and histological grade, but not related with sex, tumor size, pathological type, infiltration depth, and lymphous node infiltration (Table 2).

While in esophageal cancer, 13.33% hMSH2 mRNA positive tissues was detected hMSH2 promotor methylation, lower than that in hMSH2 mRNA negative tissues (52.95%) (Table 3).

4 Discussion

Human MMR gene is capable of correcting defects in DNA bio-synthesis, increasing DNA duplication "confidence level", and is critical to prevent random mutation. The loss of MMR gene may result in massive DNA duplication errors and increase the instability of tumor microsatellite. Additionally, it may cause a number of key genes to be inactivated due to the mutation. Therefore, it's recognized as one of the possible causes for tumorigenesis[4]. Human MMR gene was first discovered in germs. These genes were named as "mut" genes, i.e. mutator genes.

Table 1. Expression of hMSH2 mRNA and the methylation of hMSH2 gene promotor in different tissues of esophageal cancer

Histological type	*n*	hMSH2 mRNA		Methylation of hMSH2 gene promotor	
		Positive (*n*, %)	Negative (*n*, %)	Positive (*n*, %)	Negative (*n*, %)
Cancer tissues	32	15 (46.88)*	17 (53.12)	11 (34.38)*	21 (65.26)
Normal tissues	32	27 (84.38)	5 (15.62)	0 (0)	32 (100)

*: *vs*. normal tissue, $P < 0.05$.

Table 2. The hMSH2 mRNA expression and hMSH2 gene promotor methylation in different pathological types of esophageal cancer tissues

Clinical features		hMSH2 mRNA expression		hMSH2 gene promotor methylation	
		Positive	Negative	Positive (*n*, %)	Negative (*n*, %)
Age	< 70 years old	62.3 ± 6.4 (years)	63.3 ± 8.4 (years)	5 (20.0)*	20 (80.0)
	≥70 years old			6 (85.7)	1 (14.3)
Sex	Male	9	10	7 (36.8)	12 (63.2)
	Female	6	7	4 (30.8)	9 (69.2)
Cancer size	< 5 cm	9	8	5 (29.4)	12 (70.6)
	≥ 5cm	6	9	6 (40.0)	9 (60.0)
Tumor location	Top	0	4	7 (35.0)	13 (65.0)
	Middle	9	7		
	Lower	6	6	4 (33.3)	8 (66.4)
Pathological type	Squamous carcinoma	12	13	8 (32.0)	17 (68.0)
	Adenocarcinoma	3	2	3 (42.9)	4 (57.1)
	Squamous adenocarcinoma	0	2		
Invasion depth	Mucosa	1	0	6 (31.6)	13 (68.4)
	Muscularis	10	8		
	Full thickness	4	9	5 (38.5)	8 (61.5)
Lymphatic metastasis	Present	12	11	9 (39.1)	14 (60.9)
	Absent	3	6	2 (22.2)	7 (77.8)
TNM stage	I – II	11	11	4 (18.2)#	18 (81.8)
	III – IV	4	6	7 (70.0)	3 (30.0)

*: *vs*. ≥ 70 years old, $P < 0.05$; #: *vs*. stage III – IV, $P < 0.05$.

Table 3. Relation of hMSH2 promotor methylation and its mRNA expression

	Methylation (+)	Methylation (–)
mRNA (+)	2 (13.33%)	13 (86.67%)
mRNA (–)	9 (52.95%)	8 (47.05%)
Total	11 (34.40%)	21 (65.60%)

Four different mut genes were found in *Backwoods coli*, and they were named as MutS, MutL, MutH, and MutU, respectively. After that, 6 types homologous genes were discovered in Saccharomycetes and they were named as MSH1 – MSH6. So far, 6 homologous genes were found in human gene and they're hMSH2, hMSH3, hMSH6, hMLH1, hPMS1, and hPMS2. Among them, hMSH2 was the first MMR gene to be identified. It's also the first to

be isolated. hMSH2 is located in 2p21 – 22. Its protein functions to detect the base pair mismatches. Wang *et al* reported DNA microsatellite instability lymphoma was detected in new-born rat whose hMSH2 gene was inactivated. This is the most direct proof regarding the relationships between human mismatch gene inactivation and the tumor onset[2].

Among those hereditary tumor syndrome, such as HNPCC, gastric cancer and lung cancer, hMSH2 mutation is ranked top in MMR gene mutations[5–7]. Rass K *et al*[8] did research on the melanoma and they found hMSH2 gene had great importance on carcinogenesis, cancer stabilization and infiltration. Li *et al*[9] reported that the expression of hMSH2 gene significantly decreased 22.6% in the esophageal cancer tissues. In this research, the positive rates of hMSH2 mRNA in esophageal cancer tissue and normal tissue were 46.88% and 84.38% ($P < 0.05$), respectively. Further research showed that the expression of hMSH2 mRNA didn't correlated with esophageal cancer patient's age, sex, carcinoma size and location, pathological type, tissue-inflitration, lymph node metastasis, and tumor infiltration depth ($P > 0.05$). It indicates that the loss of hMSH2 might be a forecast of esophageal cancer.

The hMSH2 mRNA positive cells are mainly gland cells in the esophageal mucosa gland, however, no hMSH2 mRNA expression was detected in muscle tissues. Almost all normal mucosa cells expresed hMSH2 mRNA, while only part of the gland cells with cancerous lesion expressed. This indicated that the positive level in cancerous tissues was lower than that of normal tissues. This was consistent with Zhang's research[10], who reported that the positive expression level of normal stomach tissue was higher than that in gastric carcinoma tissues.

It's also reported that 60% of human gene promotor has CpG island. The methylation of these CpG islands controls the DNA expression by alterating DNA structure, conformation and stability, and by participating in DNA and protein interaction. Therefore, the detection of aberrant methylation has been a hot research area for discovering the cause of tumorigenesis. Recently, many researchers focus on the MMR gene hMSH2 methylation in tumor development. Fang *et al* found that 68 gastric carcinoma samples had hMSH2 gene methylation[11]. Wang *et al* did research on 14 female non-cellule lung cancer patients and found that 28.6% hMSH2 promotor was methylated. They thought promotor methylation was the major cause of hMSH2 inactivation[12]. Herman *et al* found there was no high promotor methylation in *Backwoods coli*[3]. They used MSP method to detect hMSH2 gene promotor methylation and found that the positive rate was 34.4%. No methylation was found in normal tissuein in our results. The hMSH2 mRNA detection indicated that hMSH2 promotor methylation is somehow related to the low expression of hMSH2 mRNA in the esophageal cancer tissue.

Additionally, we found that the hMSH2 promotor methylation had no obvious correlation with the tumor size, location, infiltration depth and patient's sex ($P > 0.05$), however, it had close relationship with patient's age and TNM stage. The hMSH2 promotor methylation in elder patients (age $\geq$ 70) was much higher than younger patients (age < 70). In addition, the methylation level (70%) in patients with stage III – IV was much higher than level I – II ($P < 0.05$). This indicated that hMSH2 gene promotor methylation possibly got involved in the development of esophageal cancer, especially for elderly people. The samples of clinical pathological cases of esophageal cancer was not big enough, so it's necessary to add more research samples for the further research on hMSH2 gene expression and its relationship with CpG island methylation in esophageal cancer.

Besides, the hMSH2 methylation in the group of the positive hMSH2 mRNA expression was 13.33%, lower than the hMSH2 mRNA negative group (52.95%). This indicated that the abberant mutation of MMR gene hMSH2 is highly related to hMSH2 promotor methylation.

5 Conclusion

In our research, we found that 53.12% of esophageal cancer was hMSH2 mRNA negative and the 34.38% of the samples was detected that the hMSH2 gene promotor was methylated. The difference may tell us abnormal promotor methylation is one of the reasons to block mRNA copy. However, further research is required to discover the causes of the loss of expression in hMSH2 gene and the underlying mechanism of the abnormal promotor methylation that blocked mRNA copy.

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