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Recommended Citation

Katz, J.; Onate, M.; Pauley, Kaleb M.; Bhattacharyya, I; and Cha, S., "Presence of *Porphyromonas gingivalis* in gingival squamous cell carcinoma" (2011). *Science and Mathematics Faculty Publications*. 188.
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Presence of *Porphyromonas gingivalis* in gingival squamous cell carcinoma

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Periodontal disease has been recently linked to a variety of systemic conditions such as diabetes, cardiovascular disease, preterm delivery, and oral cancer. The most common bacteria associated with periodontal disease, *Porphyromonas gingivalis* (*P. gingivalis*) has not yet been studied in the malignant gingival tissues. The objective of this study was to investigate the presence of *P. gingivalis* in specimens from squamous cell carcinoma patients. We have performed immunohistochemical staining to investigate the presence of *P. gingivalis* and *Streptococcus gordonii* (*S. gordonii*), a non invasive oral bacteria, in paraffin embedded samples of gingival squamous cell carcinoma ($n=10$) and normal gingiva ($n=5$). Staining for *P. gingivalis* revealed the presence of the bacteria in normal gingival tissues and gingival carcinoma, with higher levels (more than 33%, $P<0.05$) detected in the carcinoma samples. The staining intensity was also significantly enhanced in the malignant tissue by 2 folds ($P<0.023$) compared to specimens stained for the non-invasive *S. gordonii*. *P. gingivalis* is abundantly present in malignant oral epithelium suggesting a potential association of the bacteria with gingival squamous cell carcinoma.

Keywords: *Porphyromonas gingivalis*; gingival squamous cell carcinoma; periodontitis; oral cancer

International Journal of Oral Science (2011) 3: 209-215. doi: 10.4248/IJOS11075

Introduction

In recent years, periodontal disease has been linked to many systemic conditions such as cardiovascular disease, low-birth weight complications in pregnancy, diabetes, pulmonary disease and cancer. The scientific rationale behind the proposed association is the long chronic nature of inflammatory process underlying periodontitis [1-2]. Recent studies support a strong relationship between certain microorganisms and various forms of cancer. The link between viral agents such as Epstein-Barr virus (EBV) and nasopharyngeal carcinoma and Burkitt's lymphoma is well documented [3]. The role of HPV in oral and cervical squamous cell carcinoma is

increasingly appreciated [4]. Some bacteria such as *Helicobacter pylori* (*H. pylori*) have been associated with gastric cancer [5].

More recently, a link between periodontal disease and cancer has been suggested through several studies looking both at specific types of cancers and at the overall total cancer rate and the relationship to periodontal disease [6]. Oral cancer, gingival squamous cell carcinoma in particular, has been known to mimic advanced periodontal disease in clinical appearance showing similar symptoms of swelling, bleeding, tooth mobility, deep periodontal pockets, and bone destruction [7]. Many cases of gingival squamous cell carcinoma presenting clinically similar features to inflammatory periodontal or periodontal/endodontic lesions have been reported [8]. In some cases these lesions were probably overlapping.

Porphyromonas gingivalis (*P. gingivalis*) is a common gram negative anaerobic oral bacteria strongly associated with periodontal disease. Although this bacterium has

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Received 19 February 2011; Accepted 13 September 2011

been proven to invade gingival epithelial cells *in vitro*, studies on presence of the bacteria at different sites of oral epithelium *in vivo* are very scarce [9]. *P. gingivalis* is known for its capacity to penetrate and invade various epithelial cells. It has a sophisticated mechanism that enables it to modify the host cell defense including alteration of some host specific genes. While inside the cell, the bacteria have been shown to affect cell cycle related molecules at different stages. Most importantly, *P. gingivalis* has been shown to prevent apoptosis of epithelial cells, an inherent protective mechanism of cells affected by cancerous processes [10-11]. It successfully invades, multiplies and survives in the cytoplasm of infected cells and spreads to adjacent cells [12]. *P. gingivalis* has also the ability to protect itself from both the humoral and cellular immune system, can suppress all three mechanisms of complement activation and is resistant to killing by oxidative stress [10]. *P. gingivalis* can block the apoptotic pathway in gingival epithelial cells through manipulation of the JAK/Stat pathways that controls the intrinsic mitochondrial cell death pathways and prevent programmed host cell death [13]. In addition, *P. gingivalis* may inhibit host cell death by prolonging cell survival and increasing cell proliferation [14]. It was shown to be capable to attach to various gingival carcinoma cell lines [15]. A significant association between presence of *P. gingivalis* and infection with EBV, a known virus associated with head and neck cancer, has also been reported [16]. Other bacteria such as *H. pylori* were shown to be associated with inflammation of the gastric mucosa, leading to gastritis, peptic ulcers, gastric carcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphomas [17].

Our long term hypothesis is that the bacteria may be associated with the etiopathogenesis of squamous cell carcinoma in the gingiva. The purpose of the present study was, therefore, to investigate the presence of *P. gingivalis* in gingival squamous cell carcinoma.

Materials and Methods

Tissue acquisition

After an institutional review board (IRB approval), we randomly selected de-identified biopsy paraffin embedded blocks ($n=10$) of squamous cell carcinoma of the gingiva archived at the Division of Oral Pathology University of Florida. These specimens had a varying degree of differentiation. Five additional blocks diagnosed as a non-neoplastic gingival tissue for comparison of staining were used. Two slides were prepared from each block and each slide contained two serial sections of 4 μ m thickness for staining with a primary antibody or rabbit

IgG (negative control). Control and subject slides were graded and analyzed for intensity of *P. gingivalis* staining. Since we have used archived samples in a retrospective study, information regarding age, gender, and smoking habits were not available.

Antigen retrieval

Tissues were fixed in formalin and then embedded in paraffin. The sectioned slides were deparaffinized using CitriSolv (Fisher Scientific, Pittsburgh, USA), hydrated by submersing in three separate concentrations of ethanol (100%, 95%, and 70%), and rinsed continuously in distilled water for 5 min. Afterward, antigen retrieval was performed by incubating slides in a 1 L antigen retrieval citra plus solution (BioGenex, San Ramon, USA), according to the manufacturer's recommendation. Briefly, the slides were placed into the citrate-based antigen retrieval solution at 95 °C for 20 min. The heated slides were cooled down to room temperature for another 20 min, rinsed with distilled water for 5 min, and then blocked for 30 min with 1.5% Normal Goat Serum (Vector Laboratories, Burlingame, USA).

P. gingivalis and *Streptococcus gordonii* Staining

Rabbit *P. gingivalis* polyclonal antibodies (a generous gift from Dr. Richard Lamont) were raised against formalinized cells of the type strain ATCC 33277 [18]. This antibody does not react with human cells or with other bacteria at dilutions of 1 : 500 or greater. Control antibodies raised to *Streptococcus gordonii* (*S. gordonii*) strain G9B were also utilized [19]. Tissue sections were incubated with antibodies at a 1 : 1000 dilution for one hour at room temperature, followed by biotin-conjugated secondary antibody for one hour at room temperature, streptavidin-peroxidase for 30 min at room temperature, and enzyme substrate (Vector Laboratories, Burlingame, USA). As an additional control, sections were also incubated with phosphate buffered saline (PBS) only, followed by incubation with biotin-conjugated secondary antibody, streptavidin-peroxidase, and enzyme substrate. PBS washes (three washes, 5 min each) were performed between each incubation. Sections were counterstained with methyl green and visualized by light microscopy (Olympus BH2, Center Valley, USA) according to the procedure for paraffin-embedded sections [20].

Data analysis

The sections were evaluated by two pathologists who had been calibrated by comparisons of printed images in high magnification of immunohistochemistry staining over a period of few weeks. The kappa statistic was used to assess interobserver variability based on the values

of >0.75 which indicate excellent agreement beyond chance. Staining intensity was rated according to the following scale: no visible staining=0, faint staining= 1+, moderate staining=2+, and strong staining=3+. Extensiveness was graded semiquantitatively as 0%, < 10%, 10%–25%, 25%–50%, 50%–75%, 75%–90%, and >90% of positively stained cells, per high-power field. To compare all of the available data, we assigned an overall score to each case by multiplying the intensity score by the mean percentage of cells stained (extensiveness). This method has been used and verified by others [21-22]. The overall score was used as the basis for statistical comparison for Table 1. The Wilcoxon signed ranks test for related samples was used to analyze the difference in the stain intensity between the samples stained with antibody to *P. gingivalis* and those stained with antibody to *S. gordonii*.

Table 1 Grading of *P. gingivalis* and *S.gordonii* immunostaining

Diagnosis based on histology	^a Intensity for <i>P. gingivalis</i>	^a Intensity for <i>S.gordonii</i>
SCC extremely well diff.	+	-
SCC well diff.	++	++
SCC mod. diff.	+	-
SCC mod. diff.	++	++
SCC mod. diff.	++	-
SCC mod. to poorly diff.	+++	++
Papillary SCC well diff.	+	+
Papillary SCC well diff.	+	+
Papillary SCC well diff.	+	-
Early verrucous carcinoma	++	-

SCC: squamous cell carcinoma; diff.: differentiated; mod.: moderately. ^aThe intensity and extensiveness were determined per high-power field. -: no staining; +: faint staining; ++: moderate staining; +++: strong staining.

Results

Clinical and histological features of gingival squamous cell carcinoma

Clinical gingival carcinoma demonstrates irregular granular and ulcerative lesions (black arrows), which can be easily misdiagnosed for periodontal disease (Figure 1A). Neoplastic epithelium in the section of well differentiated carcinoma is apparent, forming islands and extensions invading into the subjacent connective tissue

(Figure 1B) and visualizing discernible squamous eddies. Figure 1C indicates that moderately differentiated carcinoma with some of the islands of invasive epithelium displaying loss of cellular differentiation (arrows) and other areas exhibit well formed keratin pearls and squamous eddies. Poorly differentiated squamous carcinoma with normal appearing epithelium (asterisk) surrounded by islands of closely clustered, highly basophilic and pleomorphic epithelium with minimal keratinization invades into the subjacent connective tissue (Figure 1D).

P. gingivalis is detected in the specimens of gingival squamous cell carcinoma

To detect presence of *P. gingivalis*, the specimens were stained with an antibody specific for *P. gingivalis* (Figure 2). *P. gingivalis* staining was positive in a sample of well differentiated gingival carcinoma where prominent cytoplasmic staining is noted in the malignant epithelial cells (Figures 2A, 2B) while rabbit IgG controls C and D showed no staining as expected except for the edges of the specimen shown in Figure 2C.

P. gingivalis invades gingival squamous cell carcinoma more significantly than S. gordonii

In order to prove the specificity of staining for *P. gingivalis*, immunohistochemical staining for non-invasive bacteria *S. gordonii* was performed on human biopsy specimens (Figure 3). Figure 3A indicates *P. gingivalis* staining in a well-differentiated squamous cell carcinoma specimen of the gingiva exhibiting florid cytoplasmic staining of invasive malignant cells whereas *S. gordonii* staining is devoid of any staining, shown in Figure 3B. *P. gingivalis* staining demonstrates strong positive cytoplasmic reactivity again in malignant epithelial cells in a sample of moderately differentiated squamous cell carcinoma of the gingival in Figures 3C and 3D in contrast to Figure 3B exhibiting absence of reactivity with antibody specific for *S. gordonii*. The intensity of the sections stained with *P. gingivalis* were significantly enhanced compared to those stained with *S. gordonii* (Table 1, $P<0.023$). When each group was averaged for intensity, the *P. gingivalis* group had a 2-fold increase in the staining intensity compared to specimens stained for *S. gordonii*.

Normal gingival tissue exhibits significantly weak staining of P. gingivalis compared to gingival carcinoma tissues

To further examine the specificity, non-neoplastic gingival tissues were stained for *P. gingivalis* and *S. gordonii* (Figure 4). This gingival tissue was positive for *P. gingivalis* (Figure 4A). *S. gordonii* staining of gingival

tissue from normal gingival tissue showed weak staining (Figure 4C). Negative control gingival epithelium exhibiting absence of staining with rabbit IgG (Figure 4B).

The overall staining of the carcinoma group was 33% more intense than the normal gingival control group ($P < 0.05$)

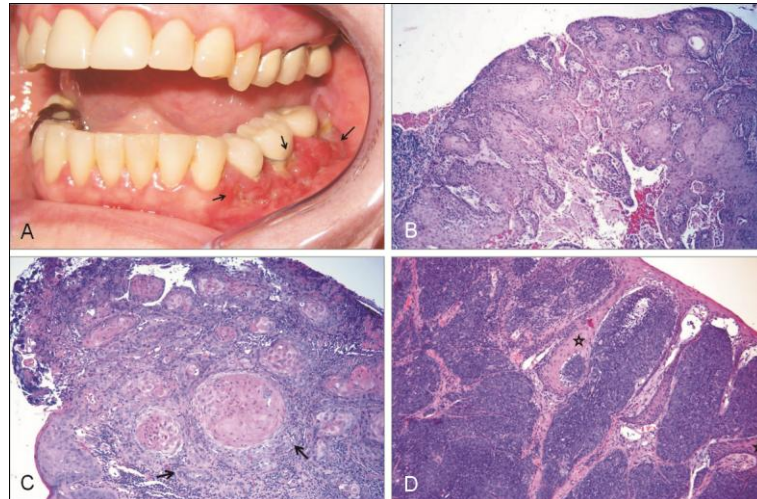


Figure 1 Clinical and histological features of gingival squamous cell carcinoma. (A) A clinical image of gingival carcinoma exhibiting irregular granular and ulcerative lesions (arrows) with recession. (B) A histology image of well differentiated squamous cell carcinoma with neoplastic epithelium forming islands and extensions that invade into the subjacent connective tissue. H&E stain, $\times 10$. (C) An image of moderately differentiated carcinoma with some of invasive epithelium islands displaying loss of cellular differentiation (arrows) and other areas exhibiting well formed keratin pearls and squamous eddies. H&E stain, $\times 10$. (D) Poorly differentiated squamous carcinoma with normal appearing epithelium (asterisk) surrounded by islands of closely clustered, highly basophilic and pleomorphic epithelium with minimal keratinization invading into the subjacent connective tissue. H&E stain, $\times 10$.

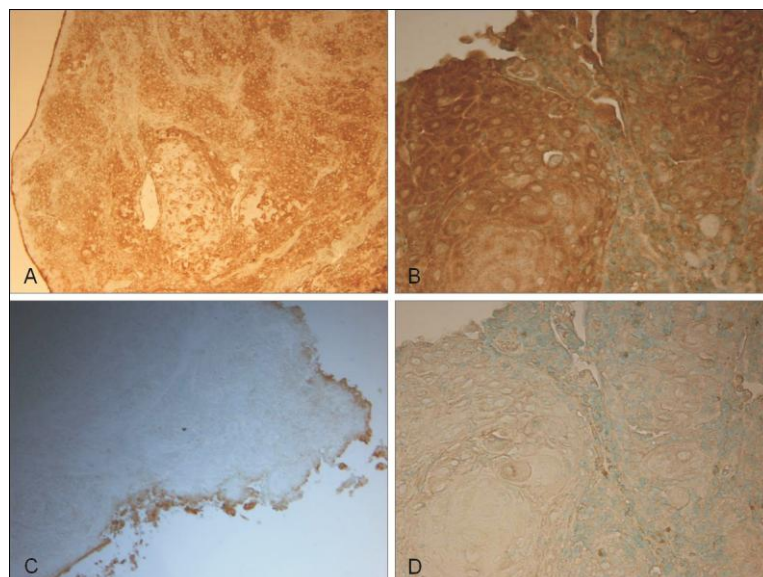


Figure 2 Detecton of *P. gingivalis* in well differentiated gingival carcinoma specimens. (A) Strongly positive staining of *P. gingivalis* in well differentiated gingival carcinoma specimens. H&E stain, $\times 10$. (B) Strongly positive staining of *P. gingivalis* in well differentiated gingival carcinoma specimens. H&E stain, $\times 40$. (C) Negative control exhibiting absence of staining with rabbit IgG. H&E stain, $\times 10$. (D) Negative control exhibiting absence of staining with rabbit IgG. H&E stain, $\times 40$.

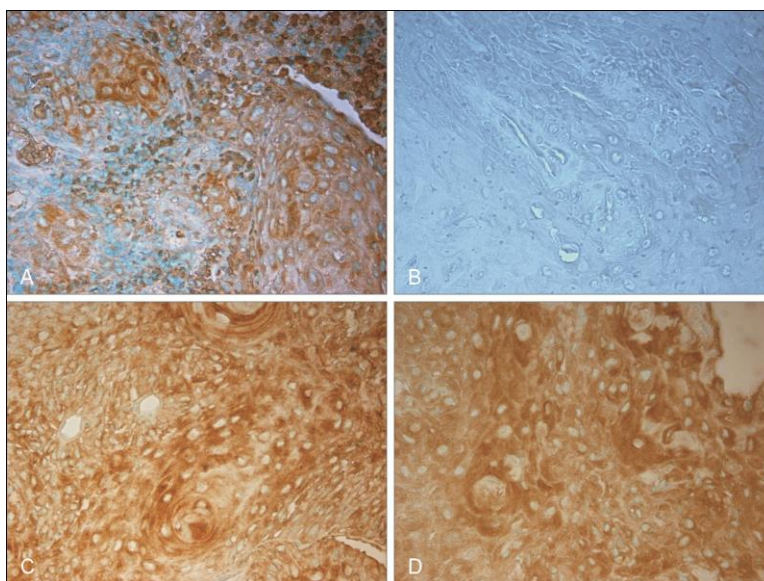


Figure 3 Strong staining of *P. gingivalis* in gingival carcinoma specimens in comparison with *S. gordonii* staining. (A) *P. gingivalis* staining demonstrates strong positive cytoplasmic reactivity in malignant epithelial cells in a sample of well differentiated squamous cell carcinoma of the gingival. H&E stain, $\times 40$. (B) An image exhibits absence of reactivity with antibody specific for *S. gordonii*. H&E stain, $\times 40$. (C) Photomicrograph of *P. gingivalis* antibody staining exhibits floriid cytoplasmic staining of invasive malignant cells in a specimen of moderately differentiated gingival squamous cell carcinoma. H&E stain, $\times 40$. (D) Photomicrograph of *P. gingivalis* antibody staining exhibits floriid cytoplasmic staining of invasive malignant cells in a specimen of moderately differentiated gingival squamous cell carcinoma. H&E stain, $\times 40$.

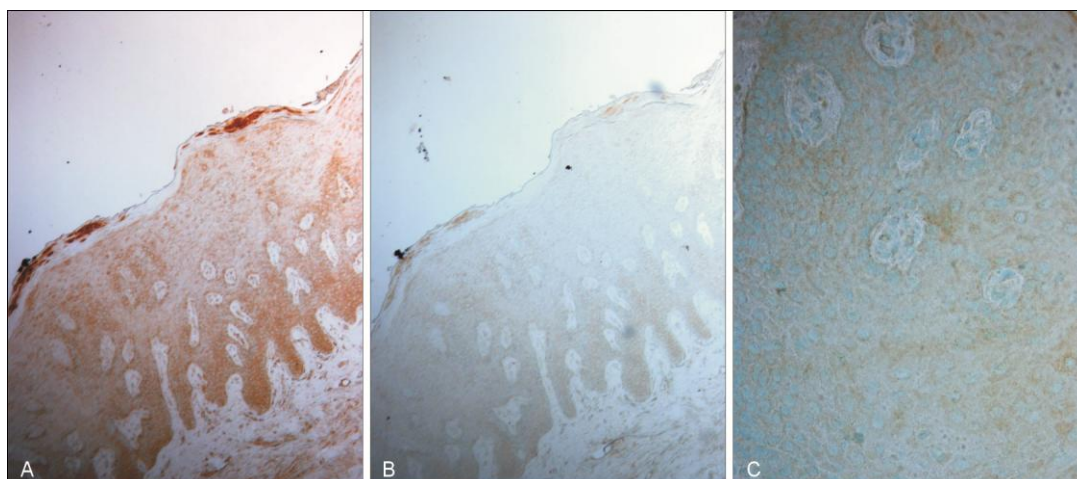


Figure 4 Normal gingival tissue staining for *P. gingivalis*. (A) Demonstrates a section of hyperplastic non-neoplastic gingival epithelium exhibiting positive staining with *P. gingivalis* antibody. H&E stain, $\times 10$. (B) Negative control with same section of gingival hyperkeratosis demonstrating absence of staining with rabbit serum H&E stain, $\times 10$. (C) Normal gingival tissue exhibiting weak staining with *S. gordonii*. H&E stain, $\times 20$.

Discussion

In the current study, we have identified significant staining specific for *P. gingivalis* in gingival carcinoma

specimens, and to a lesser extent in the normal gingival tissue. Staining was mainly localized in malignant epithelial cells in most cases. This finding indicates that at histological site, the bacteria have the capacity to

invade both normal and neoplastic cells *in vivo*. It is not clear if *P. gingivalis* invaded the malignant cells before or after their transformation. We noticed that poorly-differentiated gingival carcinoma specimens tend to show stronger *P. gingivalis* staining compared to well-differentiated tissues. Whether there is any reliable association between the degree of differentiation and *P. gingivalis* involvement still needs to be further investigated with a larger sample size. In order to examine specificity of *P. gingivalis* staining, we further stained the specimens for *S. gordonii*. The latter is known to be a non-invasive component of the oral flora. As illustrated in Figure 3, the *P. gingivalis* staining was stronger in gingival carcinoma compared to specimens stained for *S. gordonii*. According to our statistical analyses, the intensity of the section stained with *P. gingivalis* was significantly enhanced by 2-fold compared to *S. gordonii* stained sections. In the past, we have demonstrated the presence of *P. gingivalis* in the trophoblasts of placenta from both normal and chorioamnionitis. Interestingly, the bacteria invaded the normal trophoblast cells as well, although it was to a lesser extent [23], similar to the invasion of the normal gingival and malignant oral epithelium presented here.

Connections between oral cancer and tooth loss or periodontal disease have been evaluated in several studies [24–26]. Most found a significant increase of oral cancer risk in patients with increased tooth loss or other parameters of periodontal disease even after adjustment for tobacco and alcohol. Perhaps the most informative studies of these are two by Tezal *et al* [25–27], since objective measures of periodontal disease were used in these as opposed to tooth loss as a measurement tool.

The significance of our finding of increased *P. gingivalis* presence in the gingival carcinoma tissues compared to normal gingiva is not clear. Nonetheless, it is evident that *P. gingivalis* penetrates both normal and malignant epithelium at a larger extent than non-invading bacteria such as *S. gordonii*. Although there is a possibility that *P. gingivalis* may be involved in tumorigenesis by inhibiting host cell death and inducing cell proliferation, it may be presumptuous to conclude its carcinogenic potential in gingival tissue without further direct evidence.

In this retrospective study of archived samples no information is provided on co morbidity factors such as age, gender, smoking and alcohol consumption. These variables may be important as they may affect the ability of the bacteria to invade the gingival tissue and potentially impact the malignant process. Further studies using *P. gingivalis* DNA probes in carcinoma tissues at different disease stage in a large cohort is warranted to

determine its significance in gingival squamous cell carcinoma etiopathology.

Acknowledgments

This work supported by Kaleb M. Pauley, is supported by National Institute of Health/National Institute of Dental and Craniofacial Research training grant T32DE007200.

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