Expression of MMP-2 and MMP-9 and Its Correlation with Invasion and Metastasis in Human Esophageal Squamous Cell Carcinoma

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Abstract: Objective. To investigate the significance of MMP-2 mRNA and MMP-9 mRNA expression in human esophageal squamous cell carcinoma (ESCC). **Methods.** MMP-2 mRNA, MMP-9 mRNA and proteins were examined by immunohistochemistry, *in situ* hybridization, RT-PCR, zymographic analysis and Western blot for 41 cases of ESCC. **Results.** The expression rate and value of MMP-9 was significantly higher than that of MMP-2 in tumor tissues. **Conclusions.** MMP-9 has higher sensitivity and specificity in predicting the biologic behavior of invasion and metastasis in ESCC. [Life Science Journal. 2006;3(3):13-18] (ISSN: 1097-8135).

Keywords: MMP-2; MMP-9; esophageal carcinoma; invasion; metastasis

Abbreviations: BM: basement membrane; ECM: extracellular matrix; ESCC: esophageal squamous cell carcinoma; MMP: matrix metalloproteinase; PAGE: polyacrylamide gel electrophoresis; SDS: sodium dodecyl sulfate; TBS: Tris-HCl buffered saline

1 Introduction

Esophageal carcinoma is one of the most common cancers and acts as the fourth leading cause of cancer death in China. It is characterized by poor prognosis and rapid clinical progression with a high frequency of lymph node metastasis and recurrence. The transition from *in situ* to invasive tumors is a very complicated process. Proteolysis of extracellular matrix (ECM) is essential step in tumor invasion and metastasis. Numerous proteolytic enzymes including the matrix metalloproteinase (MMP) have been implicated in this process. Reports showed that both MMP-2 and MMP-9 were highly expressed in esophageal tumor tissues[1,2]. In the current study, MMP-2 mRNA and MMP-9 mRNA and their proteins were examined by immunohistochemistry, *in situ* hybridization, RT-PCR, zymographic analysis and Western blot in 41 cases of ESCC as well as the matched normal mucosa tissues, to compare the potential value of MMP-2 and MMP-9 in estimation of the biologic behavior of ESCC.

2 Materials and Methods

2.1 Tissue samples

41 specimens of patients with esophageal squamous cell caricinoma were collected from the First Affiliated Hospital of Zhengzhou University and the Henan Tumor Hospital. All of them were identified by pathology. The resected specimens including the ESCC samples and the normal adjacent tissues were snap-frozen in liquid nitrogen.

2.2 Immunohistochemistry

Specimens were fixed with 10% neutral buffered formaldehyde solution and embedded in low-melting paraffin. Sections of esophageal tumors were immunostained with monoclonal antibodies to MMP-2 and MMP-9. Immunohistochemistry for the individual MMP was performed by an alkaline phosphatase anti-alkaline phosphatase technique. After the immunohistochemistry, the sections were examined under microscope. The MMP status of the tumors was assessed as positive if any of the tumor cells showed significant immunostaining. Negative controls were done by replacing the primary antibody with TBS and by liquid phase pre-absorp-

tion of primary antibody with the corresponding immunogen at 10 nmol/ml antibody. The positive controls for both MMP-2 and MMP-9 were lung containing intra-alveolar macrophages.

2.3 *In situ* hybridization

In situ hybridization was performed on sections (4 μm). After deparaffinization and rehydration all samples were treated with proteinase K and washed in 0.1 M triethanolamine buffer containing 0.25% acetic anhydride. Sections were hybridized overnight at 50 ℃ to 55 ℃ with ^{35}S-labelled RNA probe. After hybridization, slides were washed under stringent conditions and treated with RNase to remove unhybirdized probe. Previously positive samples for each anti-sense probe were used as positive controls. The slides were independently assessed by two experienced investigators.

2.4 RT-PCR

Total RNA was extracted from shock-frozen tissue samples with RNA extract kit. First strand complementary DNA was synthesized from 2 μg of DNA-free total RNA in a 20 μl system of: 1 mmol/L dNTP; 10 U RNAsin; 20 mmol/L DTT; 1 μmol Random Hexamer Primer and 100 U MM-LV. Follow the procedure of 37 ℃ 10 min, 42 ℃ 1 hour and 95 ℃ for 5 min. PCR was done in a 50 μl system including both MMP and β-actin primers. The annealing temperatures of MMP-9 and MMP-2 were 66 ℃ and 65 ℃, respectively. Raw data from each samples, were quantified using the eagle eye system (Stratagene, American). Data from MMP cDNA were normalized to the respective content of β-actin cDNA. T/N>2.0 was recognized as positive.

2.5 Zymographic analysis

Equal amounts of total lysates of esophageal tissue samples were used for determining protein amounts with the Bio-Rad DC protein assay kit and stored at −20 ℃ until assayed. MMP activity in the lysates was assessed by gelatin zymography: lanes were loaded with 2 μg of total protein each. The concentrated media were run on nonreducing sodium dodecyl sulfate (SDS)-polyacrylamide gel (10%) containing 1 mg/ml gelatin. After electrophoresis in 25 mM Tris base, 250 mM glycine, and 1% SDS, the gel was washed at room temperature in solution (2.5% Triton X-100, 5 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.5), and was incubated again in the same buffer twice for 1 hour each. After rinsing the gel extensively with six changes of distilled water, it was incubated overnight at 37 ℃ in 5 mM $CaCl_2$ and 50 mM Tris-HCl, pH7.5, followed by Coomassie blue staining and destaining in solution of methanol, acetic acid and water(50:10:40). Gelatin zymography depicts MMP as negatively staining bands of gelatinolytic activity.

2.6 Western blot

Tissue lysates were separated by SDS-PAGE using separating gels and stacking gels of 3% polyacrylamide. Lane was loaded with 2 μg of total protein each. After electrophoresis, the proteins were transferred to PVDF membrane. Protein bands were localized by staining with Ponceau S. Blots were blocked with Tris-buffered saline-NaCl, pH 7.6, containing 10% bovine serum albumin, 20 mM Tris, 137 mM NaCl, and 0.1% Nonidet P40; washed; blocked with 10% milk and incubated with antibodies against MMP-2 or MMP-9. Experiments were done in triplicate. The bands were scanned by computer analysis. Data from all tissue samples analyzed were normalized by setting the protein amount in the healthy tissue to 1.0 in each sample[3].

2.7 Statistical analysis

The SPSS statistical package program was used for all analysis. Associations between the variables were tested by χ^2 test, Student's t test. $\alpha = 0.05$ were set significant.

3 Results

3.1 Immunohistochemistry

The results revealed that there were 26 cases of positive reaction for MMP-9 in 41 ESCC and 5 cases of positive reaction in matched normal mucosa tissues. The percentage of positive cases in ESCC was significantly higher than that in matched normal mucosa tissues ($P<0.01$). Faint staining was also found in monocyts, fibroblasts, endothelial and smooth muscle cells. Positive reaction for MMP-9 showed a tendency to be stronger in deeply invading nests, especially in peripheral fronts. On the contrary, MMP-2 levels were lower and positive reaction in carcinoma cells was generally weaker than that in stromal cells. There were distinct difference between the MMP-9 and MMP-2 expression in ESCC ($P<0.01$) (Table 1, Figure 1 and Figure 2).

Table 1. Expressions of MMP-2 and MMP-9 by immunohistochemistry

MMP type	Cases (n)	Expression	
		Positive cases (n)	Positive rate (%)
MMP-9	41	26	63.41
MMP-2	41	7	17.07

$P<0.01$

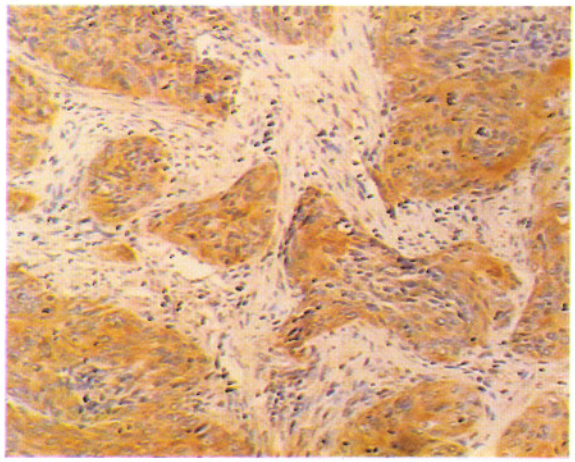

Figure 1. Detection of MMP-9 protein in ESCC by immunohistochemical staining SP×200

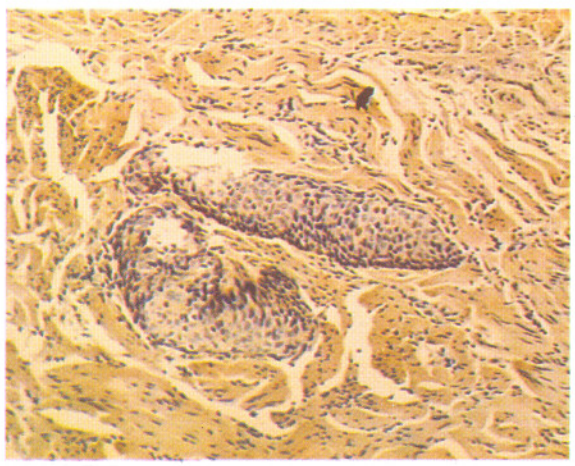

Figure 2. Detection of MMP-2 protein in ESCC by immunohistochemical staining SP×200

3.2 *In situ* hybridization

The results revealed that there were 22 cases of positive reaction for MMP-9 mRNA in 41 ESCC and 5 cases of positive reaction in matched normal mucosa tissues. The percentage of positive cases in ESCC was significant higher than that in matched normal mucosa tissues ($P<0.01$). Faint staining was also detected in macrophages, fibroblasts, endothelial and smooth muscle cells but stronger staining was observed in plasmacytes. Positive reaction for MMP-9 mRNA showed a tendency to be stronger in deeply invading nests, especially in peripheral fronts. On the contrary, MMP-2 levels were lower and positive reaction in carcinoma cells was generally weaker than that in stromal cells. There was distinct difference between the MMP-9 mRNA and MMP-2 mRNA expression in ESCC ($P<0.01$) (Table 2, Figure 3 and Figure 4).

Table 2. Expressions of MMP-2 mRNA and MMP-9 mRNA by *in situ* hybridization

MMP type	Cases (n)	mRNA expression	
		Positive case (n)	Positive rate (%)
MMP-9	41	22	53.66
MMP-2	41	4	9.76

$P<0.01$

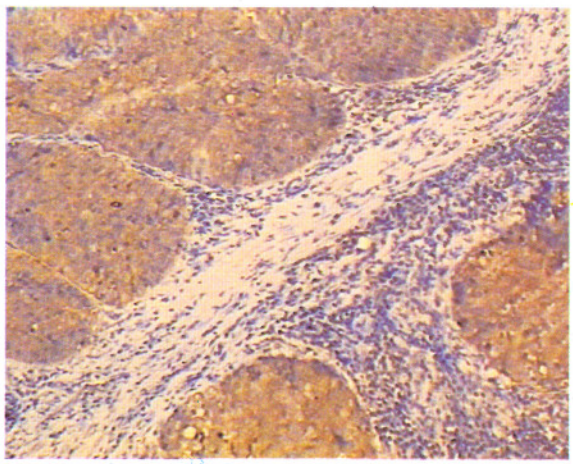

Figure 3. Detection of MMP-9 mRNA in ESCC by *in situ* hybridization×200

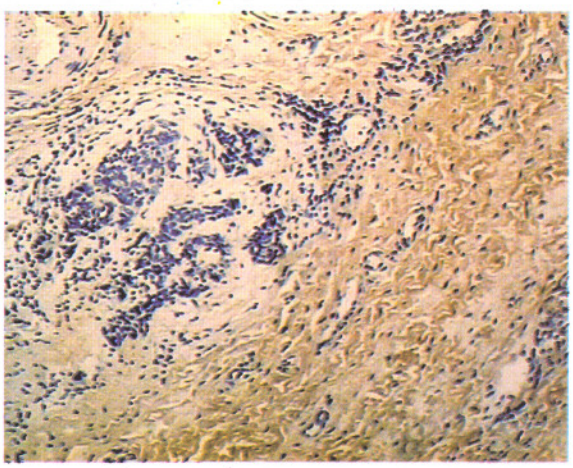

Figure 4. Detection of MMP-2 mRNA in ESCC by *in situ* hybridization×200

3.3 RT-PCR

RT-PCR inspect showed that the positive rate and semi-quantitative value of MMP-9 mRNA were 80.49% (33/41) and 0.57 ± 0.43 in ESCCs, respectively. The positive rate and the semi-quantitative value of MMP-2 mRNA were 58.54% (24/41) and 0.21 ± 0.21 in ESCC, respectively. The positive rate and the semi-quantitative value of MMP-2 mRNA were distinctly lower than those of MMP-9 mRNA ($P<0.05$, $P<0.01$) (Table 3, Figure 5 and Figure 6).

Table 3. Expressions of MMP-2 and MMP-9 mRNA by RT-PCR

MMP type	Cases (n)	mRNA expression		
		Positive case (n)	Positive rate(%)	Absorbance value
MMP-9	41	33	80.49#	0.57±0.44*
MMP-2	41	24	58.54#	0.21±0.21*

#: *vs*. MMP-9, $P<0.05$; *: *vs*. MMP-9, $P<0.01$

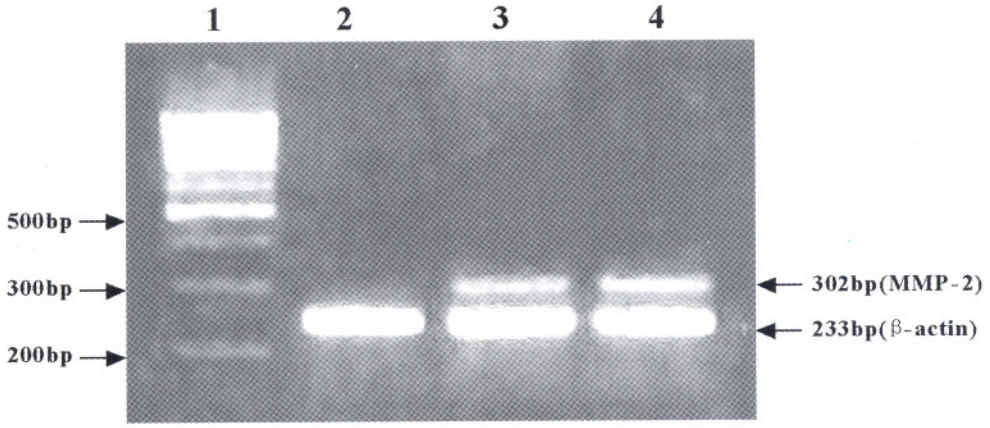

Figure 5. Detection of MMP-2 mRNA in ESCC, tissue adjacent to tumor and matched mormal mucosa tissue by RT-PCR
Lane 1: Marker; Lane 2: normal tissue; Lane 3: tissue adjacent to tumor; Lane 4: ESCC

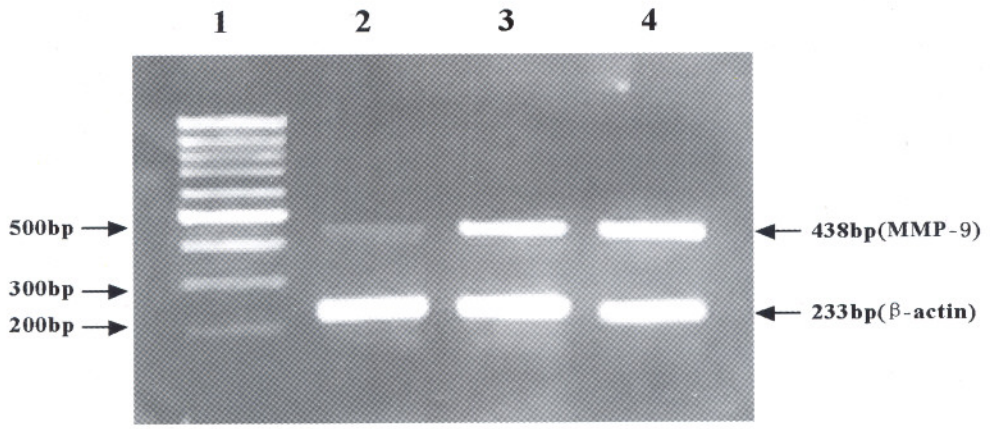

Figure 6. Detection of MMP-9 mRNA in ESCC, tissue adjacent to tumor and matched mormal mucosa tissue by RT-PCR
Lane 1: Marker; Lane 2: normal tissue; Lane 3: tissue adjacent to tumor; Lane 4: ESCC

3.4 Zymographic analysis

By zymographic analysis, there were small amount secretions of proMMP-9 (92 kD) and proMMP-2(72 kD) in matched normal mucosa tissues. The activated MMP-2(62 kD) was detected in 31 biopsy specimens, but no activated MMP-9 (82 kD) could be found. The semi-quantitative values of proMMP-9, activated MMP-2 and proMMP-2 in ESCC were 2.52±0.75, 1.92±0.42 and 1.51±0.42, respectively. There were distinct differences between proMMP-9 and activated MMP-2 ($P<0.01$), proMMP-9 and proMMP-2 ($P<0.01$). The activated MMP-9 could be detected in 24 biopsy specimens (Figure 7).

3.5 Western blot

By Western blot analysis, the semi-quantitative values of MMP-9 and MMP-2 in ESCC were 3.02±0.56 and 2.26±0.51, respectively. There were distinct differences between MMP-9 and MMP-2($P<0.05$) (Figure 8).

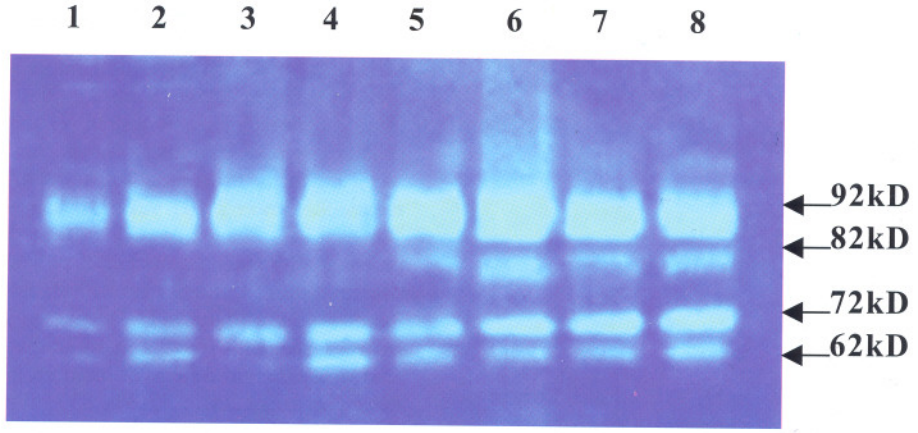

Figure 7. Detections of MMP-2, MMP-9 in ESCC and matched normal mucosa tissues by zymographic analysis
Lane 1: standard of collagenase IV; Lane 2 – 4: normal tissues; Lane 5 – 8: ESSC

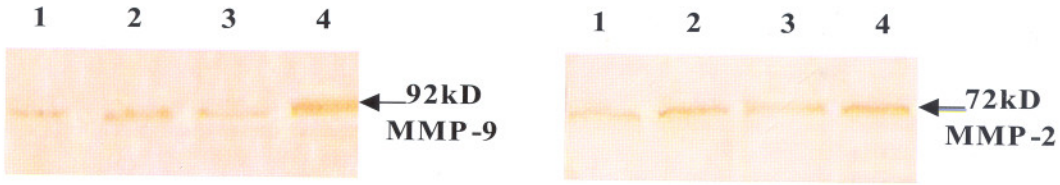

Figure 8. Detections of TIMP in ESCC by Western blot
Lane 1, 3: matched normal mucosa tissues; Lane 2, 4: ESSC

4 Discussion

Matrix metalloproteinase (MMP) is a family of Zn^{2+} metalloproteinase, involved in degradation of the extra cellular matrix macromolecules. They have been classified into four classes based on substrate specificities such as collagenases, gelatinases, stromelysins and membrane-type MMP. Collagenases, gelatinases and stromelysins are solubale while membrane-type metalloproteinsaes attach to the surface of the cell[4]. MMP-2 and MMP-9 are capable of cleaving basement membrane type Ⅳ collagen, but their enzymatic activity is far greater against gelatins, hence they are often referred to as gelatinases. MMP-2 and MMP-9 are also responsible for further degradation of the large 3/4 and 1/4 collagen fragments and other proteins including fibronectin, laminin, and elastin[5]. Reports showed that both MMP-2 and MMP-9 played an important role in tumor invasion and metastasis[6-9].

In the current study, MMP-2 mRNA, MMP-9 mRNA and their proteins were examined by immunohistochemistry, *in situ* hybridization, RT-PCR, zymographic analysis and Western blot for 41 cases of ESCC as well as matched normal mucosa tissues, in order to compare the potential value of MMP-2 and MMP-9 in estimation of the biologic behavior of ESCC. Our results showed that the expression rate and semi-quantitative value of MMP-9 was significantly higher than those of MMP-2 in tumor tissues, which suggested that MMP-9 had higher sensitivity in predicting the biologic behavior of invasion and metastasis in ESCC.

Most of MMP are secreted as inactive zymogens (proMMPs), and extracelluar activation mechanisms are required for their function. Through separating the Zn^{2+} and cysteine, the Zn^{2+} active center are exposed. Activation of MMP shows waterfall effect[10]. Zymographic analysis is a special technique to detect the activity of MMP by polyacrylamidedel electrophoresis (PAGE), which can differentiates proenzyme and activated enzyme and detects a group of MMP with identical substrate at the same time. It has the behavior of convenient and susceptible[11].

In our study, there was small amount of secretion of proMMP-9 (92 kD) and proMMP-2 (72 kD) in all 41 cases of normal mucosa tissues. The activated MMP-2 (62 kD) was detected in 31 biopsy specimens, but no activated MMP-9 (82 kD) was found. Also, the expression of proMMP-9 was significantly higher than both proMMP-2 and activated MMP-2 in ESCC tissues. The activated MMP-9 could be detected in 24 biopsy specimens of all 41 cases of ESCC tissues. All these strongly suggested that MMP-9 has higher sensitivity and specificity in predicting the biologic behavior of in-

vasion and metastasis in ESCC.

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