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A Study on the Presence of *Porphyromonas Gingivalis* in Oral Squamous Cell Carcinoma

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Abstract

Background - Oral squamous cell carcinoma is the most common cancer of the oral cavity with multifactorial aetiology and tobacco consumption is the main risk factor. *Porphyromonas gingivalis* is the key periodontal pathogen causing periodontitis affecting the supporting tissue of the teeth. It has been linked to oral cancer development and progression by several in vitro studies but the role is not well defined.

Objective- To study the presence of *Porphyromonas gingivalis* in oral squamous cell carcinoma

Materials and Methods - This study included total of 60 tissue samples which consists of 30 samples from oral squamous cell carcinoma and 30 non-cancerous normal tissue samples from oral cavity. The tissue specimens were analysed for the presence of *Porphyromonas gingivalis* by Reverse Transcription Polymerase Chain Reaction.

Results - *Porphyromonas gingivalis* was detected in 15 oral squamous cell carcinoma tissue samples. No control tissue specimen was found to be positive for *Porphyromonas gingivalis*.

Conclusion – Present study findings reveal *Porphyromonas gingivalis* could play a role in the pathogenesis of oral squamous cell carcinoma

Key Words: oral cancer, oral squamous cell carcinoma, *Porphyromonas gingivalis*, periodontitis,

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Introduction

Worldwide, oral cancer accounts for 2%-4% of all cancer cases. In some regions, the prevalence of oral cancer is higher, reaching the 45% of all cancers around in India. In 2004-2009 over 300,000 new cases of oral and oropharyngeal cancer were diagnosed worldwide [1].Oral cancer includes a group of neoplasms affecting any region of the oral cavity, pharyngeal regions and salivary glands. However, this term tends to be used interchangeably with oral squamous cell carcinoma (OSCC), which represents the most frequent of all oral neoplasms [2]. It is estimated that more of 90% of all oral neoplasms are OSCC. The highest incidence and prevalence of oral SCC is found in the Indian subcontinent where the risk of developing oral SCC is increased by the very prevalent habits of chewing tobacco, betel quid and areca-nut [3].

Increasing evidence has revealed a significant association between microorganisms and oral squamous cell carcinoma [4].Several epidemiological and clinical studies have found a positive relationship between periodontal disease or tooth loss and the progression of cancers such as oral cancer, gastric cancer, pancreatic cancer, and even gastric precancerous lesions [5].

In a study where meta-analysis was applied, patients with periodontitis had a 2.66-fold higher risk for developing oral cancer, and periodontitis was an independent risk indicator. The microbiome in chronic and severe manifestations of periodontal disease is enriched for Gram-negative anaerobic bacteria [6].The key periodontal pathogens involved in the pathogenesis of chronic adult periodontitis are *Porphyromonas gingivalis* (*P.gingivalis*), *Tanerella forsythia* (*T.forsythia*) and *Treponemadenticola* (*T.denticola*).Among these, *Porphyromonas gingivalis* is a keystone periodontal pathogen which can invade epithelial cells, and interfere with host immune responses and the cell cycle machinery. Ecological imbalance with dominance of periodontal pathogens especially *P.gingivalis* stimulates chronic inflammation by release of inflammatory cytokines and evasion of immune host immune response resulting in the gradual degradation of periodontal tissues [7].Upon alcohol drinking, *P. gingivalis* would dehydrogenate ethanol to acetaldehyde, which is a carcinogenic derivative and capable of contributing to DNA damage, mutation and excessive proliferation of the epithelium [8].

By modulating the tumour microenvironment it is believed that chronic or dysregulated inflammation contributes to tumour growth and invasion. Evidence on the role of oral bacteria in the pathogenesis of OSCC is relatively scarce, hence to exploration of contributing role of main periodontal pathogens in the development and progression of OSCC is of current interest to the researchers. With this background a preliminary investigation on the presence of *P.gingivalis* in OSCC tissue samples was done.

Materials and Methods

A total of 60 tissue samples consisting of 30 oral squamous cell carcinoma samples and 30 non-cancerous normal oral cavity tissue samples were included in this study. Ethical clearance was obtained before proceeding with the study from the Institutional ethical committee. After obtaining informed consent from the patients and control subjects, tissue samples were harvested. The resected tumour and control tissues were washed twice with sterile 1X PBS (Phosphate Buffered Saline) and transferred into a 2ml microfuge tube containing Trizol reagent and stored at -20°C until further usage [9].

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Total RNA isolation

The grounded tissue was centrifuged at 12, 000 rpm for 15 minutes at 4°C and the supernatant was collected without disturbing the pellets.200 ul of chloroform was added to the tube followed by mixing the contents by inverting and was kept for 2 minutes incubation at room temperature.The contents were centrifuged at 12, 000 rpm for 15 minutes at 4° C and upper aqueous layer was collected in a fresh tube. To this tube added equal volume of ice cold Isopropanol and incubated at room temperature for 10 minutes followed by centrifuging the tube at 12, 000 rpm for 10 minute at 4° C. Supernatants were discarded and washed the pellets with 70% Ethanol, simultaneously air dried the pellets to remove the ethanol.

Pellets were Re-suspended with sterile RNAse free water and stored at -80° C. Concentrations were checked in Nano-drop and integrity in 10% formaldehyde agarose gel electrophoresis.The yield of total RNA obtained was determined spectrophotometrically at 260/280 ratio [10].

cDNA synthesis and detection of P. Gingivalis genes through PCR

First strand cDNA synthesis was performed with revertaid reverse transcriptase (Thermo-scientific, Germany) PCR was performed with red dye master mix (Ampliqon, Odense, Denmark). PCR cycling parameters consist of the following steps: initial denaturation at 94°C for 2 min, cycling parameters of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s (40 cycles) with a final extension at 72°C for 5 min followed by an agarose gel analysis to confirm the presence of bacterial species specificity of the PCR. The products obtained from each reaction were resolved by electrophoresis in 1.5% agarose gels in Trisacetate-EDTA buffer (TAE). Digital images of the ethidium bromide stained gels were obtained with the gel documentation system [11].

Results

The prevalence of *P. gingivalis* was analysed in 30 tissue samples obtained from 20 males and 10 females, with different periodontal conditions. Same number of healthy tissues was kept as controls.The mean age of the patient was 55 for male and 47 for female. Following is the details of clinical stage of oral cancer lesion among patients i.e. Stage – 3 (n=16), Stage - 2 (n = 10), Stage – 4 (n=3), Stage – 1 (n=1) respectively.

PCR experiment for the detection of *P. gingivalis* was able to yield a specified amplicon using the template DNA.*P. gingivalis* was detected in 14 (46.6 %) among 30 subjects.

A single band with the expected size was obtained in every reaction, as shown in Fig. 1 for the positive control reactions.Genotypic analysis was performed by PCR using primers pair's specific for *P. gingivalis*. *P. gingivalis* is abundantly present in malignant oral tissue suggesting a potential association of the bacteria with oral squamous cell carcinoma.

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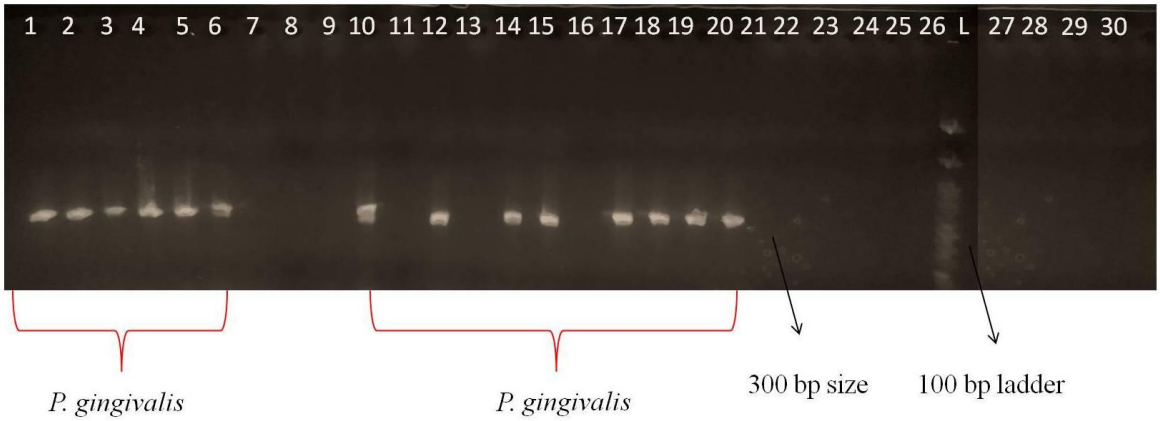

Fig. 1. PCR analysis of *P. gingivalis* for serotype-specific DNA. Lane L, molecular size marker (100-bp DNA ladder)

Discussion

The correlation between bacterial infection and the development of cancer has been a focus of cancer research in recent years. *P. gingivalis* is a Gram-negative anaerobe living in oral gingival epithelial cells as one of the three major pathogenic bacteria of the "red complex" in the oral cavity [12]. Multiple clinical and experimental studies have revealed various degrees of associations between *P. gingivalis* and cancers of the oral cavity and gastrointestinal tract, including oesophageal cancer and pancreatic cancer. The present data confirm the relationship between *P. gingivalis* and OSCC in a small group of population.The detection of *P. gingivalis* was performed by amplification of the *P. gingivalis* species specific gene because this method has a higher sensitivity and specificity than other techniques such as culture.

Connections between oral cancer and tooth loss or periodontal disease have been evaluated in several studies. 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 [13].

The significance of our finding of increased *P.gingivalis* presence in the oral cancer tissues compared to normal tissue is clear. Chronic periodontitis not only affects the occurrence and development of oral cancer but also contributes to the metastasis of this cancer. In 2011, Katz et al. found that *P. gingivalis* expression was much higher in OSCC tissue than normal tissue by comparing samples from 10 cases of OSCC and 5 healthy subjects using immunohistochemical analysis[14]. Although the sample size of that study was small, this was the first report of a significant positive correlation between *P. gingivalis* detection and the occurrence of OSCC. In addition, Chang et al. found that the detection rate of *P. gingivalis* in gingival squamous cell carcinoma was about 45%, that of tongue squamous cell carcinoma was about 40%, and that of normal gingivalis tissue was about 20% by analysing the samples from patients with OSCC in China; the difference between OSCC and normal tissue was statistically significant [15].

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