Expression and Clinical Significance of REGγ in Gastric Cancer Tissue and Variously Differentiated Gastric Cancer Cell Lines

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OBJECTIVE  To evaluate the REGγ expression in gastric cancer tissue and gastric cancer cell lines of various differentiation levels and its clinical significance.

METHODS  Immunohistochemistry was used to detect the expression of REGγ protein in 70 specimens of gastric cancer and 30 specimens of normal gastric mucosa. The relationship between the expression of REGγ protein and the biological behaviors of gastric cancer was analyzed. RT-PCR and Western blot were used to detect the mRNA level and the protein expression of REGγ in normal gastric cell line GES-1, well differentiated gastric cancer cell line MKN-28, moderately differentiated gastric cancer cell line SGC-7901 and poorly differentiated gastric cancer cell line BGC-823.

RESULTS  The expression rate of REGγ protein in gastric cancer tissue (52/70, 74.29%) was significantly higher than that in normal gastric tissue (12/30, 40%) (P < 0.01). The expression rate of REGγ was correlated with tumor size (P < 0.01), lymph node metastasis (P < 0.05), differentiation degree (P < 0.01), infiltration depth (P < 0.01) and distant metastasis (P < 0.05). RT-PCR analysis showed that the expression of REGγ mRNA was 0.459 ± 0.079 in the normal gastric mucosa cell line, 0.588 ± 0.118 in the well differentiated gastric cancer cell line, 0.715 ± 0.066 in the moderately differentiated gastric cancer cell line, and 0.873 ± 0.099 in the poorly differentiated gastric cancer cell line, showing a negative correlation between REGγ mRNA expression and differentiation level (P < 0.05). Western blot analysis showed that the expression of REGγ protein was 0.712 ± 0.065 in the normal gastric mucosa cell line, 1.176 ± 0.185 in the well differentiated gastric cancer cell line, 1.533 ± 0.127 in the moderately differentiated gastric cancer cell line, and 2.061 ± 0.398 in the poorly differentiated gastric cancer cell line, showing a negative correlation between REGγ protein expression and differentiation level (P < 0.05).

CONCLUSION  REGγ is expressed in gastric cancer tissue and normal gastric tissue. In gastric cancer tissues, REGγ expression is positively correlated with the tumor size, lymph node metastasis, differentiation degree, infiltration depth and distant metastasis. Detecting the expression of REGγ mRNA and protein is helpful for early diagnosis and predicting prognosis of gastric cancer.

KEY WORDS: gastric tumor, REGγ, immunohistochemistry, gene, protein.

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Introduction

Proteasome participates in important life processes, such as cell cycle,
cell regulation, apoptosis, metabolic regulation, signal transduction, genetic transcription, as well as receptor endocytosis, antigen presentation and immune response etc. by mediating degradation of various target proteins in the cells. REG, also known as the 11S or PA28, is a proteasome reactivator, which specifically binds and activates the 20S proteasomes, and promotes the degradation of substrate peptides such as p21, SRC-3/AIB1[1-2] etc. via the mode of ATP and non-ubiquitin dependence. Up to now, 3 of the REG family members, i.e., REGα, REGβ and REGγ, have been found. REGγ, also noted as the PA28γ, PSME3 and Ki antigen, exists in worms, insects and higher animals[3]. It is an isogenetic hetero-tamer formed by seven 28KD subunits[3], and has a role in improving hydrolysis of the proteasomes. The role of REG in the occurrence and progression of gastric cancer remains unclear. In our experiment, the significance of REGγ in occurrence and progression of gastric cancer and its value as a way of auxiliary diagnosis were investigated based on the expression levels of REGγ mRNA and protein.

Materials and Methods

Clinical data

Data were collected from 70 patients with gastric cancer, who were admitted to the First Affiliated Hospital of Chongqing Medical University during a period from December 2006 to December 2007. Among the patients, 43 were male and 27 female, with the mean age of 60.01 ± 11.45, ranging from 27 to 83 years old. The samples taken from the surgery were fixed in 10% of formalin over 5.0 cm away from the tumor site or from the samples of excised gastric tissue of gastric ulcer patients. Of the samples in surgery of gastric cancer which is collected as the controls, either from the cutting edge of excised samples in surgery or outside of the serous membrane (T3 + T4); iii) degree of differentiation: good differentiation (well or moderately differentiated) or poor differentiation (poorly or undifferentiated); iv) distant metastasis: existence or not existence; v) distant metastasis: existence or not existence.

RT-PCR detection of REGγ mRNA expression

Plasmid Purification Mini Kit, bought from the Shanghai Huashun Bioengineering Co. Ltd., was used. The RNA of cultured cells was extracted based on the specification of the kit, and finally DEPC (diethyl pyrocarbonate) treated fluid was used to solve the RNA. The specific A260/A280 of totally extracted RNA ranged from 1.8-2.0. PrimeScript™ RT-PCR kit (TaKaRa Co.) was used to synthesize the cDNA sample which was stored at 4°C. The upstream primer: 5'-GCT GCG AAG GTC AAA CCC ATA-3' and downstream primer: 5'-GCA GGC TAA TTG CAG AGA CCA AC-3' with 222 bp in length of the amplified product were applied for the amplification of REGγ. The β-actin upstream primer: 5'-GCT GTC CCT GTA TGC CCT CT-3', downstream primer: 5'-TTG ATG TCA CGC ACG ATT T-T3' with 222 bp in length of the amplified product were also used in the procedure. Substances used in the reaction system included reaction solution 50 μl, cDNA 5 μl, primer with concentration of 0.1 nmol/L, and Taq enzyme 2.5 U. Reaction condition required initial denaturation of DNA from the Cell Lines Bank, Chongqing Medical University Preclinical Medicine Institute, and were conventionally cultured in an incubator with 5%CO2, at 37°C.

Immunohistochemical staining

Immunohistochemical staining was conducted based on the instruction of the kit. Rat-antihuman REGγ IgG polyclonal antibody (Invitrogen) was diluted at a ratio of 1: 80, and incubated overnight at 4°C followed by DAB coloration. The results of the processes showed that the criterion for the scoring of dyeing shade and relative strength were as follows. Different colors indicated different scores, i.e. brown color scoring 3 points, brownish-yellow color scoring 2 points, stramineous scoring 1 point and colorless scoring 0 point. The results of cell counting under a microscope with same object lens were shown as follows. With the scores of 4 points, 3 points, 2 points, 1 point and 0 point, the rates of pigmented cell reached over 75%, 51%-75%, 11%-50%, 10% or less, and negative counting in a field of vision, respectively. The product of multiplication with 2 scores indicated that the range of the score between 0 and 3 meant a “+” negative expression, while 3 scores meant a “+++” expression. Scores of 4 meant a “+++” and scores of 5 or more signed “++++” expression. The expressions ranging from “+” to “+++” were positive expressions. The parameters of the clinicopathologic signature analysis of gastric cancer were as follows: i) tumor diameter: ≤ 4 cm and > 4 cm; ii) degree of differentiation: good differentiation (well or moderately differentiated) or poor differentiation (poorly or undifferentiated); iii) depth of infiltration: inside the serous membrane (Tis + T1 + T2) or outside of the serous membrane (T3 + T4); iv) lymphatic metastasis: existence or not existence; v) distant metastasis: existence or not existence.
at 94°C for 1 min, denaturation at 94°C for 30 s, annealing at 57°C for 30 s, primer extension at 72°C for 1 min, with 30 cycles in total, and final extension at 72°C for 5 min. The electrophoresis reaction of the products gained from the reactions was conducted in 1% agarose gel. The target fragment amplification band and β-actin amplification band were analyzed using ultraviolet gelatium image analysis system, and the relative amount of REGγ mRNA was signified by specific value of gray-scale value between the 2 bands.

**Western Blot determination of the expression of REGγ protein**

The above-mentioned cell protein was extracted based on the instruction of the cell lysis buffer, which was bought from the Beyotime BioTech Inst., Haimen Jiangsu, China, and Bradford method was used for determining the protein content of the cells. A 12% of SDS-PAGE was used for electrophoretic separation of equivalent sample lysates, and transmembrane was conducted on PVDF membrane, with electronic transmembrane for 1 h (250 mA), and blockade of the filter membrane for 1 h. First antibody was added in the filter membrane (rat-antihuman REGγ polyclonal antibody was used as the first antibody, with a working concentration of 1:50), and was incubated overnight at 4°C followed by DAB developing. Analysis of the result was conducted using gelatium imaging system.

**Statistical analysis**

All data were analyzed by SPSS13.0 statistical software. Variance analysis followed by a Chi-square test was performed to determine statistical differences in the expressions of REGγ protein with different stages of gastric cancer and relationship between the different expressions and clinicopathologic factors. Data were presented as mean ± standard deviation. A value of $P < 0.05$ was considered statistically significant.

**Results**

**Expressions of REGγ protein in normal gastric mucosa and gastric cancer**

It could be seen under a light microscope that REGγ protein mainly expressed at the cell nucleus, presenting a brownish-yellow color. The expression of REGγ protein could be observed both in normal gastric mucosa and gastric cancer. The expressions of REGγ protein in can-

### Table 1. Immunohistochemical results of REGγ expression in different gastric mucous membranes.

<table>
<thead>
<tr>
<th>Histological types</th>
<th>n</th>
<th>REGγ expression</th>
<th>Positive rate (%)</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>++</td>
<td>Positive rate (%)</td>
<td></td>
</tr>
<tr>
<td>Gastric cancer</td>
<td>70</td>
<td>18</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>Normal gastric mucosa</td>
<td>30</td>
<td>18</td>
<td>11</td>
<td>0</td>
</tr>
</tbody>
</table>

Comparison between gastric cancer and paraneoplastic normal gastric mucosa, $P < 0.01$.

### Table 2. Relationship between the expression of REGγ and clinicopathologic parameters of gastric cancers.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n</th>
<th>REGγ expression</th>
<th>Positive rate (%)</th>
<th>$\chi^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T \leq 4$</td>
<td>28</td>
<td>12</td>
<td>16</td>
<td>57.1</td>
<td></td>
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<tr>
<td>$T &gt; 4$</td>
<td>42</td>
<td>6</td>
<td>36</td>
<td>85.7</td>
<td></td>
</tr>
<tr>
<td>Lymph metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>32</td>
<td>4</td>
<td>28</td>
<td>87.5</td>
<td>5.389</td>
</tr>
<tr>
<td>No</td>
<td>38</td>
<td>14</td>
<td>24</td>
<td>63.2</td>
<td></td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Better</td>
<td>27</td>
<td>12</td>
<td>15</td>
<td>55.6</td>
<td>8.072</td>
</tr>
<tr>
<td>Poor</td>
<td>43</td>
<td>6</td>
<td>37</td>
<td>86.0</td>
<td></td>
</tr>
<tr>
<td>Infiltration Depth</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inside serosa</td>
<td>31</td>
<td>14</td>
<td>17</td>
<td>54.8</td>
<td>11.016</td>
</tr>
<tr>
<td>Outside serosa</td>
<td>39</td>
<td>4</td>
<td>35</td>
<td>89.7</td>
<td></td>
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<tr>
<td>Distal metastasis</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Yes</td>
<td>28</td>
<td>3</td>
<td>25</td>
<td>89.3</td>
<td>5.497</td>
</tr>
<tr>
<td>No</td>
<td>42</td>
<td>15</td>
<td>27</td>
<td>64.3</td>
<td></td>
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</table>
The expression of REGγ protein was higher in gastric cancer than that in normal gastric mucosa ($\chi^2 = 10.71$, $P < 0.01$), and there was statistically significant difference in the expressions between the normal gastric mucosa and gastric cancer (Table 1).

**Expression of REGγ protein in gastric cancer and relationship between the expressions of REGγ protein and clinicopathologic parameters**

There was a correlation between the expressions of REGγ protein and the clinicopathologic factors of gastric cancer, such as tumor size, lymphatic metastasis, differentiation and infiltration, as well as distant metastasis (Table 2).

**Expression of REGγ mRNA**

Experimental results of RT-PCR were as follows. The total RNAs of 4 cell lines were extracted 3 straps from each cell line, i.e. RNA28S, 18S and 5S and all extracted RNA could be seen in electrophoresis (Fig.2). RT-PCR was conducted, with the above mentioned total RNA as template and the primers of REGγ and β-actin were used. The result of RT-PCR showed that the RT-PCR products with normal gastric mucosa cell lines GES-1, well-differentiated gastric cancer cell lines MKN28, moderately differentiated cell lines SGC-7901 and poorly differentiated cell lines BGC-823 presented specific strap at 780 bp or so, which was in conformity with the size of REGγ fragment. It meant that the expression of REGγ occurred in all the 4 cell lines. The results of semi-quantitative analysis of REGγ mRNA detected by RT-PCR were $0.459 \pm 0.079$, $0.588 \pm 0.118$, $0.715 \pm 0.066$, and $0.873 \pm 0.099$, among which the gene expression of REGγ was relatively higher in the BGC-823 cell lines, lower in the SGC-7901 and MKN28 cell lines, and the lowest in the GES-1 cell lines. There was statistically significant difference between the REGγ expressions ($P < 0.05$). The result of electrophoretic separation
of RT-PCR product REGγ is shown in Fig.3.

**Discussion**

Proteasome is a macromolecular compound which has various proteolytic functions. REG/11S is a non-ATP dependent proteasome activator. REGγ is one of the REG family members. It was first found in the study conducted by Li et al.[1] that REGγ/20S proteasome compound could have selectively effects on the steroid coactivating factor-3 (SRC-3), resulting in a degradation compound could have selectively effects on the steroid coactivating factor-3 (SRC-3), resulting in a degradation

The expression of REGγ protein occurred in normal gastric mucosa cell lines and the gastric cancer cell lines at various differentiation levels. The results of semi-quantitative analysis for GES-1, SGC-7901, MKN-28, BGC-823 cell lines detected by western blot were 0.712 ± 0.065, 1.176 ± 0.185, 1.533 ± 0.127 and 2.061 ± 0.398, respectively, and the results among these groups had statistically significant difference, with the value of P < 0.05. This showed that there was an increased expression of the REGγ protein in normal gastric mucosa cell lines and the well-, moderately and poorly differentiated gastric cancer cell lines. However, the expression of REGγ was higher in the cancer cell lines than in the normal cell lines.

**Relationship between expression of REGγ protein and differentiation level of gastric cancer cell lines (Fig.4)**

Western blot was used to determine the extracted proteins of cell lines, and the results of the determination were as follows. The expression of REGγ protein occurred in normal gastric mucosa cell lines and the gastric cancer cell lines at various differentiation levels. The results of semi-quantitative analysis for GES-1, SGC-7901, MKN-28, BGC-823 cell lines detected by western blot were 0.712 ± 0.065, 1.176 ± 0.185, 1.533 ± 0.127 and 2.061 ± 0.398, respectively, and the results among these groups had statistically significant difference, with the value of P < 0.05. This showed that there was an increased expression of the REGγ protein in normal gastric mucosa cell lines and the well-, moderately and poorly differentiated gastric cancer cell lines. However, the expression of REGγ was higher in the cancer cell lines than in the normal cell lines.

Recent studies showed that REGγ improves the ubiquitination of the MDM2-mediated p53, and facilitated degradation of the tumor suppressor protein p53[5], thus preventing the cell cycle course and causing apoptosis[6]. REGγ could also be combined in the body with the cell cycle regulatory factors such as p21, p16 and p19 etc., and accelerate their degradation[5]. In addition, REGγ plays important roles in keeping the stability of cyto-centrum and chromosome in karyomitosis, and formation of the nuclear stigma (NS) that was also called interchromatin granule clusters (IGCs)[8]. Because of the role of REGγ in cell cycle and apoptosis, cell cycle and apoptosis play an important role in progress and development of tumors. Therefore, further studies on the function of REGγ in gastric cancer may need to confirm the regulation role of REGγ protein in the cell cycle and apoptosis, helping researchers to find new therapeutic target of gastric cancer.

Our findings indicate that the positive rate of REGγ protein expression is significantly higher in gastric cancer than that in normal gastric mucosa, P < 0.05. This suggests that the high expression of REGγ protein may be related to the occurrence and progress of gastric cancer. Some researches showed that REGγ gene could facilitate proliferative activity of breast cancer cells, thus accelerating the cell growth[9]. Okamura et al.[10] reported that the expression of REGγ protein was significantly higher in the thyroid cancer than that in normal thyroid tissue. Roessler et al.[11] also reported that the (expression) level of REGγ protein was higher in the serum of colorectal cancer patients than that in the serum of the healthy subjects and patients with benign intestinal diseases. Further analysis has shown that REGγ protein can be expressed in gastric cancer at various differentiation levels. The expression of REGγ protein is higher in the cancerous tissues with lymph node and distant metastases than those in cancerous tissues without, suggesting the high expression of REGγ protein may have a definite correlation with the metastases of gastric cancers. We have also found that the expression of REGγ protein is obviously higher in the cancers with deep infiltration and a poor differentiation level than those of the cancers with a superficial infiltration and well differentiation level, P < 0.05, demonstrating there is a definite relationship between the expression level of REGγ protein and infestation of tumors.

After culture and incubation of the normal gastric mucosa cell lines and the well-, moderately and poorly differentiated gastric cancer cell lines, the results of the determinations using RT-PCR and Western blot indicated that REGγ mRNA and REGγ protein could be expressed in normal gastric mucosa cell lines, and the well-, moderately and poorly differentiated gastric cancer cell lines, suggesting that REGγ is necessary in cell growth. With a decreased level of differentiation of the cell lines, the expression level of REGγ mRNA and REGγ protein was increased, indicating that there is a correlation between REGγ expression level and differentiation degree in the gastric cancers. And it may be regarded as a tumor marker which may predict the prognosis of gastric cancers. So, we deem that REGγ is one of the risk factors in occurrence and progression of gastric cancers, and it can reflect the malignancy of gastric cancer and prognosis to a certain extent. It can be regarded as one of the indices in judging the prognosis of the gastric cancer patients. Current findings revealed that proteasome inhibitor would be used as a method in treating malignant tumors[12]. Inhibiting the REGγ activity may depress the activity of proteasomes to certain extent, as a result, the possibility of proliferation, metastasis and infiltration of gastric cancers may be lowered. This will arouse the researchers to explore a new way to treat gastric cancers.

**References**


