SALIVARY GLUTATHIONE AND URIC ACID LEVELS IN PATIENTS WITH HEAD AND NECK SQUAMOUS CELL CARCINOMA

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Abstract: Background. We evaluated the concentrations of glutathione and uric acid, low molecular weight antioxidants, in saliva of patients with head and neck squamous cell carcinoma (HNSCC), in order to identify differences with normal subjects and to obtain information about biochemical alterations of human saliva during carcinogenesis.

Methods. We compared 50 HNSCC patients, divided in 2 subsets on the basis of tumor site, with a control group of 77 subjects, without a previous diagnosis of HNSCC, matched for age, sex, alcohol consumption, and smoking status.

Results. At tests for equality of means by Welch and Brown-Forsythe, differences between groups resulted probable for saliva levels of glutathione ($p = .004$ and $p < .001$ respectively) but not for saliva levels of uric acid ($p = .228$ and $p = .122$ respectively). Comparing groups by Tamhane test, the patients with oral or pharyngeal cancer had significantly higher salivary levels of glutathione than both controls and patients with laryngeal cancer.

Conclusions. Salivary glutathione levels may be an index of oxidative stress at the level of the upper airways and in particular of oral cavity and pharynx. Therefore, high salivary glutathione may be an epidemiological marker to identify subjects with an increased risk of developing HNSCC, to submit to strict follow-up and chemoprevention. Metabolic alterations of saliva could be both an epidemiological marker and a target for chemoprevention of oral and oropharyngeal carcinogenesis.

Keywords: head and neck; oxidative damage; chemoprevention; salivary antioxidant system

Head and neck squamous cell carcinoma (HNSCC), as defined here, includes squamous cell carcinomas of the oral cavity, pharynx, and larynx. Approximately 39,000 cases of HNSCC are estimated to occur in the United States in 2005, with 11,000 deaths. HNSCCs represent about 3% of all malignant tumors in the United States. However, in other parts of the world, such as India, Southeast Asia, and Brazil, the disease is much more prevalent. Standard therapeutic approach, focused on surgery, irradiation, and chemotherapy, alone or in combination, has been in part modified in the last 30 years, but overall survival of HNSCC patients has not been substantially improved. Efforts toward early detection and prevention have not been entirely successful. For patients affected by early-stage carcinomas, with a high disease-specific survival rate, second primary tumors represent the first cause of death. On the other hand, patients...
with advanced head and neck SCC have a high risk of primary-treatment failure and death. Smoking and alcohol intake are the best defined risk factors for HNSCC. It has been proposed that low vegetables intake and hypofolatemia, infections by Epstein-Barr virus (especially for nasopharynx) and human papillomaviruses (for oropharynx and other sites), betel quid chewing, marijuana smoking, and acid and alkaline reflux also play a role.

DNA damage, occurring as the result of chemical attack, in large part by products of oxidative metabolism and in particular by reactive oxygen species (ROS), is probably the most frequent potentially mutagenic spontaneous event. 

When the level of ROS exceeds the antioxidant capacity of the cell, the intracellular redox homeostasis is altered and oxidative stress ensues. To cope with an excess of free radicals produced upon oxidative stress, humans have developed sophisticated mechanisms in order to maintain redox homeostasis. These protective mechanisms scavenge or detoxify ROS, block their production, or sequester transition metals that are the source of free radicals, and include enzymatic and nonenzymatic antioxidant defenses. Oxidative stress is considered to play a pivotal role in the pathogenesis of aging and several degenerative diseases, such as atherosclerosis, cardiovascular disease, type 2 diabetes, and cancer. In fact, it has been demonstrated that free radical generating compounds and reactive oxygen generating systems can promote tumor progression both in vitro and in vivo models, and also in the head and neck. Reactive oxygen species has been hypothesized to have a role in cigarette smoke-, alcohol-, and betel quid–associated carcinogenesis as well.

Antioxidants have been shown to inhibit tumor progression in several models, also of head and neck carcinogenesis. Glutathione and uric acid are low molecular weight antioxidants. In particular, glutathione can directly scavenge free radicals or act as a substrate for glutathione peroxidases and glutathione S-transferases during the detoxification of hydrogen peroxide, lipid hydroperoxides, and electrophilic compounds. Electrophilic attack upon a tissue nucleophile is the most frequent chemical reaction mediating DNA damage from exogenous carcinogens. Therefore, the protective role of glutathione is not exclusively related to its direct antioxidant effect.

In the present work, we evaluated glutathione and uric acid concentrations in saliva of HNSCC patients, in comparison with normal subjects, to obtain information about salivary antioxidant status of such patients and about biochemistry of saliva in head and neck carcinogenesis.

### MATERIALS AND METHODS

**Patients.** Fifty consecutive untreated patients with primary HNSCC (Table 1) were enrolled in our Department of Otolaryngology. They were categorized according to the site of origin of malignancy in patients with SCC of oral cavity-pharynx and of the larynx and were classified according to the stage of the disease: early (T1-T2, N0), locally advanced (T3-T4, N0), and regionally advanced (N+). The male/female ratio for our study, saliva had a much higher risk than in other HNSCCs.

<table>
<thead>
<tr>
<th>Disease stage</th>
<th>Oral and pharyngeal cancers</th>
<th>Laryngeal SCCs</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early</td>
<td>10 (52%)</td>
<td>20 (64%)</td>
<td>–</td>
</tr>
<tr>
<td>Locally advanced</td>
<td>4 (21%)</td>
<td>7 (23%)</td>
<td>–</td>
</tr>
<tr>
<td>Regionally advanced</td>
<td>5 (27%)</td>
<td>4 (13%)</td>
<td>–</td>
</tr>
</tbody>
</table>

Abbreviation: SCC, squamous cell carcinoma.
more relevant role in determining the chemical environment of oral and oropharyngeal mucosa than laryngeal mucosa.

We compared the patients with a control group of 77 subjects with no previous diagnosis of HNSCC, matched for age, sex, alcohol consumption, and smoking status (Table 1), because both alcohol and tobacco smoke are known to influence antioxidant status of saliva and are at the same time the most relevant behavioral risk factors for HNSCC. Furthermore, we excluded from our study subjects with an estimated habitual alcohol consumption higher than 35 g of alcohol, or higher than 4 glasses of alcoholic beverages, per day, because marked alterations of saliva composition have been described in heavy drinkers due to poor oral hygiene and to a different microbial flora of the mouth as well as to malnutrition, and thus might have been a relevant confounding factor in our study.

Controls were subjects from the same geographical area as patients, treated at the same hospital for conditions unrelated to cancer. Trauma patients were excluded from the control group because accidents are often related to alcohol abuse. We eliminated bias resulting from a change in behaviors caused by a chronic disease, requiring that hospitalization of the controls was attributable to a condition diagnosed in the last 6 months. Both control subjects and patients were enrolled after obtaining informed consent to the use of their saliva samples for an experimental study, in accordance with the Helsinki Declaration of 1975 as revised in 1983. No subjects included in the study had received vitamin supplements in the previous 6 months. Both patients and controls had normal renal and hepatic function.

**Chemicals.** Tri-n-buthylphosphine, dimethylformamide, 7-fluoro-2,1,3-benzoxadiazole-4-sulfonamide (ABD-F), reduced glutathione were from Sigma (St. Louis, MO). Acetonitrile was from Fluka (Buchs, Switzerland). All general chemicals and reagents were of the highest purity available.

**Preparation of Saliva Samples.** Saliva samples were collected in Salivette disposable tubes (Sarstedt, Verona, Italy) according to the manufacturer’s instructions. In brief, a cotton swab was placed under the tongue for 5 minutes and then centrifuged in the Salivette tube (1000 g for 10 minutes). All saliva samples were kept at 4°C (Crioplast container; LP Italiana, Milan, Italy). Saliva samples were collected in the early morning, just after waking up, before breakfast. About 1.5 mL of saliva were collected and analyzed for uric acid and glutathione.

**High Performance Liquid Chromatography Apparatus.** The high performance liquid chromatography (HPLC) apparatus consisted of a Jasco PU-980...
pump connected to a Jasco FP-920 fluorescence detector (Jasco, Tokyo, Japan) and equipped with an Alltech Allsphere ODS-2, 150 × 4.6 μm², 5.0-mm particle size column (Alltech Italia S.r.l., Milan, Italy) provided with a guard column packed with the same matrix as the separative column and equilibrated with 20% acetonitrile in 0.050 M phosphate buffer, pH 3.0.

**Determination of Uric Acid.** Uric acid was measured by an enzymatic colorimetric method (Uric acid plus; Boheringer Mannheim, Mannheim, Germany) in an automatic analyzer (Hitachi 917).

**Determination of Glutathione.** Glutathione was measured by HPLC according to the method of Araki and Sako.40 In brief, 300 μL of saliva was treated with 30 μL of 10% (v/v) tri-n-butylphosphine in dimethylformamide for 30 minutes at 4°C in order to reduce thiols and to decouple them for proteins. The solution was then mixed with 300 μL of a 10% trichloroacetic acid solution containing 1 mM Na₂EDTA under vigorous vortexing, followed by centrifugation at 2000g for 5 minutes; 250 μL of 1.125M borate buffer, 20 μL of 1.55M NaOH, and 20 μL of ABD-F (1 mg/mL) were then added to 100 μL of the clear supernatant. The mixture was incubated in a shaking water-bath for 10 minutes at 50°C to accomplish complete derivatization of glutathione and other thiols. At the end of the reaction, the solution was cooled and 20 μL injected into the HPLC apparatus. Glutathione concentration was then measured fluorometrically at excitation wavelength of 385 nm and emission wavelength of 515 nm.

**Statistical Analysis.** Statistical analysis was performed using SPSS 12.0. The α level was fixed at 0.05. We first verified that values of glutathione and of uric acid had a normal distribution within the 2 groups of patients and the control group. By Levene test, we assessed that variances of the values both of glutathione and of uric acid were not homogeneous between groups. Therefore, we performed Welch and Brown-Forsythe test and compared the 3 groups by Tamhane test. Finally we looked for correlations between the variables under study by Spearman’s test.

**RESULTS**

Detailed results are reported in Table 2 and Figure 2. At tests for equality of means by Welch and Brown-Forsythe, differences between groups resulted probable for salivary levels of glutathione ($p = .004$ and $p < .001$ respectively) but not for salivary levels of uric acid ($p = .228$ and $p = .122$, respectively). Comparing groups by Tamhane test, the patients with oral or pharyngeal SCC have significantly higher salivary levels of glutathione than both controls and patients with laryngeal SCC. We examined all the variables under study by Spearman’s correlation. A significant correlation has been observed between salivary uric acid level and patient sex (Spearman’s rho coefficient

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**Table 2.** Results for salivary levels of glutathione.

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Salivary glutathione, mg/dL</th>
<th>95% CI for differences</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral and pharyngeal SCCs vs Controls</td>
<td>13.1 ± 12.4</td>
<td>2.73–17.74</td>
<td>.006</td>
</tr>
<tr>
<td>Oral and pharyngeal SCCs vs Laryngeal SCCs</td>
<td>13.1 ± 12.4</td>
<td>2.12–17.25</td>
<td>.01</td>
</tr>
<tr>
<td>Laryngeal SCCs vs Controls</td>
<td>3.4 ± 2.9</td>
<td>−0.84–1.96</td>
<td>.7, no significance</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; SCC, squamous cell carcinoma.

Note: Groups are compared by Tamhane test.

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**FIGURE 2.** The box plots represent salivary concentration of glutathione in the 3 groups. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
being uric acid levels significantly higher in males as shown in Figure 3. That is not an unexpected finding as salivary levels of uric acid are influenced by serum levels and hyperuricemia is strongly associated with male sex.

We did not find other significant correlations among variables in the groups under study. Tobacco smoke is known to influence antioxidant status of saliva and in particular glutathione levels, nevertheless, in the present study, no statistically significant differences emerged, probably because of the small number of nonsmokers.

DISCUSSION
Saliva is the first biological medium confronted by external materials, covering and protecting at the same time mucosa of the upper digestive tract, and in particular the oral cavity and pharynx. The carcinogen effect of various agents is exerted at least in part through alterations of chemical composition of human saliva. During evolution, various defensive mechanisms have developed in the saliva to destroy penetrating bacteria, viruses, or fungi and to protect mucosa and teeth against chemical or mechanical damage. Among various distinct salivary protective systems, we can identify an immunological one, both specific and non-specific, based on secretory immunoglobulin (Ig)A and on a network of proteases and inhibitors, with a balance between cathepsins and cystatins, and a soft tissue integrity preservation system, in which epidermal growth factor (EGF) plays a pivotal role. Recently the importance of another salivary protective system, the antioxidant one, has become obvious. Notably, human saliva has a total antioxidant capacity higher than blood plasma. Salivary antioxidant system includes various mainly water-soluble molecules and enzymes; the most important seem to be uric acid, which accounts for about 70% of the total salivary antioxidant capacity, and peroxidase, of which 2 isoforms can be identified. Other detectable salivary enzymes are catalase, glutathione peroxidase, glutathione reductase, lact dehydrogenase, aspartate aminotransferase. Peroxidase catalyzes the oxidation of thiocyanate ion, a detoxification product of cyanide present in parotid saliva, by hydrogen peroxide, producing powerful antibacterial compounds and thus also participating in the immunological defense system. Glutathione levels in human saliva are quite low if compared with other body fluids, and it does not seem to have a fundamental role in the human antioxidant system.

The present study is only a preliminary evaluation of 2 compounds involved in such antioxidant system, in order to define its role in head and neck carcinogenesis and the potential clinical implication. To obtain definitive results, it is necessary to enlarge our series of HNSCC patients and to study the other main molecules involved in the system (both low molecular weight compounds and proteins). Nevertheless, some considerations can already be formulated.

Concentration of uric acid, the main salivary antioxidant, seems to be quite stable in controls and in patients with HNSCC. Glutathione concentrations are characterized by a larger range of variation, in particular within the group of patients with SCCs of oral cavity and pharynx. The increase in salivary glutathione in oral-pharyngeal SCCs may be secondary exclusively to metabolism or lysis of malignant cells, as it is known that it is mainly intracellular in biological systems. A second hypothesis, supported by the observation of an increase secondary to cigarette smoking, is that it is actively secreted in saliva. The parotid gland secretes most of the enzymes and low weight molecules involved in the antioxidant system, and we can thus hypothesize that it is also responsible for the increase of glutathione concentration in our patients, even if such an assumption needs to be demonstrated (for example, by catheterizing the duct of Stenone to selectively parotid saliva).

We observed significant alterations in salivary glutathione of patients with oral and pharyngeal

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0.34, p = .002\]

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\text{FIGURE 3. The box plots represent salivary concentration of uric acid in males and females.}
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SCCs when compared with controls, who do not, on the other hand, significantly differ from laryngeal SCC patients. The marked difference between the 2 groups of patients is probably related to the fact that, while the growth medium of oral and pharyngeal carcinomas during all the phases of progression is constituted by saliva, laryngeal SCCs, and in particular glottic ones, have a less constant contact with salivary fluid. The present study is merely observational and the results remain absolutely preliminary, because of the small number of patients and because several aspects of our findings deserve further investigation; nevertheless, some considerations can be formulated.

The increase in salivary glutathione in oral-pharyngeal SCCs may be secondary to metabolism of malignant cells, which modifies salivary environment, but glutathione concentration is too constant and ranges too widely to be effectively used as diagnostic marker; in other words, evaluating salivary glutathione cannot help diagnose oral or pharyngeal SCCs. We plotted a receiver operating characteristic (ROC) curve, with an area under the curve of only 0.54613, confirming the poor diagnostic value of glutathione levels.

Alternatively, we could suppose that the rise in salivary glutathione levels may precede cancer development. In this case, we would expect the same findings also in precancerous conditions of the oral cavity and pharynx (dysplastic lesions). Such a hypothesis is indirectly confirmed by the observation of higher salivary glutathione levels in smokers than in nonsmokers, probably as a way to know that in smokers higher glutathione is not associated with an analogue increase in total antioxidant capacity, which in some reports is even decreased if compared with controls. That is probably because the increase we measured is in the total concentration of glutathione and not specifically in the reduced quote and because glutathione levels are not comparable with those of other antioxidants, as for example uric acid. The inducible increase of glutathione we observed is thus not sufficient to protect head and neck mucosa, but it might be an index of oxidative stress at the level of the upper airways and in particular of the oral cavity and pharynx. Therefore, high salivary glutathione may be the result of an unsuccessful attempt to protect mucosa from oxidative stress and we may use it as an epidemiological marker to identify subjects with an increased risk of developing oral and pharyngeal SCC, to submit to strict follow-up and chemoprevention. The perspective of a local chemoprevention by changing saliva composition is quite intriguing and has already begun to be tested with preliminary encouraging results. Acetaldehyde is highly toxic, mutagenic, and carcinogenic in different cell cultures and animal models. It is the first metabolite of ethanol, and it has been hypothesized that the major part of the carcinogenic potency of alcohol is mediated via this compound; furthermore, it is present in cigarette smoke and is released also in betel quid chewing. It has been recently reported that after alcohol intake, up to two thirds of carcinogenic acetaldehyde can be removed from saliva with a slow-releasing buccal l-cysteine drug formulation. l-cysteine is a nontoxic compound with antioxidant activity, able to inactivate acetaldehyde reacting covalently. So, first, studying saliva we could obtain epidemiological metabolic (as in this case) or molecular (for example by microsatellite analysis) markers to identify high-risk subjects, and then, modifying saliva, we could perform an effective chemoprevention on the same subjects. Such a chemopreventive approach is particularly interesting because it would probably support a physiologic function of saliva, which has been demonstrated to reduce the mutagenicity of well known oral carcinogens, probably also by inhibiting ROS production, and to have an intrinsic role in protecting against cancer and in delaying carcinogenesis in animal models.

REFERENCES


