

Expression and Clinical Significance of REG γ in Gastric Cancer Tissue and Various Differentiated Gastric Cancer Cell Lines

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OBJECTIVE To evaluate the REG γ 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 REG γ protein in 70 specimens of gastric cancer and 30 specimens of normal gastric mucosa. The relationship between the expression of REG γ 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 REG γ 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 REG γ 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 REG γ 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 REG γ 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 REG γ mRNA expression and differentiation level ($P < 0.05$). Western blot analysis showed that the expression of REG γ 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 REG γ protein expression and differentiation level ($P < 0.05$).

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

KEY WORDS: gastric tumor, REG γ , 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., REG α , REG β and REG γ , have been found. REG γ , also noted as the PA28 γ , PSME3 and Ki antigen, exists in worms, insects and higher animals^[3]. It is an isogenetic heptamer formed by seven 28KD subunits^[4], 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 REG γ in occurrence and progression of gastric cancer and its value as a way of auxiliary diagnosis were investigated based on the expression levels of REG γ 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 followed by the procedures of paraffin imbedding, serial section, routine H&E staining and pathological analysis. In accordance with the classification of tumors issued by WHO, the cases were diagnosed as follows: well-differentiated adenocarcinomas in 11 cases (15.7%), moderately differentiated cancer in 16 cases (22.9%), poorly differentiated cancer in 28 cases (40.0%) and undifferentiated cancer in 15 cases (21.4%). Information of the extent of canceration, lymphatic metastasis, depth of infiltration and extra-gastric metastasis, were noted based on intraoperative record and postoperative pathological examination. In addition, 30 normal gastric tissues were collected as the controls, either from the cutting edge of excised samples in surgery of gastric cancer which is over 5.0 cm away from the tumor site or from the samples of excised gastric tissue of gastric ulcer patients. Of the 30 gastric tissues, 12 were taken from male subjects and 18 from female. The age of these subjects ranged from 23 to 40 years old.

Cell lines

The normal gastric mucosa cell lines GES-1, the well-differentiated gastric cancer cell lines MKN-28, the moderately differentiated cell lines SGC-7901, the poorly differentiated cell lines BGC-823 were all obtained

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 REG γ 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 signified “+++” 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.

RT-PCR detection of REG γ 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 A_{260}/A_{280} 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 780 bp in length of the amplified product were applied for the amplification of REG γ . The β -actin upstream primer: 5'-GCT GTC CCT GTA TGC CCT CT-3', downstream primer: 5'-TTG ATG TCA CGC ACG ATT T-3' 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 μ l, cDNA 5 μ l, primer with concentration of 0.1 nmol/L, and Taq enzyme 2.5 U. Reaction condition required initial denaturation of DNA

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 1.5% of agarose gel. The target fragment amplification band and β -actin amplification band were analyzed using ultraviolet gelatum image analysis system, and the relative amount of REG γ mRNA was signified by specific value of gray-scale value between the 2 bands.

Western Blot determination of the expression of REG γ 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 REG γ 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 gelatum 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 REG γ protein with different stages of gastric cancer and relationship between the different expressions and clinicopathologic factors. Data were presented as mean \pm standard deviation. A value of $P < 0.05$ was considered statistically significant.

Results

Expressions of REG γ protein in normal gastric mucosa and gastric cancer

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

Table 1. Immunohistochemical results of REG γ expression in different gastric mucous membranes.

Histological types	n	REG γ expression				Positive rate (%)	χ^2
		-	+	++	+++		
Gastric cancer	70	18	15	22	15	74.3	10.71
Normal gastric mucosa	30	18	11	1	0	40.0	

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

Table 2. Relationship between the expression of REG γ and clinicopathologic parameters of gastric cancers.

Characteristics	n	REG γ expression		Positive rate (%)	χ^2	P
		Negative	Positive			
Tumor size (cm)					7.197	0.007
T \leq 4	28	12	16	57.1		
T > 4	42	6	36	85.7		
Lymph metastasis					5.389	0.02
Yes	32	4	28	87.5		
No	38	14	24	63.2		
Differentiation					8.072	0.004
Better	27	12	15	55.6		
Poor	43	6	37	86.0		
Infiltration Depth					11.016	0.001
Inside serosa	31	14	17	54.8		
Outside serosa	39	4	35	89.7		
Distal metastasis					5.497	0.019
Yes	28	3	25	89.3		
No	42	15	27	64.3		

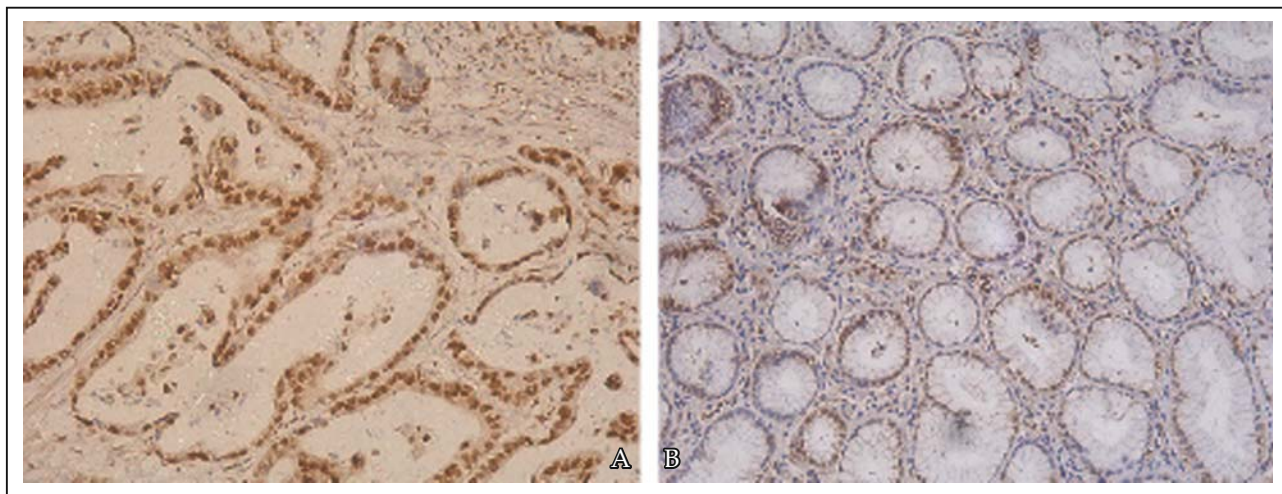


Fig.1. Expressions of REG γ in gastric cancer (A) and in normal gastric tissue (B) ($\times 200$).

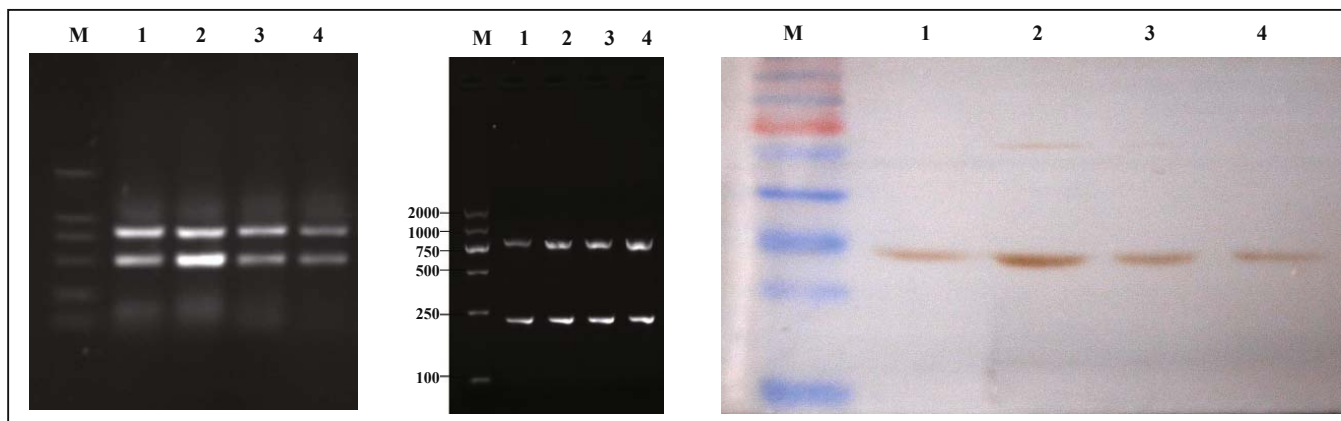


Fig.2. Denaturing gel electrophoresis of RNA. M, Marker; 1, BGC823; 2, SGC-7901; 3, MKN-28; 4, GES-1.

Fig.3. Expression of REG γ mRNA in four cell lines. M, Marker; 1, GES-1; 2, MKN-28; 3, SGC-7901; 4, BGC823.

Fig.4. Expression of REG γ protein in four cell lines. M, Marker; 1, GES-1; 2, BGC823; 3, SGC-7901; 4, MKN-28.

cer are shown in Fig.1. The expression of REG γ 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 REG γ protein in gastric cancer and relationship between the expressions of REG γ protein and clinicopathologic parameters

There was a correlation between the expressions of REG γ 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 REG γ 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 REG γ 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 REG γ fragment. It meant that the expression of REG γ occurred in all the 4 cell lines. The results of semi-quantitative analysis of REG γ mRNA detected by RT-PCR were 0.459 ± 0.079 , 0.588 ± 0.118 , 0.715 ± 0.066 , and 0.873 ± 0.099 , among which the gene expression of REG γ 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 REG γ expressions ($P < 0.05$). The result of electrophoretic separation

of RT-PCR product REG γ is shown in Fig.3.

Relationship between expression of REG γ 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 REG γ 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 REG γ protein in normal gastric mucosa cell lines and the well-, moderately and poorly differentiated gastric cancer cell lines. However, the expression of REG γ was higher in the cancer cell lines than in the normal cell lines.

Discussion

Proteasome is a macromolecular compound which has various proteolytic functions. REG/11S is a non-ATP dependent proteasome activator. REG γ is one of the REG family members. It was first found in the study conducted by Li et al.^[1] that REG γ /20S proteasome compound could have selectively effects on the steroid coactivating factor-3 (SRC-3), resulting in a degradation of SRC-3. This suggests that REG γ does play an important role in stabilization of protein in the body. Other studies showed that REG γ improved 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]. REG γ could also be combined in the body with the cell cycle regulatory factors such as p21, p16 and p19 etc., and accelerate their degradation^[2]. In addition, REG γ 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)^[7,8]. Because of the role of REG γ 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 REG γ in gastric cancer may need to confirm the regulation role of REG γ 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 REG γ protein expression is significantly higher in gastric cancer than that in normal gastric mucosa, $P < 0.05$. This suggests that the high expression of REG γ protein may be related to the occurrence and progress of gastric cancer. Some researches showed that REG γ gene could fa-

cilitate proliferative activity of breast cancer cells, thus accelerating the cell growth^[9]. Okamura et al.^[10] reported that the expression of REG γ 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 REG γ 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 REG γ protein can be expressed in gastric cancer at various differentiation levels. The expression of REG γ protein is higher in the cancerous tissues with lymph node and distant metastases than those in cancerous tissues without, suggesting the high expression of REG γ protein may have a definite correlation with the metastases of gastric cancers. We have also found that the expression of REG γ 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 REG γ 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 REG γ mRNA and REG γ protein could be expressed in normal gastric mucosa cell lines, and the well-, moderately and poorly differentiated gastric cancer cell lines, suggesting that REG γ is necessary in cell growth. With a decreased level of differentiation of the cell lines, the expression level of REG γ mRNA and REG γ protein was increased, indicating that there is a correlation between REG γ 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 REG γ 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 REG γ 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.

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