Over-expression of gastrin-releasing peptide in human esophageal squamous cell carcinomas

Ming Zhu Fang¹, Changgong Liu¹, Yunlong Song¹, Guang-Yu Yang¹, Yan Nie¹, Jie Liao¹, Xin Zhao², Yutaka Shimada³, Li-Dong Wang² and Chung S.Yang¹,4

1Susan Lehman Cullman Laboratory for Cancer Research, Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ 08854-8020, USA, 2Laboratory for Cancer Research, College of Medicine, Zhengzhou University, Zhengzhou, Henan 450052, China and 3Department of Surgery and Surgical Basic Science, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan 4To whom correspondence should be addressed Email: csyang@rci.rutgers.edu

Gastrin-releasing peptide (GRP) is known as an autocrine growth factor for a number of gastrointestinal cancers. There is, however, little information on the expression of GRP in the squamous epithelia and squamous cell carcinoma, particularly in the esophagus. With a differential display approach, up-regulated GRP was observed in human esophageal squamous cell carcinoma (ESCC) samples obtained from a high-risk area for esophageal cancer, Linzhou in northern China. Up-regulation of phosphoglycerate mutase and P311 HUM (3,1) and down-regulation of keratin 13, cystatin B, endoglin and annexin I were observed. Using a reverse transcription-polymerase chain reaction (RT-PCR) method, significant over-expression of GRP was observed in 10 out of 12 ESCC samples (83.3%) and all four ESCC cell lines. In situ hybridization, GRP mRNA expression was detected in nine out of 21 (42.8%) samples with basal cell hyperplasia (BCH), five out of seven (71.4%) samples with dysplasia (DYS) and 17 out of 24 (70.9%) ESCC samples. In contrast, GRP was expressed only in three out of 16 (18.7%) normal epithelium. Digital image analysis revealed that the mean value of GRP expression index, determined by intensity and area ratio of staining, was 0.19 in normal epithelium, 1.23 in BCH, 2.94 in DYS and 2.38 in ESCC, showing a progressive increase. Studies on ESCC cell lines showed GRP increased cell growth in a dose-dependent pattern in GRP receptor-positive ESCC cells, but not in GRP receptor-negative ESCC cells. GRP (1 mM) also increased cyclooxygenase-2 protein expression by 3.4-fold. This is the first demonstration that GRP is over-expressed in ESCC, and its over-expression may play a role in ESCC development and growth.

Introduction

Human esophageal cancer is the sixth most common cancer in the world, of which esophageal squamous cell carcinoma (ESCC) accounts for >90% of the cases. Epidemiological studies have identified the use of tobacco and consumption of alcohol as major risk factors for ESCC (1). Nitrosamines, mycotoxins, physical injury and chronic inflammation have also been suggested to be possible etiological factors (2). With similar life styles, environment and genetic backgrounds, the specimens obtained from the high-risk area, Linzhou (formerly named Linxian) in Henan, China provided a good opportunity to study its molecular alterations in the development of ESCC. Our previous studies suggest that inactivation of p53 and Rb tumor suppressor systems are frequent events in esophageal carcinogenesis (3,4), and p53 mutation is likely to occur in the early stage (5,6). Loss of heterozygosity and microsatellite instability of the tumor suppressor gene cluster 9p21 (p14ARF, p15INK4b, and p16INK4a) and hypermethylation of p16INK4a, p14ARF and HLA class I genes are also frequent early events in ESCC carcinogenesis (7-9). High levels of epidermal growth factor receptor have also been observed in some ESCC cases (10). The involvement of the ras and myc genes in ESCC have been studied extensively, but point mutations of the H-, K- or N-ras genes and the amplification of the c-myc gene have rarely been detected in samples from different high-risk areas (11-14). Recently, dozens of up-regulated or down-regulated genes have been observed in ESCC with cDNA microarray, of which oncogenes Fra-1 and Neogenin are over-expressed in ESCC (15-17). However, the major growth promoting factors in esophageal squamous carcinogenesis are not known. In this study, we used a differential display approach to study up-regulated and down-regulated genes to explore novel mechanisms that may be involved in human esophageal carcinogenesis; of the several up-regulated genes, gastrin-releasing peptide (GRP) was selected for detailed investigation.

GRP, a 27 aa small peptide, is the mammalian homolog of amphibian tetradecapeptide, bombesin (18). Human GRP has been isolated, and the gene has been localized to chromosome 18p26 (19). GRP acts as a neurotransmitter in the brain, as a paracrine hormone in the gastrointestinal tract and as a growth factor in the developing lung (20). In the mammalian gastrointestinal tract, GRP induces secretion of hormones, gastric acid and mucin; regulates smooth muscle contractions; and modulates neuronal firing rate (21). High levels of GRP immunoreactivity occur in the human fundus, antrum, pylorus and pancreas, whereas lower levels occur in the duodenum, jejunum, terminal ileum and colon (22). GRP acts by binding to the GRP receptor (GRP R), a specific G-protein coupled receptor with seven transmembrane spans (23). GRPR is widely expressed in the central and enteric nervous systems, where they act to alter a number of normal physiological processes including satiety, thermoregulation, circadian rhythm, smooth

Abbreviations: BCH, basal cell hyperplasia; Cox-2, cyclooxygenase 2; DIG, digoxigenin; DYS, dysplasia; ESCC, esophageal squamous cell carcinoma; EST, Expressed Sequence Tags; GRP, gastrin-releasing peptide; GRPR, gastrin-releasing peptide receptor; RT-PCR, reverse transcription–polymerase chain reaction.
expressed in human gastrointestinal, ovarian and prostate cancers (25,27).

GRP over-expression has been reported in lung, breast, renal, prostate, thyroid, stomach, intestine and colon cancers and malignant melanoma (24,26). There is, however, little information on the expression of GRP in the squamous epithelia and squamous cell carcinoma, particularly in the esophagus. Recent studies have shown that GRPR are aberrantly expressed in human gastrointestinal, ovarian and prostate cancers (25,27–29). Nevertheless, the GRPR expression in esophageal cancer is not clear.

Evidence from studies on small cell lung cancer and non-small cell lung cancer indicates that GRP stimulates growth of cells in vivo and in vitro in either autocrine or paracrine fashion (30–32). GRP is also a potent mediator for Swiss 3T3 mouse fibroblast and for N-ras transfected NIH 3T3 cells (33). Millar and Rozengurt reported that arachidonic acid release along with prostaglandin E2 formation is an early signal in the mitogenic response to GRP (34). Recently, it was reported that GRP induced activation protein 1 (AP-1) gene expression and binding activity (35). GRP-induced AP-1 transcription factor also mediated cyclooxygenase-2 (Cox-2) expression in intestinal epithelial cells (36). Monoclonal antibodies against GRP or GRPR antagonists could inhibit GRP functions (37–39). Therefore, GRP was suggested as a possible tumor therapy target (37).

In this report, we demonstrated the over-expression of GRP mRNA in ESCC with differential display and confirmed the over-expression with RT–PCR. Studies with in situ hybridization indicated the progressive increase of GRP expression in esophageal squamous carcinogenesis. In GRPR-positive ESCC cells, GRP increased cell proliferation and Cox-2 expression.

Materials and methods

Specimen preparation

Primary ESCC specimens containing neighboring normal epithelial tissues were collected from patients in Linzhou People’s Hospital, Linzhou, Henan, China. The samples were frozen in liquid nitrogen within 1 h after surgical resection, and were stored in liquid nitrogen, dry ice or in −80°C freezers before use. For each specimen, several pieces of tissues, some from the tumor mass and others from normal esophageal mucosa, were dissected and embedded with tissue freeze medium (OTC). Normal, basal cell hyperplasia (BCH), dysplasia (DYS) or ESCC samples were diagnosed histopathologically on slides stained with hematoxylin and eosin (H&E) by two pathologists (G.-Y.Y. and J.L.).

Total RNA was extracted from ESCC tissues and adjacent non-tumorous tissues using the RNA Mini kit (Qiagen, Valencia, CA) following the manufacturer’s instructions and then stored at −80°C. Frozen embedded freezing tissues were cryosectioned to a thickness of 10 μm and placed on RNase-free slides. After air-drying, these slides were stored at −80°C before in situ hybridization.

ESCC cell lines, KYSE 150, 190, 450 and 510 (40), were maintained in RPMI 1640 and Ham F12 mixed (1:1) medium containing 5% fetal bovine serum. Total RNA was also extracted using the RNA Mini kit after cells were cultured to 80% confluence. Cells were seeded in 8-well slide chambers at 1 × 10^4 cells/well and placed on the slides. After air-drying, these slides were stored at −80°C before in situ hybridization.

Differential display

Total RNA prepared from two pairs of ESCC and adjacent non-tumorous epithelial samples were used to synthesize the first strand cDNA with an Advantage^TM RT-for-PCR kit (Clontech, Palo Alto, CA) following the protocol provided in the kit. Twenty-four PCR reactions were conducted with three anchor primers combined with eight arbitrary primers (GenHunter, Nashville, TN). PCR products were resolved on 6% polyacrylamide gel. Differentially expressed bands were cut out, purified using the Qiagen Gel Extraction kit, and re-amplified with corresponding differential display primers. Re-amplified PCR products were sequenced using the Automatic Sequencer, maintained and conducted by the DNA Core Facility of the UMDNJ-Robert Wood Johnson Medical School. The Gene Bank Blast program was used for identification of the gene sequence.

RT–PCR

Reverse transcription was performed using the Advantage^TM RT-for-PCR kit. PCR primers for GRP, whole length GRPR and β-actin control were designed by Primer 3 software, developed and maintained by the Whitehead Institute/MIT Center for Genomic Research (Table I). In order to produce a genespecific mRNA signal, the total RNA was treated with DNase I to remove genomic DNA contamination. Diethylpyrocarbonate-treated water was used as a negative control and total RNA from human small cell lung cancer tissue (Novagen, Madison, WI) was used as positive control for GRP mRNA expression. PCR cycle numbers (typically 27–30 cycles) were determined to keep the reaction in the linear stage. PCR products were run in 2% agarose gel and confirmed by sequencing using an Automatic Sequencer. Signal intensities were quantified using densitometry (Bio-Rad, Hercules, CA). The level of GRP mRNA was quantified by the intensity ratio of the target signal to the β-actin control under the same PCR reaction conditions. We used the cut-off of (density of GRP/density of actin)human/(density of GRP/density of actin)normal >10 to distinguish significant over-expression.

In situ hybridization

A non-radioactive in situ hybridization protocol was adapted for the determination of GRP mRNA expression (41,42). In brief, GRP cDNA was produced by RT–PCR using the total RNA from human small cell lung cancer as a template and ligated into pcRII-TOPO vector, then transformed into competent E.coli using the TOPO TA cloning kit from Invitrogen (Carlsbad, CA). The cloned human GRP cDNA was amplified, purified and linearized with HindIII or NotI for antisense or sense cRNA probe. Digoxigenin (DIG)-labeled antisense and sense cRNA probes were prepared using a DIG T7/SP6 transcription kit (Boehringer-Mannheim, Indianapolis, IN) from linearized plasmid human GRP cDNA. The quality and specificity of the probe were determined on the positive control slides made from the human pancreas (Novagen) (data not shown). The sense probe or probe vehicle was used as a negative control to verify the specificity of the antisense cRNA probe.

The sliced tissue was fixed in 4% paraformaldehyde, digested with proteinase K and then acetylated in freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine buffer. Hybridization buffer (50% denitrogenated formamide, 25 mM phosphate buffer, pH 7.4, 2 × standard saline citrate, 2 × Denhardt’s solution, 10% dextran sulfate, 0.02 M dithiothreitol, 0.4 mg/ml yeast RNA, 250 μg/ml salmon-sperm DNA in diethylpyrocarbonate-treated water) containing 400 ng/ml of probe was placed onto the permeabilized section and covered with a paraffin coverslip. Then the slides were denatured at 80°C and hybridized at 42°C overnight. After post-hybridization washing in 1 × SSC containing 2% normal sheep serum and 0.05% Triton X-100 for 2 h, the slides were blocked for 30 min in 0.1 M maleic acid buffer (pH 7.5) containing 2% normal ship serum and 0.3% Triton X-100, and then incubated for 1 h with a sheep anti-DIG antibody (1:1000) and in streptavidin-alkaline phosphatase conjugate for 20 min. Finally, the color was developed in a NBT/BCIP chromogen solution.

Table I. RT–PCR primer sequences

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Sequence</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRP mRNA expression</td>
<td>3’-GGG ACC GTG CTG ACC AAG ATG TAC C-5’</td>
<td>575 bp</td>
</tr>
<tr>
<td></td>
<td>3’-AGC ATT AAT TGG AAG ACT CTT TGC-5’</td>
<td></td>
</tr>
<tr>
<td>β-Actin control</td>
<td>3’-GTC CAC CTT CCA GCA GAT GT-5’</td>
<td>1 kb</td>
</tr>
<tr>
<td></td>
<td>3’-ATG CTA CTA CCT CCC CTG TG-5’</td>
<td></td>
</tr>
<tr>
<td>ISH probe</td>
<td>3’-AGT GAG CTC CCG CTG GTC-5’</td>
<td>192 bp</td>
</tr>
<tr>
<td></td>
<td>3’-TCA GGC TCC CTC TCT CAG AA-5’</td>
<td></td>
</tr>
<tr>
<td>GRPR expression</td>
<td>3’-CCT CTA CAA CGT CAC GTA C-5’</td>
<td>587 bp</td>
</tr>
<tr>
<td></td>
<td>3’-ATA CCG AAG GAA AGA CCA G-5’</td>
<td></td>
</tr>
<tr>
<td>ISH, in situ hybridization</td>
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<td></td>
</tr>
</tbody>
</table>
Over-expression of GRP in ESCC

Between each reaction, the slides were washed three times for 5 min each in 0.1 M maleic acid buffer (pH 7.5) containing 0.1% Triton X-100. After being washed in tap water, the slides were then mounted with Aqua-mounting medium (Fisher, Houston, TX).

The stained sections were reviewed under a Nikon microscope. The pathological diagnosis of normal, BCH, DYS and ESCC was performed on an adjacent H&E stained slide by two pathologists. In visual analysis, in situ hybridization staining intensity was graded as −, −/+ , + and ++ . Intense purple staining (+/++) was defined as positive. Background (−) or faint staining (−/+ ) was defined as negative. For digital image analysis, five images were picked randomly within encircled areas containing normal, BCH, DYS or ESCC at a magnification of ×200. The mean optical densities from the positive signal and background were measured using Photoshop 5.0 program and the intensity ratio was calculated. We also measured the area with Imagine Plus 4.0 program to calculate the area ratio of the positively stained area to the total area. Finally, the expression index was calculated by multiplication of the intensity ratio and area ratio. The Student’s t-test with unequal variance was used to compare the GRP expression index in different groups. Differences with P < 0.05 were regarded as significant.

Cell proliferation assay

ESCC cell lines, KYSE 150, 190, 450 and 510, were used to determine the effect of GRP on cell growth with the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. In brief, cells were seeded at 1 × 10^4 cells/well in a 96 multi-well plate with serum-free RPMI 1640 media. After 24 h culture in a 37°C, 5% CO2 incubator, the cells were treated with 1, 10, 100 and 1000 nM GRP for 48 h. Then the cells were incubated with 1 mg/ml MTT for 4 h at 37°C, the insoluble formazan was formed dissolved in DMSO, and A570 was measured with an Enzyme Precision Microplate Reader (Molecular Devices, USA). Each group had six wells in replicate. The Student’s t-test was used to analyze significant differences between the treatment and control groups.

Western blot

Cultured cells were collected, and protein was prepared with radioimmuno-precipitation assay lysis buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 0.4 mM phenylmethylsulfonyl fluoride, 0.3 μM aprotinin, 4 μM leupeptin, 3 μM pepstatin A and 10 μM indomethacin. The protein concentration was determined using Bradford analysis kit (Bio-Rad). Proteins (40 mg/lane) were loaded on 4–15% acrylamide gel and blotted onto a nitrocellulose membrane (Amersham, Heights, IL). After incubation in blocking buffer (5% non-fat milk), the membranes were incubated with mouse anti-human Cox-2 monoclonal antibody (1:1000) (Cayman, Ann Arbor, MI) overnight at 4°C. After washing with TBS containing 0.1% Tween 20, the membrane was then incubated with sheep anti-mouse horseradish peroxidase-labeled secondary antibody and visualized using the electrochemiluminescence detection kit (Amersham). The band intensities were determined with a densitometry (Bio-Rad). Average intensity was calculated from triple experiments.

Results

Differential display

Differential display was performed on the total RNA prepared from two pairs of esophageal tumors and adjacent non-tumorous epithelial samples. The displays of two of the 24 PCR reactions are shown in Figure 1. Some individual variability between the two non-tumorous epithelial samples or between the two tumor samples was also observed. All 17 differentially expressed bands between non-tumorous epithelial samples and tumors in 24 reactions were cut and re-amplified with the original primers. After sequencing and conducting a Blast search in the Gene Bank, 12 differentially expressed genes were identified (Table II). The differentially expressed genes were obtained consistently from two independent differential display experiments. Among the differentially expressed genes, seven sequences were genes with known names and functions, including GRP, phosphoglycerate mutase, P311 HUM (3.1), keratin 13, cystatin B, endoglin and annexin I; four were expressed sequence tags (ESTs), AA885875, AA807512, KIAA0648 and KIAA0112; and one had no EST available. Expressions of six genes, including GRP, phosphoglycerate mutase, P311 HUM (3.1), AA885875, AA807512 and the one with no EST available, were up-regulated. The other six genes, including keratin 13, cystatin B, endoglin, annexin I, KIAA0648 and KIAA0112, were down-regulated. Of these, GRP was selected for further studies.

Over-expression of GRP in ESCC samples and cell lines

Expression of GRP was investigated using RT-PCR in 12 ESCC samples together with adjacent non-tumorous epithelial tissues for comparison (Figure 2). Ten (83.3%) cases had significant over-expression of GRP mRNA in tumor tissues over the paired non-tumorous samples (of these, only seven pairs of samples, #1, 3, 4, 6, 7, 8 and 9, are shown in Figure 2). Three out of the 12 (25.0%) adjacent non-tumorous samples

Table II. Differentially expressed genes in ESCC in differential display

<table>
<thead>
<tr>
<th>Gene or gene bank accession number</th>
<th>Expression pattern in tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Phosphoglycerate mutase</td>
<td>Up-regulation</td>
</tr>
<tr>
<td>2. Gastrin-releasing peptide</td>
<td>Up-regulation</td>
</tr>
<tr>
<td>3. P311 HUM (3.1)</td>
<td>Up-regulation</td>
</tr>
<tr>
<td>4. Unknown gene, no EST available</td>
<td>Up-regulation</td>
</tr>
<tr>
<td>5. Unknown gene, EST AA885875</td>
<td>Up-regulation</td>
</tr>
<tr>
<td>6. Unknown gene, EST AA807512</td>
<td>Up-regulation</td>
</tr>
<tr>
<td>7. Keratin 13</td>
<td>Down-regulation</td>
</tr>
<tr>
<td>8. Cystatin B</td>
<td>Down-regulation</td>
</tr>
<tr>
<td>9. Endoglin</td>
<td>Down-regulation</td>
</tr>
<tr>
<td>10. Annexin I</td>
<td>Down-regulation</td>
</tr>
<tr>
<td>11. KIAA0648</td>
<td>Down-regulation</td>
</tr>
<tr>
<td>12. KIAA0112</td>
<td>Down-regulation</td>
</tr>
</tbody>
</table>

*Genes up- or down-regulated in two ESCC samples in the 24 PCR reactions are listed.
expressed GRP mRNA; of which one had DYS (#5) and another two had BCH (only one sample, #2, is shown in Figure 2). In the cases shown, GRP expression in adjacent non-tumor-ous epithelium was more than the paired tumor. GRP expression was also detected in all four ESCC cell lines examined (data not shown). GRP mRNA expression levels of KYSE 150 and 450 cells were higher than KYSE 190 and 510 cells.

**GRP expression pattern in different stages of ESCC carcinogenesis**

To determine the GRP expression pattern in different stages of esophageal squamous carcinogenesis, we analyzed GRP mRNA expressions using *in situ* hybridization in the frozen sections, including 16 normal esophageal epithelial samples, 21 BCH, seven DYS and 24 ESCC samples. Results showed that GRP was over-expressed in 17 out of 24 (70.9%) ESCC samples with moderate to strong positive staining (Table III). GRP was expressed in the cytoplasm and around the nuclei of positive cells distributed in the periphery or throughout the cancer nest in ESCC samples (Figure 3Ad). In nine out of 21 (42.8%) BCH and five out of seven (71.4%) DYS samples, GRP was expressed mostly in proliferating cells with moderate to strong staining intensity (Figure 3B) compared with the negative control (data not shown). Only three out of 16 (18.7%) normal epithelium weakly expressed GRP in the cytoplasm of cells that were located mainly in the basal and parabasal cell layers (Figure 3Ab). Mean expression index from digital image analysis revealed a gradual increase from normal to DYS (normal epithelium, 0.19; BCH, 1.23; DYS, 2.94). The mean expression index of ESCC (2.38) was similar to DYS (Table IV). All four ESCC cell lines, KYSE 150, 190, 450 and 510, also expressed strongly GRP mRNA. The representative positive staining of KYSE 150 cells is shown in Figure 3Af. Taken together, these results indicate that GRP was over-expressed in ESCC, and the expression progressively increased in BCH and DYS, suggesting that GRP is involved in the early stage of esophageal carcinogenesis.

**Stimulation effect of GRP on ESCC cell proliferation**

To further study GRP functions in ESCC, we determined GRPR expression in ESCC cell lines, KYSE 150, 190, 450 and 510, using RT-PCR with full-length GRPR specific
Over-expression of GRP in ESCC

Table IV. Results of digital image analysis for GRP expression

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Intensity ratio</th>
<th>Area ratio</th>
<th>Expression index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n = 16)</td>
<td>2.228 ± 0.258</td>
<td>0.078 ± 0.019</td>
<td>0.189 ± 0.051</td>
</tr>
<tr>
<td>BCH (n = 21)</td>
<td>3.358 ± 0.451</td>
<td>0.303 ± 0.056</td>
<td>1.229 ± 0.267</td>
</tr>
<tr>
<td>DYS (n = 7)</td>
<td>4.417 ± 0.713</td>
<td>0.390 ± 0.127</td>
<td>2.944 ± 0.696</td>
</tr>
<tr>
<td>ESCC (n = 24)</td>
<td>4.737 ± 0.551</td>
<td>0.488 ± 0.074</td>
<td>2.35 ± 0.495</td>
</tr>
</tbody>
</table>

*Value presented as mean ± SE. Intensity = density of positive signal/density of background. Area ratio = positive area/total area. Expression index = intensity ratio × area ratio.

Statistically significant difference (P < 0.05) between normal and BCH, DYS or ESCC and between BCH and DYS or ESCC, respectively. Student’s t-test with unequal variance was used for pair comparison.

Fig. 4. GRPR expression in ESCC cell lines determined by RT-PCR. β-Actin was used as an internal control. M, DNA marker; 1, 2, 3 and 4, human ESCC cell lines, KYSE 150, 190, 450 and 510, respectively.

Fig. 5. GRP increased ESCC cell growth rate. Subconfluent, serum-starved human ESCC cell lines, KYSE 150, 190, 450 and 510 cells, were treated with 1, 10, 100 and 1000 nM GRP for 48 h, and then cell growth rate was determined with MTT assay. *Indicates significant difference (P < 0.01) between control and test groups. Values are presented as mean ± SD (n = 6).

Fig. 6. GRP induced Cox-2 protein expression in ESCC cells. Subconfluent, serum-starved human ESCC cell lines, KYSE 150 and 510 cells, were treated with 10, 100 and 1000 nM GRP for 6 h, and then protein expression levels were determined with western blot analysis. Representative pictures are shown. Band intensity was measured with densitometry and relative intensities (RI) are indicated.

Discussion

With differential display and sequencing, we found up- or down-regulated alterations of 12 genes in ESCC (Table II). The results on the over-expression of GRP and down-regulation of Keratin 13, Annexin I and Cystatin B are consistent with the results of our preliminary results with northern blot analysis (data not shown). Keratin 13 and Annexin I may be a differentiation marker in normally differentiated squamous epithelial cells (43,44), and their expressions are expected to be lower in ESCCC, which are less differentiated. Cystatin B has been reported recently to be involved in esophageal tumor invasion and metastasis (45). GRP, a paracrine and autocrine growth factor involved in several tumors, appears to be the most interesting gene over-expressed in ESCC. Therefore, GRP was selected for further study. The other genes may be subjects for future investigation.

GRP over-expression in ESCC was confirmed by RT-PCR. Over-expression of GRP was observed in 10 out of 12 ESCC samples and in a few adjacent non-tumorous samples with precancerous lesions. High expression levels of GRP were observed in all four ESCC cell lines studied. Our results from in situ hybridization studies showed that GRP mRNA existed in the cytoplasm, and the positive cells were distributed in the periphery or throughout the cancer nest in ESCC. ESCC is known to be developed through a multistage process including BCH and DYS; severe DYS is considered an immediate precursor of ESCC (17). The observation that GRP was over-expressed progressively from BCH to DYS and ESCC (Table IV), suggests that GRP is involved in the early stage of esophageal squamous carcinogenesis. This is the first demonstration of GRP over-expression in esophageal carcinogenesis.

In the present study, most normal epithelium did not express GRP, but some normal epithelial samples expressed GRP in cells located in the basal cell layer (Figure 3Ab). The cells in the basal layer of the esophageal epithelium have proliferation potential and they are frequently hyperproliferative in many individuals in the high-risk population in Linzhou, China. Therefore, GRP-positive cells in the basal cell layer in apparently normal epithelium may represent cells that start to proliferate. GRP may function as an autocrine growth factor for esophageal epithelial cells similar to that demonstrated for fetal lung development (20). Staniek et al demonstrated the
expression of GRP and GRPR on basal and parabasal layer cells of human skin, suggesting that GRP modulates epidermal cell functions (46). Recently, GRP was reported to promote re-epithelialization during cutaneous wound healing (47).

The present results showed that GRP expression was closely associated with cell hyperproliferation, and increased in BCH, DYS and ESCC during esophageal squamous carcinogenesis. GRP significantly increased the cell proliferation rate of the GRP-positive KYSE 150 cells. The most well-studied GRP-induced signal transduction pathway involves AP-1 activation, which promotes cell proliferation (35). Activated AP-1 gene may also elevate Cox-2 expression (36). The induction of Cox-2 expression by GRP was demonstrated in GRP-positive ESCC cells (Figure 6), suggesting GRP can activate the Cox-2 pathway, which can contribute to cell proliferation and tumorigenesis (48).

In summary, the present results show that GRP is over-expressed in ESCC and suggest that GRP is involved in early stage esophageal squamous carcinogenesis. GRP stimulates cell growth and increases Cox-2 protein expression in GRP-positive ESCC cells. More detailed research is needed to further elucidate the role of GRP function in esophageal carcinogenesis associated with cell hyperproliferation, and increased in BCH, DYS and ESCC during esophageal squamous carcinogenesis. Considering GRP is a small peptide, one of the possibilities may be to assess the GRP levels in serum samples from patients or high-risk population for biomarker evaluation. Our cell line study also revealed the important role of GRPR in cell proliferation. Monoclonal antibodies against GRP and GRPR antagonists have been utilized in a clinical trial in lung cancer (37–39). Our results may lead to practical applications in the development of preventive or therapeutic agents for human ESCC.

Supplementary material

Supplementary material can be found at: http://www.carcin.oupjournals.org/

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