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RESEARCH ARTICLE

HELICOBACTER PYLORI AS A RISK INDICATOR OF ORAL SQUAMOUS CELL CARCINOMA – A PCR BASED STUDY

[1]Dr. Archana A. Gupta, [1]Dr Supriya Kheur, [1]Dr. Mamatha, G. S., *,[2]Dr. Lakshmi Shetty and [3]Dr. Mohit Kheur

[1]Department of Oral Pathology and Microbiology, Dr D. Y. Patil Vidyapeeth, Dr. DY Patil Dental College and Hopsital, Pune
[2]Department of Oral and Maxillofacial Surgery, Dr. D.Y.Patil Vidyapeeth, Dr.D.Y.Patil Dental College and Hospital, Pune, India
[3]Department of Maxillofacial Prosthetics and Rehabilitation, Rangoonwala College of Dental Sciences, Pune, India

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ABSTRACT

Traditionally oral cancer has always been associated with tobacco and areca nut chewing habit. An emerging concept is that tumor development and progression is also largely dependent on the cross - talk between immune system and tumor cells. Amongst internal agents, tumor associated macrophages and fibroblasts, play a pivotal role. Cytokines released by fibroblasts crucially affect the carcinoma cell behaviour and the role of chronic inflammation in tumor progression has now widely accepted. Amongst external agents causing infections like, fungal agents (candidiasis) and viruses (HPV and EBV), with oral cancer have already been discussed by many researchers. It is the bacterial population (H. pylori) in microenvironment, which is now continuously increasing the concern of the scientists towards itself. H. pylori association has already been established with gastric, pancreatic and hepatocellular cancers. It is present in oral cavity with GCF and plaque as its ecological niche and is shown to release inflammatory mediators like cytokines, when associated with chronic gingivitis and periodontitis. These inflammatory mediators when accumulate in the microenvironment of tissues for prolonged periods of time have the capacity to induce cell proliferation and to promote prolonged cell survival through activation of oncogenes and inactivation of tumor suppressor genes. This results in genetic instability with increased risk of oral cancer. Association of H. pylori is still a grey area of study as not many studies have been done in respect to the same. Hence, this study is done to outline the association between H. pylori, its role in chronic inflammation and finally the progression of the disease towards oral cancer.

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INTRODUCTION

Head and neck cancer is one of the ten most common types of cancer worldwide, afflicting >500,000 individuals each year(1). Squamous cell carcinomas present the predominant histological type among malignant tumors of the head and neck. Oral squamous cell carcinoma (OSCC) represents 95% of all forms of head and neck cancer, and during the past decade its incidence has increased by 50% (2,3). Snuff and alcohol consumption are associated with 90% of patients that exhibit oral cancer (1) and the two factors appear to have a synergistic effect (4).Whole upper aerodigestive tract, including oral cavity, oropharynx, hypopharynx, and larynx are affected by carcinomas caused by alcohol and tobacco use. Other possiblerisk factors reported for OSCCinclude viral infections (5,6), infection with Candida species (7), periodontitis (8, 9), poor oral hygiene (10), poor dental status (11) and chronic bacterial infections and inflammation (12). Several mechanisms for possible bacterial associationin carcinogenesis may include chronic infection by evasion of immune system and immune suppression (13), or induction of chronic inflammation (14), or direct or indirect interference

****Corresponding author: Dr. Lakshmi Shetty,***
Department of Oral and Maxillofacial Surgery, Dr. D.Y.Patil Vidyapeeth, Dr.D.Y.Patil Dental College and Hospital, Pune, India.

with eukaryotic cell cycle and signalling pathways (5), or via metabolism of potential carcinogenic substances (15). Diversified microflora(11) that colonize host tissues and co-aggregate with one another (16) harbors the oral cavity. Any loss in integrity of oral epithelial barrier exposes the underlying tissues to various aerobic and anaerobic microflora of oral cavity (17). The signalling pathways of cell proliferation and survival are affected by microbial endotoxins (lipopolysaccharides), enzymes (proteases, collagenases, fibrinolysin and phospholipase) and their metabolic by-products (hydrogen sulfide, ammonia and fatty acids) that may directly induce mutations in tumor suppressor genes and proto-oncogenes (5, 15). Reactive species (hydrogen peroxide and oxygenradicals), reactive nitrogen species (nitric oxides), reactive lipids and metabolites (malondialdehyde and 4-hydroxy-2 -nonenal) and matrix metalloproteases are generated when microorganisms and their products activate neutrophils, macrophages, monocytes, lymphocytes, fibroblasts and epithelial cells. These compounds lead to nuclear translocation of the transcription factor NF-kB and cytokines production directly affect tumor growth by activating tumor cell toll-like receptors (TLR) and can induce DNA damage in epithelial cells. (18, 19, 20). These cytokines, produced in dysregulatedfashion, have roles in cell growth, invasion and interruption of tumor suppression, immune status and even survival (21). However, it is unclear whether these mediators are critical for the development and/or growth of tumors and/or whether they constitute a permissive environment for the progression of malignancies (22). Serum, saliva, and tissue specimens of patients with oral cancer show elevated levels of certain proinflammatory, proangiogenic NF-kBdependent cytokines TNF-α, IL-1, IL-6, IL-8, GM-CSF and VEGF (23, 24).

Helicobacter pylori have been classically associated with bacterial infection and cancer. Several clinic entities such as atrophic gastritis, gastric and duodenal ulcers, gastric MALT lymphoma, and gastric cancer have been related to Helicobacter pylori (*H. pylori)*infection(25). Interest in the possible relationships between bacteria and the different stages of cancer development has been increased since the classification by the World Health Organization of *Helicobacter pylori* as a definite (class 1) carcinogen. Various authors reported *H. pylori*presence in oral cavity could be the source for stomach infection and re-infection even after an eradication therapy (26, 27). Conditions such as gastroesophagealreflux and poor hygienecan facilitate the oral colonization by *H. pylori*, (28). At the same time, some authors found cultural and living conditions also to be responsible in the development of infection by this bacterium (26). However, a confirmed relationship between gastric symptoms andthe presence of *H. pylori* in the oral cavity has not been yet established. There are conflicting results reported in the literature on the isolation of *H. pylori* from dental plaque. Several studies indicate a low prevalence of *H. pylori* in the oral cavity of their patients and consider that it is not a significant environment for this bacterium (29). Some studies suggest that *H. pylori* has only a transient presence in the oral cavity and also demonstrate the antagonist effects of some oral bacteria to *H. pylori*, which could inhibit colonization by this organism in the oral cavity (30). On the other hand, authors who found this bacterium in almost all of their study population consider that the oral cavity may act as a reservoir for re-infection of the stomach and that*H. pylori* is part of the normal micro biota in the mouth (31).

Therefore, the aim of the present study is to access the presence of *H.pylori*in patients suffering from oral cancer. Different samples including dental plaque which is considered as the ecological niche of the bacteria, taken from the site adjacent to cancer lesion and excised tissue samples have been taken. Samples were subjected to molecular method of Polymerized Chain Reaction (PCR) to access the association of H. pylori with Oral carcinogenesis.

MATERIALS AND METHODS

A written informed consent was taken from the patients clinically and histopathologically diagnosed with Oral Squamous Cell Carcinoma.

The patients were divided according to convenient sampling method into two subgroups.

- Group A: n = 40, Patients clinically and histopathologically diagnosed with Oral Squamous Cell Carcinoma
- Group B: n = 10, Normal healthy individuals with gingivitis and periodontitis, who chew betel nut, will form the control group of our study.

The demographic data of patients describing the division of patients according to age, sex, site of involvement, staging and grading of tumoris recorded (Table 1)

The inclusion and exclusion criteria decided for the study is described as follows:

Inclusion Criteria:

1. Patients with mild to moderate gingivitis and periodontitis
2. Patients who are clinically and histopathologically diagnosed with Oral Squamous Cell Carcinoma who are willing to participate in the study.

Exclusion Criteria:

1. Patients receiving treatment with H2-receptor antagonist, proton pump inhibitors or antibiotics within the preceding six months.
2. Patients suffering from any other major immuno-compromised state.
3. Patients who are not willing to participate.

The study was conducted on the lesional tissue samples of the patients who were undergoing surgery in the Department of Oral and Maxillofacial Surgery in Dr. D. Y. Patil Dental College and Hospital. (Fig1). Part of the sample was processed for routine H/E procedure and part of the specimen was dropped in the transport medium and sent for PCR analysis to correlate the association of H. pylori with Oral Squamous Cell

Carcinoma in Microbiology Department of Maratha Mandal Dental College, Belgaum. The DNA extraction was done by Modified Proteinase-K Method. Collected sample in TE buffer was centrifuged at 5,000 rpm for 5 min. Supernatant was discarded. 500µl fresh T.E. buffer was added and centrifuged for 3-4 minutes. The above procedure was repeated for 2-3 times with fresh T.E. buffer. Supernatant was discarded, and 50 µl lysis buffer I was added, it was Vortexed and kept for 5 min. After that 50 µl Lysis buffer II and 5 µl Proteinase – K(100ug/ml) was added and vortexed vigorously. It was kept in water bath for 2 hrs and then kept in boiling water bath for 10 minutes to deactivate the enzyme. The tubes were centrifuged at 5000rpm for 3 min. Supernatant containing DNA was taken in fresh tube and the DNA was stored at -20°C until PCR. (Fig.2)

Procedure of PCR

The DNA samples obtained were subjected to a realtime PCR, performed in an iCycler IQ (Bio-Rad, Hercules, CA, USA), data acquisition and analysis were carried out using the Real-Time Detection System Software (Bio-Rad). The RT-PCR was targeted at the 26 KDa Helicobacter species-specific antigen (SSA) gene (O'Toole *et al*., 1991) and a double-stranded DNA-specific dye SYBR Green I was used. The 50 µL reaction mixture was composed of follows: 25 µL of iQ SYBR® Green Supermix (Bio-Rad), 50 nM of each primer

- (forward: 5'-TGGCG TGTCTATTGACAGCGAGC-3',
- reverse: 5'-CCTGCTG GGCATACTTCACCATG-3') (32)

1 µL of extracted DNA (200 ng).The reaction was cycled with preliminary denaturation for 5 min at 95 °C, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 65 °C for 30 s and primer extension at 72 °C for 30 s. This was followed by melting point analysis of the double-stranded amplicons consisting of 40 cycles of 1 °C decrement (45 s each) beginning at 95 °C. Positive controls consisted of genomic DNA extracted from H. pylori reference strains 26695 and J99, kindly provided by dr. Johannes G. Kusters (Erasmus Medical Center, Rotterdam, The Netherlands) and negative controls were provided by DNA isolated from blood samples and gastric mucosa of uninfected mice (strain C57BL/6,CEMIB/ UNICAMP, Campinas, SP, Brazil). Products obtained were analyzed on 2% agarose gel electrophoresis performed in Tris-acetate-EDTA buffer. Gel was stained with 0.5 µg/ml ethidium bromide and visualized on an ultraviolet transilluminator. The expected product after amplification was 300 base pairs in length.

The armamentarium used for the study includes

- **For H/E staining**
  - Histological Slides
  - Hematoxylin and Eosin staining solutions
  - Coupling jars
  - Coverslips
  - DPX as mountant
- **For Molecular analysis**
  - Tweezer
  - BP handle with blade
  - Transport medium in Eppendorf tubes,
  - Primers for molecular analysis

C – Reactive protein analysis

The RapidTex CRP Test is based on the latex-agglutination method introduced by Singer *et al* in 1957(100). The principle of this test is based on the immunological reaction between CRP as an antigen and the corresponding antibody coated on the surface of biologically inert latex particles. All reagents, controls and serum samples are brought to room temperature. CRP latex reagent was shaken gently before use. A drop of reagent was delivered to the test circle. Using the disposable pipettes, one drop of the undiluted patient serum was added onto the same circle and both were mixed together with the paddle end of the pipette. Positive and negative controls should be run with each series of test serums in the same way as in Step 2. Slide is rotated back and forth for 2 minutes and results are read under an indirect oblique light source. Positive reaction is indicated by agglutination. And no agglutination is interpreted as a negative result. (Fig.3,4)

Measuring Lymphocyte density

Excised tissue samples sent for routine histopathological procedure were stained and examined for lymphocytes density under image analyser. The chronic inflammatory cells, lymphocytes were counted in five representative areas of the sample. An average of the lymphocyte density calculated in five areas of the sample was taken and the results were recorded. (Fig. 5,6)

RESULTS

According to the demographic analysis of the study, amongst 40 patients, histopathologically diagnosed as Oral Squamous Cell Carcinoma, 28 were males and 12 females. The number of cases were being divided depending on the age group of patients where maximum number of cases (19) fall between 50 to 60 years of age. Maximum number of patients has the habit of tobacco and guthka chewing, tobacco smoking and alcohol drinking. Only one patient diagnosed with oral squamous cell carcinoma did not harbour any of these habits. Amongst the site evaluation, 30 cases were found to be on the gingivobuccal complex which is known to be the most common site for occurrence of oral squamous cell carcinoma in Indian Subcontinent, followed by tongue and buccal mucosa. According to the TNM staging, 29 cases belong to the stage IV and 11 cases belong to stage III of oral squamous cell carcinoma. According to histopathological grading of tumor, 22 cases belong to well differentiated, 16cases belong to moderately differentiated and 2 cases belong topoorly differentiated oral squamous cell carcinoma. The demographic analysis of the study has been represented in tabular form and graphically below. (Fig. 7 – 12) Preoperatively, C reactive protein levels of the patients were determined following the above described procedure. However, only 25% of the cases showed agglutination after reacting with the reagent. Initially, a pilot study was planned on first 10 Gingival Crevicular Fluid samples of oral squamous cell carcinoma.

Table 1. Demographic data of study

S. No.	Gender	Habit	Site	Staging	Grade
			Gingival Crevicular Fluid		
1	M	Y	Gingivobuccal complex (GB)	3	Well
2	F	Y	GB	4	Well
3	M	Y	Alveolus	4	Moderate
4	M	Y	GB	4	Moderate
5	M	Y	Palate	4	Well
6	M	Y	Tongue	3	Well
7	F	Y	GB	4	Well
8	F	Y	Alveolis	4	Well
9	F	Y	GB	4	Well
10	M	Y	GB	3	Well
			Excised OSCC tissue sample		
11	M	Y	GB	4	Well
12	M	Y	GB	3	Moderate
13	M	Y	GB + Palate	4	Well
14	M	Y	Tongue	3	Well
15	M	Y	Buccal Mucosa	4	Moderate
16	M	Y	GB	4	Well
17	F	Y	Retromolar	3	Well
18	F	Y	GB	4	Moderate
19	M	Y	GB	3	Moderate
20	M	Y	Tongue	4	Well
21	M	Y	GB	4	Moderate
22	F	Y	Buccal Mucosa + Floor of Mouth	4	Well
23	F	Y	GB	3	Moderate
24	F	Y	Labial Mucosa	4	Well
25	F	Y	GB	4	Well
26	F	N	Buccal Mucosa	3	Well
27	M	Y	GB	4	Well
28	F	Y	GB	4	Moderate
29	M	Y	Palate	4	Well
30	M	Y	Buccal Mucosa	3	Well
31	M	Y	GB	4	Moderate
32	M	Y	Labial Mucosa	4	Well
33	M	Y	Tongue	4	Poor
34	F	Y	GB	4	Well
35	M	Y	Floor of Mouth	4	Moderate
36	M	Y	Tongue	4	Moderate
37	M	Y	GB	4	Well
38	M	Y	Buccal Mucosa	3	Well
39	M	Y	Tongue	4	Well
40	M	Y	GB	3	Well
41	M	Y	Palate	4	Moderate
42	M	Y	GB	4	Poor
43	F	Y	Tongue	4	Well
44	M	Y	Retromolar	4	Moderate
45	M	Y	GB	3	Moderate
46	M	Y	Labial Mucosa	4	Well
47	F	Y	Buccal Mucosa + Floor of Mouth	4	Moderate
48	M	Y	GB	3	Well
49	M	Y	Buccal Mucosa + Floor of Mouth	4	Moderate
50	F	Y	GB	4	Moderate

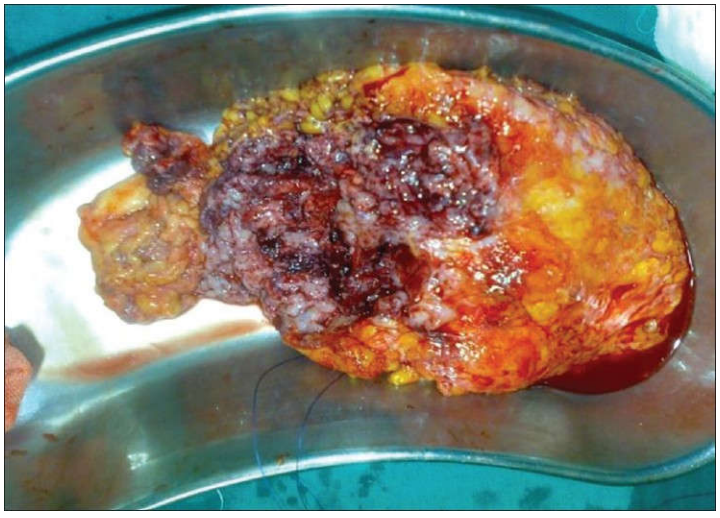


Fig. 1.

GCF samples were collected in transport medium and sent for molecular analysis. However, none of the sample revealed H. pylori in Gingival Crevicular Fluid. Probably the sample size may be too small that it might have been got diluted in the transporting medium (Fig. 13, 14). Hence, we changed the course of our study and we started taking excised tissue samples of Oral Squamous Cell Carcinoma patients undergoing surgery. Forty excised tissue samples were taken before putting it into formalin and were stored in the transport medium to send it for molecular analysis. Out of forty cases five cases showed presence of H. pylori in the study. Also, 10 samples of normal gingival tissue of the pateints undergoing gingivectomy for orthodontic or other similar causes have been taken as control, where only one sample showed positive results for the presence of H. pylori.Below are the photographs representing the presence and absence of bands corresponding to H. pylori compared to the controls. (Photographs 1 – 3) Histopathological evaluation of the slides for lymphocytes density was done with image analyser. The lymphocytes density of the samples ranged between 135 – 170/mm^2. However, no significant differences were recorded amongst the samples regarding lymphocytes density.

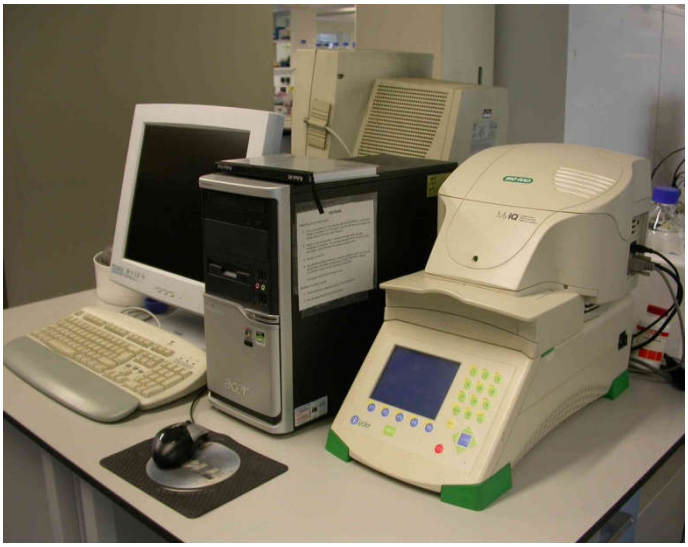


Fig.2.

Fig.3.

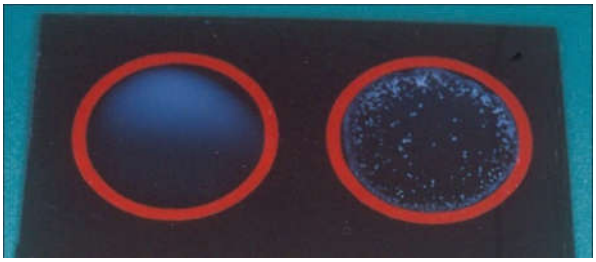


Fig.4.

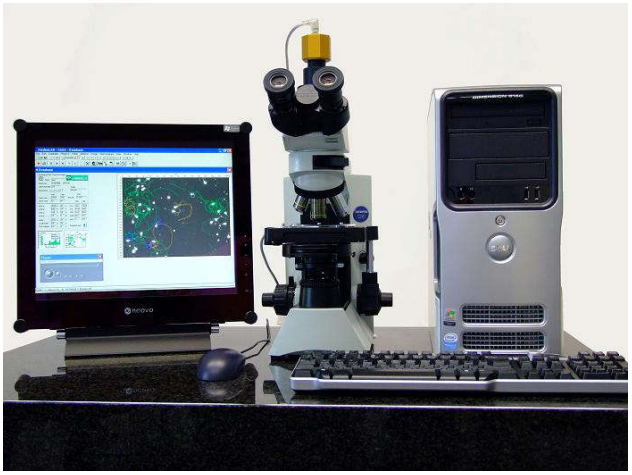
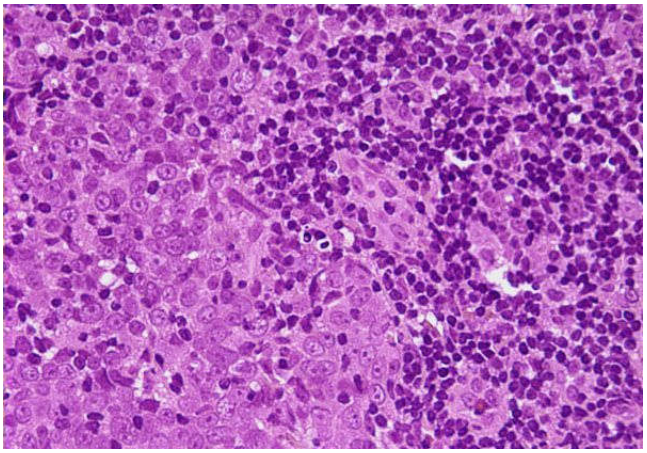


Fig.5.



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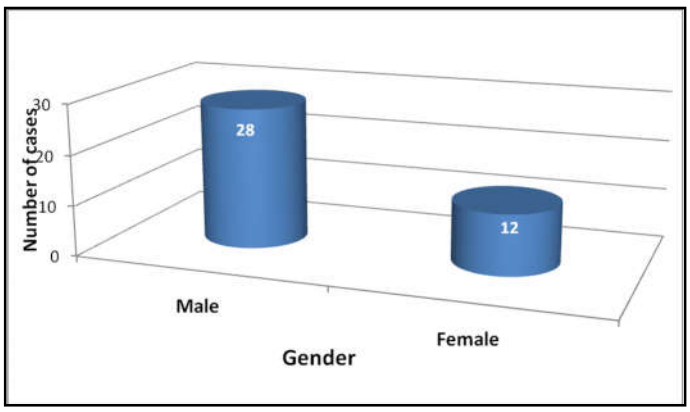


Fig.7.

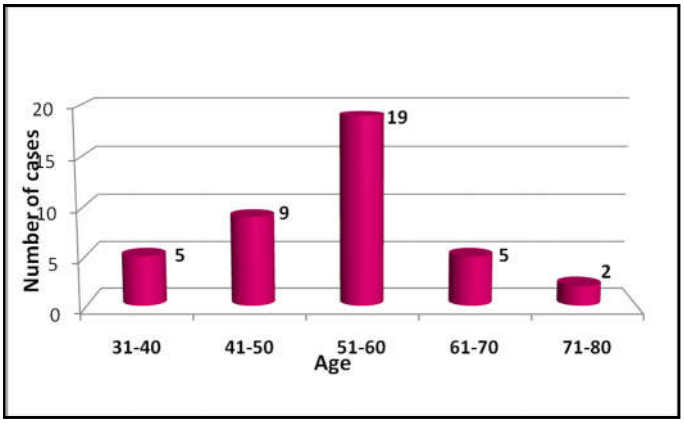


Fig.8.

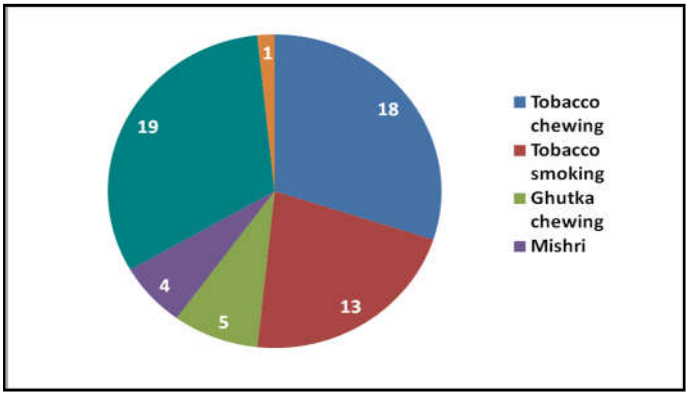


Fig.9.

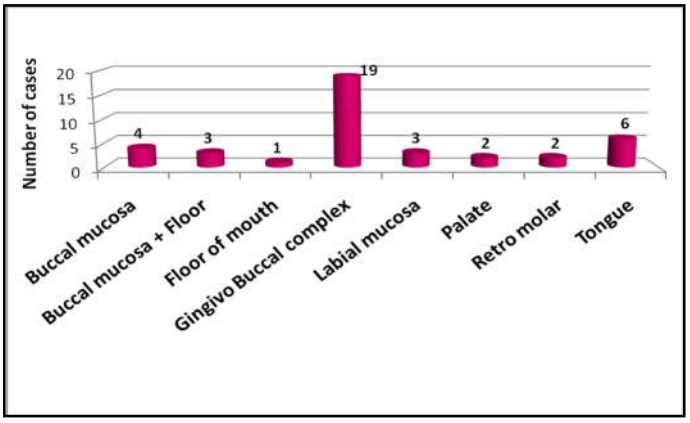


Fig.10.

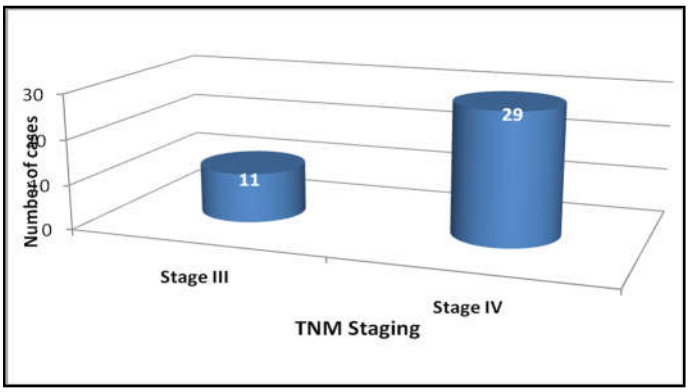


Fig.11.

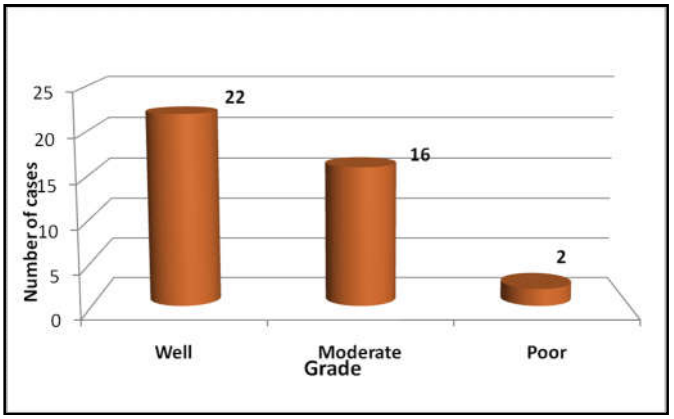


Fig.12.

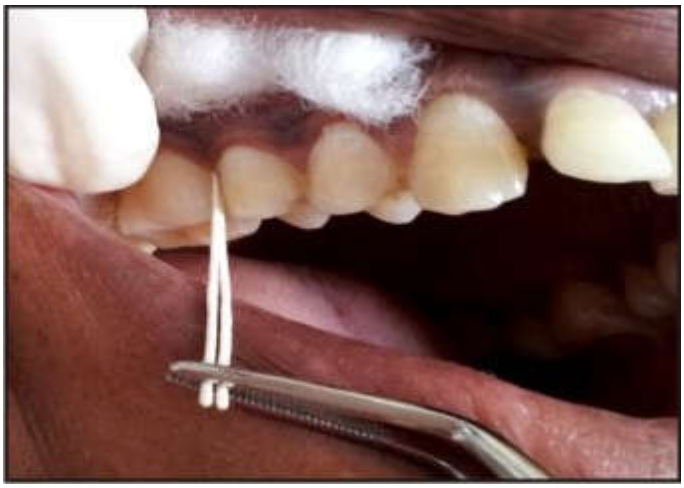
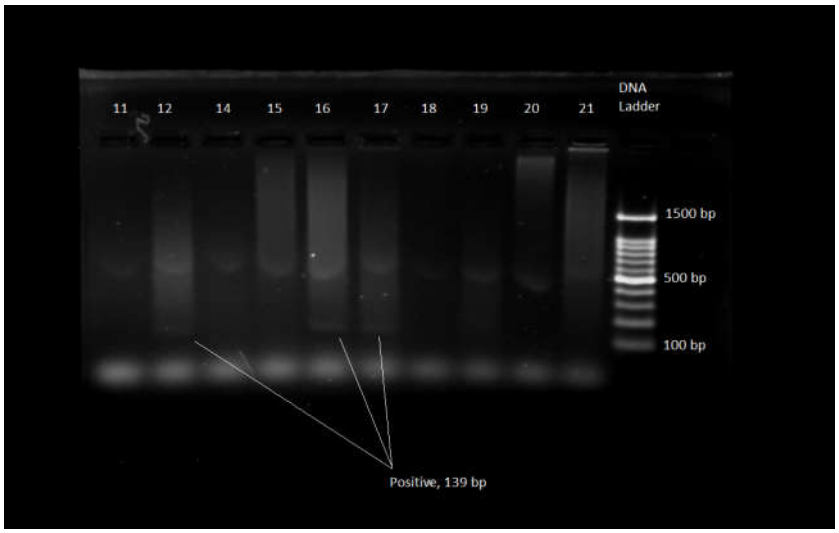


Fig.13.

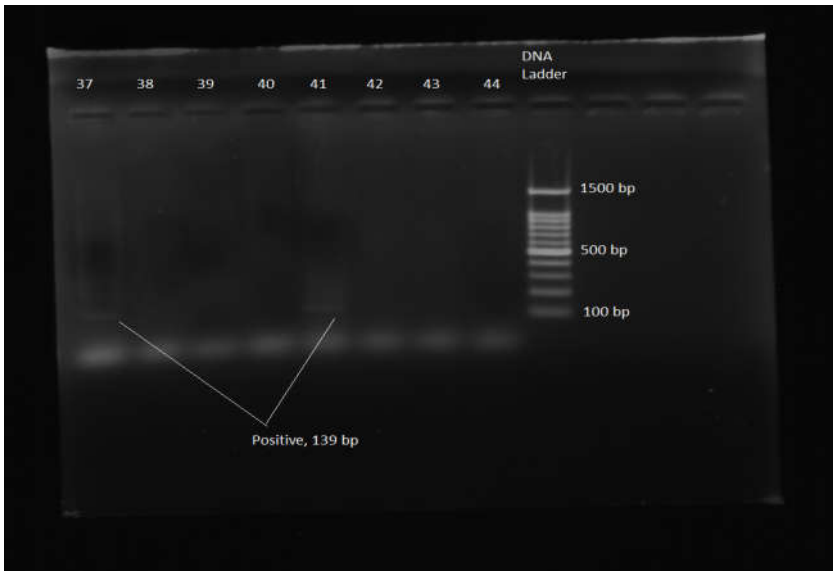
Fig.14.

DISCUSSION

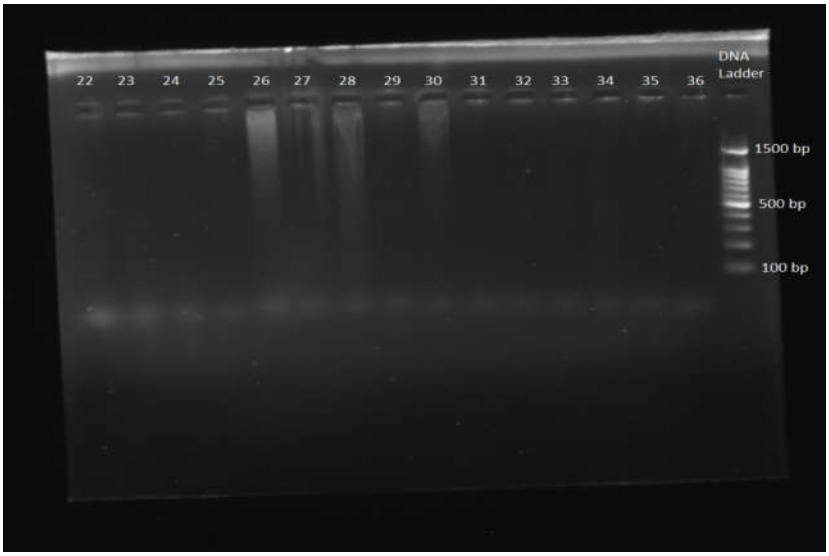
The established risk factors for Oral Squamous Cell Carcinoma include tobacco, alcohol and areca nut. (33 - 38) However, the never decreasing mortality rate of the cancer. (39) prompted the scientists to search for the less explored etiological factors. These factors include the infectious agents, inflammation and chronic trauma. Amongst infectious agents decent amount of studies are available on virus and candida serving as potential causes of Oral Cancer. (40 – 51)



Photograph 1:PCR analysis representing Lanes 12, 16 and 17 show positive samples and Lanes 11, 14, 15, 18, 19, 20 and 21 show negative samples against the DNA ladder



Photograph 2: PCR analysis representing Lanes 37 and 41 shows positive samples and Lanes 38, 39, 40, 42, 43, 44 show negative samples against the DNA ladder



Photograph 3: PCR analysis representing All Lanes showing negative samples against the DNA ladder

A fair amount of discussion is available on chronic trauma and inflammation resulting from chronic trauma which also came to be known as the potential causes of Oral Squamous Cell Carcinoma.(52 – 57) The Gingivocrevicular Fluid is the ecological niche for various microrganisms present as commensals in the oral cavity due to which the role of persistent inflammation as a cause of oral cancer came into play. Amongst the infectious causes the least discussed factor is the bacterial cause of oral carcinogenesis. H. pylori have been persistently present in the oral cavity in gingivocrevicular fluid as a commensal. The role of H. pylori in gastric inflammation leading to gastric cancer has been discussed in a good amount of studies.(58 – 61) Association of H. pylori has also been established in periodontitis. Keeping these facts in view, the possible role of H. pylori in oral carcinogenesis followed by chronic periodontitis has been hypothesized. Hence, the present study has been planned to study the association of H. pylori with Oral Squamous Cell Carcinoma.

The demographic data of the study revealed the predominance of old age and male predilection in the cases reported. The data is in accordance with earlier studies which also reported the same predominance regarding age and sex of the individuals suffering from Oral Squamous Cell Carcinoma. (62 – 64) Maximum percentage of cases in the study revealed the involvement Gingivobuccal complex as the most common site due to the tobacco chewing habit of the individuals in the Indian subcontinent. The cancer of Gingivobuccal complex has also been reported as the Indian Cancer by Kumar V. *et al* in 2013 (65) However, in other studies reported by Hirota S K *et al* (66)and Pires F R *et al* (62),floor of the mouth and ventral surface of the tongue were considered the most commonly involved sites for oral cancer. The cases reported to us for study were mostly in the stage III and stage IV of the tumor. The reason for the same is because most individuals in Indian subcontinent presenting with the disease report very late to the doctor in hospital. Similar is the case with grading of tumor where most of the cases reported presented with well differentiated Oral Squamous Cell Carcinoma followed by moderate and poorly differentiated ones. Panahi*et al*, in 2011 indicated the presence of *H. pylori* in dental plaque. However, the numberof organisms is very low in individual samples.Also, the numbers appear to vary from one site to anotherwithin the mouth. The presence of this organism inplaque may be the resultof gastroesophageal reflux. The presence of H. pylori in dental plaque showed the male predominance as reported in our study also. (67)

Fernando *et al*., in 2009 reviewed that people with gum disease are more likely to test positive for *H.pylori*. They also concluded with their study on SriLankan population that betel chewing may predispose to colonization with *H. pylori* in digestive tract through swallowing the quid or during betel chewing. (68) These studies made us to start our study from collecting the GCF samples of the patients. However the absence of the bacteria in our samples made us hypothesize that tobacco chewing by the individuals reported might have altered the ecological niche i.e. GCF of the bacteria resulting in its absence from the same. The negative results of pilot study made us to change the course of our study from taking the GCF samples to excised tissue samples obtained after the surgical treatment of the patients in which 23% of cases revealed the positive results. Possible association of *H. pylori* with oral cancer hasbeen suggested by Dayama*et al*., in 2011 who carried out a hospital-based,case-control pilot study of 20 patients with newly diagnosed oral cancer and 20 healthy controls without any cancer to evaluate associations between *H. pylori* infection and oral cancer using culture and 16sRNA polymerase chain reaction (PCR) technique for bacterial identification. They could find the positive results in only about 3% of cases. The male predominance and the mean age of around 48 years correspond well with the present study. However, the habit predisposition, differentiation and staging of the tumorhas not been reported in the study and additional studies in larger populations were recommended to confirm and to quantify this possible association more accurately. (69) Gupta AA*et al*, in 2014 also published a short review article stating the possible role of H. pylori in oral cancer (70)

The possible mechanism of action of H. pylori promoting Oral Carcinogenesis has been discussed as follows:

Cytotoxin-associated antigen A (CagA), cag-pathogenicity island (cagPAI), vacuolatingcytotoxin (VacA), and outer membrane proteins (OMPs) are the four major virulence factors identified fromH. pylori.These virulence factors activate multiple intracellular pathways in epithelial cells, such as mitogen-activated protein kinases (MAPK), NF-κB, activator protein (AP)-1, Wnt/β-catenin, signal transducer and activator of transcription 3 (STAT3) and phosphatidylinositol 3-kinase (PI3K). Activated NF-κB mediates the expression of interleukin (IL)-6, -8. Epigenetic changes like DNA methylation and histone modifications caused in epithelial cells also indicate H. pylori infection(71).These expressions lead to increased inflammatory cytokine production, immune cell infiltration, affecting host cell apoptosis, proliferation and differentiation, finally resulting inepithelial cell oncogenic transformation (72)

Preoperative CRP levels and Prognosis of OSCC

A positive association between CRP levels and periodontitis has already been discussed by Anitha G. *et al* in 2013. (73). Also, Kumar AC *et al* in 2011 and Wang CS *et al* has discussed the association of the protein with precancerous and malignant conditions respectively. (74, 75) Hence, the possible mechanisms to explain such association in oral squamous cell carcinoma are as follows:

a) Tumor growth can cause tissue inflammation and hence increase CRP levels
b) CRP acts as an indicator of immune response to tumor antigens
c) Evidence of cancer cells increasing the production of inflammatory proteins
d) Some cancerous cells cell lines secrete Interleukin-6 (IL-6) and IL-8, which in turn induce the production of CRP.

However, the present study showed contradictory results where a strong positive association of CRP with oral cancer could not be found.

Summary and Conclusion

Through the above reported study following points can be concluded:

1. Though GCF is the ecological niche of the H. pylori which is the reported commensal of oral cavity. Its role in promoting oral carcinogenesis cannot be established through the present study. The reason for which can be the alteration of the ecological niche of the bacteria due to tobacco chewing habit.
2. However, the role of bacteria in promoting oral carcinogenesis can be established through excised tissue samples where 23% of reported cases showed the positive results.
3. The present study showed a male predominance of Oral Squamous Cell Cacrcinoma
4. The mean age of occurrence of Oral Squamous Cell Carcinoma according to the present study came out to be about 54 years.
5. Most commonly involved site of Oral Squamous Cell Carcinoma in the present study is the Gingivobuccal complex followed by buccal mucosa and floor of the mouth.
6. Most of the cases reported in present study belong to Stage III of the Oral Squamous Cell Carcinoma.
7. Maximum amount of cases reported in the present study were well differentiated cases of Oral Squamous Cell Carcinoma.
8. No correlation was found between the histopathological (lymphocytes density) and the haematological (CRP) parameters with the H. pylori as the etiological factor in Oral Squamous Cell Carcinoma.

However, more studies with higher range of samples are recommended to further evaluate the exact role of the species in promoting oral carcinogenesis. Further studies should also segregate the groups according to the habits associated oral cancer and non habit induced oral cancer.

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