ORIGINAL RESEARCH

Prognostic significance of MMP 2 & sE- cadherin in saliva with MMP 9 in serum of OSCC cases- Original Research

Sonalee Shah[1], Alka Hande[2], Madhuri Gawande[3]

Abstract

Introduction: The investigation and application of prognostic biomarkers are crucial for addressing the prognostic difficulties in oral cancer programs. The formation of invasive lesions is governed by paracrine/autocrine signaling molecules, such as the proteolyzed extracellular domain of E-cadherin, which disrupts apoptosis through its physical interaction with EGFR. Proteinases initiating the "degradation of the basement membrane in the local milieu, particularly MMP 2 & MMP 9, facilitate tumor invasion.

Aim: Aim of this study is to evaluate and compare the effectiveness of E-cadherin, MMP 2 in saliva & MMP 9 in serum as biomarkers for assessing the metastatic and recurrence potential of OSCC.

Materials & Method:

Study Variables- Quantification of MMP 2 & E-cadherin in saliva & Quantification of MMP 9 in serum. Study Groups- Group I - Controls: 29; Group II – OSCC patients: 32. Ethical committee consent: It was obtained prior to onset of study from college ethical committee vide letter no. DMIMS (DU)/IEC/Dec-2019/8667 dated 31/12 2019. Methodology: Saliva & serum were collected from OSCC patients & controls after consent, centrifuged, refrigerated and analysed by ELISA method with respective parameter kits. Results were statistically analysed by SPSS software.

Results: Most cases 16/32 cases were in 4th to 6th decade of life (50%). The mean±SD of Cases and Controls had no statistically significant difference at $p>0.05$ level for MMP 9 in serum. The mean±SD of Cases and Controls, for sE- cadherin in saliva showed a statistically significant difference at $p < 0.05$ level. For MMP 2, the mean ± SD for the cases and for the controls demonstrated no statistically significant difference at $p>0.05$ level.

Discussion: Both markers in saliva showed a mean value that was higher than mean value of controls but only E-cadherin had "a statistically significant difference. The mean serum MMP 9 value was lower in cases than in controls. Determining prognosis therefore should be done with combined evaluation of all three parameters which will allow better predictability of metastases and recurrence "likelihood.

KEYWORDS: Oral squamous cell carcinoma (OSCC), Gelatinases, E-cadherin, Saliva, Serum, ELISA, OSCC prognosis, Matrix metalloproteinase 2 & 9(MMP 2, MMP 9)

Introduction

Squamous cell carcinoma (SCC) of the oral cavity mucosa, commonly known as oral cancer, ranks as the second principal contributor to mortality.[1] Over 90% of head-and-neck malignancies are OSCCs and from them a large majority, affects the lips, tongue, cheeks, mouth floor, hard and soft palate, and gingiva. Despite innovations in treatment modalities, oral cancer still has a 50% 5-year survival rate worldwide.[2,3]

According to GLOBOCAN 2022, India contributes nearly one-third of all global OSCC cases, and OSCC itself represents around 30% of the country's total cancer burden. The central Indian region—covering Uttar Pradesh, Madhya Pradesh, Rajasthan, and Chhattisgarh—records the highest rates of oral cancer. This disease burden is worsened by delayed

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detection and poor survival outcomes.[4] The age-standardized mortality rate (ASMR) for oral cancer rose from 5.32 to 5.92 between 1990 and 2021[5]. Consequently, oral cancer management presents a significant health problem for developing countries experiencing fast economic change[4, 5].

A crucial recommendation to address the significant gap in the management of oral cancer treatment is post-treatment follow-up, according to the NAMS task force report on oral cancer, 2025. Individuals with oral cancer are susceptible to locoregional recurrences and secondary cancers.[5] Addressing these gaps requires prognostic biomarker research and application.

E-cadherin, a 120kDa glycoprotein of epithelial surface, consists of three key domains: the cytoplasmic, transmembrane, and extracellular regions[6]. It regulates essential physiological activities such as polarity, differentiation, and cell movement[7]. The formation of invasive lesions, necessitates the presence of tumor-promoting paracrine/autocrine signalling molecules like the proteolyzed extracellular domain of E-cadherin, that with EGFR functions as growth factor and inhibits apoptosis and facilitates cell proliferation[6,7]. Signalling by sE-cad may potentially disrupt apoptosis.[8-9]

Due to chronic overabundance of MMP 2& 9 in the local environment, there is degradation of the epithelial basement membrane and tumor invasion is facilitated.[6-10]

MMP-2 is generated by fibroblasts, keratinocytes, endothelial cells, chondrocytes, and monocytes, whereas MMP-9 is produced by alveolar macrophages, polymorphonuclear leukocytes, and osteoclasts[10]. MMP2 & 9 break down denatured collagen and a wide array of extracellular matrix (ECM) molecules.[10-11]

A non-significant improvement in prognosis of OSCC patients after diagnosis and post-treatment is attributable not only to, delay in diagnosis but also to the fact that, tumors with identical staging & grading behave differently and give variable prognosis.[12,13]

Therefore, use of saliva and/or serum based diagnostics will assist in early detection of OSCC and also aid in predicting the prognosis as well as serving to monitor for likely recurrences.

A prospective non-interventional analytical study was performed in the Department of Oral Pathology at Sharad Pawar Dental College & Hospital, Datta Meghe Institute of Higher Education and Research.

Materials & Method

Aim: In this study, we quantified the salivary concentration of MMP 2 & soluble E-cadherin and serum concentration of MMP 9 in OSCC pre-treatment patients & healthy controls and compared & correlated them to draw inference about the biological behaviour of tumor and hence the probable prognosis.

Objectives of Study:

- To assess the expression of soluble E-cadherin & MMP2 in saliva of Controls by ELISA method
- To assess the expression of MMP 9 in serum of Controls by ELISA method
- To assess the expression of soluble E-cadherin & MMP2 in saliva of Oral Squamous cell Carcinoma (OSCC-PRE-TREATMENT) patients by ELISA method
- To assess the expression of MMP 9 in serum of Oral Squamous cell Carcinoma (OSCC-PRE-TREATMENT) patients by ELISA method
- To correlate & compare the expression levels of soluble E-cadherin, MMP2 in saliva of Controls and OSCC patients (PRE-TREATMENT group)
- To correlate & compare the expression levels of MMP 9 in serum of Controls and OSCC patients (PRE-TREATMENT group)

Research Study Design

a. Statistical design for Sample Size:

Sample size commensurates with the study design.

Justification: Based upon the results from Nosratzehi T et al and using an

Alpha (α) level of 0.05 as well as Beta(β) level of 0.20 i.e. power= 80%

- The minimum projected sample size is 26 subjects per group.
- Sample size calculation had been carried out by employing G*Power Version 3.1.9.2
- Analysis: A priori : Compute required sample size
- lnput: Tail(s) = One
- Effect size d = 0.7040469
- α err Prob = 0.05
- Power(1-β err Prob) = 0.80
- Allocation ratio N2/N1 = 1
- Output : Non-centrality parameter δ =2.5384772
- Critical t = 1.6759050
- Sample size Per group = 26

** Calculated on the basis of mean difference of MMP-2 levels in saliva of Oral Squamous cell carcinoma and Healthy subjects.

b. Variables of study-

Quantitative estimation of MMP 2 & E-cadherin in saliva

Quantitative estimation of MMP 9 in" serum

c. Sample size- 32(OSCC)+ 29 (Controls)

d. Study Groups-Group I - Controls (No habits; No diseases): 29; Group II – OSCC patients (PRE-TREATMENT): 32

e. Sampling method-Simple Random sampling

f. Study Settings: Study samples were collected with prior informed consent from OPD patients at Sharad Pawar Dental College, Wardha.

g. Ethical committee consent: It was obtained prior to onset of study from college ethical committee.

h. Study Duration: Study duration from first visit of patient to 12 month follow-up visit was approximately 2 years until all samples were collected. This was followed by a month of test implementation and result data compilation into charts.

Materials

The Human Matrix Metalloproteinase 2 /Gelatinase A GENLISA ELISA KIT

The Human Matrix Metalloproteinase 9/Gelatinase B GENLISA ELISA KIT

The Human E-cadherin GENLISA KIT

ELISA reader

Methodology

a) Saliva sample collection and Analysis

Collection of salivary and serum samples

- OSCC patients were identified and for those fulfilling inclusion criteria
- The study is explained and details regarding the sample techniques are provided; each participant signed a written informed consent form.
- Participants sitting upright and tilted their heads slightly forward to collect saliva on the mouth floor for 10-15 minutes, collecting approximately 5 ml.
- Subsequently, intraorally held saliva was expectorated into a graded sterile container.
- Before analysis, the samples were centrifuged at 3000rpm for 15minutes, after which 2 ml of the clear supernatant was transferred to Eppendorf vials and kept at -20°C until analysis was conducted.

b) Serum sample collection and Analysis

1. From Controls 5ml blood had been also collected after informed consent.

2. For OSCC patients, 5mL of venous blood was collected from all subjects prior to radiation and chemotherapy, as well as from the control group, which was let to clot. Then Serum of the patient was obtained from blood collected for routine referral haematological investigations in the department after centrifuging the same 3000 rpm for 15 minutes.

3. Serum was then transferred to 5 ml sterile, stoppered tubes & stored at −20 °C until the analysis was done.

4. Sample Handling-Each sample was clarified by centrifuging at 3000 rpm at room temperature for 15 mins and stored.

c) Reagent Preparation (all reagents should be diluted immediately prior to use):

1. Label all aliquots prepared with the kit's Lot Number and Expiration Date, and store them under the specified conditions.

2. Permit all reagents to attain ambient temperature before use.

3. To prepare 500 ml of Wash Buffer (1X), dilute 25 ml of (20X) Wash Buffer in 475 ml of deionized water.

4. Streptavidin: HRP Conjugate and Biotinylated Matrix Metalloproteinase-2 / Gelatinase A (MMP-2) Antibody Working Solution – Gently spin or briefly centrifuge the Streptavidin–HRP Conjugate and the Biotinylated MMP-2 Antibody before use.

5. Dilute each component to its working concentration by making a 1:100 dilution in the HRP Conjugate Dilution Buffer and the Biotin Antibody Dilution Buffer, respectively.

6. Standards Preparation: Use 0.5ml of Standard" Diluent to reconstitute the original Matrix Metalloproteinase 2 / Gelatinase A, MMP-2 Standard. Maintain standard for 10min with moderate agitation before diluting.

7. Use 1.0 milliliter of Standard Diluent to reconstitute the original E-Cadherin Standard. Use gentle agitation to keep the standard for 10 minutes before diluting.

8. Using 1.0ml of Standard Diluent, reconstitute the original Matrix Metalloproteinase 9/Gelatinase B Standard. Make additional dilutions after gently stirring the standard for ten minutes.

d) Assay Procedures:

The Human MMP-2 & E-cadherin ELISA kit (Boster Biological Technology, Pleasanton, CA, USA) had been employed to assess the MMP-2 & E-cadherin levels in the thawed samples. The sandwich ELISA kit uses a polyclonal antibody coated on a microplate that binds to MMP-2 and E-cadherin in the sample. Detection is made easier by a secondary biotinylated MMP-2 and E-cadherin specific antibody that bounds to the MMP-2 and E-cadherin bind to the plate, respectively.

[Add Standard/Sample] → [Incubate] → [Wash] → [Add Biotin-Antibody] → [Incubate] → [Wash] → [Add HRP-Streptavidin] → [Incubate] → [Wash] → [Add TMB Substrate] → [Color Change] → [Add Stop Solution] → [Read OD at 450nm]

e) Calculation of Results-

Every duplicate or triple standard and sample set had a mean absorbance computed.

Graph paper was employed to plot the average absorbance (450nm) of each standard versus its concentration on X-axis. Through the standard points, curve with the best fit was created.

Samples that were diluted were multiplied by the proper dilution factor.

For each antibody, the readings of Controls were taken as guideline readings & compared with readings of pre-treatment OSCC patients.

The readings of Controls were taken as guideline readings & compared with readings of post-treatment OSCC patients after six months and twelve months of surgery.

INCLUSION CRITERIA:

1. Controls

A) Individuals with no H/O of Alcohol or Tobacco usage

B) No H/O of sytemic or Oral debilitating disease

C) No H/O of any malignancy

2. OSCC Pre-treatment patients

a) Patients were diagnosed with OSCC through clinical oral examination.

b) Patients with HP confirmed Primary OSCC who have not received any prior treatment

c) The study comprised participants aged 18 to 70 years.

d) Both male and female participants are selected.

e) Participants with history of smokeless tobacco / areca nut consumption, smoked tobacco consumption and alcohol abusers

EXCLUSION CRITERIA

- Patients with inflammatory oral lesions, autoimmune disorders, previous malignancies, hepatitis, periodontal disease, systemic diseases, or human

immunodeficiency virus infection, along with pregnant or breastfeeding women, were excluded from the study.

- Patients not willing to participate.
- Patients with history of other tumors
- Patients indicated only non-surgical treatment
- Patients and subjects with recent trauma, infection, burning, or laceration.
- Patients with previous history of surgery or chemo-radiotherapy
- Patients taking medication that could alter salivary characteristics

Justification: Currently, no research exists about the assessment of serum MMP-9 and salivary concentrations of MMP-2 and sE-cadherin in individuals with OSCC. This case–control study assessed saliva and serum protein levels in pre-treatment OSCC patients and compared them with healthy individuals. Salivary MMP-2, sE-cadherin, and serum MMP-9 will be evaluated as biomarkers for oral squamous cell carcinoma to help predict patient outcomes and long-term survival.

Results

The gathered data were entered into Microsoft Excel 2016 and analyzed using IBM SPSS Statistics for Windows, Version 29.0 (IBM Corporation, Armonk, NY). Descriptive statistics included frequencies and percentages for categorical variables, and means with standard deviations for continuous variables. The independent sample t-test was applied to compare unrelated groups, while the paired sample t-test evaluated paired observations to identify significant bivariate differences. One-way ANOVA with Tukey's post-hoc test was utilized for multivariate comparisons, and repeated measures ANOVA with Bonferroni correction was performed for reducing Type I error in multiple comparisons. Pearson's Correlation assessed the factors' relationship. The Chi-Square test was implemented for determining the significance of qualitative categorical data, while Fisher's Exact test was utilized for 2x2 tables with cell frequencies below 5. To evaluate the proteins' case prediction abilities, the Receiver Operating Characteristic (ROC) curve was employed, including sensitivity, specificity, and cut-off values. All the statistical techniques above use a significance threshold of.05.

Table 1 and Fig 1, show the study sample distribution among study groups where Cases is 52.5%, Controls is 47.5%.

Table 2 & Fig-2 show that, when age distribution was evaluated, the number of individuals of age <= 50 years was 45/61 (73.8%) of total sample, and for > 50 yrs age it was 16/61(26.2%) of total sample. The mean age of our cases was 48.97 yrs. & median was 45 years.

Table 1: Study Groups' distribution

Groups distribution		
	Frequency	Percent
Cases	32	52.5
Controls	29	47.5
Total	61	100.0

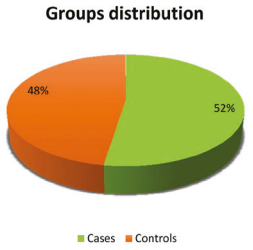

Fig 1 – Graph of study sample distribution

Table 2: Age distribution among study sample

Age distribution		
	Frequency	Percent
<= 50 yrs	45	73.8
> 50 yrs	16	26.2
Total	61	100.0

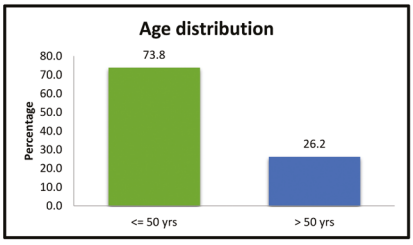

Fig 2-Age distribution among study sample

Table 3: Comparison of Age between Groups

AGE GROUPS	CASES	CONTROLS
21-40	09	21
41-60	16	6
61-80	7	2

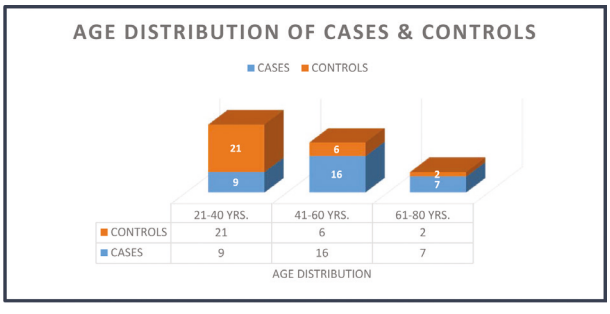

Fig 3- Graph of comparison of Age between groups

Table 3 and Fig 3 shows the distribution chart of total sample into three age groups of 20 years each from 20 years onwards. It is evident from **Table 3 and Fig 3** that, most cases 16/32 cases, were in 4th to 6th decade of life (50%) and Cases were more 09/32 cases in 2nd to 4th decade (28.13%), suggesting ***changing trend of increase in occurrence at a younger age.***

Overall cases were more in 4th decade & later 23/32(68.75%) but still incidence showed an increase in 2nd to 4th decade.

When comparison of Age & Gender between Groups was done by Pearson's Chi-Square test it showed no statistical significance.

The mean age of OSCC cases was 49.25years and median age was 49. 31 years.

On comparison of gender between age groups as in Fig-4, it was noted that females were more prone to OSCC occurrence in 41-60 years age group than the males & the M:F ratio was 7:9 (43.75% : 56.25%) so, significantly more percentage of females with OSCC were seen in this age group than in other 2 age groups. It was notable that the young age (21-40 yrs.) group M:F ratio was 7:2 and in middle age (41-60 yrs.) group M:F ratio was 7:9. Thus, it showed an equivalent occurrence of OSCC in males in these two age groups. In the elderly age (61-80 yrs.) group the M:F ratio was 4:3 and it showed an equivalent gender distribution.

- The Pearson's Chi-Square test indicated that the Age between Groups yielded χ^2=2.308, p=0.129, demonstrating no statistically significant correlation between Age and Groups.
- Analysis of Gender across Groups using Pearson's Chi-Square test yielded χ^2=0.794, p=0.373> 0.05, indicating no statistically significant correlation between" Gender and Groups.

Table 4 & Fig-5 show the comparison of MMP 9 Result between Case & Control Groups by Independent sample t-test where Pre-Rx, t-value = 0.621, p = 0.268>0.05. It shows that mean±SD of Cases is (1122.18±420.17) and Controls is (1188.47±419.67), which demonstrated no statistically significant difference at p>0.05 level. Median value" of case group was 1259.05pg/ml which was higher than mean of controls.

However, among OSCC cases, 18/32 cases, ie, (56.25%) cases showed a rise in MMP 9 levels in their serum in comparison to mean serum level of MMP 9 in controls (1188.47 pg/ml). The median value of cases was 1259.05 pg/ml.

MMP 9 was seen to be elevated in more patients of more than > 50 years of age, and so in this age group, 8/11 cases (72.72%) cases with more in males 5/8 cases, showed elevated serum MMP 9 than the mean of controls.

On the other hand, in patients below or equal to 50 years age, 10/21 cases (47.61%) patients with more in females (6/10), had higher serum MMP 9 levels than mean of controls.

18 cases showed elevated MMP 9 above mean of controls and from them, 14 cases (77.77%) showed elevated level above the predicted cut-off value of this parameter in our study. Additionally, 10/14 cases with increase above cut-off value also

Table 4: Comparison of MMP 9 Result between Groups by Independent sample t-test

MMP 9 Result	Groups	N	Mean	SD	t-value	p-value
OSCC cases	Cases	32	1122.18	420.17	0.621	0.268 #
	Controls	29	1188.47	419.67		

No Statistical Significance at p > 0.05 level

Table 5: Comparison of E-cadherin Result between Groups by Independent sample t-test

E-cadherin Result	Groups	N	Mean	SD	t-value	p-value
OSCC cases	Cases	32	4340.907	2693.449	-2.120	0.038 *
	Controls	29	2972.143	2309.760		

* Significant at p < 0.05

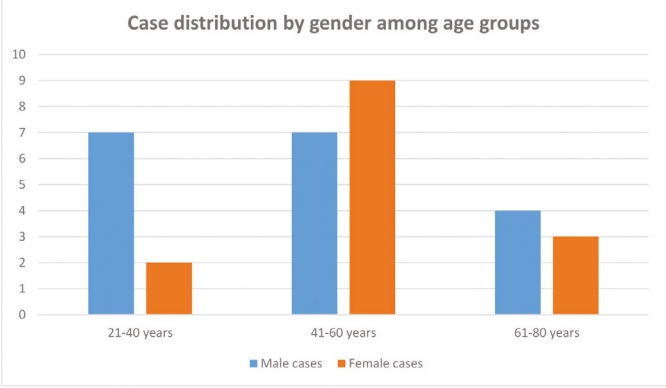

Fig 4 – Graph showing Case distribution among gender by Age groups

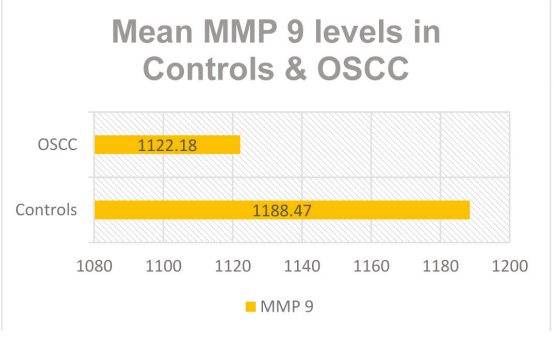

Fig-5: Graph of comparison of MMP9 result between OSCC cases & Controls

showed a simultaneous increase in E-cadherin level.

Table 5 & Fig-6 show the comparison of sE-cadherin Result between OSCC Case & Control Groups by Independent sample t-test where Pre-Rx t-value=-2.12, p=0.038<0.05. It shows that, the mean±SD of Cases is (4340.91±2693.45) and Controls is (2972.14±2309.76), which therefore exhibited statistically significant difference at p<0.05 level.

There were 22 /32 patient samples (68.75%), that showed an increase in sE-cadherin in pre-treatment samples above the mean value of control group.

sE-cadherin levels were more persistently higher in those upto 50 years of age 12/22 samples (54.54%) and 10/22 (45.45%) showed an elevated level in those above 50 years age. Also, 90.9% (20/22 samples with elevated sE-cadherin) showed levels that were above the cut-off value of our study. 8/32(25%) samples show simultaneous elevation of MMP 9 & sE- cadherin level increase above their individual cut-off values predicted in our study.

Table 6 and Figure 7 illustrate the comparison of MMP 2 results between the OSCC case and control groups using an independent sample t-test. For the pre-treatment phase, t-value is -0.288 and p-value is 0.775, indicating p>0.05. Mean ± SD for the cases is (24.52 ± 9.40) and for the controls is (23.52 ± 17.17), demonstrating no statistically significant difference at p>0.05 level.

When MMP 2 levels of cases were compared with mean value of the same in controls, it was noted that, 18/32 (56.25%) cases had an elevated value. From these 18 samples, when

Table 6: Comparison of MMP 2 Result between Groups by Independent sample t-test

MMP 2 Result	Groups	N	Mean	SD	t-value	p-value
OSCC cases	Cases	32	24.524	9.403	-0.288	0.775 #
	Controls	29	23.517	17.173		

No Statistical Significance at p > 0.05 level

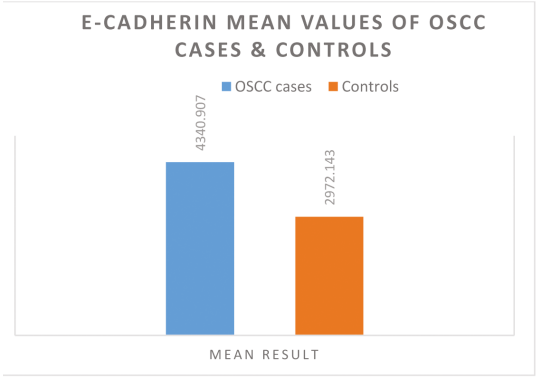

Fig-6: Graph of comparison of sE-cadherin result between OSCC cases & Controls

Table 7: Correlations of OSCC MMP 9 Result, OSCC E-cadherin Result with OSCC E-cadherin Result, OSCC MMP 2 Result in Groups by using Pearson Correlation analysis

Correlations				
Groups			OSCC E-cadherin Result	OSCC MMP 2 Result
Cases	OSCC MMP 9 Result	r-value	-.242	-.110
		p-value	0.182 #	0.549 #
		N	32	32
	OSCC E-cadherin Result	r-value	1	-.394*
		p-value		0.025 *
		N		32
Controls	OSCC MMP 9 Result	r-value	.420*	-.529**
		p-value	0.023 *	0.003 **
		N	29	29
	OSCC E-cadherin Result	r-value	1	-.521**
		p-value		0.004 **
		N		29

** Highly Statistical Significance at p < 0.01 ,* Significant at p < 0.05 and # No Statistical Significance at p > 0.05 level

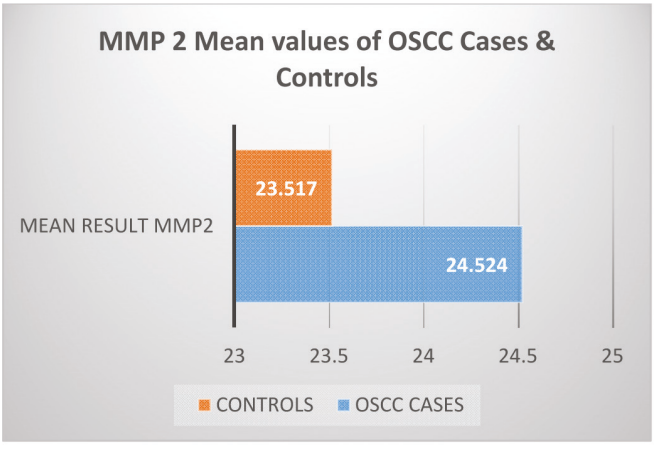

Fig-7: Graph of comparison of MMP 2 result between OSCC cases & Controls

its elevation was compared between age groups, 12/18 cases (66.66%) of patients in ≤ 50 years age group suggesting that, MMP 2 elevation being more common in younger age group.

In 6/18 (33.33%) of patients in older age group showed elevated salivary MMP 2 levels as compared to mean of controls.

Additionally, all parameters were above their respective controls' mean value in six cases out of thirty –two (18.75%), whereas ten out of thirty –two cases (31.25%) showed an increase of both MMP 9 & 2. In 5/32(15.63%) cases sE-cadherin & MMP 2 were simultaneously elevated but not MMP 9.

Table 7 shows Correlations of OSCC MMP 9 Result, OSCC E-cadherin result with OSCC E-cadherin Result, OSCC MMP 2 Result in Groups by using Pearson Correlation where all the variables showed highly statistically significant positive & negative Correlation at $p < 0.01$ level and statistically significant positive & negative Correlation at $p < 0.05$ level in controls. In cases , only E- cadherin & MMP 2 results showed a correlation.

Table 8 and Figure 8 illustrate the efficacy assessment for predicting cases based on MMP-9 results employing "Receiver Operating Characteristic (ROC) curve". The "area under the curve is 0.418, with a p-value of 0.2720, exceeding 0.05 at a 95% confidence" level. The values range from 0.273 to 0.563, indicating no statistical significance, with a cutoff of 1295.70. The sensitivity is 40.6%, and the specificity is 41.4%.

On evaluation of our result, it was seen that, 14/18 (77.77%) cases that showed MMP 9 level above control mean also showed it to be elevated above the predicted cut-off value level of this parameter in our study and 16/18 (88.88%) cases showed a value above mean value of controls as well as median value of cases. Additionally, 10/14 cases with increase above cut-off value also showed a simultaneous increase in E-cadherin level.

Table 9 and Figure 9 illustrate the efficacy assessment for predicting cases based on sE-cadherin results applying the "Receiver Operating Characteristic curve (ROC)". The area under the curve is 0.652, having p-value of 0.0420 and a 95% confidence interval of 0.509 to 0.794, showing statistical significance. The threshold is 4086.40 pg/ml, with 62.5% sensitivity and 62.1% specificity.

Notable was the observation that, in our study, 90.9% (20/22 samples with elevated sE-cadherin) showed levels that were above the cut-off value of our study.

Additionally, 8/32(25%) samples show simultaneous elevation of MMP 9 & sE- cadherin level increase above their individual cut-off values predicted in our study.

On evaluation of the efficacy assessment to predict the cases by MMP 2 result protein by using Receiver Operating Characteristic curve (RoC) as shown in Table 10 & Fig 10, we obtained cutoff value of 23.5755 and sensitivity of 54.5% with specificity of 79.3%. Therefore, 17/18 (94.44%) MMP2 readings that were higher than the Mean of controls were also higher than the estimated cut-off value of the parameter.

Thus, two (sE-cadherin & MMP 2) out of the three studied parameters showed more than 90% values above their obtained cutoff value, indicating that, these parameters could be useful in predicting the disease status of OSCC patients. All three parameters were elevated above their respective mean value of controls in at least 18/32 (56.25%) cases.

Table 8: Efficacy assessment to predict the cases by MMP 9 protein result by using Receiver Operating Characteristic curve (RoC)

Area Under the Curve				
Area	Std. Error	p-value	95% C.I	
			LB	UB
.418	.074	0.272 #	.273	.563

No Statistical Significance at $p > 0.05$ level

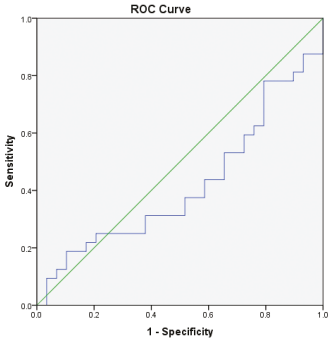

Fig-8: ROC curve of MMP 9 in serum of OSCC cases

Table 9: Efficacy assessment to predict the cases by sE-cadherin Result protein by using Receiver Operating Characteristic curve (RoC)

Area Under the Curve				
Area	Std. Error	p-value	95% C.I	
			LB	UB
.652	.073	0.042 *	.509	.794

* Statistical Significance at $p < 0.05$ level

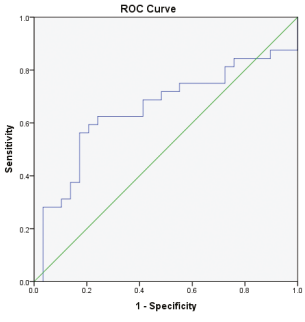

Fig-9: ROC curve of sE-cadherin in saliva of OSCC cases

The ROC curve analysis (Table 11 & Fig 11) showed that, in distinguishing oral squamous cell carcinoma (OSCC) from healthy controls, MMP2 (AUC 0.653, p=0.042) and E-cadherin (AUC 0.648, p=0.049) have statistically significant diagnostic performance.

MMP9 (AUC 0.423, p=0.307) does not have statistically significant diagnostic performance, however, that indicates a negligible probability of metatstases and is therefore considered a good prognosis indicator for our study sample cases.

Summary of Results:

Most cases 16/32 cases were in 4th to 6th decade of life (50%). Among cases, 18/32 cases ie. (56.25%) cases showed an elevated MMP 9 levels in their serum as compared to the mean serum level of MMP 9 in controls (1229.44 pg/ml). Of these, 8 cases were more than 50 years in age.

The mean±SD of Cases was (4340.91±2693.45) and Controls was (2972.14±2309.76), for sE- cadherin in saliva and it was a statistically significant difference at $p < 0.05$ level.

By Independent sample t-test, none of the case groups showed statistically difference with mean value of controls.

Discussion

In our investigation, regardless of whether the age data was categorized or not for OSCC cases, the average age of OSCC occurrence was 49 years. Our study found that 41-60 years age group were most affected. Similar findings were reported by Shenoi R et al.[12] Like our study, Sharam S et al.[13] and Gupta et al.[14] found early cases. 9 of 32 patients (28.13%) were under 40, 7 men (77.77%) and 2 females (22.22%). The rising prevalence of oral cancer in younger demographics is typically ascribed to the indiscriminate consumption of chemicals, primarily tobacco and tobacco-related products, over an extended duration, resulting in genetic harm. Immune surveillance diminishes at the age of 20. The reduced immunity, combined with the indiscriminate use of toxic substances, accounts for age-adjusted rates (AAR) of oral cancer occurrence at a notably early age. Chattopadhyay et al.[15] and Mathew et al.[16] conducted an epidemiological study on mouth cancer in India, revealing that in poor nations, oral cancer may disproportionately impact younger men and women compared to the western world. At diagnosis, the youngest male patient was 24 and the youngest female was 40.[12-16]

The male-to-female ratio was greater than that documented in the majority of research, and within the 41-60 years age group, a larger prevalence was observed among females than males. Babu A et al.[17] found that the predominant age group for oral cancer in males is 45 to 49 years, while in females, it is 50 to 64 years.

In our study the mean value of MMP 9 in OSCC cases was 1081.50±459.13 pg/ml and the median value was 1259.05pg/ml. The mean of controls was 1229. .44±360.82 pg/ml. So, unlike other studies in our study the mean value of cases was lower than that of controls. However, the median value of cases & the cut-off value (1295.70pg/ml) both were higher than the mean of controls. Thus, these two values might be more reliable in predicting the disease outcome and prognosis of OSCC patients. Studies by Dalirsani Z et al., Benitha G et al., & Lotfi A et al. [18-20] showed a higher value among cases. In the study by Lotfi et al.[20] MMP-9 in oral cancer from T1 –T3 stage were reported to be 1119.80 ± 307.71 to 1483.75 ± 134.85ng/mL. In the study by Saini J et al. also the MMP 9 serum concentration in OSCC patients from Stage I to IV ranged from 762.9 ± 161.5 to 866.1 ± 184.0 ng/mL. [18-23] In the study by Chaudhary et

Table 10: Efficacy assessment to predict the cases by pre MMP 2 resultprotein by using Receiver Operating Characteristic curve (RoC)

Area Under the Curve				
Area	Std. Error	p-value	95% C.I	
			LB	UB
.628	.074	0.086 #	.483	.774

No Statistical Significance at $p > 0.05$ level

Table 11: Comparison of Area under curve of all study variables

Test Variables	Area	P value	Significance
E-CADHERIN	.648	.049*	Significant
MMP 9	.423	.307	Non-significant
MMP2	.653	.042*	Significant

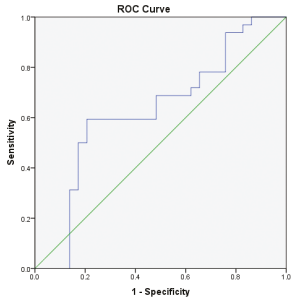

Fig-10: ROC curve of MMP 2 in saliva of OSCC cases

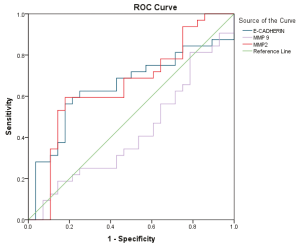

Fig-11: ROC curve of MMP 9 in serum& MMP 2 and sE- cadherin in saliva of OSCC cases

al.,[45] the median level of serum MMP 9 in OSCC cases was 6491.8 pg/ml (2794.4 to 11004.8) and control group median was 4138.6 pg/ml(2213.0 to 12829.4). In our study serum MMP 9 median value was 1295.70 pg/ml for OSCC cases (123.09 to 1768.7).

Many studies have related MMP-9 expression to oral cancer metastasis, showing that higher levels of MMP-9 are associated with regional lymph node or distant metastases. In our investigation, 14 out of 32 (43.75%) OSCC cases exhibited high MMP 9 levels beyond both the control mean and the case median, suggesting potential metastases, typically to regional lymph nodes, even if not detectable.

In contrast, Guttman et al. found no correlation between tongue cancer patients' MMP-9 expression and neck metastasis[27]. MMP-9 expression is significantly reduced post-surgery in oral cancer patients. [24–28] Our findings confirm MMP 9's role as a predictive biomarker of clinical and prognosis significance, indicating OSCC patients' oral cancer progression.

Our Study Findings For MMP 9	*Other Study Findings For MMP 9*
Mean of Pre-Rx cases < Mean Controls	Mean of Pre-Rx cases < Mean Controls Feng et al (2019) Mean controls< Mean Cases [Ikebe et al. (1999) & Lotfi et al (2015)[20]]

The OSCC group and the non-OSCC group had statistically significant differences in saliva sE-cadherin levels, according to López-Verdín S et al.[9] The mean values of the OSCC cases and the control group differed statistically significantly, according to our analysis.[9] These results were similarly consistent with those of Al Kasam et al.[29], who investigated head and neck cancer and discovered a statistically significant difference in sE-cadherin levels between the blood plasma of cancer patients and healthy individuals. However, we examined sE-cadherin in saliva in both our work and the investigation by López-Verdín S et al.[9]. According to our research, the identification of elevated levels of sE-cadherin in oral cancer patients allows us to propose that the biological behavior of OSCC can be predicted by using saliva as a tool for E-cadherin dysregulation study.

A large majority of patients in the Pre-treatment group, irrespective of age or gender showed an increase in soluble E-cadherin level beyond the mean of this parameter in controls suggesting that, it is not only the primary change in a carcinogenic tissue but also a persistent change during the various phases of carcinoma evolution.

In the study by Brouhxon et al.[30] they observed that sEcad promotes SCC invasion by increasing the secretion and activation of the pro-invasive enzymes MMP-2 and MMP-9, and further verified that this effect relies on MMP activity through the use of the MMP inhibitor GM6001. These findings align with earlier evidence showing that sEcad elevates MT1-MMP, MMP-2, and MMP-9 secretion in transformed lung cancer cells.

Additionally, in the study by Aznab et al.[31] on soluble E-cadherin in the saliva of intestinal carcinomas, it revealed that the case group had a significantly higher salivary level of soluble E-cadherin than the serum level, while the control group had a lower level than the case group.[31]

The research conducted by Vajaria B N et al.[32] demonstrated a substantial positive connection between reduced E-cadherin and plasma total MMP 2 as well as plasma active MMP 9. Their investigation also observed a negative connection between plasma total MMP-9 and E-cad mRNA. The study revealed that OSCC patients with Total MMP2 and Total MMP9 values beyond their respective ROC cutoff values exhibited reduced overall survival compared to those with values below the ROC cutoff. These findings align with our study's results, leading us to expect a poor prognosis and an increased likelihood of metastases and/or recurrence in these patients.[32]

Our Study Findings For E- Cadherin	**Other Study Findings For E- Cadherin**
Statistically significant difference (0.038) in soluble E-cadherin salivary level in the Pre-Rx case group compared with control group at p< .05	Statistically significant differences in the saliva sE-cadherin levels between the OSCC group and the group without OSCC [López-Verdín S(2017)9, Al Kassam et al. (2007)[29]]

MMP-2 and MMP-9 are type IV collagenases that degrade extracellular matrix and basement membrane structural proteins, according to Lotfi A et al.,[20] in initiation and progression of OSCC. Increased MMP expression has been related to tumor growth and metastasis in many studies. Lotfi et al.,[20] found that, oral cavity SCC patients had significantly higher serum MMP-2 and MMP-9 levels than healthy people. This was in contrast to our study in which we got the mean value of OSCC cases to be lower than mean value of controls. However, in our cases the median value was 1259.05 pg/ml which was above the mean value of controls. Also, more than 75% (14/18) of our cases with elevated values had value of MMP 9 that was higher than mean of controls, cut-off value and median of cases.[20]

MMP 2 is an additional biomarker in saliva of persons with chronic inflammation from many causes, including OSCC, and its measurement indicates the destruction of extracellular matrix components, promoting tumor invasion, angiogenesis, and metastasis. Significantly higher levels of MMP 2 have regularly been detected in the saliva of OSCC patients and connected with poor clinical outcomes, as shown by researchers such as Monea M et al.[36], Spitzer T et al.[37], Feng Y et al.[39], and Patel BP et al.[38].

Dalirsani Z et al.[18] however, observed no significant differences in MMP-2 levels between OSCC patients and healthy

controls in head and neck squamous cell carcinoma (HNSCC), suggesting MMP-2 may not always be a definitive biomarker across all populations or settings. However, our study focused specifically on OSCC, a distinct subset of HNSCC and in our study also the mean value of MMP2 of OSCC cases was higher than the mean value of controls although the difference was not statistically significant. However, in 18/32 (56.25%) of our cases, MMP 2 value was elevated above mean value of controls. From these 18 samples, when its elevation was compared between age groups, 12/18 cases (66.66%) of patients in ≤ 50 years age group suggesting that, MMP 2 elevation being more common in younger age group. According to Feng et al. and Nosratzehi et al.[39, 48], the mean saliva MMP 2 level in OSCC patients was 0.878 ± 0.315 pg/ml. Cai et al.[45] noted no difference in saliva MMP2 expression between OSCC patients and controls. Our research demonstrated no statistically significant difference in saliva MMP2 expression between OSCC cases and controls, however it was higher in cases. According to Dalirzani et al.[18], the average saliva MMP 2 level was 2.17 ± 0.62 ng/ml, while our study found 0.025 ng/ml ±0.009. According to Hemashree K et al.[46], OSCC patients had greater saliva MMP 2 levels than controls, which was highly statistically significant.

Our Study Findings For MMP 2	Other Study Findings For MMP 2
MMP 2 was higher in cases than in controls	MMP 2 was higher in cases than in controls [Shpitzer T et al.(2007)[37], Dalirsani et al.(2019)18 and Cai M. et al. (2022)[45]]
No statistically significant difference at $p > 0.05$ level on comparison of mean values of controls with mean values of case groups	A significant difference was not observed in the salivary levels of MMP-2 in the controls and cases in the study by Dalirsani et al.(2019)[18] and Cai M. et al. (2022)[45] A significant difference was observed, in the salivary levels of MMP-2 in the controls and cases in the study by [Hema Shree K et al. (2024)[46], Feng et al (2019)[39]]

When the cut-off value was obtained with sensitivity & specificity for MMP 9, it was noted that efficacy assessment by ROC showed no statistical significance. Cut-off was 1295.70, Sensitivity was 40.6%, and specificity was 41.4%. Patel BP et al.[38] found that plasma total MMP-9 levels were useful for monitoring oral SCC patients after therapy. They categorized patients by serum MMP 9 levels as responders or non-responders.

However, in spite of low predictability, 8/32(25%) case samples showed simultaneous elevation of MMP 9 & sE-cadherin level increase above their individual cut-off values predicted in our study. Based on this finding, we suggest that these patients should be followed up more vigorously with quarterly assessment of MMP 9 in serum alongwith sE-cadherin in saliva post-treatment, to determine the recurrence likelihood of patients based on the quantification and comparison of levels of these proteins with their pre-treatment levels as well as with mean of controls.

When cut-off value was obtained with sensitivity & specificity for sE-cadherin, it was noted that, efficacy assessment by ROC showed a statistical significance with the Cut off determined to be 4086.40 pg/ml, Sensitivity is 62.5%, Specificity is 62.1%. Another notable observation of our study was that, 90.9% (20/22 samples with elevated sE-cadherin) showed elevated levels that were above the cut-off value of our study.

Both MMP2 & 9 were elevated above the mean value of their controls in equivalent number of cases.

The research by Chang P et al.[42] noted that serum MMP 9 had an area under the curve (AUC) rate of 0.886 for differentiating OSCC from controls, while the study by Ghallab NA et al.[43] In the Chaudhary et al.[44] study, they found an AUC of 1.00. AUC was 0.554 at 95% C.I. 0.436-0.668, having cutoff value of 5929.88pg/ml, sensitivity of 55.3%, and specificity of 65.8%. In our study AUC obtained for serum MMP 9 in OSCC cases was 0.418 at 95% C.I 0.273 to 0.563 and Cut off was 1295.70 pg/ml with Sensitivity which was 40.6% and Specificity that was 41.4%.

In the research conducted by Lopez-Verdin et al.[9], a limited number of OSCC cases had elevated median levels of sE-cadherin (6460 pg/ml) compared to the control group. In our study, the median value of OSCC cases was 5436.8 pg/ml, which was surpassed in 16 out of 32 (50%) cases. According to the study by Aznab et al.[31], in cases of gastrointestinal carcinomas, the salivary level of soluble E-cadherin was considerably higher in the case group than in the control group. We infer that this result enables the differentiation of malignancies by elucidating intracellular processes that facilitate malignant pathways.

The study's limitations include a restricted sample size and breadth. Consequently, additional investigation with bigger and more diverse populations is essential for further validating its diagnostic and prognostic significance and determining its practical applicability more accurately. Notwithstanding these constraints, our data were adequate to assess the correlation and screening efficacy of salivary MMP-2 and E-cadherin, as well as serum MMP-9 for OSCC. Despite the lack of integration among the findings of various studies, all research suggests the significance of MMPs and sE-cadherin in assessing OSCC, potentially serving as early prognostic indicators, hence allowing for the development of therapeutic strategies aimed at targeting these extracellular processes.

INFERENCES:

Inferences	Observational Justifications
Better prognostic predictors	Changes more frequent and irreversible in MMP 9 & E-cadherin
Conclusive evidence cannot be drawn	No standard normal values available for the studied biomarkers

Conclusions by Objectives:

No.	OBJECTIVE	CONCLUSION
1.	To assess the expression of soluble E-cadherin & MMP2 in saliva of Controls by ELISA method	a) The expression of soluble E-cadherin in saliva of Controls by ELISA method was done and yielded a mean value of 2972.14±2309.76 pg/ml (2.97±2.3 ng/ml) for soluble E-cadherin. This was in accordance with the study by Lopez-Verdin et al.[9] in which the control mean value was of a similar range and both mean & median of controls was lower than that in the saliva of OSCC patients. b) The expression of MMP2 in saliva of Controls by ELISA method was done and yielded a mean value of 23.517 pg/ml. This was in accordance with the result of Dalirsani Z et al.[18] wherein also there was not a statistically significant difference between the mean of controls and cases in saliva, and both were in similar range although the mean & median values of controls were lower.
2.	To assess the expression of MMP 9 in serum of Controls by ELISA method	The expression of MMP 9 in serum of in serum of Controls by ELISA method was done and the mean value obtained for controls was 1188.47 pg/ml. It was noted that the mean & median of controls was higher than that of cases.
3.	To assess the expression of soluble E-cadherin & MMP2 in saliva of Oral Squamous cell Carcinoma (OSCC-PRE-TREATMENT) patients by ELISA method	The expression of soluble E-cadherin & MMP2 in saliva of Oral Squamous cell Carcinoma (OSCC-PRE-TREATMENT) patients by ELISA method was done and the mean values obtained for cases were 24.524 & 4340.9 pg/ml respectively. These values were higher than the mean values of controls, but the difference was statistically significant only for soluble E-cadherin.
4.	To assess the expression of MMP 9 in serum of Oral Squamous cell Carcinoma (OSCC-PRE-TREATMENT) patients by ELISA method	The expression of MMP 9 in serum of Oral Squamous cell Carcinoma (OSCC-PRE-TREATMENT) patients by ELISA method was done and the mean value obtained for cases was 1081.504 pg/ml. The difference between the mean for controls and cases was not statistically significant.
5.	To correlate & compare the expression levels of soluble E-cadherin, MMP2 in saliva of Controls and OSCC patients (PRE-TREATMENT group)	On comparison of the expression levels of soluble E-cadherin & MMP2 in saliva of Controls and OSCC patients (PRE-TREATMENT group) it was noted that the mean values of both these parameters showed a tendency for an elevation as compared to the mean value of controls in 23/32 (71.88%) cases for soluble E-cadherin & in 18/32 (56.25%) cases for MMP 2. So, both parameters showed a significant change in more than 55% of total cases which is quite notable.
6.	To correlate & compare the expression levels of MMP 9 in serum of Controls and OSCC patients (PRE-TREATMENT group")	On comparison of the expression levels of MMP 9 in serum of Controls and OSCC patients (PRE-TREATMENT group) it was noted that the mean value this parameter showed a tendency for elevation as compared to the mean value of controls in 18/32 (56.25%) cases which is strikingly similar to the trend noted for MMP 2 in saliva.

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