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Overexpression of hMSH2 and hMLH1 protein in certain gastric cancers and their surrounding mucosae

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Abstract. Mismatch repair (MMR) deficiency is closely related to oncogenesis, which is usually accompanied with the loss of expression of hMSH2 and/or hMLH1. These two proteins are detected in many gastric cancers (GCs), and even their overexpression have been reported in certain other cancers. We studied the protein expression levels of MMR (hMSH2 and hMLH1), PCNA and Ki67 in the cancers and surrounding mucosae (SMs) collected from the patients with GC and gastric mucosa samples from non-cancer patients (NCMs), using immunohistochemistry. Our results demonstrated that the positive MMR protein expression levels were 69.1% (132/191), 33.1% (44/133) and 7.1% (3/42) in GCs, SMs and NCMs, respectively (P<0.001); the positive PCNA protein expression levels were 92.1% (176/191), 75.9% (101/133) and 23.8% (10/42), respectively (P<0.001); the positive Ki67 protein expression levels were 79.1% (68/86), 29.2% (21/72) and 45.2% (19/42), respectively (P<0.001). In addition, the MMR protein expression significantly correlated to the level of PCNA protein expression (r_s=0.170, P=0.019), but not to the level of Ki67 protein expression in GCs. Notably, the overexpression of MMR protein was not correlated to either PCNA or Ki67 protein expression in SMs and NCMs. These results support the evidence that MMR protein expression may increase prior to gastric cancer occurrence, and in a view of early diagnosis, the detection of MMR protein by IHC may be helpful as a marker in early prediction of gastric cancer.

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Introduction

Gastric cancer (GC) is one of the most common human malignant tumors in the world, with nearly one million new patients expected to be afflicted with the disease this year (1). The incidence of GC shows considerable geographic variation. East Asia is still a high prevalence area, while the morbidity and mortality of GC is declining globally. Despite extensive research into GC, the mechanism underlying the disease remains elusive. It is believed that gastric carcinogenesis is a multistep process composed of several genetic alterations, including oncogene activation, tumor suppressor gene inactivation and DNA repair deficiency similar to other cancers (2,3). Mismatch repair (MMR) is a kind of DNA repair, which plays a critical role in the prevention of mutation and replication of fidelity assurance (4). MMR deficiency often accompanies microsatellite instability and makes some genes susceptible to mutations leading to a malignant cell transformation (5,6). There are many MMR genes that have been identified so far, hMSH2 and hMLH1 being the most important ones. hMSH2, a homologue of *Escherichia coli* (*E. coli*) MutS protein, can form heterodimers (hMutS α and hMutS ß) with substrate specificity modifying MutS homologues (hMSH3 and hMSH6). In the process of MMR, DNA base mismatches are recognized by hMutS α or hMutS ß, which interact with a protein complex containing hMLH1 and another homologue of *E. coli* MutL (hPMS2, hPMS1, hMLH3). hMutS α or hMutS ß directs the removal and replacement of base-base mispairs or larger DNA insertion/deletion loops. Thereafter, DNA-repair synthesis is carried out by DNA polymerase (7,8). Usually the protein expression of hMSH2 or hMLH1 is used as a target in judging MMR function (9). The loss of hMSH2 and/or hMLH1 expression may cause MMR deficiency, which is closely related to oncogenesis of many malignant tumors such as hereditary non-polyposis colorectal cancer (HNPCC), sporadic colorectal cancer, gastric cancer and endometrial cancer (10-13). However, hMSH2 and/or hMLH1 protein expression can be detected in many GCs and even part of HNPCCs. Furthermore, MMR protein overexpression was reported in urothelial cancer, sporadic endometrial cancer,

glioblastoma and bronchioloalveolar cancer (14-17). The reason for this phenomenon is unclear. It is usually believed that MMR protein expression is related to cell cycle or proliferation (18,19). To evaluate the role of the MMR protein over-expression in gastric carcinogenesis and its clinical significance, we investigated the synchronous expression of hMSH2 and hMLH1 in tumor samples and surrounding mucosae (SMs) collected from patients with GC and gastric mucosa of non-cancer patients (NCMs) using immunohistochemistry (IHC). In addition, the relationship between MMR protein expression and cell proliferation in different gastric mucosae was analyzed.

Materials and methods

Patients and tissue samples. In this study, a total of 191 GC, 133 SM and 42 NCM tissue samples were collected and examined from the local hospitals in the Dalian area of Northern China during the years 2001 and 2005. Of the 191 patients studied, 138 were men and 53 were women with a mean ± SD age of 61±12 years (range 25-89 years). All the patients were diagnosed by two certified pathologists independently. The control group consisted of 15 men and 27 women who were non-cancer patients with a mean ± SD age of 52±11 years (range 35-75 years). None of the patients received radio- or chemotherapy before the samples were collected. Ninety-two GC samples (41 of them with SM) were obtained from the patients who underwent surgical operations and 99 GCs (92 of them with SM) and the 42 NCMs were obtained by endoscopic mucosal biopsy. All samples were collected with the approval of The Ethics Committee of the Dalian Medical University and with the informed consent from the patient during the surgical biopsy procedures. All samples were formalin-fixed and paraffin-embedded. Clinical information and clinicopathological features of all the patients are presented in Table I. Clinicopathological examinations revealed cases with a well differentiated (n=18), medium differentiated (n=59), poor differentiated (n=88) and mucoid cell cancer (n=26) among the 191 GCs. Lymph node metastasis was examined in 127 GC patients with a positive rate of 44% (57/127). Additionally, previous *H. pylori* infection was studied in 99 GC patients and the positive rate was 52% (52/99). All SMs and NCMs were histopathologically diagnosed as either chronic atrophic gastritis mucosa or chronic superficial gastritis mucosa by two licensed pathologists.

Immunohistochemistry. Monoclonal antibodies (Abs) against human hMSH2 (1:250; clone FE11; Zymed laboratories; Invitrogen Immunodetection, USA), hMLH1 (1:50; Clone 14; Zymed laboratories; Invitrogen Immunodetection), PCNA (1:400; clone PC10; NeoMarker; USA), Ki67 (1:100; clone K-2; Zymed laboratories; Invitrogen Immunodetection) were used for primary antibodies. The IHC was performed according to the method reported by Bacani *et al* (20) with slight modifications. In brief, sections (4 μm) were deparaffinized and dehydrated. Endogenous peroxidase activity was quenched in 3% H_2O_2 for 15 min. The sections were washed in phosphate-buffered saline (PBS), pretreated with microwave epitope retrieval in 0.01 mol/l citrate buffer, pH 6.0 and cooled for 20 min naturally. After being immersed in 10% normal horse serum for 15 min at room temperature in order to reduce nonspecific background staining, the sections were incubated overnight with primary antibodies at 4˚C in a humidified chamber. After being washed with PBS, the sections were incubated with biotin-conjugated second antibody at 30˚C (PCNA at room temperature) for 15 min, then washed again and incubated with streptavidin-peroxidase at 30˚C (PCNA at room temperature) for 15 min. After further washing with PBS, sections were developed with activated 3,3'-diaminobenzidine-tetrahydrochloride (DAB) for 5 min and the reaction was stopped in water. The nuclei were visualized by counter-staining with hematoxylin. PBS replacing the primary antibody was used as a negative control. Immunoreactivity of hMSH2 and hMLH1 was evaluated as follows: -, <10% of the tumor or epithelial cells showed positive immunoreactivity; +, ≥10% of these cells showed positive immunoreactivity. Since hMSH2 and hMLH1 are required for protein to complete mismatch repair, we regarded synchronous expression of hMSH2 and hMLH1 as MMR protein-positive immunoreactivity (+); otherwise as MMR-negative immunoreactivity (-). Immunoreactivity of PCNA and Ki67 was evaluated as follows: -, <20% of the tumor or epithelial cells showed positive immunoreactivity; +, 20-50% of these cells showed positive immunoreactivity; ++, >50% of these cells showed positive immunoreactivity. All the staining results were evaluated by two independent researchers.

Statistics. The differences in the frequency of MMR protein expression among GCs, SMs and NCMs and the difference in the frequency and the interaction between MMR protein expression and clinicopathological characters were analyzed with the Chi-square test. The correlation of MMR protein expression with PCNA and Ki67 were evaluated with Spearmen correlation analysis. Statistical significance was defined as $P<0.05$. The data analysis was performed with the SPSS 10.0 software statistical package.

Results

hMSH2, hMLH1, PCNA and Ki67 protein expression in different gastric mucosae. The immunoreactions of hMSH2 and hMLH1 were localized mainly in the nuclei and occasionally in the cytoplasm (Fig. 1). The positive hMSH2 expression levels were 82.7% (158/191), 47.4% (63/133), 52.4% (22/42) (GCs vs. SMs: $P<0.001$; SMs vs. NCMs: $P>0.05$; GCs vs. NCMs: $P<0.001$), and the positive hMLH1 expression levels were 80.1% (153/191), 60.2% (80/133), 31.0% (13/42) (GCs vs. SMs: $P<0.001$; SMs vs. NCMs: $P=0.001$; GCs vs. NCMs: $P<0.001$) in GCs, SMs, NCMs, respectively. The percentages of hMSH2 and hMLH1 synchronously positive were 69.1% (132/191), 33.1% (44/133) and 7.1% (3/42) in GCs, SMs and NCMs, respectively (GCs vs. SMs: $P<0.001$; SMs vs. NCMs: $P=0.001$; GCs vs. NCMs: $P<0.001$). As shown in Fig. 1, the immunoreactions of PCNA and Ki67 were localized in the nuclei. The positive PCNA protein expression levels were 92.1% (176/191), 75.9% (101/133) and 23.8% (10/42) in GCs, SMs and NCMs, respectively (GCs vs. SMs: $P<0.001$;

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Correlation of MMR protein expression to clinicopathological features of the 191 gastric cancer patients.

Clinicopathological features	Case	MMR protein		P
		-	+	
Total	191	59	132	
Age (years)				
<60	71	21	50	NS
≥60	120	38	82	
Gender				
Female	56	19	37	NS
Male	135	40	95	
H. pylori infection				
No	47	16	31	NS
Yes	52	22	30	
Differentiation grade				
Well	18	6	12	NS
Medium	59	16	43	
Poor	88	26	62	
Mucoidal	26	11	15	
Lymph node metastasis				
Absent	57	15	42	NS
Present	70	20	50	

-, negative; +, weakly positive. NS, not significant (P>0.05).

Table II. MMR protein, PCNA and Ki67 protein expression in GCs, SMs and NCMs.

	MMR protein		Positive rate	PCNA			Positive rate	Ki67			Positive rate
	-	+		-	+	++		-	+	++	
GCs	59	132	69.1%[b]	15	53	123	92.1%[b]	18	40	18	79.1%[b]
SMs	89	44	33.1%[a]	32	50	51	75.9%[a]	42	17	3	29.2%#
NCMs	39	3	7.1%	32	6	4	23.8%	23	17	2	45.2%

-, negative; +, weakly positive; ++, strongly positive. [a]Positive rate of MMR protein in SM vs. in NCM (P<0.05); [b]positive rate of MMR protein in GC vs. in SM and in NCMs (P<0.05); #positive rate of MMR protein in SM vs. in NCM (P>0.05).

SMs vs. NCMs: P<0.001; GCs vs. NCMs: P<0.001). The positive Ki67 protein expression levels were 79.1% (68/86), 29.2% (21/72) and 45.2% (19/42) in GCs, SMs and NCMs, respectively (GCs vs. SMs: P<0.001; SMs vs. NCMs: P>0.05; GCs vs. NCMs: P<0.001) (Table II).

Correlation of MMR protein expression to PCNA and Ki67 expression in different gastric mucosae. In GCs, the rates of MMR protein expression significantly correlated to the levels of PCNA protein expression (r_s=0.170, P=0.019), while it did not correlate to the levels of Ki67 protein expression (r_s=0.072, P>0.05) (Table III). In SMs and NCMs, the rates of MMR protein expression did not correlate with the level of either PCNA or Ki67 expression (Table IV and Table V).

Correlation of MMR protein expression to clinicopathological features. The relationship between the rates of MMR protein expression levels and clinicopathological features, including age, gender, previous *H. pylori* infection, differentiation of cancer and lymph node metastasis, were analyzed and are presented in Table I. There was no statistically significant correlation between the rates of MMR protein expression levels and the clinicopathological features found in the patients studied.

Discussion

Since MMR deficiency was proven as an important agent related to HNPCC occurrence, the role of the MMR deficiency

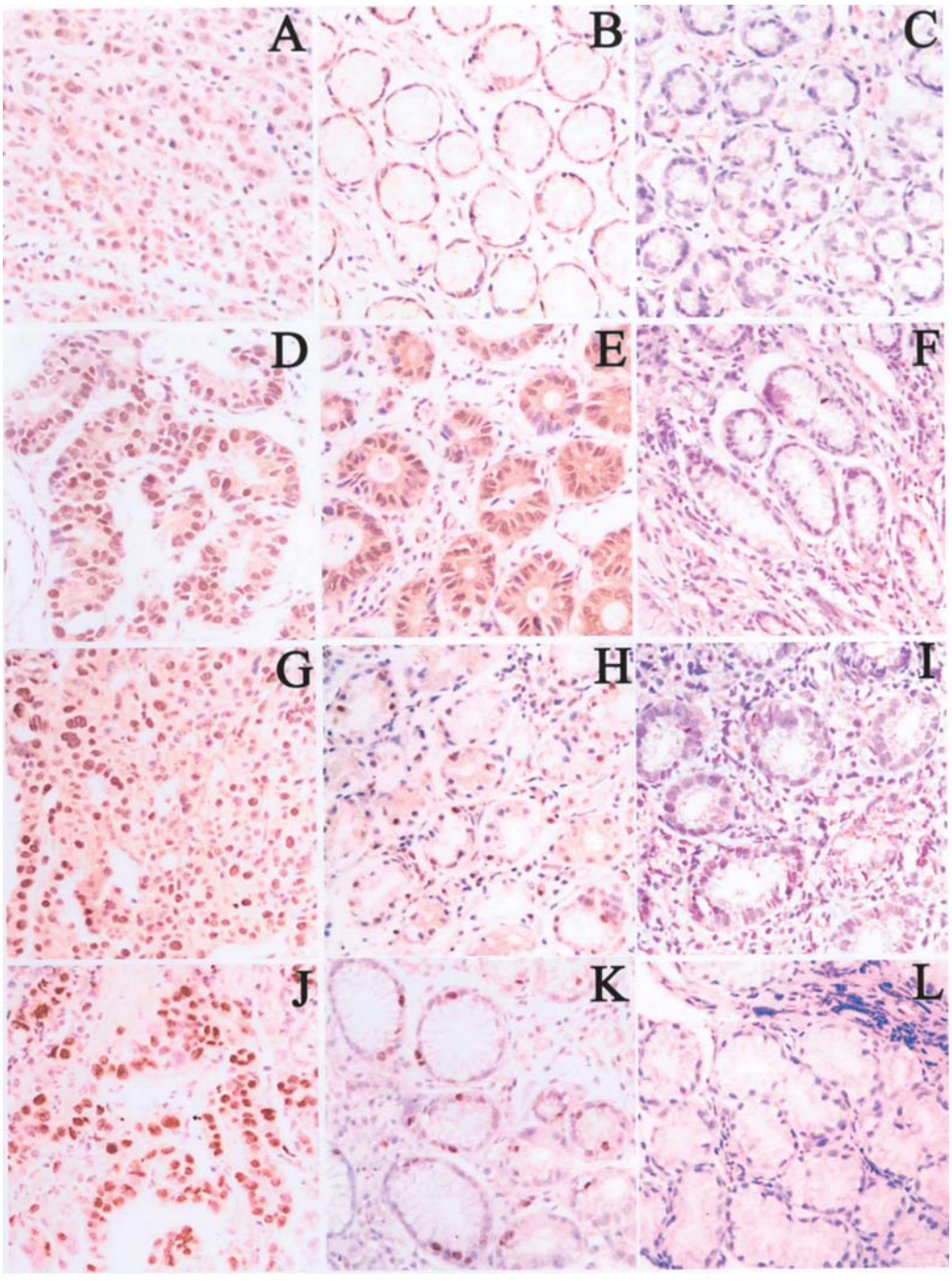

Figure 1. Immunohistochemical profiling of hMSH2 protein positive expression in GC (A), SM (B) and negative expression in NCM (C); and hMLH1 protein positive expression in GC (D), SM (E) and negative expression in NCM (F); and PCNA protein strongly positive expression in GC (G), weakly positive expression in SM (H) and negative expression in NCM (I); and Ki67 protein strongly positive expression in GC (J), weakly positive expression in SM (K) and negative expression in NCM (L). (x 200).

in gastric carcinogenesis has been increasingly investigated. It is generally believed that MMR deficiency, often showing loss of hMSH2 and/or hMLH1 protein expression, is usually caused by exon mutation or promoter methylation. The absent expression of any one of these two genes can induce MMR deficiency. IHC is a simple, convenient and useful method in detecting the presence or absence of hMSH2 or hMLH1 protein expression. In this study, we used IHC to detect hMSH2 and hMLH1 protein expression and considered the synchronous expression of the two proteins as MMR protein-positive expression. We found that the positive rate of MMR protein expression in GC samples was significantly higher than that in the samples from non-cancer gastric mucosae. One of the possible explanations would be that the overexpressed MMR protein in cancer cells of GCs was the accumulation of MMR protein without the original functions. It was reported that gene mutation may have caused a protein product overexpression several decades ago. Tumor suppressor gene p53 was one such example. Protein overexpression and gene mutation of p53 was found

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Correlation of MMR protein expression to PCNA and Ki67 expression in GCs.

MMR protein	PCNA[a]			Ki67[b]		
	-	+	++	-	+	++
-	7	21	31	7	30	5
+	8	32	92	11	20	13

-, negative; +, weakly positive; ++, strongly positive. [a]r_s=0.170 (P<0.05); [b]r_s=0.072 (P>0.05).

Table IV. Correlation of MMR protein expression to PCNA and Ki67 expression in SMs.

MMR protein	PCNA[a]			Ki67[b]		
	-	+	++	-	+	++
-	25	32	32	36	14	2
+	7	18	19	15	4	1

-, negative; +, weakly positive; ++, strongly positive. [a]r_s=0.113 (P>0.05); [b]r_s=-0.051, (P>0.05).

Table V. Correlation of MMR protein expression to PCNA and Ki67 expression in NCMs.

MMR protein	PCNA[a]			Ki67[b]		
	-	+	++	-	+	++
-	29	6	4	21	16	2
+	3	0	0	2	1	0

-, negative; +, weakly positive; ++, strongly positive. [a]r_s=-0.154 (P>0.05); [b]r_s=-0.074 (P>0.05).

simultaneously in many malignant tumors (21). Shin *et al* found that a promoter mutation of hMSH2 (G-to-C transversion at position -225) increased transcriptional efficiency by 466% in a case of HNPCC with GC (22). This suggested that hMSH2 gene mutation could up-regulate its protein expression. The other explanation is that the overexpressed MMR protein in cancer cells of GCs was the cellular adaptation to repair the increase of DNA mismatches in carcinogenesis. Generally, MMR protein expression is up-regulated in proliferating cells. hMSH2 expression was increased at least 12-times in cells with DNA replication as much as in cells at rest (G_0 stage) (7). Wilson *et al* reported that in the rapidly proliferating cells of esophageal, intestinal epithelia and endometrium in the proliferative phase, hMSH2 expression was increased, suggesting that the rapidly proliferating tumor cells may raise the expression levels of the MMR proteins (18,19). Cells of GC, as other malignant cells, are in an aggressive growth pattern and may produce more DNA mismatches than the normal gastric mucosal cells do. Therefore, it would be required to increase the expression of the MMR protein in GCs for repairing these mismatches.

We selected Ki67 and PCNA to represent the proliferation status of cells. Ki67, a proliferation-associated antigen, is strictly correlated with the progression of cell cycle and expresses in G1, S, G2 and mitosis, but not in G0 (23). PCNA is not only a co-factor of DNA polymerase δ participating in DNA replication, but a necessary factor for DNA repair, DNA methylation and cell cycle regulation. PCNA was able to bind to MSH2-MSH6 in the early stages of the MMR process and act as a processivity factor for DNA polymerase in the gap repair synthesis (24). In this study, we found that the protein expressions of MMR, PCNA and Ki67 were higher in cancer cells of GCs than those in non-cancer gastric mucosae. We also observed that in GCs, the rates of MMR protein overexpression were significantly correlated with PCNA, but not with Ki67. This correlation could be the result of PCNA participation in the MMR process. Our findings suggested that overexpressed MMR protein in GCs might be a response to the increase of DNA mismatches, which may help in minimizing gene mutations. In addition, our results suggested that there may be many DNA mismatches in GC cells and these mismatches might occur with the malignant transformation of cells, partly with cell abnormal proliferation. However, whether overexpression of MMR protein in GCs was the manifestation of stronger MMR function was not clarified. Several studies suggested that

other MMR factor MSH3 or PMS2 overexpression may produce hypermutation and damage tolerance phenotype, similar to that observed with MMR deficiency (25,26). Therefore, further study is needed.

The tissues of SM have inherited a background that is the same as that of the cancers that live in close vicinity to the cancers, therefore the SM cells seem to have a strong probability to transform malignantly. We found that the rates of MMR and PCNA protein expression levels in SMs were higher than that in NCMs, while the positive rate of Ki67 expression was similar between SMs and NCMs. We speculate that DNA mismatches might have occurred before neoplastic morphological manifestation was formed. Increased expression of PCNA, as a factor participating in the MMR process, might be a consequence of high MMR response. The detection of MMR protein expression may be used as a new predictive method for early diagnosis of GC.

In summary, the synchronous expression of hMSH2 and hMLH1 evidently increases prior to gastric cancer occurrence and detection of these proteins by IHC may help in early diagnosis of gastric cancer.

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