Recovery of salivary epidermal growth factor in parotid saliva following parotid sparing radiation therapy: a proof-of-principle study

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Background. Although radiation therapy (RT) causes permanent xerostomia, parotid-sparing radiation therapy (PSRT) ensures recovery of saliva quantity over time. Salivary epidermal growth factor (EGF) is produced primarily by parotid glands.

Objectives. The aim of this study was to determine whether salivary EGF can be detected in parotid saliva after PSRT and whether protein secretion is time dependent.

Study design. Salivary EGF concentration (pg/mL) was determined by enzyme-linked immunosorbent assay in stimulated parotid saliva before RT and at 3, 6, and 12 months after RT from 22 patients with head and neck cancer treated with PSRT.

Results. Saliva samples were from 17 men and 5 women (age ranges 23-70 years and 46-71 years, respectively). At 6 months after RT, EGF concentration was 407 pg/mL lower than at baseline (P = .045). Twelve months after PSRT, parotid glands produce substantial amounts of EGF and other proteins, eventually approximating pre-RT levels, with recovery of salivary function.

Conclusions. This proof-of-principle study shows that even proteins in picogram quantities, such as EGF, can be detected in saliva after PSRT. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;111:64-70)
flow primarily of stimulated saliva, from glands receiving reduced radiation, recovers over time up to 2 years after the end of treatment. This is associated with gains in quality of life. Patients with limited or no salivary flow can have trouble eating, swallowing, and speaking. In addition to improvement in quality of life, recovery of salivary flow also improves oral health. Saliva has many functions, including mucosal repair, dental remineralization, antimicrobial action, buffering, and lubrication. These functions are mediated by different components of saliva, including cytokines and glycoproteins. Therefore, an important consideration is the quality of recovered saliva in this patient population. However, until now the quality of saliva produced after PSRT has not been assessed.

To evaluate the quality of saliva, it must first be determined if proteins that are secreted in pg/mL amounts can be detected in parotid saliva after 3DRT. Salivary epidermal growth factor (EGF) was selected as a representative protein for analysis in the present proof-of-principle study. This protein was chosen because it is known to be produced by parotid glands and plays an important role in oral mucosal wound healing and maintenance. Furthermore, there is evidence that levels of salivary EGF fluctuate during conventional radiation therapy and are associated with severity of radiation mucositis. With conventional RT, salivary studies can be challenging, because salivary flow significantly decreases or ceases completely after treatment. In contrast, salivary glands receiving <26 Gy using PSRT demonstrate an initial salivary flow decrease during and immediately after RT that gradually increases in the months after the end of treatment. The objectives of the present study were to determine whether salivary EGF can be detected in stimulated parotid saliva after 3DRT and whether protein secretion is time dependent.

**MATERIAL AND METHODS**

**Study population**

Subjects were enrolled in protocol UMCC-9427, “Partial parotid sparing using 3-dimensional planning in patients undergoing bilateral neck radiation for head and neck cancer,” at the University of Michigan Comprehensive Cancer Center from 1994 to 1997. This study has previously been described. The experimental protocol, consent form, and the present retrospective analysis of the deidentified saliva samples collected under the protocol were approved by the University of Michigan Institutional Review Board for the protection of human subjects.

**3D radiation treatment planning**

All patients underwent immobilization and full 3D treatment planning as previously described. The goal of the treatment planning was full exclusion of the contralateral parotid gland at the side of the neck least involved with the tumor. All patients were treated with continuous conventional fractionation and received 1.8-2.0-Gy fractions, 1 fraction per day, 5 fractions per week, for 7 weeks.

**Parotid saliva collection and flow measurements**

Bilateral parotid saliva was collected before radiation treatment and at 3, 6, and 12 months after completion of RT over a course of time from 1994 to 1997. Subjects were instructed to refrain from eating, drinking, smoking, and oral hygiene for a minimum of 90 minutes before saliva collection. All saliva was collected between 8:00 and 12:00 a.m. to control for circadian variation in salivary gland function. Parotid saliva was collected from both parotid gland orifices using a Carlson-Crittenden cup. Salivary flow was stimulated by applying 2% citric acid swabbed onto the anterior dorsolateral surfaces of the tongue at 30-second intervals for 2 minutes for equilibration. This was followed by a 2-minute collection period during which gustatory stimulation was maintained. After saliva collection, the volumes of all saliva samples were determined gravimetrically on an analytical balance, assuming a specific gravity of 1.0. Saliva samples were aliquoted and stored at −80°C until protein analysis. An aliquot was thawed before use for this study.

**EGF assays**

Right and left stimulated parotid saliva samples were pooled for each subject at each time point. This was done because of limited saliva production by irradiated glands. EGF concentration was determined using an enzyme-linked immunosorbent assay (R & D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Briefly, this is a sandwich assay in which a recombinant human EGF was used as standards. The standard curve range was 3.9-250 pg/mL. Standards and the samples to be quantified were run in parallel on the same plate. The samples were diluted to fall within the range of the standards. In initial studies, these dilutions were determined for saliva samples. For diluted samples, the dilution factor was considered when calculating the final concentration.

Total protein concentration was determined by the Bradford assay (BioRad Laboratories, Hercules, CA). Bovine serum albumin was used as a standard accord-
ing to the manufacturer’s recommendation. The standard curve range was 2.5-12.5 mg/mL with $R^2$ values $>0.900$. Standards and the samples to be quantified were run in parallel on the same plate. Pre- and post-treatment saliva samples for a given subject were assayed concurrently to control for variation in experimental conditions. Quantifications were performed in triplicate and the average value for each sample was used in the data analysis. The standard deviation for each average was $<0.0100$ mg/mL.

### Data analysis

Descriptive statistics were calculated for EGF concentration, total EGF, total protein concentration, and flow rate for each time point in the longitudinal study. The relationships between time after completion of RT and EGF concentration, total EGF, total protein concentration, and flow rate were analyzed in the longitudinal data by using generalized estimating equations. Within-subject changes in EGF concentration, total EGF, protein concentration, and stimulated parotid salivary flow rate compared with baseline were analyzed for each time point.

### RESULTS

Longitudinal saliva samples were available for 22 subjects: 5 women and 17 men. Overall mean age was 57.8 years (range 38-76 years). There were 16 subjects with oral and oropharyngeal squamous cell carcinomas, 1 skin squamous cell carcinoma of the ear, and 1 laryngeal squamous cell carcinoma, and the rest were salivary gland neoplasms. The majority were advanced with regional node involvement at the time of treatment. None had distant metastases (Table I). On average, patients received a mean dose of 21.9 Gy during treatment. There was great variation of EGF concentration, total EGF, protein concentration, and stimulated parotid salivary flow rate between subjects at each time point (Fig. 1). This could be due to the relatively small sample size. EGF concentration, total EGF, protein concentration, and stimulated parotid salivary flow rate showed decreasing trends after RT, but they returned to values close to baseline at 12 months after RT (Table II). Mean difference from baseline was calculated for EGF concentration, total EGF, protein concentration, and stimulated parotid salivary flow rate (Table III). Mean difference from baseline of EGF concentration was significantly different ($P = .045$) only at 6 months after RT. Mean differences from baseline of total EGF and protein concentration were not significantly different at any time point. Mean difference from baseline of stimulated parotid salivary flow rate was significantly different from baseline ($P = .022$) only at 3 months after RT.

### DISCUSSION

Parotid-sparing RT is associated with gradual recovery of parotid salivary flow, approaching pretreatment levels, within the first 2 years after the end of treatment.12,13,16 This translates into improved quality of life.17,18 The present study is

<table>
<thead>
<tr>
<th>Subject</th>
<th>Gender</th>
<th>Age, y</th>
<th>Tumor site</th>
<th>Stage†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>63</td>
<td>Right base of tongue, scc</td>
<td>T3N2bM0</td>
</tr>
<tr>
<td>2</td>
<td>Female</td>
<td>46</td>
<td>Left tonsil, scc</td>
<td>T1N2aM0</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>55</td>
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<td>N/A</td>
</tr>
<tr>
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<td>Male</td>
<td>70</td>
<td>Right submandibular gland, scc</td>
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<tr>
<td>5</td>
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<td>Right tonsil and bilateral vocal cords, scc</td>
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<td>Left pyriform sinus, scc</td>
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<tr>
<td>7</td>
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<td>Left floor of mouth, scc</td>
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<tr>
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<td>67</td>
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<td>T4N2bM0</td>
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<tr>
<td>9</td>
<td>Female</td>
<td>76</td>
<td>Left supraglottic larynx, scc</td>
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<td>53</td>
<td>Right parotid, acinic cell ca</td>
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<td>14</td>
<td>Male</td>
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<td>Right base of tongue, scc</td>
<td>T2N1M0</td>
</tr>
<tr>
<td>15</td>
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<td>65</td>
<td>Right ear, scc, recurrent</td>
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<tr>
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<td>Right tonsil, pleomorphic adenoma, recurrent</td>
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<tr>
<td>22</td>
<td>Male</td>
<td>45</td>
<td>Base of tongue, scc</td>
<td>T1N2bM0</td>
</tr>
</tbody>
</table>

scc, squamous cell carcinoma; ca, carcinoma.

†Tumor, nodes metastases (TNM) classification, American Joint Committee on Cancer, 2004.
the first to assess whether EGF, a protein that is secreted in picogram amounts, can be detected in stimulated parotid saliva from patients treated with PSRT, measured before and up to 1 year after the end of treatment. We observed that at 12 months, EGF concentration in saliva is similar to baseline.

Table II. Mean epidermal growth factor (EGF) concentration, total EGF, protein concentration, and flow rate of all patients

<table>
<thead>
<tr>
<th>Time point</th>
<th>EGF concentration (pg/mL)</th>
<th>Total EGF (pg/min)</th>
<th>Protein concentration (mg/mL)</th>
<th>Flow rate (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean SEM</td>
<td>Mean SEM</td>
<td>Mean SEM</td>
<td>Mean SEM</td>
</tr>
<tr>
<td>Baseline</td>
<td>730 147</td>
<td>526 97</td>
<td>0.877 0.114</td>
<td>1.314 0.225</td>
</tr>
<tr>
<td>3 mo</td>
<td>634 86</td>
<td>450 78</td>
<td>0.864 0.132</td>
<td>0.875 0.132</td>
</tr>
<tr>
<td>6 mo</td>
<td>442 86</td>
<td>305 57</td>
<td>0.719 0.098</td>
<td>1.197 0.216</td>
</tr>
<tr>
<td>12 mo</td>
<td>737 166</td>
<td>519 119</td>
<td>0.942 0.184</td>
<td>1.269 0.275</td>
</tr>
</tbody>
</table>

Table III. Mean difference from baseline of epidermal growth factor (EGF) concentration, total EGF, protein concentration, and salivary flow rate in stimulated parotid saliva over time after radiotherapy

<table>
<thead>
<tr>
<th>Time point</th>
<th>EGF concentration (pg/mL)</th>
<th>Total EGF (pg/min)</th>
<th>Protein concentration (mg/mL)</th>
<th>Salivary flow rate (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean difference from baseline SEM P value</td>
<td>Mean difference from baseline SEM P value</td>
<td>Mean difference from baseline SEM P value</td>
<td>Mean difference from baseline SEM P value</td>
</tr>
<tr>
<td>3 mo</td>
<td>−178 207 .403</td>
<td>−138 149 .352</td>
<td>−0.029 0.109 .794</td>
<td>−0.529 0.211 .022</td>
</tr>
<tr>
<td>6 mo</td>
<td>−407 183 .045</td>
<td>−201 121 .097</td>
<td>−0.151 0.094 .129</td>
<td>−0.117 0.261 .660</td>
</tr>
<tr>
<td>12 mo</td>
<td>−245 247 .349</td>
<td>−64 171 .710</td>
<td>−0.083 0.082 .332</td>
<td>−0.086 0.428 .845</td>
</tr>
</tbody>
</table>

Mean difference refers to changes within each patient at each time point. Mean difference from baseline of EGF concentration is significant only at 6 months after RT. Mean differences from baseline of total EGF and protein concentration are not significant at any time point. Mean difference from baseline of salivary flow rate is significant only at 3 months.

Fig. 1. A, Epidermal growth factor (EGF) concentration variation among all patients within each time point. B, Total EGF variation among all patients within each time point. C, Protein concentration variation among all patients within each time point. D, Flow rate variation among all patients within each time point. RT, Radiotherapy.
Salivary EGF is a 53–amino acid peptide that exerts its action through binding with the EGF receptor found on many different cell types. In the oral cavity, the EGF receptor is expressed in mucosal epithelium and the underlying connective tissue. The parotid and submandibular glands secrete salivary EGF in humans. The relative proportions of EGF levels found in parotid saliva, submandibular gland saliva, and whole saliva are 6:1:4. Salivary EGF is needed for normal oral mucosal and skin wound healing.

Patients with risk factors for cancer, such as smoking, and patients with HN cancer, have low salivary EGF. Although patients with HN cancer have lower salivary EGF compared with control subjects, it is not yet known if salivary EGF changes in response to effective treatment or recurrence of HN cancer. EGF levels in stimulated whole saliva decrease in patients during and up to 3 weeks after conventional RT. Subsequently, salivary output is permanently lost or is very viscous and scant, thereby precluding EGF quantification after conventional RT.

There is a strong negative correlation between EGF levels and the severity score of oral mucositis. Higher levels of EGF, particularly in stimulated saliva before and during RT, are associated with less severe mucosal damage. Because the parotid glands are the primary source of salivary EGF, PSRT may confer benefits to patients by providing substantial amounts of salivary EGF to aid in healing of oral mucositis and continued protection of the oral mucosa over time. Oral mucositis usually appears within the second week of RT and persists up to 3 months after the end of therapy. By 6 months after RT, one would expect complete healing of acute radiation mucositis to have occurred. Perhaps the salivary EGF concentration was significantly lower than before RT because of the reduced demand at 6 months after RT. A larger study may provide more definitive conclusions of what is occurring at the 6-month post-RT time point regarding EGF.

There are many factors that could affect the present data. However, because this was a retrospective study, many of these factors cannot be explained or appropriately evaluated. Samples were collected from 1994 to 1997 and stored at −80°C, standard storage conditions for biorepository serum and saliva samples. There could be some degradation of samples over time. However, it is likely that degradation was minimal, because there were no repeated freeze-thaw cycles, because the samples were stored in single-use aliquots. Another limitation of this study is that there were no available data on the smoking status of each individual patient. Therefore, we could not evaluate whether changes in EGF were due to this comorbid condition. There was also no available information on the individual doses of radiation given to each patient. However, these patients are a subgroup of patients from a previous study for which the mean radiation dose to the spared parotids was 21.9 Gy.

Another limitation of the present study is the lack of data on parotid saliva during RT or within the first 3 months following therapy. The earliest time point at which saliva was collected was 3 months after PSRT. By this, saliva flow is significantly reduced and parotid saliva volumes are negligible. Also, patients with radiation mucositis cannot tolerate the parotid saliva collection devices placed on the involved mucosa. Therefore, we cannot compare our findings to those of other investigators who measured EGF in whole saliva during and immediately after conventional RT. Patients treated with conventional RT for HN cancer, especially oral and oropharyngeal cancer, generally develop permanent salivary gland hypofunction and xerostomia, thereby precluding parotid saliva collection for comparison to our study group at 6 or 12 months.

Another limitation is the lack of comparison of salivary EGF in patients whose parotid glands received different doses. The dosimetry data were not available for this retrospective study. Therefore, the relationship between radiation dose and EGF or protein concentration of parotid saliva cannot be determined. Furthermore, it is important to recognize that although the right and left parotid saliva samples were pooled in this study, generally there was no saliva output from the unspared glands, so in fact the saliva analyzed was only from the spared gland. To analyze the effects of dose on saliva protein composition, a large multicenter trial would be needed to have the sample size needed to power such a study.

The present study addressed EGF secretion in saliva from parotid gland, not whole saliva or saliva from submandibular glands, because the latter was not available. Recently, Murdoch-Kinch et al. have shown that sparing of the submandibular glands is possible for some patients with HN cancer. Further advances in salivary-sparing radiotherapy techniques, including efforts to minimize oral cavity dose, a surrogate for the dose to minor salivary glands, could improve the feasibility of using whole saliva or saliva from the submandibular or parotid glands as a diagnostic fluid for HN cancer patients treated with radiotherapy.

There have been few studies demonstrating that salivary EGF concentration can be affected by renal function. Guh et al. showed that in patients on hemodialysis, decreased levels of salivary EGF bioactivity were associated with peptic ulcer disease; however, salivary EGF levels were not different in the hemodialysis patients and normal control subjects. Dagogo-Jacket et al.
reported that salivary EGF concentrations were significantly higher in patients with chronic renal failure, especially those undergoing continuous ambulatory peritoneal dialysis. Therefore, if any of our patients had chronic renal failure and were undergoing dialysis, this may have caused elevated salivary EGF levels.

At 6 months after RT, parotid salivary output improves with PSRT, in contrast to the permanent dramatic decreased function observed with conventional RT. Perhaps, as the salivary glands initially shut down during RT, salivary EGF decreases coincident with decreased parotid flow due to the radiation injury to the parotid acini. Proliferation of acinar cells as well as progenitor cells in the intercalated ducts facilitates salivary gland regeneration and recovery after RT. As the parotid glands gradually recover function, starting at ~6 months after RT, parotid salivary flow increases, and salivary EGF and total protein levels start to rise, reaching pre-RT levels by 12 months after RT in parotid tissues receiving ≤26 Gy. Consistent with this notion, a recent study in a duct ligation animal model of salivary gland atrophy and regeneration showed upregulation of EGF and β2-adrenergic receptors during glandular regeneration.

The present proof-of-principle study demonstrates that proteins, such as EGF, that are normally present in pg/mL amounts in parotid saliva, can be detected and quantified in parotid saliva after PSRT. Protein composition, as well as other characteristics of saliva, such as pH and buffering capacity, could be used in the future to monitor recovery of the salivary glands after radiation injury. Additional research is needed to identify potential salivary protein biomarkers to be used in the diagnosis and surveillance of HN cancer patients after therapy. This is being investigated in ongoing studies at our institution with the goal of developing a noninvasive inexpensive saliva-based test for monitoring response to therapy and recurrence of HN cancer in patients treated with PSRT.

The significant intellectual and physical contributions to this research by Jonathan A. Ship, DMD, before his death are greatly appreciated and acknowledged.

REFERENCES


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