RESEARCH COMMUNICATION

Helicobacter pylori and Oral Cancer: Possible Association in a Preliminary Case Control Study

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Abstract

Oral cancer is the most common cancer diagnosed in Indian men and is the leading cause of cancer deaths. *Helicobacter pylori* have been reported to be present in 0-40% of the cases with head neck cancer. A higher percentage has been identified in laryngeal and pharangeal cancer. We here carried out a hospital-based, case–control 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 PCR technique for bacterial identification. *H pylori* was isolated from the culture of three cases and one control, while three cases and two control showed PCR positivity for *H Pylori* 16sRNA. The odds ratio by culture was 3.0, 95% CI 0.34-26.4 and 1.5, 95% CI 0.28-8.03 by PCR. None of the observed odds ratio was statistically significant. However, the results of this pilot study suggest a possible association of *H. pylori* with an increased risk of oral cancer. Additional studies in larger populations are necessary to confirm and to quantify this possible association more accurately.

Keywords: Oral cavity - squamous carcinoma -helicobacter - buccal mucosa

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Introduction

Human infection with *H. pylori* is common; the Centers for Disease Control and Prevention (CDC) estimates that approximately two-thirds of the world's population harbors the bacterium, with carriage rates much higher in developing nations (i.e. more than 90% for adults) comparing to developed nations (20-80%)(Taylor & Blaser, 1991). This infection has a central role in the development and progress of peptic ulcers (Dixon 1991) and has also been correlated with gastric cancer (Forman et al, 1991). *H. pylori* has been detected in oral cavity of patients with stomatitis, helitosis and periodontal disease, and has been proposed as a reservoir for gastric infection but the data are inconsistent. *H. pylori* had been isolated from the oral cavity in some cases (Krajden et al, 1989; Ferguson et al, 1993; Khandaker 1993) while attempts to culture the organism have failed (Bernander et al 1993; Von Recklinghausen et al 1994).

The relation between *H.pylori* and gastric tumor pathogenesis has been well described, being influenced by *H pylori*'s ability to modify host immune response. It is supposed that it could act in the same way in progression of oral and oropharyngeal carcinoma. Various studies identifying correlation between *H pylori* and head neck cancer has been reported (Ferguson et al 1993; Aygenc et al, 2001; Kiziley et al 2006; Fernando et al, 2009). Thus, we here investigated the presence n the oral biopsy tissues from patients and controls.

Materials and Methods

Study population

Present study was conducted at the department of Surgical Oncology, Institute of Medical Sciences, Banaras Hindu University. A total of 20 patients with oral cancer and 20 healthy individuals were studied. Patients between 18-75 years, willing to give consent; without any medical illness precluding biopsy; without oral sub-mucous fibrosis and those previously untreated were selected. Control subjects were normal individuals undergoing impacted molar removal. Specimens were collected in 2ml brain heart infusion (BHI) medium for culture and were immediately transferred to the microbiology lab. For PCR, specimens were collected in RNA litter and stored at -20 degrees Celsius before processing for culture and DNA extraction was performed. All patients completed a preset questionnaire interview about age, tobacco, smoking and alcohol habits, family history of cancer history of previous treatment, the study was approved by the Institute Ethics committee.

Laboratory Studies

Microbiological study: The oral tissue were

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homogenized in BHI broth in a glass tissue grinder and plated onto petri dishes containing freshly prepared BHI agar (Difco, USA) medium. The plates were incubated at 37 ºC in an atmosphere of 5% O_2, 10% CO_2, and 85% N_2 for 3 to 7 days. *H. pylori* colonies were identified on the basis of their typical colony morphology, gram negative spiral rods and oxidase and urease production.

DNA Isolation from Oral Tissue

Homogenized tissue sample was incubated with 1mg lysozyme at 37ºC for 60 minutes. Then 1 ml of 0.1% Triton-X and 5µl Proteinase-K was added and incubated again at 65ºC for 120 minutes. To this, equal volume of chloroform: IAA (24:1) was added and mixed by vortex for 15 minutes, centrifuged at 10,000 rpm for 10 minutes and aqueous phase was collected. Then 140 µl of phenol: chloroform: IAA (25:24:1) was added and mixed by vortexing for 15 seconds and centrifuged at 10,000 rpm for 10 minutes. Aqueous phase was collected. To this, equal volume of isopropanol was added. The solution was incubated at room temperature for 5 minutes, and then centrifuged at 10,000 rpm for 10 minutes and supernatant decanted. The pellet was washed with 200µl 70% ethanol. The above solution was again centrifuged at 10,000 rpm for 10 minutes. The pellets was dried over at 37ºC for 30 minutes, and dissolved in 50 µl in TE buffer.

DNA Isolation from Culture

Each bacterial culture was inoculated in 10ml LB broth in anaerobic environment and incubated at 37ºC in a shaker for 18 hours. Then 1.5ml of fresh grown culture was centrifuged at 10,000 rpm for 10 min. at room temperature. The cell pellets obtained were re-suspended in 576 µl TE buffere and vortexed. 30 µl of SDS (10%), 0.3 µl of Proteinase K of 20µg/ml concentration (100µm/ml final concentration and mixed gently by inversion and incubated at 37˚c for 2 hrs), 100 µl of 5M NaCl was added and vortexed for 15 sec. 80 µl of 10% CTAB was added and mixed and incubated for 10 min at 65ºC in water bath. In this equal volume of chloroform and IAA (24:1) was added and vortexed and centrifuged at 10,000 rpm for 10 min followed by collection of aqueous phase. 2μl of RNAse (approx. 30μg/ml) was added to make final concentration and incubated for 30 minutes at 37˚C. The equal volume of isopropanol was added and then solution was kept at room temperature for 5min. This solution was centrifuged at 10,000 rpm for 10 min. Pellets were washed by 70% ethanol and centrifuged at 10,000 rpm for 10 min, pallets were dried at 37˚C and dissolved in 100μl of sterilized TE buffer.

PCR amplification with Helicobacter genus-specific primers

The 16s RNA gene of genus *Helicobacter* was amplified by using primer C97: 5'GCTATGACGGGTATC3' and C98: 5'ACTTCACCCCAGTCGA3' yielding amplicon of size 400bp. PCRs were performed in an Applied Biosystems thermal cycler in thin-wall tubes; in 50μl reaction mixture volumes containing 250 mM concentrations of each primer; dNTP, 0.2mM; PCR buffer2.5 µl (1x); MgCl2, 1.1 µl (1.5 mM); 1U of Taq polymerase; and 50ng (1μl) DNA sample. Twenty nine cycles of amplification were performed in a DNA thermal cycler. Each cycle consisted of a 7 minutes for 95°C, 30s for 94°C, 30s for 61-54°C, 90s for 72ºc, 72ºc for 7 minutes and 4ºC pause. The amplified product was identified by electrophoresis in a 1.0% agarose gel. The DNA was stained with ethidium bromide and were visualized under short wavelength transilluminator and photographed in Alpha imager EC Alpha Innotech.

Table 1. Explanatory Variables for Oral Cancer Cases and Controls

Variable		Case No (%)	Control No (%)
Gender	Male	18 (90)	15 (75)
	Female	2 (10)	5 (25)
Tobacco	Daily	18 (90)	15 (75)
	Occasionally	1 (5)	2 (10)
	No	1 (5)	3 (15)
Smoking	Daily	9 (45)	6 (30)
	No	11 (55)	14 (70)
Alcohol	Daily	6 (30)	2 (10)
	Occasionally	5 (25)	3 (15)
	No	9 (45)	14 (70)
Family History Cancer	Yes	1 (5)	
	No	19 (95)	20(100)
Clinical Staging (TNM)	T2	9 (45)	-
	T3	6 (30)	-
	T4	5 (25)	-
	N0	3 (15)	-
	N1	5 (25)	-
	N2	12 (60)	-
Culture *H.pylori*	Positive	3 (15)	1 (5)
	Negative	17 (85)	19 (95)
PCR Tissue	Positive	3 (15)	2 (10)
	Negative	17 (85)	18 (90)

Results

The mean age of controls was 40.4 years (SD 10.3 range 18-58 year) and that of cases was 48.2 years (SD 11.0; range 31-75 years). There were 5 females and 15 males in control group and 2 females and 18 males in case. Family history of cancer was present only in one case while none of the controls had family history of cancer. The distribution of tobacco, alcohol habits and the stage distribution of cases is given in Table 1.

Helicobacter pylori was identified by culture in one control (1/20) and three cases (3/20). The presence of *H. pylori* was confirmed by PCR of the cultured organism and various biochemical tests detailed above. The presence of *H. pylori* by PCR alone was slightly higher in control group (2/20) while it was same in cases (3/20).

The odds of *H. pylor*i positive patients was 3.0 (CI 0.34-26.4) while it was lower for only PCR positive subjects (odds ratio 1.5, CI 0.28-8.0). Though the odds are increased from 1.58 to 3.35 times this increase in odds is not statistically significant.

Discussion

Our pilot case control study support the association of *H. pylori* with oral cancers though odds ratio is not statistically significant and the strength of the association

Table 2. Results of Studies on Head and Neck Cancer in the Literature

Author (year)	Method of detection	Specimens	No. of *H.pylori* positive subjects
Fernando et al. (2009)	Serology	Sera	14/53 (oral cancer) 10/60 (control betel nut chewer)
Kizilay et al. (2006)	Histology-HE, modified Giemsa stain	69 laryngeal SCCs 30 controls	0%
Nurgalieva et al. (2005)	Serology IgG antibodies	119 laryngeal/pharyngeal SCCs 111 controls without SCC	32.8% with SCC 27.0% controls
Kanda et al. (2005)	PCR, Culture, IHC, Serology from Urine	31 HNSCC	21 seropositive, 0 PCR, IHC, Culture
Rubin et al. (2003)	Serology	6 severe laryngeal dysplasia 5 tonsilar and 50 other SCCs	38 seropositive including all tonsillar SCCs
Aygenc et al. (2001)	Serology IgG antibodies	Sera of patients(26) with SCC of larynx and controls(32)	Seropositivity: SCC of larynx 73.1% Control 40.6 ($p < 0.05$)
Okuda et al. (2000)	RT PCR, Culture	116 stomach samples and oral swabs for 58 oral cancers	46.6% stomach samples 12.1% oral swabs positive 100% oral cancer swabs
Singh et al. (1998)	Histopathology	Oral mucosal biopsies 26 cases (benign and cancer) 26 Controls	4/26 (15.4%)
Grandis et al. (1997)	Serology IgG antibodies	21 HNSCCs 21 controls without SCC	Seropositivity: cases 57% and controls 62% ($p>0.05$)

IHC, immunohistochemistry

is undermined by small sample size and the presence of high-risk behavior in oral cancer cases. Furthermore, it was found that *H pylori* DNA was not unique to cancerous oral tissue as PCR from control was also positive for 2 out of 20 controls. Although a cause-and-effect relationship cannot be inferred from this study, our findings can serve as pilot study for further studies

The degree to which oral *H pylori* infection may interact with tobacco use, alcohol use, or both to increase the risk of squamous cell carcinomas of the head and neck has been unclear. A greater additive risk has been seen, albeit inconsistently, for patients exposed to *H pylori* and tobacco (Fernando et al., 2009) or alcohol. We found evidence of increased *H pylori* infection in tobacco and alcohol user suggesting association of *H pylori* infection with tobacco habits which may alter oral cavity flora. Discrepant findings may be explained by the heterogeneity of the case populations, with variable percentages of cancer cases attributable primarily to tobacco and alcohol use. Until specific genetic markers for the risk of an *H pylori*-associated cancer are identified, familial aggregation due to shared environmental exposures, tobacco habits and other multiple factor cannot be ruled out as an explanation for cancer causation. Poor dentition, and infrequent tooth brushing have been associated with an increased risk of oral infection because tooth loss is commonly caused by chronic bacterial infections (e.g. periodontitis), it may serve as a surrogate for chronic infection and inflammation, which may be important in the pathogenesis of cancer(Zheng et al., 1990; Maier et al, 1993; Moreno-Lopez et al., 2000). Particular *H pylori* co-infections in the stomach increase the risk of cancer, and our results suggest that bacterial co-infections could play a similar role in the oral region. The data however, is not consistant and positivity rates are varying from no detection to 35% in earlier studies (Table 2).

Although our results suggest an increased risk of oral cancer associated with *H. pylori* infection, but small sample size necessitate a cautious interpretation of the findings, which may have contributed to our inability to detect statistically significant findings. In addition, absence of data on *H pylori* associated gastritis is a limitation of our study because it is likely to explain the presence of *H pylori* in oral cavity which may prove as major confounding factor.

Oral cancer poses a great health burden to our society. Tobacco and betel nut chewing, smoking, alcohol, prolong exposure to sunlight, HPV virus infection and family history are the only risk factors that have been proven to be strongly associated with the development of oral cancer. The results of our pilot study show that odds that *H pylori* positive subject could develop oral cancer are 1.6 with PCR and 3.0 with culture. Larger studies are needed to obtain adequate numbers of patients to quantify fully the association between this *H. pylori* exposure and oral cancer. This pilot study should heighten the awareness in the population regarding dental hygiene, since infrequent tooth brushing have been associated with an increased risk of oral infection it may serve as a surrogate for chronic infection and inflammation, which may be important in the pathogenesis of cancer.

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