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ORAL MUCOUS MEMBRANE MICROBIOTA IN HEALTH AND ORAL SQUAMOUS CELL CARCINOMA

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Abstract
The incidence of oral cancer continues to rise, and survival rates have not improved appreciably over the last 20 years. In addition to the traditionally described risk factors (tobacco, alcohol and genetics), it has been suggested that the soft tissue microbiota colonizing oral squamous cell carcinoma (OSCC) lesions differs from that of healthy sites in oral cancer subjects and healthy sites in cancer-free subjects. Indeed, recent data has shown variations in oral flora in patients with oral cancer compared with healthy controls. Thus, alterations in the proportions of certain oral bacteria in soft tissue may indicate the presence of an OSCC lesion. This observation has important diagnostic and therapeutic implications for the healthcare team and may shape the future of prevention and clinical care.

Citation: Mager D, Haddad R, Wirth L, Haffajee A. Oral mucous membrane microbiota in health and oral squamous cell carcinoma. *Grand Rounds Oral-Sys Med.* 2007;1:12-18. (Digital version *Grand Rounds Oral-Sys Med.* 2007;1:12-18.)
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Key Words: Oral squamous cell carcinoma, microbiota, carcinogenesis

Introduction

Each year, nearly 30,000 Americans are diagnosed with oral cancer, and 90% of these lesions are oral squamous cell carcinoma (OSCC).[1] Despite advances in surgery, radiation and chemotherapy, the 5-year survival rate is 54%, one of the lowest of the major cancer sites, and this rate has not improved significantly in recent decades.[2-4] Worldwide, the problem is much greater, with over 350,000 to 400,000 new cases diagnosed each year.[5] Globally, oral cancer is the 8th most common malignant tumor, but incidence varies markedly.[6] The Indian subcontinent experiences very high rates, with the disease accounting for up to 40% of all malignancies.[7] The disease kills 1 person every hour, which amounts to more people than cancers of the cervix, brain, ovary, testes, liver, kidney, malignant melanoma or Hodgkin lymphoma.[5,8] Incidence increases with age in all countries, with approximately 85% of new cases of oral cancer occurring in people over 50 years of age.[6] However, the incidence of OSCC in young (<40 years) adults is increasing in the United States [9-11] and worldwide.[12]

Oral cancer detection

Early detection followed by appropriate treatment can increase cure rates to 80% or 90%, and greatly improve quality of life by minimizing extensive, debilitating treatments.[5,13] Despite the accessibility of the oral cavity to direct examination, these malignancies are often not detected until a late stage.[5,14,15] Unfortunately, even after successful treatment, oral cancer is unusual in that it carries a high risk of second primary tumors. Patients who survive a first cancer of the oral cavity have up to a 20-fold increased risk of developing a second primary oral cancer, and that risk lasts 5-10 years and occasionally longer.[16]

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recommends that doctors and dentists examine the mouth and throat during routine examinations,[2] as early lesions are often asymptomatic and may mimic benign lesions.[17,18] Screening of the general population, on the other hand, has not been shown to reduce the incidence of and mortality from oral cancer. The reasons for this include the low prevalence and incidence of OSCC, the potential for false-positive diagnoses and poor compliance with screening and referral.[6,19]

Risk factors

One-third of all cancers worldwide can be attributed to tobacco use, and in oral cancer that percentage increases to 75-80%.[20] Although tobacco and alcohol have independently been linked to increased cancer risk,[21,22] in the oral cavity these factors act synergistically.[1] Individuals who smoke and drink have 15 times the risk of developing oral cancer.[5] Ethanol itself is not carcinogenic; however, acetaldehyde, its first metabolite, is a potent carcinogen and induces chromosomal aberrations,[23] gene mutations[24] and hyperplastic and dysplastic changes in the oral mucosa.[25-28]

Oral bacteria and acetaldehyde production

The ability of bacteria to produce acetaldehyde from alcohol has been extensively researched.[28-33] The oral mucosa produces this metabolite, yet oral microorganisms are responsible for most acetaldehyde production in the oral cavity, particularly among cigarette smokers and alcohol consumers.[29,30] Homann and colleagues[30] found that smoking and heavy drinking were the most significant factors in increasing microbial acetaldehyde production. Increased salivary acetaldehyde content because of ethanol ingestion among smokers and heavy drinkers could explain the synergistic carcinogenic action of alcohol and smoking. Comparisons of several oral commensals showed that certain oral species produce more acetaldehyde than others. This is especially true of *Streptococcus viridans*[32] and *Neisseria* species.[31,33]

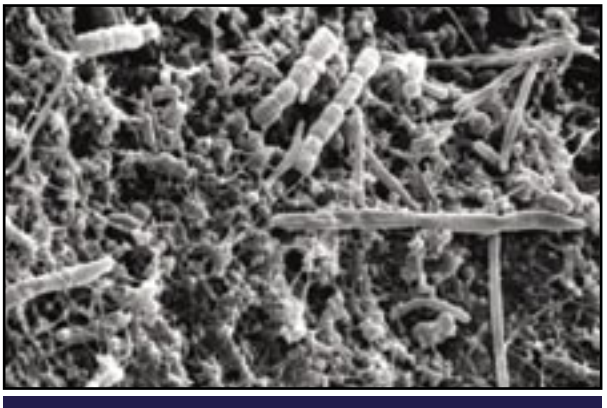

Figure 1

Scanning electron microscope (SEM) of microbial colonization on the mucosal surface of a squamous cell carcinoma involving the facial gingival of teeth #17 and #18. Diagnosis was verified by biopsy. Original magnification of x5,900. Photograph courtesy of Dr. Charles Cobb.

Colonization of OSCC lesions

Numerous studies report shifts in bacterial colonization in the presence of OSCC lesions. Increased colonization of saliva or soft tissues in oral cancer subjects has been reported. These species include *Streptococcus*, *Prevotella*, *Veillonella*, *Porphyromonas* and *Capnocytophaga*, among others.[34-38] The reasons for these elevations are unknown, but mechanistic studies indicate that shifts in colonization patterns are associated with changes in the membrane receptors expressed on cancer cells.[39-42]

Bacteria and cancer

The relationship between bacteria and cancer is currently unclear. However, certain bacterial species are consistently linked to specific cancers. For example, evidence shows that *Helicobacter pylori* is a carcinogenic agent that can lead to gastric cancer and mucosa-associated lymphoid tissue lymphomas. Similarly, chronic infection with *Salmonella typhi* is associated with increased risk of gallbladder cancer,[43,44] and endocarditis caused by *Streptococcus bovis* is significantly linked with colon carcinomas.[45]

Carcinogenic mechanisms underlying the activities of these species vary. Mechanistic studies demonstrate that some types of bacteria stimulate inflammatory mediators, induce chronic infections or produce toxins disturbing the cell cycle and thereby affecting cell growth.[45,46] These changes occur through inhibition of apoptosis,[47] stimulation of inflammatory mediators,[48,49] immune evasion[50] and production of potentially carcinogenic agents.[30] This last mechanism is potentially how oral bacteria participate in oral carcinogenesis, as in the case of acetaldehyde. In fact, reports indicate that the oral cavity produces most of the acetaldehyde in the upper respiratory tract (UAT), thereby increasing the risk of UAT cancers, particularly those seen in the oral cavity.[30] Whether specific bacterial species are markers for OSCC or involved with the development of these lesions is currently unclear.

Research initiatives for early OSCC detection

The National Institute of Dental and Craniofacial Research (NIDCR) and the Oral Cancer Foundation recommend that research efforts focus on developing novel, early detection techniques.[5,16] Currently, researchers are focusing on preventive strategies and early non-invasive diagnostic tests using saliva. As stated, some studies indicate that OSCC lesions are colonized by altered populations of microbiota.[34,37] Other investigations report bacterial deoxyribonucleic acid (DNA) or live organisms within oral cancer tissues or associated lymph nodes.[38,39,49,50] It is possible

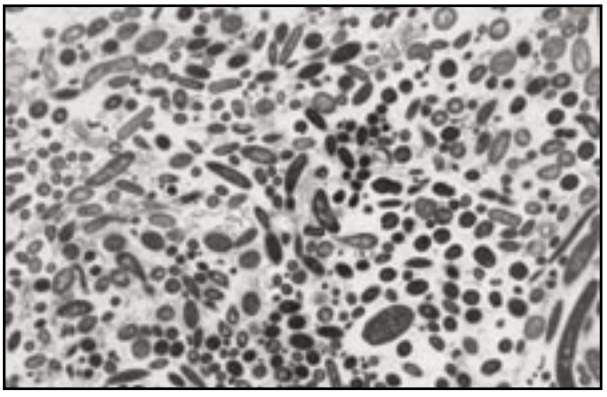

Figure 2

Transmission electron microscope (TEM) of microbial colonization from the mucosal surface of a squamous cell carcinoma involving the facial gingiva of teeth #17-#18. Diagnosis was verified by biopsy. Note the diversity of bacterial morphotypes, ranging from cocci to various rod shaped microbes. Original magnification of x3,000. Photograph courtesy of Dr. Charles Cobb.

that oral bacteria play a role in carcinogenesis; however, ecological changes may simply favor the growth of certain species. To demonstrate the potential role of oral bacteria in the initiation of oral cancer, Figures 1 and 2 provide illustration of microbial colonization of the mucosal surfaces associated with lesions of squamous cell carcinoma. Studies have compared the soft tissue microbiota of cancer-free and OSCC tissues. However, until recently little was known about the soft tissue and salivary microbiota in healthy cancer-free subjects.

Salivary and soft tissue microbiota in cancer-free subjects

In previous work from our laboratory we conducted what to our knowledge was the first comprehensive examination of intra-oral microbiota in systemically healthy subjects.[51] A total of 225 subjects were enrolled in a cross-sectional study at Forsyth Institute. Subjects provided microbiological samples of saliva and soft tissue samples from 8 oral sites. Samples were individually evaluated for levels of 40 common oral bacteria using checkerboard DNA-DNA hybridization.[52] Microbial samples were taken from the 8 tissue surfaces in 225 systemically healthy subjects using a buccal brush, and saliva was taken by expectoration.

There was a high degree of specificity in the "preferred" intraoral localization of species. This specificity in localization of individual species agreed with that described in previous studies.[53,54] The most striking finding of this study was the highly site-specific colonization patterns of the 40 bacteria at individual soft tissue locations. Even within genera such as *Streptococcus*, striking differences among species were noted. For example, *Streptococcus intermedius* was seen at low levels at all sites except the hard palate, where it was markedly elevated. *Streptococcus gordonii* colonized all sites moderately but favored the buccal mucosa and vestibule/lip, where it was found at high levels. *Streptococcus anginosus* was markedly elevated on the attached gingiva but found at relatively low levels at the remaining sample locations.

The salivary microbiota in OSCC subjects compared with cancer-free subjects

In the initial study of oral cancer subjects, we compared the salivary microbiota from the original control subjects (225) with that found in 45 subjects with a primary, untreated oral squamous cell carcinoma lesion.[37] Oral cancer subjects were enrolled at Dana-Farber Cancer Institute and Massachusetts General Hospital. DNA counts/ml of saliva were determined for each of the 40 species using checkerboard DNA-DNA hybridization. Diagnostic sensitivity and specificity in detection of OSCC using levels of salivary organisms were computed and comparisons made separately between a non-matched group of 45 OSCC subjects and 225 cancer-free subjects and a group of 45 OSCC subjects and 45 cancer-free subjects matched by age, gender and smoking history. Even after adjusting for multiple comparisons,[54] salivary counts/ml of 3 of the 40 species tested, *Capnocytophaga gingivalis*, *Prevotella melaninogenica* and *Streptococcus mitis*, were elevated in individuals with OSCC ($P<0.001$). When evaluated as diagnostic markers these species were found to predict 80% of cancer cases (sensitivity), while excluding 83% of cancer-free subjects (specificity) in the non-matched group. Diagnostic sensitivity and specificity in the matched group were 80% and 82%, respectively. We concluded that high salivary counts of these 3 species may be diagnostic indicators of OSCC.

Results from our investigation demonstrated that oral cancer subjects had elevated proportions of *C. gingivalis*, *P. melaninogenica* and *S. mitis* in saliva compared with OSCC-free subjects. In the matched group it was determined that these results were of borderline significance after adjusting for multiple comparisons. The difference in the size and demographics of the 2 populations were limitations of the study.

Despite these caveats, our data supports the notion that differences exist in microbiota colonizing soft tissues of oral cancer versus cancer-free subjects. Not only did oral cancer subjects have higher proportions in salivary and soft tissue of the 3 bacterial species, but control subjects, regardless of periodontal and smoking status, showed higher levels of *Eubacterium saburreum*. In the group matched in terms of age, smoking status and gender, *P. melaninogenica* and *S. mitis* were elevated in oral cancer subjects as noted but after adjusting for multiple comparisons these differences were of borderline significance. In

contrast, *E. saburreum* and *Actinomyces israelii* remained significantly elevated in cancer-free subjects. Interestingly, the proportion of colonized *Porphyromonas gingivalis* and *Tannerella forsythia* tended to increase in periodontitis, smoking and oral cancer subjects.

One study of oral cancer microbiota indicates that although increased numbers of both aerobic and anaerobic colony-forming units are seen at a tumor site, there are few significant differences in the distribution of bacterial species colonizing cancer lesions or contralateral healthy sites.[34] Liebermann and colleagues[55] report that in oral cancer subjects, additional bacteriological examination of the surrounding pharyngeal microbiota showed no major difference in the types of organisms associated with oral carcinoma. A relationship of bacteria to oral and other cancers has been observed in other studies. Recent reports describe significantly elevated levels of *S. anginosus* DNA and/or related *Streptococcus* species in surgical samples taken from several cancers of the upper digestive tract, including gastric, esophageal,[56] oral and head and neck[57,58] cancers, as well as cervical lymph nodes associated with oral carcinoma.[36] In some studies, *S. anginosus* DNA was repeatedly detected.[57,58] In the present investigation, *S. anginosus* was consistently present at higher levels in oral cancer subjects than in cancer-free subjects regardless of whether 225 or 45 cancer-free subjects were evaluated. However, these trends were not statistically significant.

Interestingly, when samples were compared from both lesion and healthy sites of oral cancer patients and sites from healthy subjects (the unmatched group of 225 healthy and 45 oral cancer subjects and the matched group of 45 healthy and 45 oral cancer subjects), in terms of microbial profile, healthy sites in oral cancer subjects were more similar to oral cancer lesions than to healthy sites in cancer-free subjects. The reason for this finding is unclear.

Some factors potentially affecting healthy sites in oral cancer subjects include an impaired immune system, changes in nutrient availability or altered epithelial receptors. Recent studies indicate molecular alterations in tissues surrounding OSCC lesions.[59-61] These genetic alterations support Slaughter's model of a field from which second primary recurrent tumors arise.[62] Interestingly, some researchers refer to cancer as a molecular disease of cell membrane glycoconjugates.[63-65] Particular glycoconjugates serve as receptors for specific bacteria, and recent reports support the idea that shifts in bacterial colonization of different cancer cells are associated with molecular changes in cell surface receptors.[63,66-68] As bacterial colonization has been shown to be highly site-specific and dependent upon unique receptors, it is reasonable that "field cancerization" could result in microbial shifts, which have been seen in cancer cells both *in vitro* and *in vivo*.[63,66,68]

As previously stated, certain oral species reportedly produce significantly higher levels of acetaldehyde than others, namely *Neisseria* species and *S. viridans*.[32] Muto and colleagues[31] found that non-pathogenic members of the oral *Neisseria* genus, such as *Neisseria* mucosa, produced 100 times the amount of acetaldehyde than members of several other genera. Notably, in all of our comparisons, *S. mitis*, a member of the *S. viridans* family, and *N. mucosa* were present at higher levels in oral cancer subjects. Furthermore, levels of *N. mucosa* were increased proportionally from healthy sites in cancer-free subjects, to healthy sites in cancer subjects, to sites in OSCC lesions.

Future directions

A screening test for oral cancer based on salivary counts or soft tissue proportions of bacterial species is appealing. Salivary analysis provides an inexpensive, noninvasive, and easy-to-use diagnostic aid for oral and systemic diseases and an assessment tool for risk behaviors such as tobacco and alcohol use. Detection of human immunodeficiency virus (HIV) by the presence of virus-specific antibodies in saliva, for example, has led to development of commercially available test kits.[69] If increased numbers or proportions of certain oral species are shown to be a signature of oral cancer, an early diagnostic test for OSCC may be developed, reducing the morbidity and mortality of this devastating cancer. Studies examining the validity of findings presented here are planned. If these studies are validated, it will be important to determine whether elevations in certain oral bacteria indicate the presence of oral cancer or actually contribute to carcinogenesis.

Acknowledgments

My sincere gratitude goes to Charles M. Norris, MD; Julia Kazakin, MD; Marshall R. Posner, MD, PhD; James Rocco, MD, PhD; Rosemary Costello, RN, MS, OCN; and Tina Yaskell, BS, for their unwavering support of this study. I thank Francisco Groppo, DDS, MSc, PhD, for providing invaluable assistance in analyzing the salivary data. Finally, special thanks go to the patients who enrolled in this study; it would not have been possible without them. **Supported by NIDCR grant K23 DE-00453-05**.

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