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TO STUDY THE EXPRESSION OF E-CADHERIN AND VIMENTIN IN PRE-CANCEROUS AND CANCEROUS LESIONS OF ORAL CAVITY

Pathology

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ABSTRACT

Background: The aim today is early detection of cancer and prevention of progression for a better prognosis. In this respect various markers have received attention. This study was done to evaluate the role of E-cadherin and Vimentin in early detection of cancer. It may predict tumor behavior and micro invasion, and thus the prognosis which will help in better treatment and increase patient survival. **Methodology:** The study was conducted over a 12-month period and comprised 40 patients of oral cavity lesions. Microscopic examination was done and morphological findings were noted. After histopathological diagnosis, sections from tumor were selected for staining E-cadherin and Vimentin using standard procedures. **Results:** Maximum number of patients (35%) were in the age group of 51 to 60 years. Majority of the patients were males (77.5%).12 were premalignant lesions and 28 cases were different grades of squamous carcinoma. Buccal mucosa was the commonest site involved. E-cadherin expression was shown to be higher in premalignant lesions as the degree of dysplasia rose. Vimentin staining showed decrease in expression with increase of histological grade in some cases. **Conclusion:** E-cadherin and vimentin, when combined, can be employed as key indicators of premalignant and cancerous oropharyngeal lesions. They predict the histological grade and suggest prognostic outcome.

KEYWORDS

INTRODUCTION

Head and neck cancers account for fifth most common cancers in humans worldwide. On an average 400,000 new cases are diagnosed annually (1). India is different from other countries of the world in having the largest number of patients of oral malignancy. The incidence is calculated at 12.5 per one lakh cases, this is age standardized. It also accounts for 9.4 % of all malignancies seen in India (2).

In oral cavity, 90% of the cancers arise from the mucosal lining and is of squamous cell type. Squamous cell carcinoma of the oral mucosa is a malignancy that typically affects the lower lip, tongue, floor of mouth, soft palate, and periodontal ridge in the fourth decade. Smoking and drinking are two of the most significant lifestyle factors (3). Inspite of many therapies, targeted therapy, radiotherapy and new adjuvant chemotherapy, the five years survival rate of oral cancer has been low for the past two decades. In 50 % of the cases the patient presents in advanced stages with involvement of lymph nodes (1).

The aim today is early detection of cancer and prevention of progression for a better prognosis. In this respect various markers have received attention such as E-cadherin, which is an adhesion molecule between cells. It also functions as a tumor suppressor gene. It is an adhesion molecule that is calcium dependent found on the cell surface. It is responsible for transduction of signals that control many cellular events. Amongst these events are polarity, growth and cell differentiation and migration. It also inhibits cell proliferation by upregulating the p27 via EGFR. It is encoded by the gene CDH 1, which in turn is localized on chromosome 16q-22. Decreased expression of E-cadherin is said to indicate cancer progression (4). E-cadherin is found in oral cavity, head & neck, esophageal, prostate, pancreatic, stomach, and uterine cervix carcinomas. Lack of E-cadherin development is linked to aggressive activity, increased growth, infiltration, dissemination, and a poor prognosis of disease (4).

Mahendra Singh et al in his study, demonstrated difference with regard to E-cadherin expression with degree of dysplasia and site of lesion, indicating a change towards a cell phenotype probably indicating the capacity to invade (1). Many studies have shown that E-cadherin expression reduces with progression of the grade of dysplasia. This may be used as an indicator of increased invasive ness of oral cancers. It can thus can be used as a prognostic marker (4).

Vimentin is expressed in normal mesenchymal cells. It is an intermediate filament and maintains integrity of the cell and safeguards the cell against stress. Increased expression of Vimentin is found in many cancers of epithelial tissue like prostate, GIT, CNS, breast, lung and malignant melanoma. Many authors have shown that increased expression of Vimentin is associated with tumor growth, tumor invasion and a poor prognosis. Vimentin has also been identified as a marker for epithelial mesenchymal transition. It also provides a focus for cancer care (5). Danielsson et al showed that Vimentin plays a protective role against misfolded proteins in cells (6).

This study was done to evaluate the role of E-cadherin and Vimentin in early detection of cancer. It may predict tumor behavior and micro invasion, and thus the prognosis which will help in better treatment and increase patient survival.

OBJECTIVES

To study and classify epithelial pre-cancerous and cancerous lesions of oral cavity and to study the expression of E- cadherin and Vimentin in these lesions and to correlate the two markers.

MATERIALS AND METHODS

This study was a cross-sectional study carried out in Department of Pathology over a period of one year. Written informed consent from the patients and ethical clearance from Institutional Ethics Committee was taken prior to the study.

The sample size was calculated by the formula $n = Z^2_{\alpha/2}\, pq / l^2$. Taking the prevalence as 50% at 5% level of significance and 20% relative error, the sample size was found to be 40.

Selection of Subjects:

Inclusion Criteria: All cases of oral squamous cell carcinoma who was diagnosed in the Department of Pathology in Himalayan Institute of Medical Sciences.

Exclusion Criteria: Other tumors of oral cavity and cases undergoing therapy and cases showing recurrence.

Study Tool:

- Harris Hematoxylin and Eosin (H & E). stained sections.
- E-cadherin and Vimentin antibody (Biogenex).
- Immunohistochemistry-stained sections.

Study Protocol:

All the relevant clinical details of patients were recorded on case reporting form. The specimen was fixed for 24-48 hours in 10% formalin. After fixation, the tissue was taken up for grossing which was done according to the Standard Operating Procedure being followed in the department. All relevant findings were recorded. The tissue after proper labelling was processed as per Standard Processing methods

using Automated Tissue Processor.
70% ethanol: 1 and a half hours.
80% ethanol: 1 and a half hours
90% ethanol: 1 and a half hours
Absolute alcohol I : 1 and a half hours
Absolute alcohol II : 1 and a half hours
Absolute alcohol III: 1 and a half hours
Xylene I: 1 and a half hours
Xylene II: 1 and a half hours
Xylene III: 1 and a half hours
Paraffin wax I: 1 and a half hours
Paraffin wax II: 1 and a half hours
Paraffin wax III: 1 and a half hours

Paraffin blocks were made. Sections of 4–5-micron thickness were cut with the help of microtome. Sections stained with Hematoxylin & Eosin (H&E). stain according to the standard procedure used in department of pathology. Before staining, the tissue sections were deparaffinized, dehydrated and then hydrated, a process called clearing. Deparaffinize tissue by placing on tissue warming plate at 66 degree C± 2-degree C.

Air dried deparaffinized slides was placed in:
- Xylene I : 5 minutes
- Xylene II : 5 minutes

I. Air dried
II. Slides were kept in slide carrier (20 slides was placed in one time).
III. Slide carrier was kept in absolute alcohol for 2 minutes and then in 70% alcohol
IV. Slide carrier was put under running tap water (slow stream) for 5-10 minutes.
V. Slide carrier with slides was placed in Harris Hematoxylin for 15 minutes
VI. Place in water for 1-2 minutes.
VII. One dip in 1% acid alcohol (acid is HCL).
VIII. Place under water for 10-15 minutes for bluing before dip in Eosin 2%
IX. Dip in Eosin 2% for 30 seconds to 1 minute.
X. 1-2 dips in water
XI. Now place in ascending strength of alcohol
a. 70 % alcohol : 2 minutes
b. 80 % alcohol : 2 minutes
c. 90 % alcohol : 2 minutes
d. Absolute I : 2 minutes
e. Absolute II : 2 minutes
XII. Dry slides in air
XIII. Clear with xylene I : 5 minutes
XIV. Clear with xylene II : 5 minutes
XV. Place the slide on filter paper with section downwards and press gently

Microscopic examination was done and morphological findings were noted at least by two observers in detail and diagnosis were recorded. After histopathological diagnosis, sections from tumor were selected for staining E-cadherin and Vimentin.

Immunohistochemistry staining procedure for E-cadherin and Vimentin:
A). Preparation of buffers and reagents
1. Tris buffer (pH is 9.0-9.2).

Preparation
- 6.050gm Tris (methylamine).
- 0.750gm EDTA
- 1000ml deionized water.

2. Pbs buffer (pH is 7.2-7.6).
Preparation

Stock solution A: Sodium dihydrogen phosphate	3.4gm
Stock solution B: Disodium hydrogen phosphate	12.0gm
Sodium chloride	8.5gm
Denoised water	1000ml

B). Steps of staining
- Deparaffinization was be done.
- Antigen was retrieved by microwave technique (at high power).
- It was cooled for 5-10 minutes.
- It was washed in PBS for 5-10 minutes.
- Blocking of endogenous enzyme activity was done by placing the sections in coplin jar filled with 0.3% H_2O_2 (1 ml of 30% H_2O_2 + 9 ml methanol) for 10-13minutes.
- After the incubation the slides were taken out and washed in PBS for 5 minutes.
- After blocking endogenous peroxidase activity with power block for 10-15 minutes, excess was wiped off. No washing was done at this stage.
- Now it was incubated with primary mouse monoclonal antibody to E-cadherin and Vimentin on separate slides for one hour at 25-30 degC temperature in moist chamber.
- Excess antibody was wiped off and slide was placed in PBS buffer for 5 minutes.
- It was incubated with super-enhancer and kept for 30 minutes at 25-30 degC temperature in moist chamber.
- Excess super enhancer was wiped off and placed in PBS for 5 minutes.
- It was incubated with Horse-radish peroxidase (HRP). polymer in moist chamber for 30 minutes at 20-25 degC temperature.
- Excess polymer was wiped off and placed in PBS buffer for 5 minutes.
- It was incubated with peroxidase substrate solution 33' diaminobenzidine tetrahydrochloride (DAB/AEC). 1ml of DAB buffer add 38-40 microliter DAB chromogen for 10-15 minutes at 25-30 degC temperature in moist chamber.
- It was rinsed with distilled water.
- It was counter stained with hematoxylin in just one dip followed by one dip in 1% acid alcohol before washing in running tap water for 10-15 minutes.
- Dehydration was done in upgrading alcohol. Absolute alcohol, 90%, 80% and then 70%.
- Drying and mounting was done with DPX.
- After examination under microscope results were recorded.

Evaluation of E-cadherin and Vimentin immunostaining:
The E-cadherin and Vimentin immunostaining was scored quantitatively as (30).
Intensity score-
0 – Negative
1 – Weak
2 – Moderate
3 – Strong
The staining proportion score
0 - Negative
1 - <10% positive cells
2 – 10-50% positive cells
3- 50-80% positive cells
4- >80% positive cells

Final score = Intensity score X Proportion score
Total score ranges from 0 to 12
- 0- Negative
- 1-4 low Immunoreactivity
- >4 high Immunoreactivity

Except for the routine histology investigations, the cost of which was borne by the patient, the financial cost of research investigation (Immunohistochemistry) was borne by the investigator.

Data Management & Statistical Analysis:
Statistical analysis of the observations was performed using SPSS Software (Statistical Package for Social Sciences). version 23 and Microsoft Excel. Categorical data was expressed as frequencies and continuous data as mean ± standard deviation or median. Association of categorical variables was analyzed using Pearson's chi-square test.

RESULTS
This one-year research comprised a total of 40 patients. The youngest patient was 35 years and the oldest patient was 87 years. The mean age calculated was 53.025. There was an obvious male predominance, accounting for 77.5 % (n= 31) of the cases in the study group. Tobacco was commonest was seen in22.5 % (n= 09) of the cases, followed by alcohol with smoking 20 % (n=08).

The commonest site of oral cancers was buccal mucosa accounting for 20 cases (50%). This was followed by tongue (35%). (Table 1)

Table 1: Baseline characteristics of cases (n=40).

Variable	No. of cases	Percentage
Age groups (years)		
31-40	07	17.5 %
41-50	10	25 %
51-60	14	35 %
61-70	05	12.5 %
71-80	03	7 %
>81	01	2.5 %
Total	40	100
Sex		
Male	31	77.5 %
Female	09	22.5 %
Addiction		
Only chewing tobacco	03	7.5 %
Alcohol + smoking	08	20 %
Alcohol + smoking + chewing tobacco	03	7.5 %
Only smokers	09	22.5 %
No addiction	13	32.5 %
Sites		
Tongue	14	35 %
Buccal mucosa	20	50 %
Retromolar trigone	02	5 %
Tonsillar fossa	01	2.5 %
Palate	01	2.5 %
Supraglottic biopsy	02	5 %
Total	40	100

Out of 31 males including in the study 14 had moderately differentiated squamous cell carcinoma and out of 9 females, 4 had moderately differentiated squamous cell carcinoma. Substance abuse was found both in patients with benign lesions of oral cavity and in malignancies. However, incidence of substance abuse was more in patients with malignancies. A significant association was seen between the histomorphology of cancer and gender. (Table 2)

Table 2: Histomorphology spectrum of cases and their association with gender and history of addiction

Histological subtype	Male(n=31)	Female (n= 09)
Premalignant (n= 12)		
Mild dysplasia	03	00
Moderate dysplasia	01	00
High grade dysplasia	05	00
Verrucous carcinoma	03	00
Malignant (n= 28)		
Well differentiated squamous cell carcinoma	01	04
Moderately differentiated squamous cell carcinoma	14	04
Poorly differentiated squamous cell carcinoma	04	01
Chi square = 6.40, p= 0.040 (Significant)		
Histological subtype	Addiction	No addiction
Premalignant (n= 12)		
Mild dysplasia	00	03
Moderate dysplasia	01	00
High grade dysplasia	03	02
Verrucous carcinoma	02	01
Chi square = 4.53, p= 0.209 (Not significant).		
Malignant (n= 28)		
Well differentiated squamous cell carcinoma	02	03
Moderately differentiated squamous cell carcinoma	14	04
Poorly differentiated squamous cell carcinoma	05	00
Chi square = 5.01, p= 0.081 (Not significant)		

For E-cadherin expression, out of 28 malignant cases, majority (n= 15) showed 1+ positivity followed by 2+ positivity (n= 08). Out of 12 premalignant cases, majority cases (n= 05) showed 2+ positivity followed by 1+ positivity (n= 04). Similarly, for Vimentin, out of 12 premalignant cases, majority (n= 10) did not express vimentin. Out of 28 malignant cases, majority of the cases (n= 09) showed 2+ positivity followed by 1+ positivity (n= 06). There was a statistically significant association between histological type and Vimentin intensity score. (Table 3)

Table 3: Intensity score of E-cadherin and Vimentin expression in premalignant and malignant lesions

Histological type	Intensity score			
E-cadherin				
Total (n=40)	0	1+	2+	3+
Premalignant (n=12).	01	04	05	02
Malignant (n=28)	03	15	08	02
Chi square = 1.97, p= 0.577 (Not significant)				
Vimentin				
Premalignant (n=12)	10	01	01	00
Malignant (n=28)	08	06	09	05
Chi square = 10.46, p= 0.015 (significant)				

For E-cadherin, out of 12 premalignant cases, majority cases (n= 04) showed 3+ score followed by 2+ score (n= 04). Out of 28 malignant cases, majority cases (n= 07) showed 3+ score, followed by (n= 07) cases. For Vimentin, out of 12 premalignant cases, majority cases (n= 10) showed negative expression of vimentin (score 0). Out of 28 malignant cases, majority cases (n= 10) had 2+ score (50-80 % positive cells), followed by 05 cases had 3+ score (50-80% positive cells). A significant association was observed between (Table 4)

Table 4: E-cadherin and Vimentin staining proportion score in premalignant and malignant lesions

Histological type	Immunostaining proportion score				
Total (n=40)	0 (Negative)	1+ (<10% positive cells)	2+ (10-50% positive cells)	3+ (50-80% positive cells)	4+ (>80% positive cells)
E-cadherin					
Premalignant (n=12)	01	00	04	04	03
Malignant (n=28)	03	05	07	07	06
Chi square = 2.66, p= 0.616 (Not significant)					
Vimentin					
Premalignant (n=12)	10	01	01	00	00
Malignant (n=28)	08	01	10	05	04
Chi square = 12.12, p= 0.016 (significant)					

Out of 12 premalignant lesions 2 mild dysplasia cases and 3 verrucous carcinoma cases had high E-cadherin expression. Out of 28 malignant cases, 4 well differentiated cases and 2 moderately differentiated squamous cell carcinoma cases had high E-cadherin expression.

For Vimentin, out of 12 premalignant lesions 3 cases of mild dysplasia, severe dysplasia verrucous carcinoma and one case of moderate dysplasia had no vimentin expression. Out of 28 malignant cases, 4 poorly differentiated cases and 4 moderately differentiated squamous cell carcinoma cases had high vimentin expression.

Table 5: Classification of cases according to final score of E-cadherin expression

Diagnosis	Final score		
	0 (Negative)	1-4 (Low)	>4 (High)
E-cadherin			
Premalignant (n= 12)			
Mild dysplasia	0	1	2
Moderate dysplasia	0	1	0
Severe dysplasia	1	4	0
Verrucous carcinoma	0	0	3
Malignant (n= 28)			
Well differentiated squamous cell carcinoma	0	2	4
Moderately differentiated squamous cell carcinoma	1	15	2
Poorly differentiated squamous cell carcinoma	02	03	0

Chi square = 23.00, p= 0.519 (Not significant).			
Vimentin			
Premalignant (n= 12)			
Mild dysplasia	3	0	0
Moderate dysplasia	1	0	0
Severe dysplasia	3	2	0
Verrucous carcinoma	3	0	0
Malignant (n= 28)			
Well differentiated squamous cell carcinoma	2	3	0
Moderately differentiated squamous cell carcinoma	6	8	4
Poorly differentiated squamous cell carcinoma	0	1	4
Chi square = 1.440, p= 0.837 (Not significant)			

Out of 12 premalignant cases only 05 cases showed high immunoreactivity for E-cadherin and none of the case had high vimentin expression. Only six of the 28 malignant patients exhibited significant E-cadherin positivity, while eight had strong vimentin immunoreactivity. Correlation coefficient (r = - 0.043) indicates negative correlation. There was no significant difference between the two indicators (p=0.790). (Table 6, Fig. 1)

Table 6: Comparisons between immunoreactivity of E-cadherin and Vimentin in premalignant and malignant lesions

	E-cadherin Immunoreactivity			Vimentin Immunoreactivity			P value
Immunoreactivity	Negative	Low	High	Negative	Low	High	
Premalignant (n=12)	01	06	05	10	02	00	0.000 (significant)
Malignant (n=28)	03	19	06	08	12	08	0.126 (Not significant)
Total	04	25	11	18	14	08	

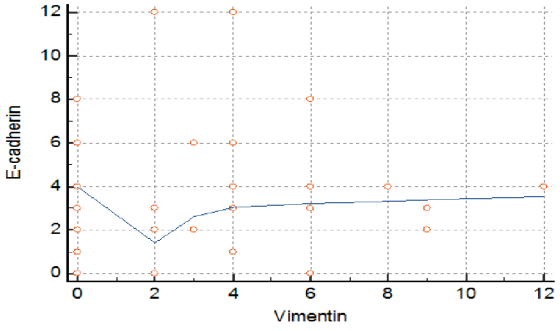

Figure 1: Correlation between E-cadherin and Vimentin staining

DISCUSSION

Cancers of oral cavity is one of the commonest malignancies in India and account for 30 % of all the cancers. Oral cancer is diagnosed very late due to which the outcome of treatment is not very good and most of the patients cannot afford the high cost of treatment (7). In rural areas of India there is inadequate medical facilities and health services. This causes a lag in assessment and treatment (8). The most crucial component in ensuring a good prognosis for oral cancer is early detection. Early detection and treatment can increase survival rates by up to 90 %. Advance in science and technology has resulted in many novel techniques for improving diagnosis of oral cancers. The conventional techniques include clinical examination, histopathological evaluation on biopsy tissue, spectroscopic and radiological techniques (9).

The use of molecular techniques to diagnose both precancerous and cancerous lesion of oral cavity can aid in identification of patient at a higher risk and also help in diagnosing malignancy itself (10). Studies have shown that a series of indicators are utilized to determine the changes in epithelial tissue of oral cavity some examples of these are E-cadherin, EMP 1, N-cadherin and 5T4. Studies have shown that of these the best molecular marker is E-cadherin to diagnose abnormal epithelium (11).

In their study, Mahendra A et al noticed that large number of cases were above 40 years (86%). and only 14 % patients were found in group one. Many authors in their study had found that the average age of patient was in between 52 to 55 years (12). Where on one hand Sreedevi A et al in his study found that the greatest incidence of oral cancers was in the sixth decade of life, another study found that the average age was as low as 36.5 years for oral malignancies. This incidence in young age was probably due to early evaluation and identification of tumors by use of the intensive diagnostic test (13). The majority of patients in the current study were between the ages of 51 and 60 years (n= 14), which is in concordance with previous studies.

Incidence of oral cancers in males was found to be 60% in a study done by Hirashi et al (14). Only single by Nafarzadeh et al in Iran, found a high incidence of oral lesions in females with a sex ration of 1.3:1 for females: males. This indicates a shifting epidemiological trend of illness, most likely owing to a larger tobacco consumption by females in the Iranian population (15). In the present study oral lesions were commonly seen in the males accounting for 77.5 % of the cases.

Lambert et al. concluded that one of the risk factors for pre-cancer to transform into malignant lesions was the continual to use of nicotine. Majority of the patients who had precancerous lesions were found to be heavy smokers. (16). Cancela et al. conducted a study in Kerela, in which they studied oral malignancies and their association with alcohol intake. They found that alcohol consumption was more in men as compared to women and these men had high risk of developing malignancies (17). In this present study tobacco use was seen in 22.5 % (n= 09) of the cases with oral lesions. Alcohol consumption with smoking was seen in 20 % (n=08) of the cases.

According to a Taiwanese survey, the tongue is the most commonly used site (39.29%). for cancers of oral cavity followed by buccal mucosa (32.14%) (18). Another study discovered that the tongue was the most prevalent location for oral cancer, followed by the buccal mucosa and gum (15). The buccal mucosa was the most prevalent location of mouth malignancies in the current investigation accounting for 20 cases (50%).

E-cadherin, like catenin, has been well known for its epithelial-specific expression and activity. Vimentin production in combination with low or absent E-cadherin interpretation is indicative of mesenchymal cells. While the opposite is true for epithelial cells. E-cadherin and vimentin expression was studied in ten cases of oral dysplasia by Khafil Akhtar et al. 80% cases of dysplasia showed strong four plus appearance of E-cadherin, while 50% of carcinoma-in-situ cases showed great four plus staining. They also observed that when there was transformation of dysplasia into advanced disease the expression of E-cadherin was much lower. They also demonstrated that vimentin expression patterns were more in dysplasia, in situ tumor, and aggressive tumors. Thus, they concluded that from dysplasia to invasive cancer vimentin production rose (2). Balasundaram et al studied sixty cases of oral malignancies out of which 15 cases showed high vimentin expression and rest 45 cases showed low expression of vimentin (19).

In the present study all premalignant lesions either showed negative or low expression of vimentin. Amongst the invasive carcinomas only four cases showed high expression. This study was only partly in concordance with other studies as most of the studies showed variable results This needs further evaluation with a bigger sample size.

CONCLUSION

E-cadherin expression was shown to be higher in premalignant lesions as the degree of dysplasia rose. Vimentin staining showed decrease in expression with increase of histological grade in some cases. E-cadherin and vimentin, when combined, can be employed as key indicators of premalignant and cancerous oropharyngeal lesions. They predict the histological grade and suggest prognostic outcome. More research on a larger group of patients is needed to understand the exact relevance of these two indicators in predicting survival rates and outcome in oral lesions.

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