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Elevated gastrin-releasing peptide receptor mRNA expression in buccal mucosa: association with head and neck squamous cell carcinoma

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Abstract

Background—Expression of gastrin-releasing peptide receptor (GRPR) is elevated in mucosa adjacent to head and neck squamous cell carcinoma (HNSCC) compared with mucosa from cancer-free controls, suggesting elevated GRPR expression may indicate presence of HNSCC.

Methods—We measured *GRPR* mRNA levels in histologically normal buccal mucosa from 65 surgical patients with HNSCC and 75 cancer-free control subjects using quantitative polymerase chain reaction (PCR). We tested for association between *GRPR* expression and HNSCC and evaluated differences in patient progression-free survival (PFS).

Results—Buccal *GRPR* expression was higher in cases but not controls who were active smokers ($p = .04$). High *GRPR* expression was associated with HNSCC (odds ratio [OR] = 3.55; 95% confidence interval [CI] = 1.15–10.93), even after adjustment for age, sex, tobacco use, and sample storage time. PFS did not differ between patients with HNSCC with high versus low *GRPR* expression ($p = .22$).

Conclusion—Elevated buccal *GRPR* expression was significantly associated with HNSCC independent of known risk factors but was not an indicator of disease prognosis.

Keywords

gastrin-releasing peptide receptor; head and neck cancer; case-control study; surrogate tissue biomarker; risk factor

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC), which includes cancers arising in the oral cavity, pharynx, and larynx, share anatomical proximity, alcohol, and smoking histories as risk factors, and several associated molecular alterations. Tobacco and alcohol use are significant risk factors for HNSCC. However, approximately 20% of patients with HNSCC

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are lifetime never-smokers. More recently, human papillomavirus (HPV) has been identified as contributing to head and neck cancers especially of the oropharynx among never smokers.[1–3] Increased expression of specific growth factor receptors is common for HNSCC,[4–6] including the gene encoding the gastrin-releasing peptide receptor (*GRPR*).[7,8] However, the relationships between *GRPR* expression and the identified HNSCC risk factors have not been clearly defined.

We previously reported elevated levels of *GRPR* mRNA in HNSCC tumors and lung cancers.[7,9] *In vivo* and *in vitro* studies by our group indicate that a gastrin releasing peptide (GRP)-GRPR ligand-receptor autocrine loop is involved in HNSCC and lung cancer proliferation.[7,10] In addition to cancer-specific overexpression of *GRPR*, we demonstrated that mucosal tissues adjacent to HNSCC have *GRPR* mRNA levels reflective of the adjacent HNSCC tumor.[7] Our group also reported that *GRPR* expression in bronchial epithelium in female never-smokers was associated with a diagnosis of lung cancer.[11] These findings suggest that elevated *GRPR* mRNA in normal oral mucosa may be associated with upper aerodigestive cancer risk and/or may indicate the presence of HNSCC.

We undertook a prospective case-control study to determine whether elevated *GRPR* mRNA expression in normal buccal mucosal tissue was associated with the presence of HNSCC. Our primary finding reported here was the observed increased *GRPR* expression in histologically normal buccal mucosa from HNSCC cases compared with cancer-free controls, with higher levels of buccal *GRPR* mRNA associated with increasing risk of HNSCC. In addition, we found *GRPR* mRNA levels were elevated in patients with HNSCC who were active smokers, but *GRPR* expression in cancer-free controls did not differ by smoking status at enrollment. Multivariable logistic regression analyses indicated that elevated buccal *GRPR* expression was significantly associated with HNSCC independent of age, sex, smoking status, pack-years of tobacco use, and sample storage time. These findings suggest that elevated *GRPR* mRNA in normal oral mucosa is a candidate biomarker for the presence of HNSCC.

MATERIALS AND METHODS

Head and neck squamous cell carcinoma case-control study subjects and tissues

From 2000 to 2004, patients with pathologically-confirmed HNSCC ($n = 160$) who were to undergo surgery with curative intent were enrolled in this Early Detection Research Network–sponsored study before surgery. Community and clinic controls without cancer ($n = 186$) were also enrolled, and study participants provided informed consent, answered an administered questionnaire, and donated buccal cells in accordance with the institutional review board–approved protocol.[12] Oral squamous cell carcinoma (OSCC) tissue and paired histologically normal tissue were obtained for a subset of cases ($n = 10$) in order to assess expression of the housekeeping gene used as the reference in quantitative polymerase chain reaction (PCR) studies. All specimens were collected and stored under the auspices of the Head and Neck Tumor Bank at the University of Pittsburgh Medical Center. Subjects were included in this study if adequate buccal material was isolated for the detection of β-glueuronidase gene *(GUSβ)* expression by quantitative PCR. Of the enrolled cases and controls, 110 HNSCC cases and 158 cancer-free controls provided buccal samples of adequate quantity and quality for analysis. The case-control study populations are described in Table 1.

Quantification of GRPR, GUSB, and ACTB expression

Total RNA from buccal cells, OSCC tissues, and histologic normal oral mucosal tissues was isolated using RNeasy kits (Qiagen; Valencia, CA). cDNA was synthesized from 1 μg of

each total RNA sample using SuperScript First-Strand Synthesis System (Invitrogen; Carlsbad, CA) and random hexamer deoxynucleotides. Quantification of *GRPR* and *GUSB*, which encodes β-glucuronidase, gene expression in buccal cell-derived cDNA was performed by TaqMan real-time quantitative PCR (q-PCR) using a 7700 Sequence Detector (Applied Biosystems [Life Technologies], Carlsbad, CA) with an initial 12-minute 95°C denaturation followed by 40 cycles of 15-second 95°C denaturation and 60-second 60°C annealing and extending. PCR amplifications of *GRPR* (forward primer: 5′-CAGGAT TGGCTGCAAACTGA-3′; reverse primer: 5′-GAGGCCT GGATATCCATTGG-3′) and *GUSB* (forward primer 5′-CTCATTTGGAATTTTGCCGATT-3′; reverse primer 5′-CCGAGTGAAGATCCCCTTTTTA-3′) resulted in 121 and 81 base-pair products, respectively. Fluorescent probes (*GRPR*: 5′-CGGCAGACAGATACAA AGCCATTGTCC-3′ and *GUSB*: 5′-TGAACAGTCACCG ACGAGAGTGCTGG-3′) were labeled with 6-carboxy-fluorescein phosphoramidite at the 5′ terminus and 5-carboxy-tetramethyl-rhodamine at the 3′ terminus. The threshold cycle value for each primer-probe pair was retrieved from the TaqMan Sequence Detector (Life Technologies), the difference in threshold cyde values (ΔCT) between *GRPR* (target gene) and *GUSB* (control gene) was determined, and the relative *GRPR* expression level was calculated as $2^{-\Delta CT}$. Expression was defined as below the limit of detection if the threshold cycle was greater than 40 cycles. To evaluate the validity of using *GUSB* as a reference gene for gene expression changes associated with HNSCC, threshold cycle values for *GUSB* in OSCC and paired histologically normal mucosa were determined as described above.

GRPR expression was quantified relative to *ACTB*, which encodes β-actin, in buccal cDNA samples with detected *GRPR* expression and material available for analysis after completion of *GRPR* and *GUSB* mRNA quantification ($n = 17$; 7 HNSCC cases and 10 cancer-free controls). PCR amplifications of *ACTB* (forward primer: 5′-ACCGAGCGCGGCTACAG-3′; reverse primer: 5′-CTT AATGTCACGCACGATTTCC-3′; fluorescent probe: 5′-TTCACCACCACGGCCGAGC-3′) were performed as previously described, except with an annealing and extending temperature of 55°C. Quantification of relative *GRPR* expression was performed as described above with *ACTB* as the control gene.

Statistical analyses

The difference between *GUSB* threshold cycle values in OSCC and paired histologically normal samples was assessed using the Wilcoxon signed rank test. Concordance of *GRPR* expression relative to *GUSB* and *GRPR* expression relative to *ACTB* in the 17 samples with quantified *GUSB* and *ACTB* was assessed using the Pearson correlation coefficient. For all other analyses, *GRPR* expression relative to *GUSB* was used. Reliability of q-PCR assay for relative *GRPR* expression was assessed by calculating the 1-way random effects intraclass correlation coefficient. Specificity and sensitivity of *GRPR* buccal expression was analyzed using the receiver operator characteristic (ROC) curve and summarized by reporting the area under the ROC curve. Differences in HNSCC cases versus control subject population characteristics and differences between subjects with measured *GRPR* buccal expression versus subjects without detected *GRPR* buccal expression were evaluated using the chi-square test, the Fisher exact test, or Wilcoxon rank sum test, as appropriate. The natural log ($\log_e$) transformation of relative *GRPR* expression levels was used in analyses.

In order to determine whether buccal *GRPR* mRNA expression levels differed by subject characteristic or risk factor exposure, candidate confounding variables including age category (younger than 55, 55–64, or older than 64 years), sex, ethnicity (white vs non-white), smoking status (never, former, or active smoker), pack-year category of tobacco-use (never smoker, <50 pack-years, or ≥50 pack-years), and alcohol consumption category (never drinker, <7 drinks per 2 weeks, or ≥7 drinks per 2 weeks) were evaluated for

association with *GRPR* mRNA expression separately for cases and controls using the analysis of variance test (ANOVA). Fifty pack-years and 7 drinks per 2 weeks were selected as cutoff points for categories because these were the median values for ever smokers and ever drinkers, respectively, for cases and controls combined. Because RNA is labile and may degrade in a nonuniform manner, buccal storage time (≤12 months, 12–24 months, and >24 months), defined as the time in months from buccal cell collection to cDNA synthesis, was also evaluated for association with *GRPR* expression. All *p* values reported were 2-sided.

Evaluation of differences in *GRPR* expression in non-cancerous buccal mucosal epithelia between cases and controls was the primary endpoint of the study; association was considered significant if the log likelihood ratio test-associated *p* value was < .05. Univariate and multivariable logistic regression models were implemented to assess the significance of the association of elevated *GRPR* buccal expression with cancer before and after controlling for possible confounding variables. Multiple logistic regression models were developed with age and sex defined a priori to be included. Sex, ethnicity, smoking status, and alcohol consumption category were treated as categorical variables, and age and buccal storage time as continuous variables. Variables found to be significantly associated with case status in univariate logistic models ($p < .25$) were considered for inclusion in the multivariable models. Candidate variables were retained in the multivariable model if the associated Wald *p* value was < .25. The Hosmer Leme-show goodness of fit test was used to assess the final multivariable logistic model. In order to account for the possible bias due to the missing *GRPR* expression quantification in many of our samples, we used multiple imputation (MI) methods to impute *GRPR* expression levels using the data structure of the existing *GUSB* and *GRPR* expression levels. Based on the recommendation for higher levels of missing data, 10 MIs were used.[13] The set of 10 regression coefficients and their respective variances were combined into an overall MI estimate of the regression coefficient and its variance using Rubin's rules.[14] This was accomplished using SAS 9.2 (SAS Institute Inc, Cary, NC). Subsequently, odds ratios (ORs) and confidence intervals (CIs) were calculated from these estimates for univariate and multivariable logistic regression models.

Differences in *GRPR* buccal expression levels in patients with HNSCC by tumor site (oral cavity, oropharynx, hypopharynx, or larynx), tumor type (primary or recurrent tumor), disease stage, and by whether or not the patient experienced disease progression within 5 years were evaluated using ANOVA. Disease progression within 5 years was defined as experiencing a new head and neck cancer, cancer recurrence, metastases, or death within 5 years of receiving first treatment associated with enrollment in this study. Date of surgery, new cancer event, death, and last follow-up were provided by the Head and Neck Cancer Organ-Specific Database, maintained by the University of Pittsburgh Head and Neck SPORE bioinformatics groups and the University of Pitts-burgh Cancer Institute Head and Neck Cancer Registrar. A distribution of HNSCC cases according to these clinical and pathological parameters is provided in Table 1.

RESULTS

Head and neck squamous cell carcinoma case-control study population displayed typical characteristics

Two hundred sixty-eight subjects (110 HNSCC cases and 158 controls; Table 1) had measurable buccal *GUSB* gene expression, indicating that the RNA isolated from these samples was of sufficient quantity and quality to detect this housekeeping gene. *GUSB* was selected as the reference gene for these studies because it was previously reported to be expressed at moderate and stable levels across several tumor types.[15,16] In order to validate *GUSB* as a reference gene in our studies, we measured *GUSB* expression in 10 paired

OSCC and histologically normal oral tissues and found that *GUSB* expression did not differ by tissue type ($p = .69$; Supplemental Figure 1).

One hundred forty-one samples (66 cases and 75 controls) also had detectable *GRPR* mRNA expression by q-PCR, whereas in 127 of the samples, *GRPR* expression levels were below the limit of detection. In order to be conservative in our primary analysis, we confined our analysis to subjects with measured *GRPR* expression and omitted 1 HNSCC case, which was classified as an outlier with relative *GRPR* expression more than 5 SDs above the mean. Of the 140 samples (65 HNSCC cases and 75 controls) with measured *GRPR* relative expression, 53 were evaluated in independent experiments, and the concordance of these independent replicates indicated that the TaqMan assay was reliable: relative *GRPR* expression levels for independent replicates had an intraclass correlation coefficient of 0.89. To further validate our findings, we measured *GRPR* expression relative to *ACTB* gene expression in 17 samples with detected *GRPR* expression for which sufficient material remained for reanalysis. *GRPR* expression measured relative to *GUSB* and *GRPR* expression measured relative to *ACTB* were highly correlated with a Pearson correlation coefficient of .96 ($p < .001$). This subset analysis further supports the use of *GUSB* as an appropriate reference gene for these studies.

The median threshold cycle value for *GUSB* for cancer-free controls was significantly less than HNSCC cases (Table 1), indicating that fewer amplification cycles were required to detect *GUSB* gene expression in buccal material obtained from controls compared to cases. This was the same for all subjects with detected *GUSB* expression as well as for the subset with measured buccal *GRPR* expression (Table 1). These data indicated that RNA isolated from controls was at least of comparable quantity and quality as material obtained from cases.

As is typical of HNSCC cases compared with cancer-free control populations, HNSCC cases in our study population were predominantly men, were more often active smokers with more pack-years of smoking, and consumed on average more alcoholic drinks in a 2-week period than cancer-free control subjects. These differences were apparent in the population including all cases and controls in this study as well as in the subpopulation including only those subjects with measured buccal *GRPR* expression (Table 1).

Those subjects with detected *GRPR* buccal expression had significantly lower *GUSB* threshold cycle values for both cases ($p = .034$) and controls ($p = .012$), indicating that *GRPR* expression was detected more often in those subjects for whom higher quantity and/or quality of RNA was obtained from the buccal swab. Importantly, an evaluation of the differences between subjects with detected buccal *GRPR* mRNA expression versus those without detected *GRPR* expression stratified by case status indicated that there were no significant differences by age, sex, ethnicity, smoking status, pack-years of tobacco use, or alcohol quantity (all $p > .10$) for cases and controls (data not shown). Cases with detectable buccal *GRPR* mRNA expression did not differ by tumor site, tumor type, disease stage, adjuvant treatment, or disease progression from those without detected *GRPR* mRNA expression ($p > .10$ for all comparisons; data not shown).

Among HNSCC cases, but not cancer-free controls, active smoking status and female sex tended to be associated with elevated buccal GRPR expression

In a large prospective cohort study of 476,211 participants, men were found to smoke more than women, but hazards for HNSCC associated with smoking were reported to be higher for women than men.[17] In at least 1 independent study, smoking-related risk for oral cancers was also reported to be higher for women than men.[18] *GRPR* resides on the portion of the X chromosome reported to escape X-inactivation,[19] and we hypothesized that *GRPR*

expression may play a role in the heightened female HNSCC risk. In order to explore the relationships between HNSCC, subject sex, and buccal *GRPR* mRNA expression, we evaluated all HNSCC cases for differences between men ($n = 81$) and women ($n = 29$) regarding subject characteristics and risk factors. We found that male HNSCC cases in our population did not significantly differ from female cases with regard to age, smoking status, pack-years of tobacco use, tumor site, tumor type, disease stage, or adjuvant treatment (all $p > .10$; Supplemental Table 1). Male cases did, however, drink significantly more alcoholic beverages in a 2-week period than female cases ($p = .046$, rank sum test). Median numbers of drinks per 2-week period were 12 and 5 for men and women cases, respectively. The mean relative buccal *GRPR* mRNA expression values for male ($n = 50$) and female ($n = 15$) HNSCC cases was 0.19 and 0.53, respectively (Table 2). Although this difference was not significant ($p = .072$; Table 2), this trend toward a higher buccal *GRPR* mRNA expression in women was not apparent in the cancer-free control population ($p = .567$; Table 2).

GRPR gene expression has been reported to be elevated with tobacco use.[11] We tested for differences in *GRPR* expression in buccal mucosal tissues by smoking status and pack-years of tobacco use category stratified by cancer status. For HNSCC cases, active smokers had significantly elevated *GRPR* buccal expression levels ($p = .043$; Table 2). However, buccal *GRPR* mRNA expression levels did not differ by smoking status in cancer-free controls, nor did *GRPR* mRNA expression differ by pack-year category or alcohol consumption category for either cases or controls (Table 2). Buccal *GRPR* mRNA expression tended to be elevated in non-white HNSCC cases compared to white cases ($p = .052$), and this tendency was not observed for cancer-free control subjects. However, the small number of non-white subjects in the study limits the power and interpretability of this finding.

Because RNA is labile, we evaluated storage time differences between cases and controls. RNA was isolated from stored buccal cell samples in batches, and cDNA was synthesized and *GRPR* mRNA expression analyzed in a timely fashion after RNA isolation. To our surprise, storage time of buccal cells before isolation of RNA and synthesis of cDNA differed significantly between cases and controls with measured *GRPR* expression (Table 1). This was determined to be primarily the result of different recruitment kinetics for HNSCC cases compared with controls. HNSCC cases were recruited steadily throughout the study enrollment period while recruitment of cancer-free control subjects was delayed relative to cases with 2 prominent periods of recruitment (data not shown). In our analysis, stratified by case status, buccal cell storage time was significantly related to quantified *GRPR* buccal mRNA levels in both HNSCC cases and controls with samples stored for short periods of time having significantly lower *GRPR* expression (Table 2). Therefore, even though the magnitude of the buccal storage time effect seems to be substantially less than the magnitude of the difference in *GRPR* expression between HNSCC cases and control subjects, we identified buccal sample storage time as a confounder in our study (Table 2).

In order to determine whether *GRPR* mRNA expression differed by tumor clinical and/or pathological characteristics, we tested for differences in buccal *GPRR* mRNA expression levels by tumor site, tumor type, and disease stage by ANOVA analysis (Table 2). Mean buccal *GRPR* mRNA expression and 95% CI of the mean for HNSCC cases by tumor site, tumor type, and clinical disease stage are provided in Table 2. Buccal *GRPR* mRNA expression did not differ by any of these clinical parameters (Table 2). Of the HNSCC cases with quantified buccal *GRPR* mRNA expression, *GRPR* mRNA levels did not differ significantly between those HNSCC cases who experienced disease progression within 5 years of first treatment ($n = 36$) and those who did not ($n = 29$; $p = .679$; Table 2). A Kaplan–Meier plot of 5-year progression-free survival (PFS) among HNSCC cases by

buccal *GRPR* mRNA level indicates that 5-year PFS did not differ by buccal *GRPR* mRNA level (Figure 1).

Elevated buccal GRPR expression was associated with head and neck squamous cell carcinoma

The median relative buccal *GRPR* mRNA expression for cases and controls were 0.026 and 0.007, respectively (Table 1), indicating that the median relative *GRPR* mRNA level for cases was almost 4 times the level of controls and significantly elevated compared to *GRPR* mRNA levels in cancer-free control subjects ($p < .001$; Figure 2A). As a measure of the power of *GRPR* mRNA levels to differentiate between HNSCC cases and cancer-free controls subjects, the area under the ROC curve was calculated and determined to be 0.69 (Figure 2B), indicating that buccal *GRPR* mRNA levels provided a modest degree of discrimination between HNSCC cases and controls (Figure 2B).

In order to evaluate HNSCC risk by *GRPR* buccal mRNA level, we estimated ORs for HNSCC by tertile of *GRPR* expression level using univariate and multiple variable logistic regression models with the lowest tertile serving as the reference group (Table 3). The estimated OR for intermediate *GRPR* mRNA level was 1.81 and the estimated OR for high *GRPR* buccal mRNA level was 5.80 in univariate logistic regression models evaluating all subjects with measured buccal *GRPR* mRNA expression (Table 3). A test of trend indicated that there was a significant increase in HNSCC risk with increasing *GRPR* buccal expression ($p < .001$; Table 3). The association between HNSCC risk and elevated *GRPR* buccal expression was also significant even after adjusting for age, sex, tobacco exposure, and buccal storage time with the OR for high *GRPR* buccal expression compared with the reference estimated to be 3.55 (95% CI = 1.15–10.93). The test of trend indicated that subjects with high levels of buccal *GRPR* mRNA had the highest risk of HNSCC followed by subjects with intermediate levels of buccal *GRPR* mRNA, even after adjusting for age, sex, tobacco exposure, and buccal storage time ($p = .027$).

In order to evaluate whether there was selection bias for samples with measured *GRPR* mRNA, we performed a 10-fold imputation of *GRPR* expression for those subjects without quantified buccal *GRPR* expression. This analysis enabled us to take advantage of inherent data structure to impute *GRPR* expression levels. Using combined measured and imputed buccal *GRPR* expression levels and the same tertile cutoff points used for evaluating subjects with measured *GRPR* mRNA, the OR point estimate for high buccal *GRPR* mRNA expression compared to the reference in the univariate model was 3.38 (95% CI = 1.72–6.67) with a significant trend across tertiles ($p < .001$). The OR for high buccal *GRPR* mRNA expression compared with the reference was estimated to be 3.39 (95% CI = 1.54–7.46) after adjusting for age, sex, and tobacco use history with a significant trend across tertiles ($p = .002$) and to be 2.27 (95% CI = −0.09–1.73) after adjusting for age, sex, tobacco use history, and buccal storage time with a p trend equal to .06. Although multiple imputation results were somewhat reduced in magnitude, the similarity of the ORs obtained from combined measured and imputed *GRPR* mRNA expression levels compared to ORs obtained from only measured samples (Table 3) suggests that biased sample selection was not a significant factor in the study.

Alcohol consumption history variables were found to not be significant in multivariable models. An analysis stratified by subject sex yielded similar results with increasing risk of HNSCC with increasing buccal *GRPR* mRNA expression levels in both men and women in univariate models and a trend toward increased risk in multiple logistic regression models adjusted for age, and smoking status with and without adjustment for buccal storage time (Table 3).

DISCUSSION

Our group's previously reported results demonstrated that head and neck tumor tissues have elevated *GRPR* mRNA levels and histologically normal tissues adjacent to head and neck tumors have elevated *GRPR* expression compared with tissues from cancer-free control subjects.[7] Our primary new finding reported here indicates that, in a prospectively collected HNSCC case-control cohort, elevated *GRPR* mRNA expression in cytobrush samples from histologically normal buccal mucosa was significantly associated with HNSCC even after controlling for the effects of possible confounding by age, sex, and tobacco use. Importantly, we found *GRPR* mRNA levels in noncancerous mucosal tissues were significantly elevated in HNSCC cases versus cancer-free controls with increasing risk of HNSCC with increasing buccal *GRPR* mRNA expression across tertiles. These findings are consistent with our previous reports describing elevated *GRPR* mRNA levels in mucosal tissues adjacent to HNSCC compared with oral mucosal tissues from cancer-free control subjects.[7] An important distinction between this study and our previously published work is the method of cytobrush collection used in this study, which is noninvasive by comparison with surgical biopsy methods used in our previous study. *GRPR* mRNA in oral mucosal tissues collected using this noninvasive method provided sufficient material for quantitative assessment from many of our subjects.

The *GRPR* mRNA level in noncancerous mucosal tissues was not correlated with clinical disease stage and was not an indication of 5-year disease progression. Therefore, our data indicate that *GRPR* mRNA expression in surrogate tissues did not reflect tumor burden or disease progression but may instead be a marker of risk exposure or a marker of host susceptibility. A prospective cohort study will be required to fully understand the relationship between *GRPR* mRNA levels in surrogate tissues and the development of HNSCC and to determine whether elevated *GRPR* mRNA in buccal cells is a risk factor for HNSCC or a biomarker for prevalent HNSCC. Nonetheless, our study indicates that *GRPR* mRNA expression level in buccal mucosa has potential value as an indicator for HNSCC risk. *GRPR* is overexpressed in many solid tumors, but only 1 other group has evaluated *GRPR* or *GRP* levels in surrogate tissues of patients with cancer to date. Uchida et al[20] reported that serum levels of pro-*GRP*, as measured by enzyme-linked immunosorbent assay, correlated with tumor *GRP* expression levels in patients with small cell lung cancer.

We found *GRPR* buccal mRNA levels did not differ significantly by subject sex. Our study finding of no difference in *GRPR* expression between men and women was surprising given that *GRPR* resides on a portion of the X chromosome that escapes inactivation. In our group's previously published study evaluating *GRPR* mRNA expression in bronchial tissues,[11] we reported *GRPR* mRNA was more frequently expressed in women than in men. In our previous bronchial study, which had a smaller sample size of 78 patients, 63 of whom had lung cancer or cancer metastatic to the lung, the presence of cancer was not separately evaluated. In fact, only 1 never smoker with lung cancer was male.[11] Therefore, in retrospect, it is possible that the associations between *GRPR* mRNA levels, female sex, and smoking observed in our previous study were actually surrogates for the underlying association between bronchial epithelial *GRPR* mRNA expression and lung cancer.

We found no association between buccal *GRPR* mRNA levels and pack-years of smoking, but we did note the elevation of buccal *GRPR* mRNA levels among active smoking HNSCC cases but not cancer-free controls. This is somewhat consistent with our previously reported findings that *GRPR* expression in bronchial epithelium was activated with tobacco use[11] and with findings that bombesin-like peptide receptors play a role in wound healing after airway injury.[21] However, the presence of association in HNSCC cases and lack of association in cancer-free controls suggests that pathologies specific to HNSCC may be a primary

determinant. It is, therefore, probable that increased *GRPR* gene expression is an early event in HNSCC and may represent a marker of field cancerization.[22]

Because elevated *GRPR* expression in buccal cells was significantly associated with HNSCC even after controlling for the effects of smoking, the data indicate that elevated *GRPR* expression contributes to HNSCC through processes that are at least in part independent of tobacco use. The formal possibility exists that increased *GRPR* expression is related to an as yet unevaluated or unidentified environmental risk factor. HPV has been identified as a risk factor for the development of HNSCC, especially oropharyngeal cancers in never-smokers. We were not able to evaluate HPV infection status in our HNSCC cases and controls because HPV as a risk factor was not appreciated at the time the cohort was collected, and this study exhausted the analyzed specimens so that further analysis for the detection of HPV-associated gene expression will not be possible. However, buccal *GRPR* mRNA level in HNSCC cases did not differ significantly by tumor site and was not elevated in never-smokers. Therefore, elevated *GRPR* mRNA expression was not associated with characteristics typical of HPV-positive tumors.

Our study has several limitations. The higher number of female control subjects reflected a greater response to research advertisements by women. Also, healthy volunteers included family members who accompanied HNSCC cases to hospital clinic visits; these family members tended to be female. Our analysis stratified by sex, indicated that the observed association of *GRPR* mRNA overexpression and HNSCC was not an artifact of dissimilarity in the sex composition of case and control populations. Our identification of buccal storage time as a confounding variable in this study underscores the necessity to immediately process and evaluate biological samples and to normalize recruitment kinetics between comparison populations. In addition, we were not able to quantify buccal *GRPR* mRNA levels in many of our HNSCC cases and controls. This was likely due to the relatively low expression of *GRPR* in the tissues assessed, heterogenous quantity and quality of RNA isolated, and the assay limits of detection. However, we have evaluated whether systematic sample loss occurred, including whether RNA recovered from HNSCC cases was of significantly better quality than RNA isolated from cancer-free controls and have presented evidence that systematic sample loss did not occur to an appreciable degree. In addition, RNA quality was not significantly better for HNSCC cases and cannot account for the observations reported here. We have carefully controlled for important covariates associated with buccal *GRPR* mRNA level, and, despite the limitations of the study, we have demonstrated an important and significant association between increased buccal *GRPR* mRNA and HNSCC.

Because of the paucity of quality reagents to evaluate *GRPR* protein expression and function when we undertook this study, we isolated only RNA from buccal samples for this study in order to maximize RNA recovery. Therefore, we were unable to evaluate *GRPR* protein expression or function. Although it is possible that *GRPR* RNA levels do not reflect *GRPR* protein levels, we speculate that elevated *GRPR* mRNA in the upper aerodigestive tract may contribute to increased cancer risk by promoting proliferation. *GRPR* mRNA is expressed at early embryonic stages in the nervous, urogenital, respiratory, and gastrointestinal systems and expression in these tissues is generally down-regulated before birth.[23–25] The *GRPR* ligand, *GRP*, is a bombesin-like peptide growth factor, and bombesin-like peptides stimulate growth of bronchial, gastrointestinal, and pancreatic epithelial cells and lead to ligand-dependent hyperplasia.[24,26–29] Increased *GRPR* expression in upper aerodigestive tract epithelium may, therefore, reflect a state that is more proliferative in nature than epithelium with low or undetected *GRPR* mRNA expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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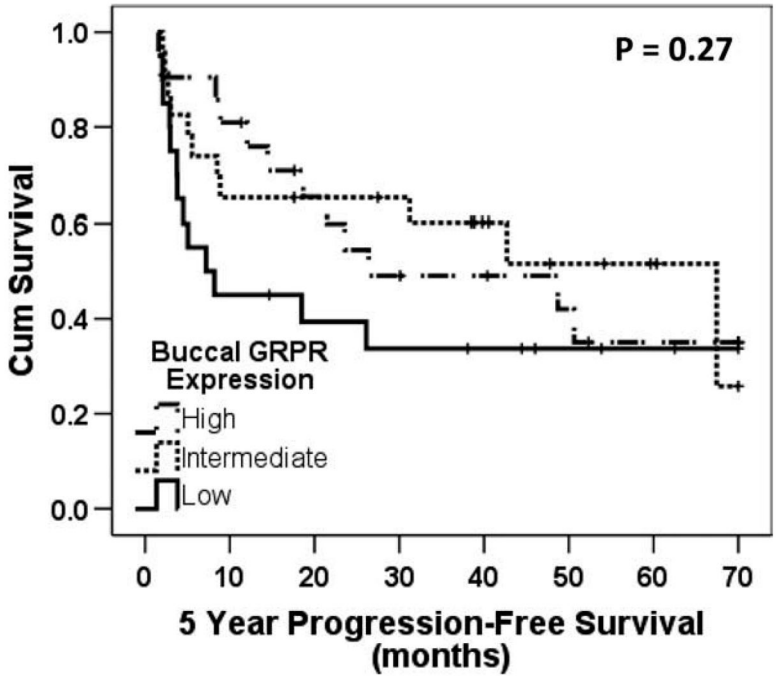

FIGURE 1.

Gastrin-releasing peptide receptor (GRPR) buccal expression level was not an indicator of progression-free survival (PFS) for head and neck squamous cell carcinoma (HNSCC) cases. A Kaplan–Meier plot of 5-year PFS by GRPR buccal expression category indicates that for 64 HNSCC cases with available follow-up, PFS did not differ significantly by GRPR buccal expression category. Log-rank test of equality of survivorship associated p value is provided.

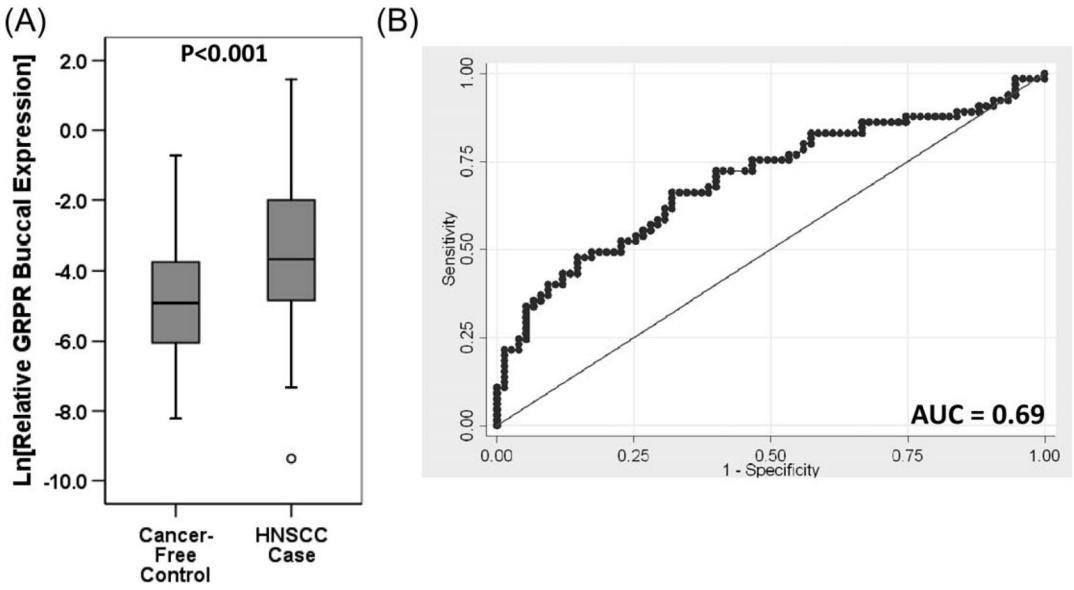

FIGURE 2.

Gastrin-releasing peptide receptor (GRPR) buccal expression is elevated in head and neck squamous cell carcinoma (HNSCC) cases compared with cancer-free control subjects. (A) Relative GRPR expression levels were measured using quantitative polymerase chain reaction (PCR) in cancer-free controls (n = 75) and patients with HNSCC (n = 65). Distributions of natural log (ln) transformed values are indicated. HNSCC cases had significantly elevated GRPR buccal expression compared with cancer-free controls ($p < .001$; Wilcoxon rank sum test). (B) The area under the receiver operating characteristic (ROC) curve (AUC) was determined using the ln-transformed relative GRPR expression values quantified in buccal cells from 65 patients with HNSCC and 75 cancer-free controls.

TABLE 1

Subject and sample characteristics.

	All HNSCC cases and controls					HNSCC cases and controls with measured buccal GRPR expression				
Characteristic	**Cases (n = 110)**		**Cancer-free controls (n = 158)**		**p value***	**Cases (n = 65)**		**Cancer-free controls (n = 75)**		**p value***
Age, y										
Median (range)	58 (26–89)		59 (25–83)		.388†	60 (24–89)		58 (25–78)		.676†
Sex										
Male	81	73.6%	69	43.7%	< .001‡	50	76.9%	32	42.7%	< .001‡
Female	29	26.4%	89	56.3%		15	23.1%	43	57.3%	
Ethnicity										
White	103	93.6%	138	87.3%	.044§	60	92.3%	65	86.7%	.565§
African American	5	4.5%	19	12.0%		4	6.2%	9	12.0%	
Other	2	1.8%	1	0.6%		1	1.5%	1	1.3%	
Smoking status										
Never smoker	13	11.8%	41	25.9%	< .001‡	6	9.2%	22	29.3%	< .001‡
Former smoker	34	30.9%	80	50.6%		24	36.9%	37	49.3%	
Active smoker	60	54.5%	36	22.8%		33	50.8%	15	20.0%	
Unknown	3	2.7%	1	0.6%		2	3.1%	1	1.3%	
Pack-years										
0	13	11.8%	41	25.9%	< .001‡	6	9.2%	22	29.3%	< .001‡
>0 and <50	29	26.4%	67	42.4%		18	27.7%	31	41.3%	
≥50	57	51.8%	41	25.9%		35	53.8%	18	24.0%	
Unknown	11	10.0%	9	5.7%		6	9.2%	4	5.3%	
Alcohol use										
Never	23	20.9%	23	14.6%	.175‡	11	16.9%	13	17.3%	.949‡
Ever	87	79.1%	135	85.4%		54	83.1%	62	82.7%	
Alcohol quantity										
0	23	20.9%	23	14.6%	.001‡	11	16.9%	13	17.3%	.102‡
<7	25	22.7%	65	41.1%		17	26.2%	30	40.0%	
≥7	51	46.4%	44	27.8%		31	47.7%	23	30.7%	

	All HNSCC cases and controls					HNSCC cases and controls with measured buccal GRPR expression				
Characteristic	**Cases (n = 110)**		**Cancer-free controls (n = 158)**		**p value***	**Cases (n = 65)**		**Cancer-free controls (n = 75)**		**p value***
Unknown	11	10.0%	26	16.5%		6	9.2%	9	12.0%	
Buccal *GUSB* CT										
Median	31.10		30.53		0.002†	30.77		29.85		.040†
Range	23.08–37.77		25.22–36.74			23.08–27.77		23.47–34.69		
Relative buccal *GRPR* Expression										
Median	0.026		0.007		< .001†	0.026		0.007		< .001†
Range	0.000086–4.3		0.00027–0.48			0.000086–4.3		0.00027–0.48		
Sample storage time, mo										
Median	11.4		13.8		.328†	25.0		12.4		< .001†
Range	2.1–37.9		0.7–26.2			2.1–37.9		0.7–26.2		
Tumor site										
Oral cavity	52	47.3%	–	–		34	52.3%	–	–	
Oropharynx	21	19.1%	–	–		13	20.0%	–	–	
Hypopharynx	8	7.3%	–	–		4	6.2%	–	–	
Larynx	25	22.7%	–	–		13	20.0%	–	–	
Other	4	3.6%	–	–		1	1.5%	–	–	
Tumor type										
Primary	99	90.0%	–	–		60	92.3%	–	–	
Recurrence	9	8.2%	–	–		4	6.2%	–	–	
Metastasis	2	1.8%	–	–		1	1.5%	–	–	
Disease stage										
0–I	21	19.1%	–	–		11	16.9%	–	–	
II	14	12.7%	–	–		9	13.8%	–	–	
III	21	19.1%	–	–		14	21.5%	–	–	
IV	42	38.2%	–	–		25	38.5%	–	–	
Unstaged¶	11	10.0%	–	–		5	7.7%	–	–	
Unknown	1	0.9%				1	1.5%			
Treatment										
RT only	25	22.7%	–	–		13	20.0%	–	–	

	All HNSCC cases and controls				HNSCC cases and controls with measured buccal GRPR expression			
Characteristic	**Cases (n = 110)**		**Cancer-free controls (n = 158)**	**p value***	**Cases (n = 65)**		**Cancer-free controls (n = 75)**	**p value***
CT only	0	0.0%	–	–	0	0.0%	–	–
CRT	20	18.2%	–	–	11	16.9%	–	–
No CRT	58	52.7%	–	–	38	58.5%	–	–
Unknown	7	6.4%	–	–	3	4.6%	–	–
Progression-free survival								
No disease progression, no.	45		–		27		–	
Median (range), mo	40.4 (5.6–82.3)		–		44.5 (11.4–82.3)		–	
Disease progression, no.	63		–		37		–	
Median (range), mo	10.2 (1.5–81.0)		–		8.2 (1.5–70.2)		–	
Lost to follow-up	2		–		1		–	

Abbreviations: HNSCC, head and neck squamous cell carcinoma; RT, radiation therapy; CT, chemotherapy; CRT, chemoradiotherapy.

*HNSCC cases versus controls.

†Rank sum test.

‡Chi-square test.

§Fisher exact test.

¶Unstaged recurrent/metastatic cancers.

**Typical number of alcohol drinks in a 2-week period.

TABLE 2

Mean relative buccal GRPR expression according to participant and sample characteristics for HNSCC cases and controls.

	HNSCC cases				Cancer-free controls			
Characteristic	**No.**	**Mean**	**95% CI**	**p value***	**No.**	**Mean**	**95% CI**	**p value***
Age, y								
< 55	22	0.11	0.033–0.18	.725	26	0.14	0.0018–0.025	.060
55–64	27	0.39	−0.038–0.82		26	0.04	0.0014–0.080	
65	16	0.28	−0.026–0.58		23	0.16	0.0082–0.23	
Sex								
Male	50	0.19	0.0087–0.37	.072	32	0.025	0.0097–0.040	.567
Female	15	0.53	−0.065–1.12		43	0.023	0.00042–0.045	
Ethnicity								
White	60	0.020	0.051–0.356	.052	65	0.023	0.0070–0.039	.456
Non-white	5	1.048	−1.23–3.33		10	0.029	−0.0026–0.062	
Smoking status								
Never smoker	6	0.097	−0.0088–0.20	.043†	22	0.012	0.0059–0.018	.632
Former smoker	24	0.062	−0.0035–0.13		37	0.032	0.0048–0.060	
Active smoker	33	0.42	0.060–0.78		15	0.020	−0.0042–0.041	
Pack-years								
0	6	0.097	−0.0088–0.20	.684	22	0.012	0.0059–0.018	.733
<50	18	0.24	−0.036–0.51		31	0.021	0.0072–0.034	
≥50	35	0.31	−0.010–0.64		18	0.046	−0.010–0.10	
Alcohol consumption								
0 drinks per 2 wk	11	0.24	−0.21–0.68	.175	13	0.016	0.0050–0.027	.567
<7 drinks per 2 wk	17	0.046	0.0088–0.82		30	0.017	0.0064–0.028	
≥7 drinks per 2 wk	31	0.30	0.038–0.56		23	0.020	0.0021–0.029	
Buccal storage time								
0–12 mo	23	0.12	−.080–0.32	.009†	37	0.011	0.0019–0.019	< .001†
>12 mo–24 mo	9	0.64	−0.46–1.75		37	0.036	0.0090–0.064	
≥24 mo	33	0.27	0.032–0.50		1	0.031	–	
Tumor site								

	HNSCC cases				Cancer-free controls			
Characteristic	**No.**	**Mean**	**95% CI**	**p value***	**No.**	**Mean**	**95% CI**	**p value***
Oral cavity	34	0.31	0.020–0.60	.118	–	–	–	–
Oropharynx	13	0.31	−0.32–0.95		–	–	–	
Hypopharynx	4	0.42	−0.082–0.92		–	–	–	
Larynx	13	0.08	0.018–0.14		–	–	–	
Tumor type								
Primary	57	0.29	0.08–0.51	.668	–	–	–	–
Recurrence	7	0.067	0.024–0.16		–	–	–	
Disease stage								
0–I	11	0.045	−0.019–0.11	.262	–	–	–	–
II	9	0.19	−0.052–0.43		–	–	–	
III	14	0.050	0.0045–0.096		–	–	–	
IV	25	0.55	0.068–1.08		–	–	–	
Unstaged≠	5	0.083	−0.63–0.23		–	–	–	
5-y disease progression								
Yes	36	0.275	0.021–0.511	.679	–	–	–	–
No	29	0.27	−0.043–0.584		–	–	–	

Abbreviations: HNSCC, head and neck squamous cell carcinoma; CI, confidence interval.

*Test of differences in means ($\log_e$ transformed) using analysis of variance (ANOVA).

†Significant at $p < .05$.

≠Unstaged recurrent/metastatic cancers.

TABLE 3

Buccal GRPR expression levels and ORs (95% CI) for HNSCC across tertiles of GRPR buccal expression levels.

	Tertiles of relative buccal GRPR expression			p value for trend
All subjects	1	2	3	
Relative *GRPR* expression mean (SD)	0.0023 (0.0016)	0.14 (0.0067)	0.40 (0.88)	
Range	0.000086–0.0055	0.0061–0.028	0.029–4.29	
Cases/controls	13/33	20/28	32/14	
OR (95% CI)*	1.00 (reference)	1.81 (0.77–4.29)	5.80 (2.36–14.24)	< .001
OR (95% CI)†	1.00 (reference)	1.27 (0.45–3.62)	3.55 (1.15–10.93)	.027
Males				
Relative *GRPR* expression mean (SD)	0.0014 (0.0082)	0.0085 (0.0029)	0.24 (0.68)	
Cases/controls	10/14	17/9	23/9	
OR (95% CI)*	1.00 (reference)	2.64 (0.84–8.31)	3.58 (1.17–10.96)	.028
OR (95% CI)‡	1.00 (reference)	1.46 (0.38–5.71)	2.18 (0.51–9.32)	.295
Females				
Relative *GRPR* expression mean (SD)	0.0027 (0.0018)	0.015 (0.0074)	0.61 (1.08)	
Cases/controls	3/19	3/19	9/5	
OR (95% CI)*	1.00 (reference)	1.00 (0.18–5.59)	11.4 (2.22–58.56)	.004
OR (95% CI)‡	1.00 (reference)	0.83 (0.13–5.40)	7.59 (1.11–51.81)	.043

Abbreviations: OR, odds ratio; CI, confidence interval.

*Univariate logistic regression model estimate.

†Multivariable logistic regression model estimate adjusted for age, sex, smoking status, pack-year category, and buccal storage time.

‡Multivariable logistic regression model estimate adjusted for age, smoking status, pack-year category, and buccal sample storage time.