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ORIGINAL RESEARCH

Study of Survival, Prognosis and Methylation Difference in Malignancies of the Oral Cavity in Different Sites

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Abstract

Background: This study was conducted to evaluate the survival and prognosis of malignancies of the oral cavity at different sites. We also wanted to study the methylation difference in oral cancer cases and compare it with normal.

Methods: The study was conducted in the Department of Otolaryngology – Head and Neck Surgery, Travancore Medical College, Kollam, over a period of 5 years from January 2017 to December 2021. 325 patients who presented with malignancies of the oral cavity were evaluated. The tumours of the salivary glands were excluded. A study on DNA methylation was conducted among 30 cases of different stages of carcinogenesis and compared with five normal cases.

Results: The males were most commonly affected at all sites, and the male-to-female ratio was 2.3:1. Subjects in the 41-60 years age group were most commonly affected, with a mean age of 55 years. A significant proportion of the patients consumed tobacco or alcohol. 76.6% of patients presented at an advanced stage (stage III or stage IV). The majority of the patients presented with complaints of ulcers, pain, or local swelling. 39% of the patients had anemia on presentation. The tongue was the most common site involved, followed by the buccal mucosa. Squamous cell carcinoma was the most common type of malignancy in the oral cavity with well-differentiated SCC present in 48.62% of the cases. Level I was the most common level of lymph nodes involved. A biopsy of the lesion and FNAC from the lymph nodes were sufficient in most of the cases to reach a proper diagnosis. Surgery with or without postoperative radiotherapy was the preferred modality of treatment in early cases, while primary radiotherapy alone was employed in the majority of cases with late-stage disease. Surgical margins were positive in 17.5% of the cases. Recurrence rates were higher when radiotherapy alone was employed. Treatment failures occurred more commonly at the primary site than at the nodal sites. Immunostaining of tissue sections with anti-5-mc antibodies showed increased staining with the progression of the stage of carcinogenesis.

Conclusion: A well-coordinated approach is necessary for the prevention, early detection and treatment to tackle the growing incidence of malignancies of the oral cavity and their associated mortality and morbidity. Early diagnosis is the key to effective treatment of oral malignancies. To optimize survival, the therapeutic approach requires careful planning on the part of an integrated team of head and neck specialists, including the surgeon and the radiotherapist.

Keywords: Survival, Prognosis, Methylation Difference, Malignancies, Oral Cavity.

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Introduction

Oral cavity cancer is largely preventable. The principal risk factors are cigarette smoking and consumption of alcohol, particularly dark spirits. In combination, alcohol and tobacco show a multiplicative relative risk. Early detection of oral cancer is important. Small lesions can be treated successfully with radiotherapy, with a 5-year survival rate of around 80%. Advanced tumours require radical combination therapy and have 5-year survival rates of 30% or lower.
A link between DNA methylation and cancer was first demonstrated in 1983, when it was shown that the genomes of cancer cells are hypomethylated relative to their normal counterparts[1]. Methylation is deregulated in cancer via global methylation and regional specific hypermethylation. Several theories have been put forth regarding mechanisms by which both phenomenon can lead to increased invasion by neoplastic cells. In general, two patterns have been observed: wide areas of global hypomethylation along the genome, and localized areas of hypermethylation in CpG islands within gene promoter regions.

Objectives of the study

1. To study the survival and prognosis of disease at different sites.
2. To study the methylation difference in oral cancer cases and compare it with normal.

Materials &methods

This study was conducted at the Department of Otolaryngology – Head and Neck Surgery, Travancore Medical College, Kollam, over a period of 5 years from January 2017 to December 2021. During this period, 325 patients who presented to us with malignancies of the oral cavity were studied.Patients who presented to this hospital with clinical features suggestive of malignancies of the lips, buccal mucosa, alveolus, tongue, floor of the mouth, hard palate, and retromolar trigone were included in the study. Patients with malignancies of the salivary glands have been excluded. The patients were followed up once a month in the first year and once in every six months thereafter. For data on patients who had already been treated in the past for malignancies in the above-mentioned sites, records of all such patients were obtained from the MRD (Medical Records Department) at periodic intervals.

Role of DNA Methylation in Oral Carcinogenesis

Paraffin-embedded tissue blocks from each of the successive stages of cancer progression were retrieved from the department archives and stained with a monoclonal antibody anti-5-methyl cytosine (Merk Ltd, USA) following which the staining reaction was carried out using the standard streptavidin – peroxidase method. The steps as given in the immunohistochemistry protocol.

Study Procedure

Sections of 4μM thickness taken on poly L lysine coated slides were dewaxed and hydrated through descending grades of alcohol and antigen retrieval was done by immersing the slides in 3.4 pH citrate buffer for 10 minutes in a microwave oven (720 watts). Sections were covered with 100 μl of hybridoma supernatant containing 5-MeCyd monoclonal antibody (5μg/ml) and incubated for 1 hour at 37 deg C, incubated with secondary antibody (Biotinylated Goat anti mouse IgG - 1:200 IN PBS containing 0.1% BSA) for 1 hour at RT (DAKO) and subsequently incubated with streptavidin - peroxidase conjugate (1:100 in PBS-BSA) - 20 minutes (DAKO) and finally treating the sections with DAB for 5 minutes. Between each step the sections were washed three times with PBS containing 0.1% Tween 20 (PBST) and pictures were taken on colour positive films.

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Inference

Immunostaining of neoplastically altered cells reveals densely labelled spots within faintly labelled areas, whereas normal nuclei are darker and uniformly stained. A total of 1000 cells per slide were measured. The intensity of colour was measured by a software program called NIH Image J. An average of intensity scores for each stage of cancer progression was recorded from 0 – 3 which were categorized into four classes. The class IV (-) meant the cells were unstained and the class I meant that the cells had the maximum intensity staining score (+++). The class II (++) and class III (+) cells had intermediate scoring intensities. The percentage of cells within each class was also recorded independently by two observers to remove any interobserver bias. All the variables through different stages of cancer progression were studied for any variation in the 5mC expressivity. The slides were photographed on a CCD camera under 10X and 40X magnification, and intensities were recorded using NIH Image J software.

Results

Surgery vs. Positive Surgical Margins vs. Recurrence and Duration of Recurrence

Of the 166 surgeries that were performed for malignancies of the oral cavity, 29 (17.47%) histopathological specimens showed a positive margin for tumor. Of the 29 patients, 20 received adjuvant radiotherapy. Of them, 6 had a recurrence in less than 6 months (30 %), 5 had recurrence a between 6 months and 1 year (25%) and 3 patients (15%) had a recurrence after 1 year.

Lips: Of the 7 surgeries done margins were positive in 1 case (14.29%) who underwent adjuvant radiotherapy. No recurrence was reported.

Buccal Mucosa: Of the 27 cases who had surgery done, 3 cases (11.11%) had positive margins. 2 of these 3 cases (66.67%) received adjuvant radiotherapy. One case (50%) developed recurrence in less than 6 months. The case without adjuvant RT (100%) developed recurrence between 6 and 12 months.

Alveolus: Of the 18 cases who had surgery done, 2 cases (11.11%) had positive margins. 1 of these 2 cases (50%) received adjuvant radiotherapy. The case with adjuvant radiotherapy developed recurrence in less than 6 months. The case without adjuvant RT (100%) was lost to follow-up.

Tongue: Of the 77 cases who had surgery done, 15 cases (19.48%) had positive margins. 12 of these 15 cases (80%) received adjuvant radiotherapy. 10 cases of 12 developed recurrence; 3 (25%) in less than 6 months, 4 (33.33%) in 6 to 12 months and 3 (25%) in more than 12 months. In the case without adjuvant RT all 3 (100%) developed recurrence within 12 months.

Floor of Mouth: Of the 19 cases who had surgery done, 5 cases (26.32%) had positive margins. 2 of these 5 cases (40%) received adjuvant radiotherapy. One case (50%) developed recurrence in less than 6 months. All three cases without adjuvant RT (100%) developed recurrence.

Hard Palate: Of the six cases who had surgery done, none had margins that were positive.

Retromolar Trigone: Of the 12 cases who had surgery done, 3 cases (25%) had positive margins. 2 of these 3 cases (66.67%) took adjuvant radiotherapy. One case (50%) developed

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recurrence in 6-12 months. One case (100%) without adjuvant RT (100%) developed recurrence within 1 year.

Treatment Failures in Oral Cavity Malignancies – Comparison between Surgeries, RT and Surgery Followed by RT

Lip: Of the 5 cases who underwent surgery alone, 2 (40%) had recurrence in the primary site, while 1(20%) had nodal recurrence. In the 3 cases who underwent primary radiotherapy 1 (33.33%) had recurrence at the primary site. In the cases where patients had surgery with postoperative radiotherapy, there was no recurrence.

Buccal Mucosa: Of the 14 cases who underwent surgery alone, 2 (14.29%) had recurrence in the primary site, while 1 (7.14%) each had nodal recurrence, both primary with regional recurrence. In the 27 cases who underwent primary radiotherapy 9 (33.33%) had recurrence in primary site, 1 (3.7%) had nodal recurrence and 2 (7.41%) had both nodal and primary recurrence. In the 13 cases who had surgery with postoperative radiotherapy 4 (30.77%) had recurrence in the primary site and 1 (7.69%) had recurrence in both the primary and nodal sites.

Alveolus: Of the 9 cases who underwent surgery alone, 3 (33.33%) had recurrence in the primary site while 1 (11.11%) had both primary and regional recurrence. In the 2 cases who underwent primary radiotherapy 1 (50%) had recurrence in the primary site and 1 (50%) had nodal recurrence. In the 8 cases who had surgery with postoperative radiotherapy 1 (12.5%) had recurrence in the primary site and 1 (12.5%) had recurrence in the nodal site.

Tongue: Of the 26 cases who underwent surgery alone, 3 (11.54%) had recurrence in the primary site, while 4(15.38%) had nodal recurrence and 3 (11.54%) had both primary and regional recurrence. In the 50 cases who underwent primary radiotherapy 7 (14%) had recurrence in the primary site, 3 (6%) had nodal recurrence and 4 (8%) had both nodal and primary recurrence. In the 46 cases who had surgery with postoperative radiotherapy 10 (21.74%) had recurrence in the primary site, 4 (8.7%) in the nodal site and 3 (6.52%) had recurrence in both the primary and nodal sites.

Floor of Mouth: Of the 11 cases who underwent surgery alone, 1 (9.09%) had a recurrence in the primary site. In the 18 cases who underwent primary radiotherapy 3 (16.67%) had recurrence in the primary site and 1 (5.56%) had nodal and local recurrence. In the 8 cases who had surgery with postoperative radiotherapy 1 (12.5%) had both recurrences in the primary and nodal sites.

Hard Palate: Of the 4 cases who underwent surgery alone, 1 (25%) had nodal recurrence. In the 6 cases who underwent primary radiotherapy 1 (16.67%) had recurrence in the primary site. In the 2 cases who had surgery with postoperative radiotherapy there was no recurrence.

Retromolar Trigone: Of the 4 cases who underwent surgery alone, 1 (25%) had primary recurrence and 1 (25%) had both primary and nodal recurrence. In the 5 cases who underwent primary radiotherapy 3(60%) had recurrence at the primary site. In the 8 cases who had surgery with postoperative radiotherapy 2 cases (25%) had recurrence at the primary site.

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	SURGERY										
	No Rec	**Rec Primary**				**Rec Nodal**				**Rcpn**	**Total**
		T1	***T2***	***T3***	***T4***	***N0***	***N1***	***N2***	***N3***		
Lip	2	2	-	-	-	-	-	1	-	-	**3**
BM	10	-	2	-	-	-	1	-	-	1	**4**
Alv	5	-	1	1	1	-	-	-	-	1	**4**
T	16	-	2	1	-	-	3	1	-	3	**10**
FOM	10	-	-	1	-	-	-	-	-	-	**1**
HP	3	-	-	-	-	-	1	-	-	-	**1**
RMT	2	-	1	-	-	-	-	-	-	1	**2**
Total	48	2	6	3	1	-	5	2	-	6	**25**
% Age	65.75	8	24	12	4	-	20	8	-	24	-
	RADIOTHERAPY										
	No Rec	**Rec Primary**				**Rec Nodal**				**Rcpn**	**Total**
		T1	***T2***	***T3***	***T4***	***N0***	***N1***	***N2***	***N3***		
Lip	2	-	-	1	-	-	-	-	-	-	**1**
BM	15	1	4	1	3	-	-	1	-	2	**12**
Alv	-	1	-	-	-	-	-	1	-	-	**2**
T	36	-	5	1	1	-	2	1	-	4	**14**
FOM	14	-	1	1	1	-	-	-	-	1	**4**
HP	5	-	-	1	-	-	-	-	-	-	**1**
RMT	2	1	-	1	1	-	-	-	-	-	**3**
Total	74	3	10	6	6	-	2	3	-	7	**37**
% Age	66.67	8.11	27.03	16.22	16.22	-	5.41	8.11	-	18.92	-
	SURGERY+RADIOTHERAPY										
	No Rec	**Rec Primary**				**Rec Nodal**				**Rcpn**	**Total**
		T1	***T2***	***T3***	***T4***	***N0***	***N1***	***N2***	***N3***		
Lip	1	-	-	-	-	-	-	-	-	-	-
BM	8	-	1	-	3	-	-	-	-	1	**5**
Alv	6	-	1	-	-	-	1	-	-	-	**2**
T	29	1	4	4	1	-	-	4	-	3	**17**
FOM	7	-	-	-	-	-	-	-	-	1	**1**
HP	2	-	-	-	-	-	-	-	-	-	-
RMT	6	-	1	-	1	-	-	-	-	-	**2**
Total	59	1	7	4	5	-	1	4	-	5	**27**
% Age	68.60	3.70	25.93	14.81	18.52	-	3.70	14.81	-	18.52	-

Table 1 - Treatment Failures in Oral Cavity Malignancies

Role of DNA Methylation in Oral Carcinogenesis

Immunostaining of neoplastically altered cells reveals densely labelled spots within faintly labelled areas, whereas normal nuclei are darker and uniformly stained. A total of 1000 cells per slide were measured. The intensity of colour was measured by a software programme called NIH Image J. An average of intensity scores for each stage of cancer progression was recorded from 0 – 3 which were categorized into four classes. The class IV (-) meant the cells were unstained, and the class I meant that the cells had the maximum intensity staining score (+++). The class II (++) and class III (+) cells had intermediate scoring intensities. The

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percentage of cells within each class was also recorded independently by two observers to remove any interobserver bias. All the variables through different stages of cancer progression were studied for any variation in the 5mC expressivity. The slides were photographed on a CCD camera under 10X and 40X magnification and intensities were recorded using NIH Image J software.

A notable significance was also noted in relation to early and advanced premalignant lesions, where a p-value of less than 0.05 was observed when applying the Mann-Whitney test between these two variables with respect to both the percentage of cells taking up the stain and the average intensity scores. It was evident from our observation using a scatter plot (Chart 8) that there was a definite fall in the percentage of class IV [-] and a reverse trend with respect to class I cells [+++] with an increase in the severity of the disease. In other words, the percentage of class IV cells [-] was higher in normal samples as compared to the percentage of class I cells [+++] which were significantly more dysplastic and cancer cells.

Photomicrographs (PM) for Immunohistochemical Analysis

[(40x) 5mC staining**]**

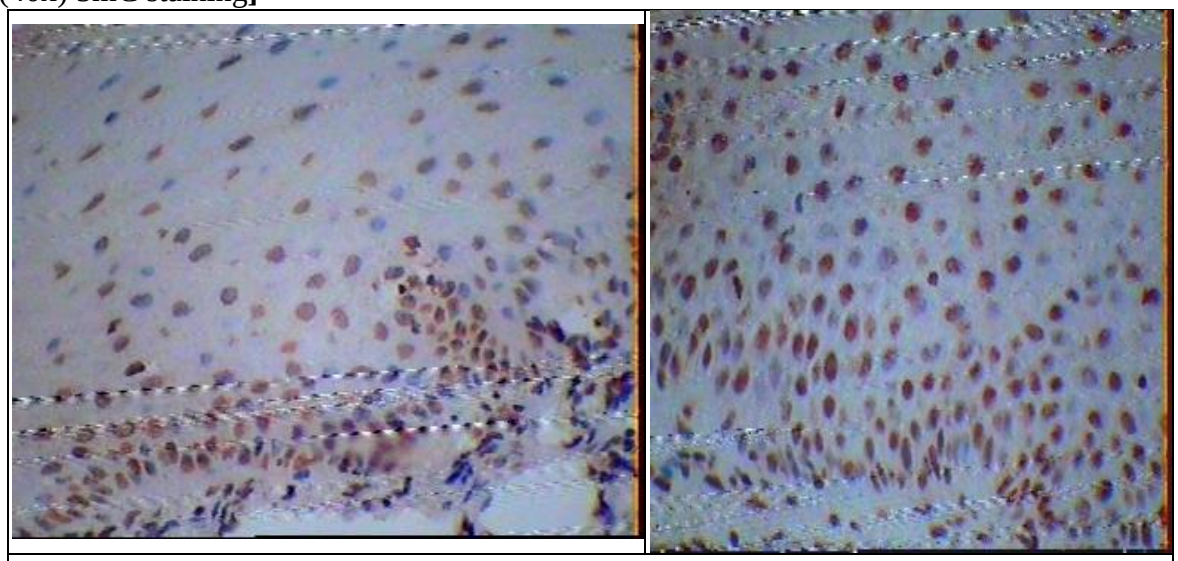

Figure 1 - Normal Mucosa Mild Dysplasia

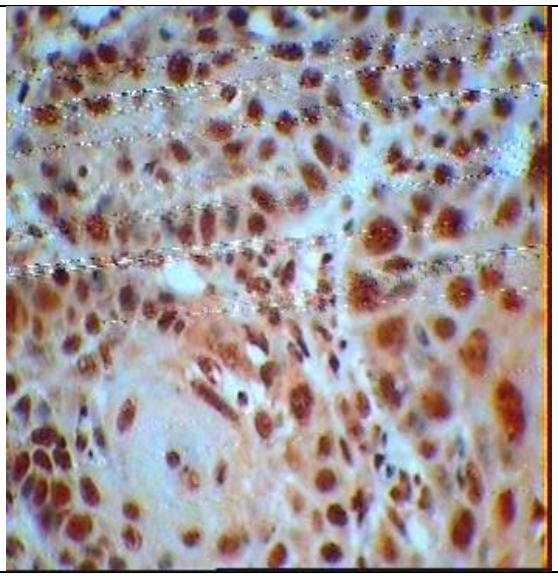

Figure 2 - Well Differentiated Squamous Cell Carcinoma

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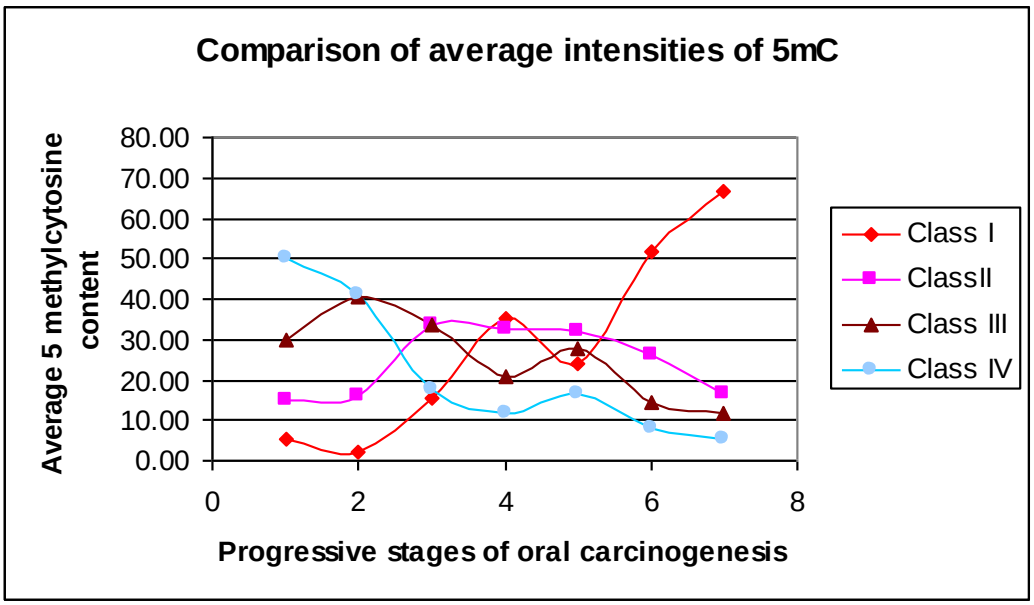

Figure 3 -Trends in Average Intensity between Classes in Different Stages of Cancer Progression

Discussion

Failures with Different Modalities of Treatment

Tongue: In the series by El-Husseiny G et al.[2] The recurrence rate was higher in patients who received radiotherapy alone. Among them, 33.3% each were local, regional, and locoregional recurrences. In the patients who underwent surgery alone, 20% had regional recurrence and 10% had locoregional recurrence. In the patients who had surgery followed by radiotherapy 19.56% had a regional recurrence. In our study, among the patients who underwent surgery alone, 15.38% had regional failure, while 11.53% each had local and locoregional recurrence. In the cases with primary RT alone, 19.44% had local recurrence.

Floor of Mouth: In the series by Ildstad ST et al.[3] 18.18% of the cases who underwent surgery alone had recurrence in the primary site. 21.90% of the cases who received primary radiotherapy alone had regional failure. Of the patients who received surgery with adjuvant radiotherapy, 11.76 % had a local recurrence.

In our study, 10% of the patients who underwent surgery alone had local failure. 21.43 % of the patients with primary RT alone had local recurrence.

	Tongue								
	S			**RT**			**S+RT**		
	Rcp	**Rcn**	**Rcpn**	**Rcp**	**Rcn**	**Rcpn**	**Rcp**	**Rcn**	**Rcpn**
El-Husseiny G et al.	5%	20%	10%	33.30%	33.30%	33.30%	15.20%	19.56%	4.30%
Our Study	11.53%	15.38%	11.53%	19.44%	8.33%	11.11%	34.48%	13.79%	10.34%
	Floor of Mouth								
	S			**RT**			**S+RT**		
	Rcp	**Rcn**	**Rcpn**	**Rcp**	**Rcn**	**Rcpn**	**Rcp**	**Rcn**	**Rcpn**
Ildstad ST et al.	18.18%	12.12%	9.08%	7.29%	21.90%	16%	11.76%	5.88%	5.88%
Our Study	10%	—	—	21.43%	—	7.10%	—	—	7.10%

Table 2 - Failures with Different Modalities of Treatment in Comparison

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Role of DNA Methylation in Oral Carcinogenesis

Immunostaining the tissue sections in each of the successive stages of tumor progression was done, thereby analyzing the global methylation status of the tissue. It is believed to be as prevalent as promoter hypermethylation. It has been proposed that global hypomethylation occurs early in tumor development and at subsequent genomic sites in the gene promoter region. Evaluation of global methylation status in different stages of oral cancer progression in tissue sections by IHC using a monoclonal antibody developed against 5mC.[1,4]

From our observations, it was clear that dysplastic lesions and SCCs of the oral cavity are significantly hypermethylated compared with the normal oral epithelium of subjects in matched controls and those without cancer. These findings are in accordance with Piyathilake CJ et al.[5] 2005 who made similar observations, further highlighting the site specific differences in methylation pattern. In their opinion, this difference points to the embryologic roots of the development of the oral mucosa and the mucosa of the anterior two-thirds of the tongue which are ectodermally derived.

Although any one gene that is hypermethylated may not be detected using 5mC antibody, cumulative changes in the methylation status of multiple genes could be discerned using immunohistochemistry. If a significant association between nonspecific global DNA hypermethylation and gene-specific methylation could be established, alterations in GDM may serve as an effective marker for methylation changes in specific genes and thereby determine the subject at risk.

Conclusion

A well-coordinated approach is necessary for the prevention, early detection and treatment to tackle the growing incidence of malignancies of the oral cavity and their associated mortality and morbidity. There should be a deliberate effort to make the public aware that oral cancer is largely preventable. They should be enlightened about the undeniable association of the disease with tobacco and alcohol. Early diagnosis is the key to effective treatment of oral malignancies. The primary physician should be alert for the early detection of oral lesions. To optimize survival, the therapeutic approach requires careful planning on the part of an integrated team of head and neck specialists including the surgeon and the radiotherapist. Though significant improvements have been made in the diagnosis and treatment of oral cancers in the last few decades, there is still a long way to go before we conquer this devastating disease.

Bibliography

1. Piyathilake CJ, Johanning GL, Frost AR, Whiteside MA, Manne U, Grizzle WE et al. Immunohistochemical evaluation of global DNA methylation: Comparison with in vitro radiolabeled methyl incorporation assay. Biotech Histochem 2000;75:251-8.
2. El-Husseiny G, Kandil A, Jamshed A, Khafaga Y, Saleem M, Allam A et al. Squamous cell carcinoma of the oral tongue: an analysis of prognostic factors. Br J Oral Maxillofac Surg 2000;38(3):193-9.
3. Ildstad ST, Bigelow ME, Remensnyder JP. Intra-oral cancer at the Massachusetts General Hospital. Squamous cell carcinoma of the floor of the mouth. Ann Surg. 1983;197(1):34-41.
4. Chen JK, Katz RV, Krutchkoff DJ. Intraoral squamous cell carcinoma. Epidemiologic patterns in Connecticut from 1935 to 1985. Cancer 1990;66(6):1288-96.
5. Piyathilake CJ, Bell WC, Jones J, Henao OL, Heimburger DC, Niveleau A, et al. Pattern of nonspecific (or global) DNA methylation in oralcarcinogenesis. Head & Neck 2005;27:1061-7.