Gastrin-Releasing Peptide Receptor-Mediated Autocrine Growth in Squamous Cell Carcinoma of the Head and Neck

Miriam N. Lango, Kevin F. Dyer, Vivian Wai Yan Lui, William E. Gooding, Christopher Gubish, Jill M. Siegfried, Jennifer Rubin Grandis

Background: Gastrin-releasing peptide receptor (GRPR)-mediated autocrine growth appears to be an early marker of susceptibility to tobacco-related lung cancers. Because expression of GRPR, however, has not been reported in squamous cell carcinoma of the head and neck (SCCHN), we investigated its expression and that of its ligand GRP in normal mucosa and SCCHN tissues and the involvement of these proteins in the proliferation of SCCHN cells. Methods: We assessed GRPR messenger RNA (mRNA) expression in specimens from 25 patients with SCCHN, six control noncancer patients, and 14 SCCHN cell lines by use of quantitative reverse transcriptase-polymerase chain reaction. We used neutralizing GRP monoclonal antibody 2A11 to block the GRP–GRPR interaction in SCCHN cell lines and xenografts and assessed the antibody’s effect on proliferation by counting cultured cells or measuring xenograft tumor volume in vivo. All statistical tests were two-sided. Results: Tumor and mucosa tissues, respectively, from SCCHN patients expressed sixfold and fourfold higher levels of GRPR mRNA than normal mucosa tissue from noncancer patients (P < .001). The levels of GRPR expression in the tumor and adjacent normal epithelium of individual patients with SCCHN were correlated (r = .652; P = .001), suggesting that increased GRPR expression is an early event in SCCHN formation. SCCHN cells expressed fivefold higher levels of GRPR mRNA than did cultured normal mucosal epithelial cells (P = .005). GRP stimulated proliferation of SCCHN cells in a dose-dependent fashion (P = .006). Neutralizing GRP monoclonal antibody 2A11 inhibited SCCHN cell proliferation in vitro and in vivo. Median survival was 54 months in patients with higher levels of GRPR mRNA and was not reached in those with lower levels. Conclusions: GRP and GRPR appear to participate in an autocrine regulatory pathway in SCCHN. Thus, strategies that specifically target GRP and/or GRPR may be effective therapeutic approaches for this disease. [J Natl Cancer Inst 2002;94:375–83]
factor for the development of squamous cell carcinoma of the head and neck (SCCHN), to our knowledge, expression of neither GRP nor GRPR in SCCHN has been reported previously. To investigate whether GRPR-mediated autocrine growth is involved in SCCHN carcinogenesis, we investigated whether GRPR stimulated growth in SCCHN cell lines and tumors, whether GRPR messenger RNA (mRNA) was expressed in SCCHN tissues and normal control mucosa, and whether blocking the interaction of GRP and GRPR affected the growth of SCCHN cells in vitro and in vivo.

**Materials and Methods**

**Patients and Tissue Samples**

Tumor tissue was collected between 1990 and 1995 at the University of Pittsburgh Medical Center (PA) from 25 patients with SCCHN who were undergoing surgical resection with curative intent and had adequate tissue for analysis. A sample of normal-appearing mucosa at the margin of the surgical resection several centimeters from the tumor was also collected and designated histologically normal mucosa. Patients with pathologically confirmed SCCHN of the upper aerodigestive tract (oral cavity, oropharynx, hypopharynx, or larynx) were eligible. Tissue was collected under the auspices of a tissue-bank protocol that was approved by the Institutional Review Board. Written informed consent was obtained from all patients. Clinical characteristics of the SCCHN patients are presented in Table 1. Previous radiation therapy failed for four patients. Of the 19 patients whose smoking histories were available, 18 patients were current smokers or former smokers and one female with an oral cavity cancer was a nonsmoker. The six female smokers in our study reported a mean smoking history of 20 pack-years, and the 13 male smokers reported a mean smoking history of 80 pack-years. Pack-years are defined as the number of packs smoked per day multiplied by the number of years over which that amount was smoked. Eight patients were alive with no evidence of disease on follow-up from 39 to 91 months after surgical resection. Eleven patients died of their disease, either from recurrence or second primary SCCHN tumors, five patients died of other causes, and one patient was lost to follow-up. The median follow-up for surviving patients was 55 months. The median survival of patients who died of disease was 20 months. Mucosa from six control noncancer patients who did not have SCCHN was also harvested. These control patients underwent unrelated head and neck surgical procedures (e.g., uvulopalatopharyngoplasty). Patients were matched with the study group with respect to age (mean age ± 5 years) and sex.

**Cells and Tumors**

Fourteen cell lines derived from patients with SCCHN were grown in Dulbecco’s modified Eagle medium (DMEM; Cellgro, Washington, DC), supplemented with 12% fetal bovine serum (Life Technologies, Inc. [GIBCO BRL], Rockville, MD), penicillin (0.5 μg/mL), and streptomycin (0.5 μg/mL; Life Technologies, Inc.). These cell lines are part of a large collection established in the Department of Otolaryngology, University of Pittsburgh (15). Cell lines UM-22A and UM-22B were derived from a primary SCCHN (22A) and a metastatic cervical lymph node (22B) (16). Cell line 1483 was derived from an oropharyngeal tumor (17). SCCHN cell lines UPCI:SCC066, UPCI:SCC078, UPCI:SCC111, UPCI:SCC105, UPCI:SCC068, UPCI:SCC104, UPCI:SCC143, UPCI:SCC099, UPCI:SCC089, UPCI:SCC149, UPCI:SCC117, UPCI:SCC049, UPCI:SCC105, and UPCI:SCC107 were purchased from the American Type Culture Collection (Manassas, VA). Twenty-four cell lines were derived from the University of Pittsburgh Cancer Institute (UPCI). The SCCHN cell lines UPCI:SCC066, UPCI:SCC078, UPCI:SCC111, UPCI:SCC105, UPCI:SCC068, UPCI:SCC104, UPCI:SCC143, UPCI:SCC099, UPCI:SCC089, UPCI:SCC149, UPCI:SCC117, UPCI:SCC049, UPCI:SCC105, and UPCI:SCC107 were purchased from the American Type Culture Collection (Manassas, VA). Twenty-four cell lines were derived from the University of Pittsburgh Cancer Institute (UPCI:SCC066, UPCI:SCC078, UPCI:SCC111, UPCI:SCC105, UPCI:SCC068, UPCI:SCC104, UPCI:SCC143, UPCI:SCC099, UPCI:SCC089, UPCI:SCC149, UPCI:SCC117, UPCI:SCC049, UPCI:SCC105, and UPCI:SCC107).

**Table 1.** Clinicopathologic characteristics of patients undergoing resection for squamous cell carcinoma of the head and neck*

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, y</th>
<th>Sex</th>
<th>Site</th>
<th>Stage†</th>
<th>Grade‡</th>
<th>ECS</th>
<th>Patient status</th>
<th>Follow-up, months§</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>55</td>
<td>M</td>
<td>Larynx</td>
<td>T3 N2c M0</td>
<td>Moderate</td>
<td>+</td>
<td>DOD</td>
<td>54</td>
</tr>
<tr>
<td>2</td>
<td>57</td>
<td>M</td>
<td>Larynx</td>
<td>T4 N2 M0</td>
<td>Moderate</td>
<td>NA</td>
<td>DOD</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>48</td>
<td>M</td>
<td>Larynx</td>
<td>T3 N3 M0</td>
<td>Moderate</td>
<td>+</td>
<td>DOD</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>56</td>
<td>M</td>
<td>Larynx</td>
<td>T3 N2c M0</td>
<td>Moderate</td>
<td>+</td>
<td>DOD</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>70</td>
<td>M</td>
<td>Hypopharynx</td>
<td>T4 N0 M0</td>
<td>Moderate</td>
<td>+</td>
<td>DOC</td>
<td>45</td>
</tr>
<tr>
<td>6</td>
<td>72</td>
<td>M</td>
<td>Larynx</td>
<td>T4 N2c M0</td>
<td>Moderate</td>
<td>–</td>
<td>DOD</td>
<td>14</td>
</tr>
<tr>
<td>7</td>
<td>55</td>
<td>M</td>
<td>Larynx</td>
<td>T3 N0 M0</td>
<td>Well</td>
<td>–</td>
<td>NED</td>
<td>48</td>
</tr>
<tr>
<td>8</td>
<td>64</td>
<td>F</td>
<td>Larynx</td>
<td>T2 N0 M0</td>
<td>Moderate</td>
<td>–</td>
<td>NED</td>
<td>39</td>
</tr>
<tr>
<td>9</td>
<td>67</td>
<td>M</td>
<td>Larynx</td>
<td>T2 N0 M0</td>
<td>Moderate</td>
<td>–</td>
<td>NED</td>
<td>53</td>
</tr>
<tr>
<td>10</td>
<td>38</td>
<td>F</td>
<td>Oropharynx</td>
<td>T3 N0 M0</td>
<td>Well</td>
<td>ND</td>
<td>NED</td>
<td>55</td>
</tr>
<tr>
<td>11</td>
<td>58</td>
<td>M</td>
<td>Larynx</td>
<td>T4 N0 M0</td>
<td>Poor</td>
<td>–</td>
<td>DOD</td>
<td>20</td>
</tr>
<tr>
<td>12</td>
<td>82</td>
<td>M</td>
<td>Larynx</td>
<td>T2 N2 M0</td>
<td>Moderate</td>
<td>ND</td>
<td>DOC</td>
<td>95</td>
</tr>
<tr>
<td>13</td>
<td>59</td>
<td>F</td>
<td>Oral cavity</td>
<td>T2 N0 M0</td>
<td>Well</td>
<td>ND</td>
<td>DOD</td>
<td>64</td>
</tr>
<tr>
<td>14</td>
<td>71</td>
<td>M</td>
<td>Oral cavity</td>
<td>T4 N0 M0</td>
<td>Well</td>
<td>–</td>
<td>NED</td>
<td>58</td>
</tr>
<tr>
<td>15</td>
<td>57</td>
<td>M</td>
<td>Larynx</td>
<td>T2 N0 M0</td>
<td>Moderate</td>
<td>–</td>
<td>DOC</td>
<td>66</td>
</tr>
<tr>
<td>16</td>
<td>53</td>
<td>F</td>
<td>Hypopharynx</td>
<td>T1 N1 M0</td>
<td>Moderate</td>
<td>–</td>
<td>NED</td>
<td>91</td>
</tr>
<tr>
<td>17</td>
<td>83</td>
<td>F</td>
<td>Oral cavity</td>
<td>T3 N0 M0</td>
<td>Well</td>
<td>ND</td>
<td>DOC</td>
<td>47</td>
</tr>
<tr>
<td>18</td>
<td>69</td>
<td>M</td>
<td>Larynx</td>
<td>T4 N0 M0</td>
<td>Moderate</td>
<td>–</td>
<td>NED</td>
<td>57</td>
</tr>
<tr>
<td>19</td>
<td>74</td>
<td>M</td>
<td>Larynx</td>
<td>T4 N1 MX</td>
<td>NA</td>
<td>NA</td>
<td>DOD</td>
<td>24</td>
</tr>
<tr>
<td>20</td>
<td>70</td>
<td>M</td>
<td>Larynx</td>
<td>T2 N0 M1</td>
<td>Moderate</td>
<td>–</td>
<td>DOC</td>
<td>47</td>
</tr>
<tr>
<td>21</td>
<td>44</td>
<td>F</td>
<td>Larynx</td>
<td>T2 N1 M0</td>
<td>Moderate</td>
<td>–</td>
<td>DOD</td>
<td>3</td>
</tr>
<tr>
<td>22</td>
<td>66</td>
<td>M</td>
<td>Hypopharynx</td>
<td>T4 N2c M0</td>
<td>Moderate</td>
<td>+</td>
<td>DOD</td>
<td>12</td>
</tr>
<tr>
<td>23</td>
<td>65</td>
<td>M</td>
<td>Oral cavity</td>
<td>T2 N0 M0</td>
<td>Moderate</td>
<td>–</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>24</td>
<td>55</td>
<td>F</td>
<td>Hypopharynx</td>
<td>T4 N1 M0</td>
<td>Well</td>
<td>–</td>
<td>NED</td>
<td>72</td>
</tr>
<tr>
<td>25</td>
<td>66</td>
<td>M</td>
<td>Oropharynx</td>
<td>T2 N0 MX</td>
<td>Poor</td>
<td>–</td>
<td>DOD</td>
<td>103</td>
</tr>
</tbody>
</table>

* M = male; F = female; T = tumor; N = node; M = metastasis; X = cannot be determined; NA = not available; ECS = presence of extracapsular spread; ND = neck dissection not done; DOD = dead of disease; NED = no evidence of disease; DOC = dead of other causes; + = ECS noted; – = no evidence of ECS.
†TNM staging (14).
‡Grade: well differentiated, moderately differentiated, or poorly differentiated.
§Clinical follow-up period in months after surgical resection.
and UPCI:SCC103 were gifts from Dr. Susanne M. Gollin (University of Pittsburgh Graduate School of Public Health, PA). Cell line OSC-19 was derived from a squamous cell carcinoma of the tongue (18). Primary mucosal cultures established from oropharyngeal mucosa harvested from control noncancer patients were used within the first three passages, as described previously (19). Cell line 201T is an NSCLC cell line that was used as a positive control for GRPR immunoblotting.

RNA Extraction and Quantitative Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was extracted from primary tissues and SCCHN cell lines with TRIZOL reagent (Life Technologies, Inc.), as described by the manufacturer. GRPR was amplified in a one-step reverse transcriptase–polymerase chain reaction (RT–PCR) with Superscript/Taq enzyme (Life Technologies, Inc.) and 0.05 μg of total RNA in each reaction. The sequence of the 5’ GRPR primer was 5’-CTCCCCGTGAAAGATGACTTG-3’, and the sequence of the 3’ GRPR primer was 5’-ATCTTTCATGGGGCATGGAG-3’. This reaction gave a 390-base-pair (bp) PCR product. The sequence of the 5’ β-actin primer was 5’-GGCCGCACACCATGTACCT-3’, and the sequence of the 3’ β-actin primer was 5’-AGGGCGCGACTCGTCACTAT-3’. This reaction gave a 202-bp PCR product. The sequence of the 5’ GRPR primer was 5’-GGGACCAGTGCGGCCAGTG-3’, and the sequence of the 3’ primer was 5’-TGCAAGGAAATTGTGGCTCT-3’. This reaction gave a 405-bp product. RT–PCR conditions for GRP were identical to those used for GRPR. The RT reaction was carried out at 50°C for 30 minutes, followed by PCR for 40 cycles at 95°C for 30 seconds, 58°C for 45 seconds, and 72°C for 45 seconds. A semiquantitative RT–PCR protocol for GRPR mRNA quantitation was developed in our laboratory. Expression levels were based on a ratio of GRPR or GRP mRNA expression to β-actin mRNA expression. Preamplification of GRPR or GRP for 17 cycles before adding (“dropping in”) a 5’ β-actin primer was followed by coamplification for an additional 18 cycles. The preamplification was determined empirically but required both PCR products to be in the linear phase of amplification. Deoxycytidine 5’-32P)triphosphate was incorporated into the PCR products. The products were separated by electrophoresis on a 7.5% polyacrylamide gel, and the gel was dried onto Whatman paper. The gel was then exposed to X-Omat film (Eastman Kodak, Rochester, NY) and subsequent analysis was performed on a phosphorimager (Molecular Dynamics, Sunnyvale, CA) and analyzed by ImageQuant software (Molecular Dynamics) as described previously (20).

Sequencing

The amplified GRPR PCR product from a representative cell line, UM-22B, was isolated from the agarose gel and purified by use of a Qiagen DNA purification column (Qiagen, Valencia, CA), as described by the manufacturer. Ten nanograms of PCR product was cycle sequenced (AmpliTaq; PerkinElmer, Foster City, CA) with 3.4 pmol of 3’ GRPR primer in an automated sequencing reaction and an ABI Prism 377 sequencer. Base-specific, laser-induced fluorescent emissions data were collected and processed by computer software after dye-labeled fragments were separated by size on a polyacrylamide gel.

Western Blotting

Lysis buffer (1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 10 mM sodium phosphate [pH 7.2], aprotinin [10 μg/mL], and leupeptin [10 μg/mL]) was used to extract total cellular protein. Cells were incubated with the lysis buffer for 5 minutes on ice, and the lysate was centrifuged for 30 minutes at 12,000g. The protein concentration in the supernatant was measured with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Fifteen micrograms of total protein was loaded onto a sodium dodecyl sulfate-10% polyacrylamide gel. After electrophoresis, the protein was transferred to a Protran membrane (Schleicher & Schuell, Keene, NH) for immunoblot analysis. GRPR antiserum was generated as described previously (21).

Growth Stimulation by GRP

The 1483 SCCHN cells were plated at 2.5 × 10⁶ cells per well in a 24-well plate in serum-free DMEM and incubated overnight. Recombinant GRP (Sigma-Aldrich, St. Louis, MO) in 0.01 M glacial acetic acid (Sigma-Aldrich) was added to the cells as indicated. Cells were then incubated at 37°C in an atmosphere of 5% CO₂/95% air. After 48 hours, a vital dye (erythrosin B) was added, and living cells, excluding the dye, were counted.

In Vitro Studies With Anti-GRP Antibody 2A11

The 1483 SCCHN cells were plated at 6 × 10⁶ cells per well in a 24-well plate in DMEM supplemented with 12% fetal bovine serum, 1% penicillin, and 1% streptomycin. Twenty-four hours later, cells were treated with either 50 μg or 100 μg of GRP-neutralizing antibody 2A11 (Abbott Biotech, Inc., Needham Heights, MA) (in 100 μL) or an equivalent volume of saline (100 μL) in DMEM supplemented with 12% fetal bovine serum, 1% penicillin, and 1% streptomycin. All assays were performed in triplicate. The GRP-neutralizing antibody binds to the carboxyl-terminal end of GRP and neutralizes the biologic effects of GRP. Every 48 hours for 8 days, cells were then trypsinized, resuspended, and counted with a hemocytometer by vital dye exclusion.

In Vivo Tumor Xenograft Studies

The 1483 SCCHN cells grow well as xenografts in nude mice. For subcutaneous implantation into mice, cells 75%–80% confluent were harvested by trypsinization, resuspended in DMEM supplemented with 10% fetal bovine serum, centrifuged at 400g for 10 minutes, and resuspended in culture medium at 1 × 10⁶ cells per milliliter. Female athymic nude mice (nu/nu, 4–6 weeks old; 20 ± 2 g [mean ± standard deviation]; Harlan Sprague-Dawley, Inc., Indianapolis, IN) were implanted with 1 × 10⁶ cells into the right flank and 1 × 10⁶ cells into the left flank in volumes of 200 μL by using a 26-gauge needle and a 1-mL tuberculin syringe. One day later, mice were randomly assigned to a treatment group of 0.5 mg of 2A11 antibody per dose or no treatment. In each experiment, each treatment group had 8–10 mice. Experiments were repeated three times to ensure reproducibility. Anti-GRP-neutralizing monoclonal antibody 2A11 was administered intraperitoneally twice a week for 5 weeks, for a total of 10 doses. Treatment was initiated 24 hours after tumor cell implantation. Bilateral tumors were measured with calipers before each injection (twice a week), and the average tumor volume for each mouse was calculated (tumor vol-
Two independent studies were considered for the statistical analysis. In the first study, the relationship between GRPR expression and tumor progression was assessed using a chi-square test. In the second study, the association between GRPR expression and patient survival was evaluated using a log-rank test. The results indicated a statistically significant difference in GRPR expression between primary tumors and normal mucosal epithelial cells. The mean GRPR expression in primary tumors was 0.411, while in normal mucosal epithelial cells, it was 0.063. These findings suggest that GRPR expression is significantly higher in primary tumors compared to normal mucosal epithelial cells.

Increased GRPR Expression in SCCHN Cell Lines

When 14 SCCHN cell lines were examined for GRPR mRNA expression, all lines expressed GRPR mRNA. The amplified GRPR fragment from a representative cell line (UM-22B) was isolated and sequenced to confirm the presence of GRPR mRNA (data not shown). No evidence of mutations was found in the amplified sequence from UM-22B cells. Polymorphisms of the GRPR gene have been previously reported, but it is not known whether these polymorphisms segregate to cancer patients (12). When GRPR expression levels (reported as a ratio of GRPR mRNA to β-actin mRNA) in these 14 SCCHN cell lines were compared with levels in primary cultures of normal mucosal epithelial cells from six noncancer patients, steady-state levels of GRPR mRNA expression were fivefold higher in the SCCHN cells, indicating that GRPR is expressed by squamous epithelial cells and is expressed at an increased level in transformed cells. The results showed that the expression of GRPR in SCCHN cells is significantly higher than in normal mucosal epithelial cells.

GRP Expression in SCCHN Cells

To determine whether an autocrine pathway involving GRP and GRPR was active in SCCHN, SCCHN cell lines were examined for GRP expression using a quantitative RT–PCR assay. The level of GRP expression was higher in SCCHN cell lines than in normal mucosal epithelial cells. To investigate whether stimulation of GRPR induces SCCHN cells to proliferate, exogenous GRP was added to cultured SCCHN cells in the absence or presence of 12% fetal bovine serum. Linear regression analysis revealed that the logarithm of the dose of GRP (0 to 0.188, 95% CI 0.063 to 0.165; P = .006) was positively correlated with the logarithm of cell count on day 8. After 8 days, proliferation of 1483 SCCHN cells was reduced by anti-GRP 2A11 in a dose-dependent manner compared with untreated cells (Jonckeere–Terpstra test, P = .001; Fig. 2, A). An isotype-matched IgG1 control mouse monoclonal antibody had no effect on the growth rate of these cells (data not shown).

Antitumor Effect of GRP-Blocking Antibody In Vitro and In Vivo

To determine whether disruption of the GRP/GRPR autocrine pathway in vitro would affect SCCHN tumor growth, 1483 SCCHN cells were treated with murine GRP-neutralizing monoclonal antibody 2A11, which binds the carboxyl-terminal region of the GRP peptide and functionally sequesters GRP (13). After 8 days, proliferation of 1483 SCCHN cells was reduced by anti-GRP 2A11 in a dose-dependent manner compared with untreated cells (Jonckeere–Terpstra test, P = .001; Fig. 2, A). An isotype-matched IgG1 control mouse monoclonal antibody had no effect on the growth rate of these cells (data not shown).

An in vivo model, nude mice bearing SCCHN xenografts, was then used to examine the effect of GRP-neutralizing antibody 2A11. Mice, bearing two xenografts each, were treated with GRP-neutralizing antibody 2A11 (0.5 mg or 1.0 mg in 200 μL given intraperitoneally twice a week for 5 weeks), beginning 1 day after implantation of the SCCHN cells. Average tumor volumes in antibody-treated mice were smaller than those in control mice (data not shown).
control mice (Fig. 2, B) by the time tumors were measurable, i.e., at approximately 14 days after tumor cell inoculation. The reduction in tumor volume was the same for groups treated with 0.5 mg or 1.0 mg of antibodies. Control mice had tumors with statistically significantly greater volumes than mice treated with either dose of the GRP-neutralizing antibody (0.5 mg, $P = .001$; 1 mg, $P = .006$). There was no statistically significant interaction between treatment group and day of tumor measurement ($P = .884$); thus, GRP/GRPR autocrine stimulation may be important in promoting cell proliferation and SCCHN tumor growth.

**Elevated GRPR Expression in the Normal Mucosa of Patients with SCCHN**

Because of the high incidence of second primary tumors in patients with SCCHN, alterations detected in the histologically normal mucosa from these patients may provide indirect evidence of early changes in SCCHN carcinogenesis. Consequently, we determined levels of GRPR mRNA expression in primary tumor tissues and adjacent normal mucosa from patients with SCCHN and found that the levels of GRPR mRNA in these tissues correlated with each other (Spearman correlation coefficient = .652, $P = .001$). These results support the concept of field cancerization and suggest that increased GRPR expression is an early event in SCCHN formation.

**GRPR Expression and Clinical and Pathologic Parameters**

Metastasis to the lymph nodes of the neck is the strongest predictor of poor outcome in patients with SCCHN. In this study, such patients had a poorer outcome than did those without clinical or pathologic evidence of such metastasis. All patients with extracapsular invasion of the cervical lymph nodes died of their disease, consistent with reports that extracapsular spread is associated with poor prognosis (22–24). The level of GRPR mRNA in primary tumor tissues from patients with SCCHN was not associated with tumor stage, tumor recurrence, or a history of prior radiation therapy. Elevated levels of GRPR mRNA in the SCCHN tumor were associated with a distal aerodigestive tract site ($P = .014$). Although there were few proximal tumors for comparison, tumors in the oral cavity had lower levels of GRPR mRNA expression than did tumors in more distal aerodigestive tract sites, such as the oropharynx, hypopharynx, or larynx. We observed a trend toward increased levels of GRPR mRNA expression in tumors with increased lymph node stage ($P = .077$). Although most patients had well-differentiated tumors, there was an association between tumor grade and level of GRPR.
expression (P = .037). The few patients with extracapsular spread after pathologic examination of the cervical lymph nodes had high levels of GRPR in their primary tumor (P = .026) and in their histologically normal mucosa (P = .034) (Fig. 3).

Our data suggest that an increased level of GRPR mRNA expression in SCCHN tissue is associated with an adverse outcome for the patient. Of 24 patients with survival information, 13 were alive with a median follow-up of 55 months. The median survival for the cohort was 84 months with a lower 95% confidence band of 54 months; there were too few events to estimate the upper confidence limit. The median survival in months after surgical resection of patients with low levels of GRPR expression (below the median) in the tumor and normal mucosa had not been reached when the data were analyzed, whereas median survivals of 54 months or 64 months were found for SCCHN patients with elevated levels of GRPR expression (above the median) in tumor or normal mucosa, respectively. Median levels of GRPR mRNA in the tumor and normal specimens were 0.421 and 0.740 (expressed as GRPR mRNA levels relative to β-actin mRNA levels), respectively, in patients who succumbed to disease and were 0.309 and 0.430 (expressed as GRPR mRNA levels relative to β-actin mRNA levels) in patients who remained free of disease.

To determine whether levels of GRPR mRNA in patients with SCCHN were associated with overall survival, Kaplan–Meier analyses were performed for 24 patients with complete follow-up and vital status information. Levels of GRPR mRNA suggested a tendency toward decreased survival but with insufficient power to detect differences (Fig. 4).

**DISCUSSION**

Previous studies have suggested that GRP and its receptor, GRPR, are early markers of susceptibility to tobacco-related cancers including NSCLC. Although tobacco exposure is a well-known risk factor for the development of SCCHN, to our knowledge, the role of GRP and GRPR in SCCHN have not been examined previously. In this study, we have observed that aero-
digestive tract tissues (i.e., primary tumor and adjacent histologically normal mucosa) from patients with SCCHN have higher levels of GRPR mRNA than do the normal mucosa from noncancer patients. Evaluation of SCCHN cell lines for the expression of GRP and GRPR mRNAs confirmed that all SCCHN cell lines expressed both ligand and receptor. Overexpression of GRP and GRPR by the same SCCHN cells suggests that GRP and GRPR participate in an autocrine regulatory pathway in SCCHN. Increased expression of GRPR in the tumor and surrounding histologically normal mucosa provides molecular evidence for the concept of field cancerization as described previously by Slaughter (25). Genetic alterations in histologically normal but “condemned” mucosa in the tumor field have been associated with tumor recurrence that may be predicted by analysis of surgical margins with sensitive molecular techniques (26). The high correlation between overexpression of GRPR in specimens of tumor and normal-appearing mucosa from the same patient with SCCHN raises the possibility that sampling the mucosa at some distance from the lesion may provide useful information about the biology of the tumor (e.g., it may predict lymph node involvement or invasion) and potentially guide therapy.

The level of GRPR mRNA expression in cultured bronchial epithelial cells from smokers has been shown previously to correlate with smoking history (11). There is further evidence that regulation of GRPR expression is sex specific. Female smokers with NSCLC had higher levels of GRPR mRNA expression at a lower mean number of pack-years of tobacco exposure than male smokers, and female nonsmokers with NSCLC had higher levels of GRPR expression than their nonsmoking male counterparts (12). The increased susceptibility of women to the carcinogenic effects of tobacco has been attributed to higher levels of GRPR in female patients when stratified for smoking history (12, 27–29). The increased baseline levels of GRPR in females has been attributed to the location of the GRPR gene in a pseudoautosomal region on the X chromosome that escapes inactivation when the rest of the X chromosome is inactivated. Sex differences also exist in the risk for head and neck cancer, with females at higher risk than males when stratified for tobacco use, suggesting that a relationship among sex, tobacco exposure, and level of GRPR mRNA expression may increase the risk of developing SCCHN. We found that the level of GRPR mRNA expression correlated with the site of the aerodigestive tract tumor. Tumors from more distal locations including the oropharynx, hypopharynx, and larynx had higher levels of GRPR expression than tumors from the oral cavity. One potential explanation for this finding may be the closer link of tumor formation in distal aerodigestive tract sites to tobacco use, whereas the more proximal sites, including the oral tongue, buccal mucosa, and hard palate, have been linked to other etiologic agents, such as human papillomavirus (31). Alternatively, tobacco-mediated induction of GRP expression in neuroendocrine cells in distal regions of the aerodigestive tract may stimulate tumor growth through a paracrine mechanism. Our results—that both GRP and GRPR mRNAs are overexpressed in SCCHN cell lines, that exogenous GRP stimulates growth, and that anti-GRP-neutralizing monoclonal antibodies inhibit cell proliferation in vitro and in vivo—are consistent with a GRP/GRPR autocrine growth pathway in SCCHN. However, the mechanism of GRP-mediated mitogenesis may be more complex in vivo than in vitro. Tobacco appears to induce GRP expression in pulmonary neuroendocrine cells. Exposure of hamsters to tobacco smoke causes hyperplasia of pulmonary neuroendocrine cells and correlates with increased levels of GRP in these animals (2). In another study, induction of pulmonary neuroendocrine cell hyperplasia in an animal model of preneoplastic lung injury was associated with increased levels of GRP mRNA expression by pulmonary neuroendocrine cells (32). Although SCCHN cells can promote their own growth through an autocrine mechanism in vitro, paracrine-mediated stimulation of SCCHN growth by neuroendocrine cells from distal aerodigestive tract sites may also contribute to tumor growth in vivo.

When human tumor cells express high levels of growth factor receptors, receptor-directed therapies may be useful anticancer strategies. Expression of bombesin-like peptide ligands and receptors has been reported in several cancers including glioblastomas (33) and carcinomas of the ovary (34), colon (35), kidney (36), breast (37), and lung (10). Mammalian forms of bombesin-like peptides have been identified as mitogens and morphogens for a variety of cancers, including NSCLC. Although classically associated with cells of neuroendocrine origin, several transformed cell types, including airway epithelial cells, respond to bombesin-like peptides (38). Immortalized human bronchial epithelial cells engineered to overexpress GRPR responded to exogenous bombesin by increasing...
calcium influx and cell proliferation (39). We reported previously that GPRP expression in lung epithelium was associated with a history of prolonged tobacco exposure and responsiveness to the mitogenic effects of bombesin-like peptides (11). In an animal model of chemical-induced oral cancer, a bombesin antagonist prevents the formation of preinvasive mucosal lesions (40). Many studies have demonstrated the antitumor effect of GRPR-specific inhibitors in preclinical animal models (41,42). A phase I clinical trial using the anti-GRP antibody 2A11 was conducted in patients with SCLC or NSCLC, with no evidence of toxicity noted (13). An antitumor effect was observed with this anti-GRP antibody in patients with SCLC where one of 12 patients who could be evaluated was disease-free for 4 months (43). In this study, we have demonstrated that treatment with anti-GRP antibody 2A11 inhibited the growth of SCCHN cells in vitro and in vivo. Therefore, strategies that specifically target GRP and/or GRPR may prove to be effective therapies for SCCHN.

REFERENCES


Fig. 4. Disease-specific survival among 24 patients with squamous cell carcinoma of the head and neck (SCCHN). The patients were divided into two groups by the median level of gastrin-releasing peptide receptor (GRPR) mRNA in the SCCHN tumor (panel A) or in the histologically normal mucosa (panel B) (P = .309 and .430, respectively; median split method). Vertical lines denote 95% confidence intervals at 2 and 5 years. The number of patients at risk in each group at 2 and 5 years is as follows: survival by GRPR mRNA in the tumor at 2 years: above median = 7; below median = 10. Survival by GRPR mRNA in the tumor at 5 years: above median = 2; below median = 4. Survival by GRPR mRNA in normal mucosa at 2 years: above median = 8; below median = 9. Survival by GRPR mRNA in normal mucosa at 5 years: above median = 3; below median = 3. All statistical tests were two-sided.


**NOTES**

Supported by grant U01CA84968 (to J. M. Siegfried and J. R. Grandis) from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

Manuscript received May 4, 2001; revised January 3, 2002; accepted January 22, 2002.