Significance of effector protease receptor-1 expression and its relationship with proliferation and apoptotic index in patients with primary advanced gastric adenocarcinoma

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Abstract

AIM: To investigate the expression of effector protease receptor-1 (EPR-1), proliferative index Ki-67 and apoptosis index in patients with primary advanced gastric adenocarcinoma and to clarify the significance of EPR-1 expression and its correlation with the proliferation and apoptosis indices.

METHODS: Using immunohistochemical staining and terminal deoxynucleotidyl transferase mediated nick end labelling (TUNEL) technique, we determined the expression of EPR-1, proliferative index (Ki-67) and apoptotic index (AI) in 120 paraffin-embedded specimens of primary advanced gastric adenocarcinoma as well as lymph node metastasis and adjacent normal tissues.

RESULTS: EPR-1 expression was distributed in the cytoplasm of normal gastric mycorderma, carcinoma cells and smooth muscle cells. The positive rate of EPR-1 expression in the primary gastric adenocarcinomas, invasion tumor node and lymph node metastasis was 65.83%, 55.29% and 68%, respectively. While the positive rate in normal gastric mycorderma and smooth muscle cells was 46.7% and 53.3%, respectively. The average positive rate of Ki-67 in EPR-1-positive tumors was 3.00% which was significantly lower than that of 8.53% in EPR-1-negative tumors, but the average AI in EPR-1-positive tumors was 1.25%, which was significantly higher than that of 0.67% observed in EPR-1-negative tumors. On the other hand, the average positive labeling index for Ki-67 (ki-67) in EPR-1-positive lymph node metastasis was 7.65%, which was significantly lower than that of 9.44% observed in EPR-1-negative lymph node metastasis. However, the average AI in EPR-1-positive lymph node metastasis tumors was 0.99%, which was significantly higher than that of 0.67% observed in EPR-1-negative lymph node metastasis.

CONCLUSION: The frequency of EPR-1 expression was significantly higher in primary gastric adenocarcinoma and in its lymph node metastasis than that in normal gastric mucosa. Expression of EPR-1 was significantly correlated with tumor histological subtypes and tumor differentiation. Weighted EPR-1 Score is positively correlated with apoptosis index, but is negatively related with proliferative index. Thus, Weighted EPR-1 Score and EPR-1 expression in gastric adenocarcinoma cells maybe a potential marker in clinical setting.

INTRODUCTION

Gastric cancer is the second most common malignancy worldwide. Approximately 95% of all malignant gastric neoplasms are adenocarcinoma. This study aimed to investigate the correlation among expression of EPR-1, proliferative index (PI, also referred to as ki-67) and apoptosis index (AI) in patients with primary advanced gastric adenocarcinoma and to clarify the significance of EPR-1 expression in human primary gastric adenocarcinoma, its lymph node metastasis and adjacent non-tumor tissues.

MATERIALS AND METHODS

Patients and tissue samples

Primary gastric adenocarcinoma specimens from 120 patients who had undergone gastrectomy in the period of January 1991 and December 1995 were selected from the files of the Pathology Department, Nanjing Jinling Hospital. None of the patients had received chemotherapy or radiotherapy before surgery. There were 72 males and 48 females, with a median age of 64.8 years (range, 45-78 years). Samples were taken from the representative cancerous lesions as well as lymph node metastasis and adjacent non-cancerous mucosa.

Specimens of the adenocarcinoma were classified into papillary adenocarcinoma, tubular adenocarcinoma, mucinous adenocarcinoma and signet-ring cell carcinoma. Each subclass had 30 cases.

For formalin-fixed and paraffin-embedded tissue specimens, consecutive 4-μm thick sections were cut and representative sections were used for immunohistochemistry (IHC) and haematoxylin-eosin (H&E) staining as well as the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) for apoptotic index. Using the H&E-stained sections, the tumor histology according to Lauren’s grade of tumor differentiation and criteria of the World Health Organization were reassessed. Tumors were staged according to TNM classification.

Immunohistochemistry

The deparaffinized and rehydrated slides were boiled in a 10 mmol/L EDTA buffer (pH 8.0) for 5 min in an autoclave at 121°C. The sections were immunohistochemically stained by
the labeled streptavidin-biotin peroxidase method (LSAB2 Kit; Dako Japan Inc., Kyoto, Japan) with the following primary antibodies: monoclonal antibodies EPR-1 (ADI, Alpha Diagnostic International, dilution 1:100), and Ki-67 (Maxim, China, dilution 1:50). The slides were immersed for 10 min in 3 mL/L hydrogen peroxide/methanol to deplete endogenous peroxidase. Then, nonspecific binding sites were blocked with 3 mL/L normal goat serum for 10 min. The primary antibody was then applied, and the sections were incubated overnight at 4 °C. After washed with PBS (0.01 mol/L pH 7.4), biotinylated goat antimouse IgG was applied onto the tissue sections and incubated at room temperature for 10 min. After washed with PBS, a streptavidin peroxidase reagent was applied and incubated at room temperature for 10 min. Finally, the reaction product was visualized using developing color by incubating the slides in a solution of 3 mL/L hydrogen peroxide and AEC chromogen. The sections were counterstained slightly with hematoxylin. Negative controls included parallel sections treated without the primary antibody, in addition to negat an adjacent section of the same block in which the primary antibody was replaced by phosphate-buffered saline (PBS).

**TUNEL staining**

For detection of apoptotic cells, apoptotic index was examined by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate fluorescence nick end labeling (TUNEL) method. TUNEL: In situ cell death detection kit POD (ISCDD, Boehringer Mannheim, Germany) was used to detect the apoptotic cell. The procedures was according to the protocol of the kit and other references. Briefly, the sections were deparaffinized, rehydrated, and washed in distilled water (DW). The tissues were digested with 20 g/mL proteinase K (Boehringer Mannheim, Mannheim, Germany) at room temperature for 15 min. Endogenous peroxidase activity was blocked by incubating it in 3 mL/L hydrogen peroxide/methanol in PBS at 37 °C for 30 min. The sections then were incubated with terminal deoxynucleotidyl transferase at 37 °C for 60 min, and dioxigenin-conjugated dUTP was added to the 3′-OH ends of fragmented DNA. Anti-digoxigenin antibody peroxidase was applied to the sections to detect the labeled nucleotides. The sections were stained with DAB and counterstained slightly with hematoxylin. The positive cells were identified, counted and analyzed under the light microscope.

**Evaluation of EPR-1 expression**

Two experienced pathologists in a blinded fashion (without knowledge of the clinico-pathological features of the tumors) examined the expression of EPR-1 and Ki-67 independently in terms of intensity and positive rate of the immunostaining in each specimen tissue section. The positive staining for EPR-1 protein was expressed as red brown granules, which were mainly protein was expressed as red brown granules, which were mainly located in cell cytoplasm at microscopy. At least 5 high-power (>400 field) fields were chosen randomly and 1 000 cells were counted. The ratio of EPR-1-positive cells was calculated by dividing the number of positive cells over the total number of cells, and was expressed as percentage.

For quantitation of the EPR-1 expression in various samples examined, a semi-quantitative scoring system was used as previously described[1]. An average percentage of positive tumor cells was determined in at least five areas at×400 magnification and assigned to one of the five categories: (a) 0≤5%, (b) 1=5-25%, (c) 2=25-50%, (d) 3=50-75%, and (e) 4≥75%. The intensity of EPR-1 immunostaining was scored on a scale of 1-3 as following: (a) weak, 1+; (b) moderate, 2+; and (c) intense, 3+. The percentage of positive tumor cells and the staining intensity were multiplied to produce a weighted score for each case. The case with the weighted score less than 1 was defined as negative, otherwise as positive.

Ki-67-positive cells showed a distinct brown staining of the nuclei with strong intratumoral heterogeneity (Figure 1) The percentages of Ki-67-positive tumor cells were evaluated with a light microscope holding a ×100 oil immersion objective by scoring a minimum of 1 000 tumor cells in randomly selected fields.

**Statistical analysis**

Data were expressed as mean±SD. The two-tailed χ² test was used to examine the correlation between various clinical or pathologic parameters and the expression of EPR-1. Statistical significance between different groups was determined by independent Student’s t-test (two-tailed). The Pearson test was used to examine linear correlation between Weighted EPR-1 Score and proliferative or apoptotic indexes. A P value less than 0.05 was considered statistically significant. All the calculations were performed by SPSS 10.0 for windows.

**RESULTS**

**Correlation between EPR-1 protein expression and clinicopathological parameters**

No significant relation was found between the expression of EPR-1 and patient age, sex, tumor location, lymph node metastasis or depth of invasion (Table 1, P>0.05).
Table 1 No Correlation between EPR-1 protein expression and clinicopathological parameters

| Clinico-pathological parameters | Cases | EPR-1 positive cases (%) | P value |
|---------------------------------|-------|--------------------------|---------|
| Age (yr)                        |       |                          |         |
| <60                             | 37    | 21 (56.8)                |         |
| ≥60                             | 83    | 56 (67.5)                | 0.258   |
| Sex                             |       |                          |         |
| Male                            | 72    | 43 (59.7)                |         |
| Female                          | 48    | 34 (70.8)                | 0.214   |
| Tumor location                  |       |                          |         |
| Upper 1/3                       | 39    | 22 (56.4)                |         |
| Middle 1/3                      | 32    | 20 (62.5)                |         |
| Lower 1/3                       | 19    | 11 (57.9)                |         |
| More than 2 locations           | 30    | 24 (80.0)                | 0.199   |
| Differentation                  |       |                          |         |
| Well                            | 60    | 46 (76.7)                |         |
| Poorly                          | 60    | 31 (51.7)                | 0.004   |
| Subtypes                        |       |                          |         |
| Papillary                       | 30    | 24 (80.0)                |         |
| Tubular                         | 30    | 22 (73.3)                |         |
| Mucinous                        | 30    | 13 (43.3)                |         |
| Signet-ring cell                | 30    | 18 (60.0)                | 0.017   |
| Invasion mucosa                 |       |                          |         |
| No                              | 66    | 40 (60.6)                |         |
| Yes                             | 54    | 37 (68.5)                | 0.369   |
| Lymph node metastasis           |       |                          |         |
| No                              | 70    | 43 (61.4)                |         |
| Yes                             | 50    | 34 (68.0)                | 0.459   |

Figure 3 Strong positive cytoplasmic staining of EPR-1 in gastric adenocarcinomas (LSAB×400).

Figure 4 Positive staining of EPR-1 in signet-ring cell carcinoma invasion in smooth muscle and smooth muscle cells (LSAB×200).

Figure 5 Moderate positive nuclear staining in benign gastric adenocytes (LSAB×200).

Figure 6 Positive cytoplasmic staining in well-differentiated lymph node metastasis (LSAB×200).

Expression of EPR-1 between histological subtypes
There were a variety of positive rates of EPR-1 protein in different histological subtypes (Figures 3, 4, 5, 6 and Table 2). The positive rate of EPR-1 expression was significantly different in the invasive tumor (P=0.007), while it was differently expressed among the 4 subtypes and adjacent non-tumor tissues in the primary carcinoma and its lymph node metastasis (P<0.05). But there was no difference of EPR-1 expression in the adjacent normal gastric mucosa cells and smooth muscle cells among the 4 histological subtypes (P=0.849 and 0.720).

Expression of EPR-1 in the highly-differentiated adenocarcinomas
The positive rate of EPR-1 expression in the primary gastric adenocarcinoma was significantly higher than that in normal gastric mucosa (Table 3, P=0.006), while no significant difference was found in the invasion tumor nodes, lymph node metastasis and smooth muscle (P=0.20, 0.632, 0.088), respectively. However, in the primary highly-differentiated gastric adenocarcinomas, the positive expression rate of EPR-1 was significantly different from that of normal gastric mucosa, invasive tumor node, lymph node metastasis and smooth muscle while no significant difference was found in the poorly-differentiated adenocarcinomas.

Relation of EPR-1 expression with AI and PI in primary adenocarcinoma
The apoptotic index for EPR-1 positive in primary adenocarcinoma group was 1.25% and was, 1.00% for EPR-1 negative group, while the proliferative index for the two group was 7.00% and 8.53%, respectively. The difference of the two indexes between the EPR-1 positive and negative groups was statistically significant (Table 4, P<0.05). The relation of EPR-1 score with AI and PI is shown in Figure 7.
Significant difference of AI and PI with EPR-1 score in primary adenocarcinoma. A: The results of Pearson correlation coefficient, $r = 0.296$, $P = 0.001 < 0.05$; B: The results of Pearson correlation coefficient, $r = -0.204$, $P = 0.025 < 0.05$. Weighted EPR-1 score was positively correlated with apoptotic index, but negatively related with proliferative index in primary adenocarcinoma.

Significant difference of AI and PI with EPR-1 score in lymph node metastasis. A: The results of Pearson correlation coefficient, $r = 0.332$, $P = 0.019 < 0.05$; B: The results of Pearson correlation coefficient, $r = -0.336$, $P = 0.017 < 0.05$. Weighted EPR-1 score was positively correlated with apoptosis index, but negatively related with proliferative index in lymph node metastasis.

| Subtypes        | Primary gastric adenocarcinoma | Normal gastric mucosa cells | Invasion node adenocarcinoma | Lymph node metastasis | Smooth muscle cell | P value |
|-----------------|-------------------------------|-----------------------------|-----------------------------|-----------------------|--------------------|---------|
| Papillary       | 24/30 (80.0)                  | 14/30 (46.7)                | 14/17 (82.4)                | 11/13 (84.6)          | 17/30 (56.7)       |         |
| Tubular         | 22/30 (73.3)                  | 15/30 (50.0)                | 12/16 (75.0)                | 11/15 (73.3)          | 15/30 (50.0)       |         |
| Mucinous        | 13/30 (43.3)                  | 12/30 (40.0)                | 10/24 (41.7)                | 3/10 (30.0)           | 14/30 (46.7)       |         |
| Signet-ring cell| 18/30 (60.0)                  | 0.017                       | 15/30 (50.0)                | 0.849                 | 0.007              | 18/30 (60.0) | 0.720 |

| Differentiation | Primary gastric adenocarcinoma | Normal gastric mucosa cells | Invasion node adenocarcinoma | Lymph node metastasis | Smooth muscle cell | P value |
|-----------------|-------------------------------|-----------------------------|-----------------------------|-----------------------|--------------------|---------|
| Well            | 46/60 (76.7)                  | 29/60 (48.3)                | 26/33 (78.8)                | 22/28 (78.6)          | 32/60 (53.3)       | 0.002   |
| Poorly          | 31/60 (51.7)                  | 27/60 (45.0)                | 21/52 (40.4)                | 12/22 (54.5)          | 32/60 (53.3)       | 0.594   |

| Differentiation | Primary gastric adenocarcinoma | Normal gastric mucosa cells | Invasion node adenocarcinoma | Lymph node metastasis | Smooth muscle cell | P value |
|-----------------|-------------------------------|-----------------------------|-----------------------------|-----------------------|--------------------|---------|
| Well            | 46/60 (76.7)                  | 29/60 (48.3)                | 26/33 (78.8)                | 22/28 (78.6)          | 32/60 (53.3)       | 0.002   |
| Poorly          | 31/60 (51.7)                  | 27/60 (45.0)                | 21/52 (40.4)                | 12/22 (54.5)          | 32/60 (53.3)       | 0.594   |

Relation of EPR-1 expression with AI or PI in cancer with lymph node metastasis

The apoptotic index for EPR-1 positive lymph node metastasis group was 0.99% and was 0.67% for EPR-1 negative lymph node metastasis group, while the proliferative index for the two groups was 7.65% and 9.44%, respectively. The difference of the two indexes between the EPR-1 positive and negative groups was statistically significant ($P < 0.05$). The EPR-1 score was shown with the AI and PI in (Table 5, Figure 8).
DISCUSSION
Effector cell Protease Receptor-1 (EPR-1) has been recognized as a receptor of the transmembrane glycoprotein coagulation factor Xa and its transcripts are present in terminal differentiated adult tissues.[1,2] Survivin gene was identified by Ambrosini et al.[3] during hybridization screening of a human EPR-1 genomic library with the cDNA of EPR-1.[4,5] The gene spanned nearly 15 kb and was co-located at 17q25 with EPR-1.[6] Survivin has been found to be a new member of the inhibitors of apoptosis proteins (IAP) family,[7,8] and is selectively overexpressed in common human cancers but not in normal adult tissues.

Transfecting with EPR-1 mRNA into cells could inhibit endogenous expression of survivin gene and induce apoptosis as well as inhibit cell proliferation.[9] Shinozawa et al.[10] used RT-PCR method to detect the expression of Survivin and EPR-1 in leukemia and lymphoma, and found that the abnormality of the expression ratio of EPR-1 and Survivin might exert anti-colon tumor proliferation functions. Yamamoto et al.[11] reported that EPR-1 in a human colon cancer cell line downregulated survivin expression, with a similar decrease in cell proliferation and an increase in apoptosis. This antitumour efficacy was further enhanced in combination with anticancer agents 5-Fu and CDDP. These findings suggest that regulation of survivin by induction of EPR-1 may have a significant potential as a therapy for human cancer. But to our knowledge, EPR-1 expression in gastric adenocarcinoma has not been reported.

In order to gain insight into the role of EPR-1 in gastric adenocarcinoma, we investigated the expression of EPR-1 in a group of gastric adenocarcinomas, and examined the relationship of its expression with cell proliferation and apoptosis index. By immunohistochemical analysis performed in 120 cases, our study shows that positive staining for EPR-1 protein was expressed as red brown granules, which were mainly located in cell cytoplasm. There was no significant relationship between the expression of EPR-1 and patient age, sex, tumor location, lymph node metastasis or depth of invasion. However, a significantly differences expressions of EPR-1 was found between histological subtypes.

Our study also explored the potential relationship between the weighted EPR-1 expression score and apoptotic/proliferative indexes, Ki-67 has been regarded as a parameter of tumor proliferation[20-29] and the TUNEL method has become a popular way to detect apoptotic cells.[30-32] Using these two reliable methods, we found that the positive rate of EPR-1 expression in the primary gastric adenocarcinoma was significantly higher than that in normal gastric mucosa and in terms with degree of differentiation, the positive expression rate of EPR-1 was significantly different among the primary gastric adenocarcinoma, normal gastric mucosa, tumor invasive node, lymph node metastasis and smooth muscle in the highly-differentiated adenocarcinomas while no significant difference was found in the poorly-differentiated adenocarcinomas.

In conclusion, EPR-1 can influence the biological behaviors of tumor cells not only by inducing their apoptosis, but by inhibiting their proliferation. Whether EPR-1 can be used as a new anti-cancer therapeutics strategy is still waiting for more evidence.

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