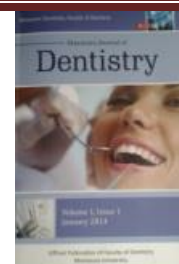# Detection of the periodontal pathogen Porphyromonas Gingivalis in Oral Squamous Cell Carcinoma

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Abstract:

Background: The concept of infection-based carcinogenesis is gaining an increasing attention. Also periodontitis has been extensively studied as possible risk factor for gastrointestinal cancers including oral cancer.

Methodology: Using conventional PCR, we detected the presence of the key periodontal pathogen Porphyromonas gingivalis (P. gingivalis) in 25 oral cancer fresh tissue specimens versus 25 healthy oral mucosal tissue..

Results: P. gingivalis showed significantly higher prevalence in control group with slightly higher loads in cancer tissue. Bacterial presence was not found to be correlated with tumor progression.

Conclusion: P. gingivalis was not found to play a possible role in the cancerous lesion. More studies should be implemented to investigate the bacterial involvement in the oral tumors.

Introduction

Oral cancer is a malignant tumor of which 90% of cases are oral squamous cell carcinomas (OSCCs)[1]. OSCC is generally considered the 6th most common cancer worldwide and is among the three most common cancers in South-Central Asia [2] . Despite therapeutic advances, the 5-year survival rate is approximately 50%, making The most significantly recognized risk factors for OSCC include tobacco smoking, alcohol consumption and chronic dental trauma[3-5]. However, around 15% of patients develop OSCC in the absence of these known risk factors, which creates the need to investigate the presence of other possible undefined contributors including the role of the oral bacteria.

Epidemiological studies for so long have been indicating that poor oral hygiene and tooth loss were significantly associated with OSCC, providing the first signs that oral bacteria
may play a role in the development of oral cancer [6,7]. Also many recent studies and meta-analyses have demonstrated a significant association of periodontal disease with oral cancer [8-10]. However, no causal relationship has been clearly demonstrated to date.

Increasing evidence is linking the infection-driven inflammation and cancers at different body sites with both viral and bacterial infections addressed. The first regarded organism as a carcinogenic agent by the WHO is Helicobacter pylori that causes gastric carcinoma[11,12]. More recently, mounting evidence has correlated oral pathogens, namely Porphyromonas gingivalis, with different types of gastrointestinal tumors[13-15].

Oral cavity biofilms can host a population of more than 500 different bacterial species [16]. Under certain conditions, the homeostatic state of oral bacterial flora can be lost, leading to an increase in the abundance of pathogenic microorganisms or the expression of virulence factors. To date, various bacterial taxa have been correlated with oral cancer and epithelial premalignant lesions[17-20]. Chronic inflammation caused by bacteria is one of the potential pathways of their contribution to various stages of oral carcinogenesis [21]. inflammation-induced DNA damage in epithelial cells caused by bacteria and endotoxins[22].

Periodontitis is a common chronic inflammatory disease. Deep periodontal pockets contain large numbers of bacteria and inflammatory factors, which can cause diseases of adjacent and remote tissues and organs[23]. Some studies have reported that there might be correlations between periodontal disease and malignant tumours, especially oral cancer[24,25]. P. gingivalis is a major periodontal pathogens that has an already established role in the initiation and progression of the periodontal disease[26]. The relationship between P. gingivalis and OSCC remains controversial. However, few clinical and experimental studies have suggested a possible connection [27]

To investigate this possibility, P. gingivalis was detected in OSCC patients through conventional PCR

METHODOLOGY

Study population

Twenty-five oral cancer tissue specimens were collected from patients with histologically verified squamous-cell carcinoma diagnosed at an earlier biopsy. Eleven males and fourteen females, with average age 54.92±12.70 years, were

being treated in Oncology center of Mansoura university between September 2017 and August 2018. Twenty-five healthy controls (12 males and 13 females; average age 34.28±1.86 years) were recruited from patients undergoing minor oral surgeries at the Faculty of Dentistry, Mansoura University.

Patient examination:

Personal, medical, dental history of patients were obtained of the database of OCMU for cancer patients. Preoperative periodontal examination was implemented to record the Plaque index (PlI), Gingival Index (GI), probing depth (PD) and clinical attachment loss (CAL) for selected teeth. Tumor stage was identified after resection according to tumor-node-metastasis (TNM) classification by American Joint Committee on Cancer/Union International Cancer Center. (8th edition, 2017).

Specimen collection:

OSCC tissue specimens were harvested from the fresh surgically resected tumors. Healthy specimens were obtained during oral surgical procedures.

DNA Extraction and PCR Assay

Experiments were conducted at Department of Medical Microbiology and Immunology, faculty of medicine, Mansoura University. All tissue specimens were homogenized as approximately 25 mg of tissue. Genomic DNA was extracted using a commercial kit QIAamp DNA mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The DNA yield, concentration and purity were assessed using the spectrophotometry (NanoDrop 2000, Thermo Scientific, Wilmington, Delaware USA).

Specific DNA primers for Porphyromonas gingivalis ATCC 33277 were supplied by invitrogen, Thermo Fisher Scientific. The forward primer: AGG CAG CTT GCC ATA CTG CG. The reverse primer is: ACT GTT AGC AAC TAC CGA TGT. PCR amplification of 16S rDNA genes was performed in a thermal cycler PTC-100 PCR Programmable Thermal Controller, MJ Research. The conditions were: 94°C 30 sec, 55 °C 30 sec, 72 °C 1 min .The Master mix used was 2x PCR Master mix Solution (i-Taq)(iNtRON Biotechnology). The reaction volume was prepared as a 25µl mixture according to the manufacturer's recommendations. The predicted amplicon length is 404 bp.

Gel Electrophoresis

The amplification products were analyzed by 1.5% agarose gel electrophoresis in 1X TAE buffer(pH 8). The used DNA marker was 10 µl of (50327) - SimplyLoad™ 100 bp DNA ladder, Lonza, Rockland, USA. Gels were run in a Bio-Rad apparatus at constant voltage (75V) for 60 minutes. Products were visualized with ethidium bromide under UV illumination..

Data analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 22.0. Qualitative data were described using number and percent. Quantitative data were described using median (minimum and maximum) for non-parametric data and mean, standard deviation for parametric data after testing normality using Shapiro–Wilk test. Significance of the obtained results was judged at the (0.05) level.

RESULTS

The gel images of the PCR yields are shown in figure (1). Analyzing the results showed that P. gingivalis was significantly higher in control group. Only 12% of cancer cases were positive for P. gingivalis in relation to 44% positive cases of control (table 1).

Table (1): PCR- detected presence of P. gingivalis in cancerous tissues of OSCC versus normal oral mucosa.

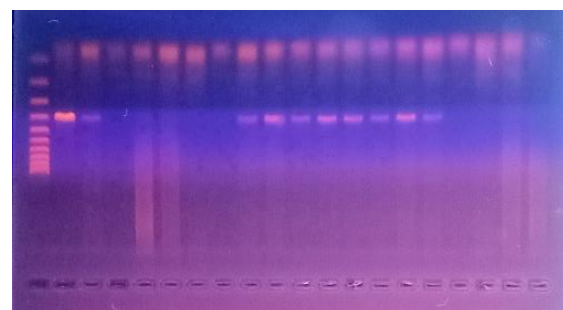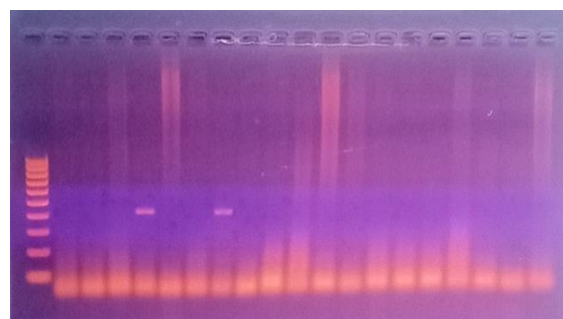| Strain	Result	OSCC				
(n=25)	Control					
(n=25)	Test of significance					
		n	%	n	%	
P.gingivalis 56.0	Negative $\chi 2=6.35$			22	88.0	14
p=0.012*						
	Positive 3		12.0	11	44.0	
*statically significant (if p<0.05) , $\chi 2$=Chi-Square test						

Figure (1) : Agarose gel electrophoresis of PCR products. Lane M shows the DNA marker (SimplyLoad™ 100 bp DNA ladder, Lonza, Rockland, USA). Lanes show specific DNA bands of the corresponding specimens (A) P.gingivalis of cancer group. (size <404 bp). (B) P.gingivalis of control group(size <404 bp).

In table 2, absolute presence the bacteria was not found to have a statistically significant difference regarding TNM staging or HP grade of the cancerous lesion (P > 0.05).

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Table (2): Relation between P. gingivalis , Tumor characteristics among OSCC cases

Tumor Clinical

Parameters
P. gingivalis
T (Tumor Size) MC
P=0.39
N (Nodal Metastasis)   MC
P=0.37
Tumor stage   MC
P=0.08
HP grade   MC
P=0.16

Positive detection of P. gingivalis showed no significant correlation with any of the clinical periodontal indices. Table (3).

Table (3): Relation between P. gingivalis & clinical indices among cases

clinical indices among Cases	P. gingivalis
presence	
absent	
n=20 Present	
n=3	
Plaque Index	
PlI	
mean±SD 1.58±0.45	2.03±0.67
Gingival index	
GI	
mean±SD 1.56±0.48	1.97±0.64
Probing depth	
PD	
mean±SD 1.64±0.54	2.06±0.61
Clinical Attachment loss	
CAL	
Median (range) 1.35	
(0.04-4.75) 2.85	
(1.38-4.45)	

DISCUSSION

In contrast to most of the previous work [28-32], we found P. gingivalis to be more frequent in controls and detected only in 12% of cancer cases. The inconsistency is probably owing to different targeted strain or detection methodology. The strain used in this study (ATCC 33277) was previously reported to be associated with oral cancer [33-35]. it may be the case that the more virulent strains of P. gingivalis like W83, W50, ATCC 49417 are more detected and involved in carcinogenesis [36,37].

Tissue specimens are preferred to saliva when studying the microbial shifts as it provides subsite specific composition [38]. PCR detection is considered ideal for bacterial identification being a widely available, sensitive, specific technique that is not influenced by microorganism growth like culture. Also it showed higher detection rate when employing fresh tissue rather than paraffin embedded tissue[39]. The possibility of tissue contamination with plaque biofilm or saliva has been overcome by sampling deeper parts of the cancerous Hooper et al performed surface decontamination of specimens by initial betadine immersion and washing with PBS and gave comparable results[40,41]. Tissue slices studies by immunohistochemistry found that the bacteria tend to extend to deeper cancer tissue layers than those found in normal tissues [32,42]. The composition of bacteria in cancer and para-cancerous tissues was found to be more complex. The cancerous tissue had more bacterial species than subgingival plaque of the same patient[42], suggesting that the higher bacterial loads in cancer tissue is not only derived from periodontally affected tissue but possibly also from other anatomical locations.

In the current study, p.gingivalis was not shown to have a significant correlation with clinical and pathological parameters of the cancerous lesion. A direct correlation for P. gingivalis positive rate and TNM stage progression and poorer tissue differentiation was reported by katz [32], chang [42] and Ganly [43]. Tumors with higher P. gingivalis loads lower periodontal disease severity. Only 3 cases were positive for P. gingivalis forming 12% of cases which renders their results not indicative.

CONCLUSION

P.gingivalis didn't show such an association with oral cancer tissue or tumor progression.

RECOMMENDATION

Future in vitro and in vivo longitudinal studies for human cohorts and improved measurements for periodontal disease are needed with more thorough focus on the progressively activated bacterial functions in relation to oral carcinogenesis.

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