Gastrin-Releasing Peptide Receptor Expression in Lung Cancer

Jane Mattei, MD, PhD; Rosane D. Achcar, MD; Carlos H. Cano, MSc; Bruno R. Macedo, MD; Luise Meurer, MD, PhD; Brenda S. Batlle; Steve D. Groshong, MD, PhD; Jane M. Kulczynski, MD, PhD; Rafael Roesler, PhD; Lissandra Dal Lago, MD, PhD; Andre T. Brunetto, MD; Gilberto Schwartsmann, MD, PhD

- **Context.**—Gastrin-releasing peptide receptors (GRPRs) activate mitogen-activated protein kinase signaling pathway primarily through epidermal growth factor receptor activation and are under investigation as a molecular target because they are overexpressed in several solid tumors.

- **Objective.**—To determine GRPR expression in both non–small cell lung carcinoma and small cell lung carcinoma, comparing results with clinical stages and demographic data.

- **Design.**—We analyzed the immunohistochemical expression of GRPR in 200 non–small cell lung carcinoma and 38 small cell lung carcinoma archival cases from 2004 to 2008.

- **Results.**—Non–small cell lung carcinoma cases tended to be higher GRPR expressers at a rate of 62.5% (weak, moderate, and strong expression in 41.5%, 13.5%, and 7.5%, respectively), compared with 52.62% in small cell lung carcinoma cases (weak, moderate, and strong expression in 34.21%, 15.78%, and 2.63%, respectively; *P* = .30). In non–small cell lung carcinoma there was a trend for higher percentages of strong expression in adenocarcinoma cases (10%; *P* = .67), and in patients with advanced stages (III and IV; 9.43% and 6.9%; *P* = .01).

- **Conclusions.**—To the best of our knowledge, this is the first study to demonstrate GRPR tissue expression in a large population of patients with lung cancer. Although GRPR expression was similar in small cell and non–small cell carcinoma, the expression was more pronounced in an advanced-stage lung cancer, particularly in adenocarcinoma cases, and may represent a potential target for the development of new treatment approaches in this population.


Lung cancer is the leading cause of global cancer death and is responsible for more than 1 million deaths per year. At diagnosis, approximately 75% of lung cancer patients exhibit locally advanced or metastatic disease. Despite recent advances in understanding the biology of lung cancer, the 5-year survival rates of patients suffering from lung cancer remain low at less than 15%; therefore, new therapeutic targets are needed to improve survival rates.

Gastrin-releasing peptide (GRP), a bombesin-like peptide growth factor, is expressed by pulmonary neuroendocrine cells and has been shown to stimulate lung development in utero and to increase growth maturation of human fetal lung organ cultures. It is also a potent mitogen for normal and neoplastic tissues, and it may be involved in growth dysregulation and carcinogenesis.

The effects of GRP are primarily mediated through binding to its receptor, GRP receptor (GRPR), which is a G protein–coupled receptor originally isolated from a small cell lung cancer cell line. Uregulation of GRP/GRPR has been reported in several cancers, including prostate, glioma, colon, head and neck, and lung cancer.

It has been proposed that GRP stimulation results in increased release of epidermal growth factor receptor (EGFR) proligands, which is metalloprotease dependent and results in activation of EGFR and mitogen-activated protein kinase downstream pathways (Figure 1). Moreover, it has been demonstrated that abrogation of EGFR by EGFR-specific tyrosine kinase inhibitors also blocks GRP-mediated mitogen-activated protein kinase activation, indicating that GRP activates mitogen-activated protein kinase primarily through EGFR. Recent advances using non–small cell lung cancer (NSCLC) cell lines have confirmed that EGFR and GRP both stimulate NSCLC proliferation, and...
inhibition of either EGFR or GRPR resulted in cell death. In addition, combining a GRPR antagonist with the EGFR tyrosine kinase inhibitors resulted in additive cytotoxic effects.\textsuperscript{14,16}

To further investigate the possible utility of GRPR as a future therapeutic target in lung cancer, we evaluated the expression of GRPR in small cell lung carcinoma (SCLC) and NSCLC. The results were correlated with sex, clinical stage, smoking status, and survival. To our knowledge, we have the largest number of SCLCs and NSCLCs evaluated for GRPR tissue expression.

**MATERIALS AND METHODS**

**Population**

We analyzed the immunohistochemical expression of GRPR in 238 lung cancer archival cases selected from the department of pathology files of Hospital das Clínicas from the University of Rio Grande do Sul (Porto Alegre, Brazil), based on the availability of formalin-fixed, paraffin-embedded tissue blocks from 2004 to 2008. The specimens were obtained from patients with adenocarcinoma ($n = 100$), squamous cell carcinoma ($n = 71$), large cell carcinoma ($n = 29$), and small cell carcinoma ($n = 38$). Results were correlated with tumor cell type, sex, tumor stage (I–IV in NSCLC cases and limited and extensive disease in SCLC cases), smoking status (smoker versus nonsmoker), and survival (date of diagnosis to date of death or date of last hospital follow-up visit).

Figure 1. Gastrin-releasing peptide (GRP)/GRP receptor (GRPR) signaling pathway. When GRP activates GRPR, inactive guanosine diphosphate (GDP)–bound $G$ protein is activated to a guanosine triphosphate (GTP)–bound state, resulting in metalloprotease-dependent increased release of epidermal growth factor receptor (EGFR) proligands, subsequent phosphorylation of EGFR, and activation of RAS/RAF/mitogen-activated protein kinase (MAPK) downstream pathway.

Immunohistochemistry Technique

Immunohistochemical stain for GRPR (rabbit polyclonal antibody, OPA1-15619, 1:200 dilution; Affinity Bioreagents, Golden, Colorado) was performed on formalin-fixed, paraffin-embedded tissue blocks from all cases sectioned at 4-μm thickness. After dewaxing, inactivating endogenous peroxidase activity, and blocking cross-reaction with normal serum, sections were incubated overnight at 4°C with the primary antibody diluted solution. Secondary detection was achieved by subsequent application of biotinylated antibody, streptavidin horseradish peroxidase conjugate (LSAB, Dako, Carpinteria, California) and diaminobenzidine tetrahydrochloride/H₂O₂ (Kit DAB, Dako). Images were obtained with an Aperio scanscope (Scanscope Aperio Technologies, Inc, Vista, California) connected to a personal computer HP xw 4300 Workstation (Hewlett-Packard Development Co, LP, Palo Alto, California). Representative photographs were taken to illustrate the findings.

Immunohistochemistry Evaluation

Double-blinded histopathology review was performed in tissue from archival cases of NSCLC (n = 200) and SCLC (n = 38). All tumor cases had one slide of tissue available for review. Five entire high-power fields (×40) containing clusters of malignant cells were identified per slide and scored for intensity and percentage of GRPR staining expression. The mean of the 5 intensities and percentages for each power field was recorded. The mean values per case were averaged and calculated for each diagnostic group. Intensity of tumor cells’ positive staining was recorded on a scale of 0 to 3 (0 = absent expression; 1 = mild expression; 2 = moderate expression; and 3 = intense expression; Figure 2, A through D). Percentage of stained tumor cells was recorded on a scale of 0 to 3 (0 = absent expression; 1 = less than 25% of cells; 2 = 25%–50% of cells; 3 = 50%–100% of cells; Table 1). Gastrin-releasing peptide receptor antibody–stained nonneoplastic pancreatic tissue was used as positive control.

Statistical Analysis

The significance of differences observed between the groups was calculated using SPSS version 19.0 (SPSS Statistics for Windows, Version 19.0, IBM Corporation, Armonk, New York.) Correlations between GRPR expression and clinicopathologic variables were analyzed by using the Pearson χ² test and Fisher exact test. Overall survival was calculated by the Kaplan-Meier method and differences between groups were assessed by the log-rank test and Fisher exact test. Patients were censored for survival at date of death or date of last hospital follow-up visit. A multivariate analysis using the Cox proportional hazards model was also performed in order to adjust for the effects of age, sex, and stage of disease. Statistical differences were accepted at \( P < .05 \) and were 2-sided.

The study proposal was reviewed and approved by institutional review board and audit committees from University of Rio Grande do Sul (Porto Alegre, Brazil).

RESULTS

Patient Demographics

The tumor specimens were obtained from adenocarcinoma (n = 100), squamous cell carcinoma (n = 71), large cell carcinoma (n = 29), and small cell carcinoma (n = 38). The male to female ratio was 1.2:1. Review of the available data showed that 200 patients (84.03%) were smokers, 8 (3.36%) were nonsmokers, and 30 (12.61%) had unknown smoking status. Of NSCLC patients, 37 (15.54%) had stage I or II

Table 1. Gastrin-Releasing Peptide Receptor Immunohistochemical Analysis Correlating Percentage and Intensity of Tumor Cell Expression

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Negative</th>
<th>1–25</th>
<th>25–50</th>
<th>50–100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma</td>
<td>32</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Squamous</td>
<td>31</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Large cell</td>
<td>12</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Small cell</td>
<td>18</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Immunostaining and measurement of stain were performed as described in “Materials and Methods.”

Figure 3. Gastrin-releasing peptide receptor (GRPR) expression in non–small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) and clinical stage (I, II, III, and IV for NSCLC and extensive disease [ED] for SCLC). A significant association (\( P = .01 \)) with more pronounced intensity of GRPR expression and advanced clinical stages was identified.

CLINICAL STAGE vs. GRPR EXPRESSION

No Weak Moderate Strong

disease, 53 (22.26%) stage III, and 110 (46.21%) stage IV. Of SCLC patients, 3 (1.26%) had limited disease, 33 (13.86%) had extensive disease, and 2 (0.84%) had unknown disease extension. The average survival was 10.65 months in the female population and 10.02 months in the male population, with an overall survival rate of 10.3 months. Clinical data are summarized in Table 2.

### GRPR Expression and Clinical Stage

Absence of GRPR expression was more prominent in early stages I and II (62.16%) compared with patients with advanced disease (34% for clinical stage III, 32.35% for clinical stage IV, and 43.24% for extensive disease). Moderate expression was more conspicuous in clinical stage IV (18.63%) and extensive disease (16.21%). Strong expression was more pronounced in clinical stages III (9.43%), IV (6.9%), and extensive disease (5.4%). There was significant association (P = .01) between strong intensity of GRPR expression and higher clinical stage, independent of tumor cell type (Figure 3). It should be noted that because we had only 3 cases with limited disease, this population may not be representative.

### GRPR Expression and Cell Type

Although the overall GRPR percentage of cell expression was similar between NSCLC (62.51%) and SCLC (52.62%; P = .22), the intensity of GRPR expression tended to be more pronounced in NSCLC (41.5% weak, 13.5% moderate, 7.5% strong) compared with SCLC (34.21% weak, 15.78% moderate, 2.63% strong; P = .30; Figure 4).

Similar weak expression of GRPR was seen among all NSCLC cell types, ranging from 40.84% to 42%. Moderate GRPR expression was seen in 16% of adenocarcinomas, 11.26% of squamous cell carcinomas, and 10.34% of large cell carcinomas. Strong GRPR expression was seen in 10% of adenocarcinomas, 4.22% of squamous cell carcinomas, and 6.89% of large cell carcinomas (P = .67). Adenocarcinoma cases tended to be stronger GRPR expressers (Figure 5).

### GRPR Expression and Sex and Smoking Status

Distribution of GRPR expression was comparable between male (weak 41.66%, moderate 12.87%, and strong 6.06%) and female subjects (weak 38.67%, moderate 14.46%, and strong 6.65%). Comparative NSCLC and SCLC GRPR expression is shown in Figure 4.

<table>
<thead>
<tr>
<th>Clinical Data</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, No. (%)</td>
<td></td>
</tr>
<tr>
<td>Female 106 (44.9)</td>
<td></td>
</tr>
<tr>
<td>Male 132 (55.1)</td>
<td></td>
</tr>
<tr>
<td>Age, mean, y</td>
<td>63.08</td>
</tr>
<tr>
<td>Female 63.44</td>
<td></td>
</tr>
<tr>
<td>Male 61.08</td>
<td></td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
</tr>
<tr>
<td>Smoker 200 (84.03)</td>
<td></td>
</tr>
<tr>
<td>Never smoker 8 (3.36)</td>
<td></td>
</tr>
<tr>
<td>Unknown 30 (12.6)</td>
<td></td>
</tr>
<tr>
<td>Cell type</td>
<td></td>
</tr>
<tr>
<td>Non–small cell lung cancer 200 (84.03)</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma 100 (42.01)</td>
<td></td>
</tr>
<tr>
<td>Squamous 71 (29.83)</td>
<td></td>
</tr>
<tr>
<td>Large cell 29 (12.18)</td>
<td></td>
</tr>
<tr>
<td>Small cell lung cancer 38 (15.96)</td>
<td></td>
</tr>
<tr>
<td>Disease stage</td>
<td></td>
</tr>
<tr>
<td>I and II 37 (15.54)</td>
<td></td>
</tr>
<tr>
<td>II 53 (22.26)</td>
<td></td>
</tr>
<tr>
<td>IV 110 (46.21)</td>
<td></td>
</tr>
<tr>
<td>Limited disease</td>
<td>3 (1.26)</td>
</tr>
<tr>
<td>Extensive disease</td>
<td>33 (13.86)</td>
</tr>
<tr>
<td>Unknown 2 (0.84)</td>
<td></td>
</tr>
<tr>
<td>Survival, mo</td>
<td></td>
</tr>
<tr>
<td>Female 10.65</td>
<td></td>
</tr>
<tr>
<td>Male 10.02</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.** Non–Small Cell Lung Carcinoma and Small Cell Lung Carcinoma Clinical Data Correlating Sex, Age at Diagnosis, Smoking Status, Cell Type, Stage of Disease, and Survival

---

**Figure 4.** Gastrin-releasing peptide receptor (GRPR) expression in non–small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC). There was no significant difference in GRPR expression between NSCLC and SCLC.
moderate 15.09%, and strong 7.54%). The absence of GRPR was seen in 39.39% of male and 38.67% of female subjects. The differences in GRPR expression between male and female subjects were not statistically significant ($P = .91$; Figure 6).

Among 238 subjects, 200 were smokers, 8 were non-smokers, and 30 had unknown smoking status. Because of the low number of nonsmoker subjects, it was not possible to correlate GRPR expression with smoking status.

### GRPR Expression and Survival

Two hundred thirty-eight patients were evaluated for overall survival until mortality or the date of the last follow-up visit if the patients were still alive, with an average follow-up of 15.2 months. The median overall survival time was 8.97 months (95% confidence interval, 5.6–12.3 months) for absent GRPR expression, 11.50 months (95% confidence interval, 3.6–19.3 months) for weak and moderate expression, and 10.6 months (95% confidence interval, 0.4–20.7 months) for strong GRPR expressers ($P = .77$). The Kaplan-Meier curve was adjusted for clinical stage (Figure 7).

### COMMENT

It has been demonstrated that GRPR activates mitogen-activated protein kinase signaling pathway primarily through EGFR activation and that combining GRPR antagonist with EGFR tyrosine kinase inhibitors results in additive cytotoxic effect in tumor cell lines. Gastrin-releasing peptide receptor synthetic antagonists, such as RC3095, have been developed as anticancer candidates, and have shown antitumor activity in both in vivo and in vitro murine and human tumor models, producing long-lasting tumor regression. In 25 patients with advanced refractory solid malignancies enrolled in a phase I trial of RC-3095 conducted at Hospital das Clínicas, in Porto Alegre, Brazil, RC-3095 was well tolerated.

Gastrin-releasing peptide receptor was originally isolated in SCLC by Cuttitta et al, and most previous studies have reported GRPR expression in up to 70% of small cell lung cancer and 10% to 20% in NSCLC. Conversely, in our study we noted GRPR expression in NSCLC and SCLC at similar rates of 62.51% and 52.62% respectively ($P = .30$). Among NSCLC cases, adenocarcinoma subjects tended to be GRPR overexpressers at a rate of 68% (41.5% weak expression, 13.5% moderate expression, and 7.5% strong expression) compared with other NSCLC cases ($P = .67$). Although we acknowledge that the differences seen in our study were not statistically significant, this pattern of GRPR upregulation may indicate that GRPR represents a future target for adjuvant therapy in lung cancer, particularly in adenocarcinoma cases.

The association between GRPR expression, smoking status, and sex is not clear. Some studies suggest that the GRPR gene is expressed more frequently in females than in males in the absence of smoking and that expression is activated earlier in females in response to tobacco exposure. On the other hand, GRP expression has also been reported to be increased with tobacco use. Higher levels of GRP protein have been detected in bronchoalveolar lavage and urine specimens of asymptomatic long-standing smokers when compared with nonsmoking control subjects. Although our study is limited with respect to smoking status because of a low number of nonsmoker subjects, we noticed no difference in GRPR expression between males and females, suggesting that GRPR plays a similar role in development of lung cancer independent of sex.

Gastrin-releasing peptide receptor expression was noted in bronchial epithelium and submucosal gland epithelial cells (not quantified) from small cell and non–small cell...
carcinoma cases. The role of extratumoral GRPR expression is unclear. However, Egloff and colleagues\(^2\) evaluated GRPR mRNA levels in histologically normal bronchial epithelial cells from 224 lung cancer patients and 107 surgical cancer-free controls and demonstrated that GRPR expression in noncancerous bronchial epithelium was significantly associated with lung cancer independent of age, sex, or smoking status. Egloff et al\(^2\) showed a 71% rate of GRPR expression in nonneoplastic bronchial cells from lung cancer samples compared with only 38% of cancer-free controls, suggesting that increased GRPR expression in normal epithelial mucosa may be an early event in lung cancer formation.

Gastrin-releasing peptide receptor overall percentage and intensity of tumor cell expression staining were more pronounced in clinical stages III and IV and extensive disease compared with GRPR expression in clinical stages I and II. These differences were statistically significant \(P = .01\) and were independent of tumor cell type, sex, and survival. The impact and implications of increased GRPR expression in patients with advanced lung disease deserve future studies and may indicate that GRPR may be a potential therapeutic target in this population.

To our knowledge, this study is the first to correlate GRPR tissue expression and distribution with the clinical stages of lung cancer in a large small cell and non–small cell population. We detected GRPR expression in NSCLC and SCLC at similar rates. Adenocarcinomas and advanced lung cancer disease subjects tended to be GRPR overexpressers, suggesting that GRPR may be a potential future therapeutic target in this population. The GRPR pathway is known to interact with the EGFR pathway in lung cancer cells by increasing the release of EGF proligands, which could act to further promote cancer in patients who develop EGFR mutations. Molecular profiling of lung cancer patients with GRPR upregulation deserves future study.

**References**


![Figure 7. Overall survival of lung cancer and gastrin-releasing peptide receptor (GRPR) expressers calculated using the Kaplan-Meier survival curves. There was no significant correlation with survival and GRPR expression.](http://example.com/figure7.png)


