Original Article

Expression And Clinical Significance of Glucose Regulated Proteins GRP78 And GRP94 in Human Colon Cancer

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ABSTRACT

Objective: To investigate the expression of glucose regulated proteins GRP78 and GRP94 in human colon cancer.

Methods: Tissues of resected primary colon cancer, colon adenoma and normal tissue were investigated. Protein expression was detected with immunohistochemical staining. mRNA expression levels of GRP78 and GRP94 were determined by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) after mRNA extraction.

Results: The expression of GRP94 and GRP78 was significantly higher in colon cancer when compared to those in colon adenoma and normal tissue (P<0.01). GRP94 mRNA and protein expression was found to be in close relationship with the grade of differentiation, Dukes stages, lymph node involvement and remote metastasis in colon cancer (P<0.01), but no relationship with gender and age (P>0.05). GRP78 mRNA and protein expression increased with cancer progression along the normal tissue-adenoma-cancer sequence, but showed no association with grade of differentiation, Dukes stages, lymph node involvement, remote metastasis, gender and age (P>0.05). The mRNA expression of GRP78 and GRP94 was consistent with the proteins (P<0.01), but there is no correlation between overexpression of GRP78 and GRP94 (P>0.05), and the patients with both strong GRP78 and GRP94 protein expression did not show advanced tumor stages (P>0.05).

Conclusion: Overexpression of GRP78 and GRP94 was found in colon cancer. Overexpression of GRP94 was closely related to cellular differentiation, Dukes stages, invasion and metastasis.

Key words: Colon cancer; GRP78; GRP94; Colon adenoma; Dukes stages; Metastasis

INTRODUCTION

As an endoplasmic reticulum (ER) chaperone, GRP78 and GRP94 are key components of the unfolded protein response, promoting cell survival under ER stress, which were discovered in the late 1970s together as cellular proteins induced by glucose starvation. GRP78 and GRP94 are members of the HSP70 and HSP90 gene family respectively[1]. GRP78 is also referred to as immunoglobulin heavy chain binding prote in (BiP) and GRP94 is also referred to as GP96. Both GRP78 and GRP94 play an essential role in ER linked nuclear signaling, protein folding, sorting and secretion, protection against Ca^{2+} depletion stress, and antigen presentation. Pathological conditions, such as acidosis, hypoxia or hypothermia could induce the up-regulation of the two proteins[2].

Although GRP78 and GRP94 expression are maintained at low basal level in major adult organs such as brain, lung, and heart, the expression could be significantly induced in tumors[1]. Recently,

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many studies have demonstrated that higher GRP78 and GRP94 levels correlate with higher pathological grade and aggressive behaviour in liver[3], gastric[4], lung[5], breast[6, 7], prostate[8], esophageal and[9] carcinomas. Colon cancer is one of the most malignant cancers and there might be a close relationship between the occurrence of colon cancer and the overexpression of GRP78 and GRP94. However, limited information is available currently on the expression of GRP78 and GRP94 in colon cancer tissues. In this study, we detected the expression of GRP94 and GRP78 on mRNA and protein levels in colon cancer with or without metastasis.

MATERIALS AND METHODS

Specimens

Specimens of 95 patients with primary resected colon cancer, colon adenoma and normal colon tissues from Shengjing Hospital Affiliated to China Medical University, were investigated. The patients consisted of 47 males and 48 females, ranging from 38 to 89 years old. All cases were diagnosed by routine pathological examination, including 10 normal cases, 21 colon adenomas and 64 colon cancer. Well-differentiated type (grade I) was found in 22 cases, moderately-differentiated type (grade II) in 28 cases, and poorly-differentiated type (grade III) in 14 cases. According to the revised Dukes stages, stage A was determined in 7 cases, stage B in 27 cases, stage C in 20 cases, and stage D in 10 cases. In these cases, 56 had regional lymph node metastasis, and 22 had remote metastasis.

Immunohistochemistry

An S-P Kit (Zhongshan Golden Bridge Biotech Co., Ltd) was used for immunohistochemical analysis. 4 mm thick tissue sections were cut. All sections were deparaffinnized and dehydrated with graded alcohol. Endogenous peroxidase was then blocked with 3% hydrogen peroxide for 10 min at room temperature, then heated for 30 min at 95°C to repair antigens and finally rinsed in PBS. Nonspecific protein staining was blocked by goat serum. Subsequently, sections were treated with the primary antibodies against GRP78 and GRP94 (Santa Cruz, CA, USA) diluted to 1:100 with PBS, overnight at 4°C in a humidified chamber. After a complete wash in PBS, the slides were treated with goat anti-mouse antibody (1:100) for 45 min at 37°C. After a complete wash in PBS, the slides were developed in 0.05% freshly prepared diaminobenzedine solution (DAB, Sigma Co.) for 8 min, and then counterstained with hematoxylin, dehydrated, airdried, and mounted. Ten visual fields were randomly selected from each section, and at least 100 cells were counted in each field. The immunoreactivity of GRP94 or GRP78 was classified into four categories: −, no expression, around 0%−5% of cells stained; +, weak expression, around 6%−25% of cells stained; ++, moderate expression, around 26%−75% of cells stained; and +++, strong expression, more than 75% of cells stained.

RT-PCR

Total RNA was isolated from the Specimens using Trizol (Invitrogen, CA, USA), and 1 mg of total RNA was converted to cDNA using a First Strand cDNA Synthesis Kit (Takara, Japan). Then, PCR reactions were performed in volume of 20 ml. 1 ml aliquot of the cDNA was used for PCR amplification with the following primers. GRP94 primers were 5'-CAGTTTTGGATCTGCTGTGG-3' and 5'-CAGCTGTAGATTCCTTTGC-3' which produced to a 270 bp fragment. PCR conditions were 95°C for 1 min, 55°C for 30 s and 72°C for 30 s, for 30 cycles. Under the same PCR conditions, β-actin were amplified as an internal control for RT-PCR analysis from the same cDNA samples, and the primers were 5'-AAGGATTCCTATGT-GTGGG-3' and 5'-CATVTVTTGCTCGAAGTC-3' with a product of 535 bp. GRP78 primers were 5'-GATAATCAACCAACTGTTAC-3' and 5'-GTAT CCTCTTCACCAGTTGG-3' that amplified to a 577 bp fragment. PCR conditions were 95°C for 1 min, 55°C for 45 s and 72°C for 45 s, for 30 cycles. Under the same PCR conditions, β-actin was amplified with primers 5'-TCGTCACCAACT-GGGACGACATGG-3' and 5'-CATCTTGATCTT-CATTGTGCT-3' that amplified a 750-bp fragment. Semi-quantitative data about the PCR products were obtained by comparing the intensity of PCR band of GRP94 and GRP78 with that of internal control of β-actin using gene tools software.

Statistical Analysis

Data are presented as $\bar{x}\pm s$. The comparison between different tissues and differentiation grades were assessed by Student's t test. The comparison between different pathological types and clinical stages were assessed by analysis of variance. The immunohistochemistry results of GRP94 and

GRP78 was assessed by rank sum test. The correlation between GRP94 and GRP78 were assessed by Spearman correlate analyses. All the analyses were performed with SPSS 13.0 software. A value of $P<0.05$ was recognized as statistically significant.

RESULTS

Expression of GRP94 and GRP78 Proteins

Cytoplasmatic staining was detectable in colon cancer, colon adenomas and normal colon tissues for both GRP78 and GRP94. The main intensity of staining of colon cancer tissues was "++" to "+++", for adenoma tissues was "+" to "++", for normal tissues was "–" to "+" (Figure 1), there was a statistically significant difference ($P<0.01$). Staining results are given in Table 1 and Figure 1. GRP94 and GRP78 expression increased with progression along the normal tissue-adenoma-cancer sequence. Overexpression GRP94 was found to be closely linked to Dukes stages, grades of differentiation, lymph node involvement and Remote metastasis in colon cancer ($P<0.01$). No correlation was found between GRP94 expression and gender or age ($P>0.05$). In contrast, GRP78 expression was not significantly associated with tumor stage and pathological grades ($P>0.05$) (Table 1).

Expression of GRP94 and GRP78 mRNA

Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis showed that GRP78 and GRP94 mRNAs were detectable in cancer, adenoma and normal tissues. Table 2 shows that the expression level of GRP94

Table 1. Expression of GRP94 and GRP78 protein

Tissue	GRP94						GRP78				
	n	–	+	++	+++	*P* value	–	+	++	+++	*P* value
Normal	10	6	4	0	0		4	6	0	0	
Colon adenoma	21	11	4	5	1		10	6	3	2	
Colon cancer	64	7	8	30	19	$P<0.01$	10	12	26	16	$P<0.01$
Tumor differentiation											
Well	22	5	3	10	4		4	3	9	6	
Moderate	28	2	4	17	5		3	6	12	7	
Poor	14	0	1	3	10	$P<0.01$	4	3	5	2	$P>0.05$
Dukes stages											
A	7	2	1	2	2		2	2	3	0	
B	27	5	4	16	2		4	5	11	7	
C	20	0	3	10	7		3	3	9	5	
D	10	0	0	2	8	$P<0.01$	1	2	3	4	$P>0.05$
Lymph node metastasis											
Yes	30	0	3	12	15		6	5	11	8	
No	34	7	5	18	4	$P<0.01$	4	7	15	8	$P>0.05$
Remote metastasis											
Yes	10	0	0	2	8		2	3	4	1	
No	54	7	8	28	11	$P<0.01$	8	9	22	15	$P>0.05$
Gender											
Male	47	12	7	22	6		13	14	12	8	
Female	48	12	8	13	15	$P>0.05$	11	10	17	10	$P>0.05$
Age											
<60	44	14	7	16	7		9	12	11	12	
>60	51	10	9	19	13	$P>0.05$	15	12	18	6	$P>0.05$

mRNA was significantly higher in colon cancer than in normal colon tissues and colon adenomas (P<0.01), and also significantly higher in colon adenomas than in normal colon tissues (P<0.01). Furthermore, for GRP94, significant higher mRNA levels were found in the poorly differentiated tumors as compared to moderately and high differentiated tumors (P<0.01), and in C stage and D stage tumors as compared to A stage and B stage tumors (P<0.01). However, the GRP78 mRNA level was equal in colon cancer tissues in all stages and failed to show any association to pathological grades, lymph node involvement and Remote metastasis (P>0.05) (Table 2).

Table 2. Expression of GRP94 and GRP78 mRNA

Tissue	n	GRP94 ($\bar{x}\pm s$)	P value	GRP78 ($\bar{x}\pm s$)	P value
Normal	10	0.42±0.08		0.69±0.10	
Colon adenoma	21	0.76±0.21		1.20±0.32	
Colon cancer	64	1.15±0.36	P<0.01	1.68±0.45	P<0.01
Tumor differentiation					
Well	22	0.92±0.25		1.55±0.39	
Moderate	28	1.11±0.19		1.70±0.44	
Poor	14	1.32±0.28	P<0.01	1.66±0.42	P>0.05
Dukes stages					
A	7	0.88±0.23		1.44±0.34	
B	27	1.05±0.24		1.78±0.40	
C	20	1.22±0.19		1.71±0.43	
D	10	1.34±0.26	P<0.01	1.64±0.39	P>0.05
Lymph node metastasis					
Yes	30	1.26±0.30		1.73±0.42	
No	34	1.05±0.31	P<0.01	1.69±0.40	P>0.05
Remote metastasis					
Yes	10	1.38±0.22		1.60±0.41	
No	54	1.12±0.35	P<0.01	1.71±0.43	P>0.05
Gender					
Male	47	0.91±0.34		1.49±0.50	
Female	48	1.04±0.41	P>0.05	1.62±0.48	P>0.05
Age					
<60	44	0.89±0.33		1.52±0.51	
>60	51	1.03±0.40	P>0.05	1.72±0.43	P>0.05

Combined Expression of GRP78 and GRP94

The majority of the cases with high expression of GRP94 and GRP78 mRNA had strong immunostaining, and low expression of GRP94 and GRP78 mRNA showed light immunostaining, the results of Spearman correlate analysis showed a positive correlation between the expression of mRNA and protein (P<0.01) (Table 3). Meanwhile, we analyzed the relation of GRP78 expression with GRP94 in colon cancer. The results of Spearman correlate analysis revealed no correlation between the overexpression of GRP78 and GRP94 (P>0.05), and the combined expression of GRP78 and GRP94 did not show any correlation with Dukes stages (P>0.05) (Table 4).

Table 3. Correlation between GRP78 and GRP94 mRNA and protein expression

	n	Protein	mRNA ($\bar{x}\pm s$)	P value
GRP94	40	- — +	0.76±0.09	P<0.01
	55	++—+++	1.68±0.27	
GRP78	48	- — +	0.99±0.11	P<0.01
	47	++—+++	1.77±0.22	

Table 4. Combined GRP78 and GRP94 protein expression and Dukes stages

	HighGRP78/high GRP94	High GRP78/low GRP94	Low GRP78/high GRP94	Low GRP78/low GRP94	*P* value
Total	33	9	16	6	*P*>0.05
Dukes A	1	2	2	2	
Dukes B	13	5	6	3	*P*>0.05
Dukes C	13	2	4	1	
Dukes D	6	0	4	0	

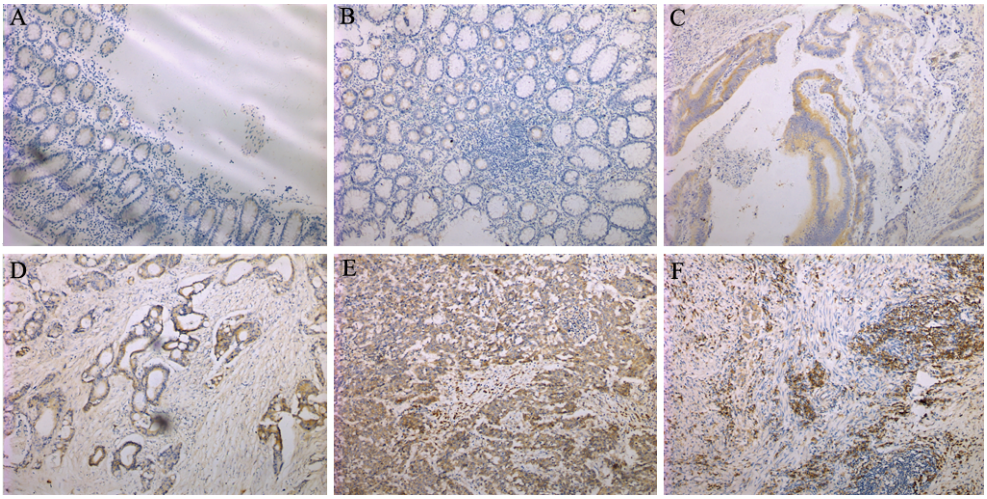

Figure 1. Expression of GRP94 and GRP78 in different tissues. A: "–" expression of GRP94 in normal tissue; B: "+" expression of GRP94 in colon adenoma; C: "++" expression of GRP94 in colon cancer (well-differentiated); D: "++" expression of GRP94 in colon cancer(moderately-differentiated); E: "+++" expression of GRP94 in colon cancer (poorly-differentiated); F: "++" expression of GRP78 in colon cancer (poorly-differentiated) (A–F, ×100).

DISCUSSION

GRP78 and GRP94 are the best investigated members of the family of glucose-regulated proteins (GRPs) in current studies[1, 10]. They were first described as a set of proteins whose synthesis was enhanced when cells were deprived of glucose. Because of their ability to assist in protein folding and assembly, the GRPs are referred to as molecular chaperones[1]. In tumor cells, besides hypoxic acidic or the above mentioned glucose starvation conditions, the induction of GRP94 or GRP78 might also represent a defensive mechanism for the survival of cancer cells exposed to these stress conditions or to the immunological response of the host[10].

In this study we examined GRP78 and GRP94 expression in primary resected cancer, adenomas and normal tissues of the colon on levels of mRNA and protein by RT-PCR and immunohistochemistry. Both RT-PCR and immunohistochemistry revealed increased GRP94 and GRP78 expression with progression along the normal tissue-adenoma-cancer sequence, and a positive correlation was detected between the mRNA levels and the protein levels of GRP94 and GRP78. GRP94 mRNA and protein expression was found to be in close relationship with the grades of differentiation, Dukes stages, lymph node involvement and remote metastasis in colon cancer. High GRP94 expression was detected in the poorly differentiated cancer and high-staging colon cancer with lymph and remote metastasis, indicating that overexpression of GRP94 might impose impact on the growth, invasion, metastasis, and progression of colon cancer. Determination of GRP94 mRNA and protein levels might be valuable in evaluating the grade of differentiation and clinical stage of human colon cancer. GRP78 expression in colon cancer failed to show any association with the grade of differentiation, Dukes stages, lymph node involvement and remote metastasis, although it was significant higher in colon cancer compared to that in adenomas and normal tissues. Our results

suggest that the overexpression of GRP78 might correlate to the occurrence of human colon cancer, but not to cellular differentiation, invasion or metastasis.

It has been confirmed in previous studies that GRP78 and GRP94 play an important role in tumor occurrence and progression, and cancer cell protection through multiple mechanisms, and increased cytoplasmic GRP78 and GRP94 expression corresponded well with progression from normal tissue to carcinoma[11–15]. GRP78 and GRP94 expression was highly up-regulated in varieties of cancer cell lines and human cancer specimens, including breast cancer, lung cancer, esophageal cancer, gastric cancer，liver cancer and prostate cancer, correlating with malignancy, metastasis and drug resistance[3–10]. As to human colon cancer, GRP78 and GRP94 expression was examined in only two studies. Xing X[16] reported that the expression of GRP78 protein was increased with progression along the normal tissue-adenoma-carcinoma sequence, but no difference in the relative mRNA expression levels of GRP78 was determined between normal and colon tumors. Wang XP[17] found that GRP94 expression was significantly higher in tumor compared to that in adjacent tissues, and correlated to cellular differentiation and metastasis.

Fu Y[10] reported that GRP78 shares common transcriptional regulatory elements with the GRP94 promoter and is coordinately regulated with GRP94, and induction of GRP94 is commonly associated with the upregulation of GRP78 in tumor cells. Wang Q[5] reported that there was a positive correlation between GRP94 and GRP78 in human lung cancer, but Langer R[9] found that combined expression of GRP78 and GRP94 did not show any correlation with pathological parameters in human esophagus cancer. In the present study, we analyzed the relation of GRP78 expression with GRP94 in colon cancer. The results revealed no correlation between overexpression of GRP78 and GRP94, and combination of GRP78 and GRP94 expression did not show any correlation with Dukes stages, indicating that GRP78 and GRP94 expression was not coherent in colon cancer. However, further studies are needed before the mechanism could be clarified. Furthermore, we analyzed the relation of GRP78 and GRP94 expression of protein and mRNA in colon cancer, and a positive correlation between the expression of mRNA and protein was determined, revealing that the expression of GRP78 and GRP94 were up-regulated at both the transcriptional and protein levels.

In conclusion, by detecting the expression of GRP78 and GRP94 in primary resected human colon cancer, we found that the overexpression of GRP94 and GRP78 was in close relationship with the occurrence and progression of human colon cancer. GRP94 and GRP78 were considered as the objective and effective markers in colon cancer diagnosis[18, 19]. The overexpression of GRP94 was closely related to cellular differentiation, Dukes stages, invasion and metastasis, and aggressive behaviors of colon cancer[18]. Inhibiting GRP94 or GRP78 expression may inhibit human colon cancer growth or increase sensitivity of radiotherapy and chemotherapy[20–22].

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