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# Prognostic significance of E-cadherin, β-catenin and cyclin D1 in oral squamous cell carcinoma: a tissue microarray study

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**Summary.** Objective. To study the prognostic significance of E-cadherin, β-catenin, and cyclin D1 expression in oral squamous cell carcinoma.

Subjects and Methods. The study included 65 subjects with histologically confirmed squamous cell carcinoma. TMA blocks were prepared for immunohistochemical quantification of the expression of the three markers using IHC profiler and Immune ratio plugin of Image J.

Results. E-cadherin expression was significantly correlated with histological grades and the metastasis status ($p<0.05$), whereas β-catenin expression was significantly correlated with smoking and tumor recurrence ($P<0.05$). Cyclin D1 expression was significantly correlated with depth of invasion and tumor recurrence. ($p<0.05$). Advanced tumor stage and depth of tumor invasion increases the risk of recurrence or death by 2.5 times (OR=2.53 and 0.84 respectively).

Conclusion. High expression of β-catenin and cyclin D1 are significantly correlated with tumor recurrence and old age. Depth of invasion, low histological grade and old age were a significant predictor for the risk of having tumor recurrence and cancer related death.

**Key words:** E-cadherin, β-catenin, Cyclin D, OSCC, Cell adhesion

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## Introduction

In several parts of the world, oral cancer is one of the most common cancers. In 2020, the GLOBOCAN database recorded 377,713 new cases and 177,757 deaths among males and females, according to the most recent reléase (https://globocan.iars.fr/). Oral cancer was the eighth most common cancer in men, with 3.8/100000 people in high/very high HDI countries and 10.2/100000 people in low/medium HDI countries, with a death rate of 1.4-5.7/100000 people. Oral cancer is the 15th most common cancer in women, with 1.7/100000 people in high/very high HDI countries and 3.6/100000 people in low/medium HDI countries, with a death rate of 0.6-2.2/100000 people. (Sung et al., 2021) Around half of the oral cancer cases came from Asia, with India, Sri Lanka, and Pakistan having the highest rates, owing to the popularity of betal nut chewing (Ferlay et al., 2021).

Squamous cell carcinoma (SCC) accounts for 90 to 95 percent of all oral cancers. Oral cancer is among the top ten primary locations, according to the Ministry of Health and the National Cancer Registry of the United Arab Emirates (UAE), with 114 (3.16%) cases each year, and is ranked No. 7 among male cancer cases (81cases 4.91%) (Radwan et al., 2018).

Advanced oral cancer is treated by a multimodal approach that includes surgery, radiation, and chemotherapy. Despite major advances in these modalities, long-term survival rates for these patients have remained stable over the last 30 years.The majority of advances in studying this condition have arisen in the last few decades, but none have resulted in clinically significant findings. The lack of progress in improving care effectiveness highlights the pressing need for more successful treatments, as well as scientifically appropriate biomarkers to stratify patients and increase treatment results (Alsahafi et al., 2019).

SCC prognosis is determined by a number of factors, including tumor size, grade, and level. The most important prognostic markers, though, are loco-regional recurrence and lymph node metastases, which are difficult to assess based on the primary tumor's scale (Rosado et al., 2013).

The TNM classification is a worldwide normative staging method for determining the magnitude of cancer and is a significant prognostic factor in forecasting patient outcomes. The 8th version of the TNM staging scheme responds to the need to categorize the prognosis of patients with various clinical and biological activities, taking into account the extent of invasion as a better predictive criterion than tumor thickness, which was previously used to classify prognostic risk groups based on tumor size (Denaro et al., 2018). OSCC behaviour is difficult to predict using only routine clinical and histopathological criteria, resulting in a very low OSCC survival rate.

The discovery of certain prognostic tumor biomarkers linked to OSCC clinical results has resulted from advances in cancer knowledge at the regulatory protein expression stage.

Cell adhesion molecules are considered to be significant prognostic factors in solid tumor invasion and metastasis (Pukkila et al., 2001). E-cadherin and β-catenin are two proteins that help normal cells adhere together (Kemler, 1993). Cadherins are a wide family of membrane-associated glycoproteins that regulate cell-to-cell adhesion (Gumbiner, 2005; Halbleib and Nelson, 2006; Lien et al., 2006). Impaired expression of cadherin and catenin causes cell adhesion dysfunction, leaving tumor cells less articulate and invasive. Loss of E-cadherin expression has been discovered to increase the likelihood of these cells metastasizing locally or to a distant area. E-cadherin deficiency has been linked to poorly differentiated cancers, lymph node metastases, and a poor prognosis (Downer and Speight, 1993). In oropharyngeal SCC, however, no connection was found between E-cadherin expression and the occurrence of metastases (Ukpo et al., 2012) .

Catenin is another cell adhesion molecule essential for the functions of E-cadherin. When the expression of E-cadherin fails, β-catenin expression shows a high invasive feature of SCC (Liu et al., 2010). Reduced membranous expression of both E-cadherin and β-catenin has been linked to low survival and nodal metastasis in some trials (Rosado et al., 2013). Other studies reported that cyclin D1 is a target of β-catenin in breast cancer (Lin et al., 2000), or colon cancer (Tetsu and McCormick, 1999) and the overexpression of cyclin D1 is caused by activated β-catenin, eventually related with poor prognosis (Miyamoto et al., 2003).

Cyclin D1 is a central player in oral cancer growth, as it promotes cell proliferation, migration, and differentiation. The rate of amplification of the cyclin D1 gene is more than two times greater than in other cancers (Ramos-García et al., 2017). This amplification of the cyclin D1 gene can lead to overexpression of this protein, which is sometimes linked to prognostic markers such as high T and N stages, advanced level, low differentiation, and decreased survival (Ramos-García et al., 2018).

Using tissue microarray immunohistochemistry, this paper investigated the expression of E-cadherin, β-catenin, and cyclin D1 in various grades and stages of OSCC, as well as their association with physiological, pathological, and survival rates.

## Materials and methods

### *Patients and tissue specimens*

A total of 65 patients with oral SCC were collected from Tawam Hospital (Al-Ain, UAE). They had been treated between January 2010 and March 2019. From the patients' records, detailed clinicopathological knowledge was collected. The H&E-stained tissue sections were first examined by two specialist histopathologists (NH and AQ) to validate the previous histological diagnosis. The tumors originated from the tongue, floor of the mouth, cheek, gingiva, palate, or retromolar region. Since the vermilion boundary of the lip and the pharyngeal complex are not considered members of the oral cavity, they were excluded. Health documents were used to obtain a history of tobacco and alcohol use.

### *Histopathological evaluation*

Histopathology records were used to document information on histologic category, pTNM level of primary tumor according to AJCC classification, extent of invasion, resection margin, perineural invasion, lymphovascular invasion, and extra nodal expansion.

### *Tissue microarray*

A specialist pathologist tested the original Hematoxylin and Eosin (H&E) slides from surgical specimens. All cases were selected and retrieved under permission from the Tawam Hospital Ethical committee (REC: AA/AJ/556). The tumors' representative areas (Invasive front) were labelled, and one core (0.5 cm) was punched out of donor paraffin blocks to conduct the experiment. TMA cores of the 65 sample were mounted on 4 praffin blocks (16-17 cores / block). TMA blocks that eventually reflected a minimal tumor region and blocks from referral cases were eliminated after being compared to the initial H&E slides.

### *Immunohistochemistry*

Paraffin sections were cut at 4 μm thickness, placed on positively charged slides and dried in an oven for 30 min. at 70°C. Deparaffinization, rehydration and target retrieval were performed in the PT Link (Dako) using 3-in-1 procedure. Antibodies were detected using visualization system (EnVision FLEX, Dako) and chromogen (DAB) at 25°C. Meyer's Hematoxylin was

used as a counterstain. IHC staining for 3 antibodies; E-cadherin (mouse monoclonal antibody, clone 36, Ventana Medical System, Dilution 1:50), and cyclin D1 (rabbit monoclonal antibody, clone SP4-R, Ventana Medical system, Dilution 1:150), were performed on a Benchmark-ULTRA fully automated staining instrument (Ventana Medical systems) using Ultra View Universal DAB Detection kit (Ventana). The antibodies were detected by DAB and then counterstained with Meyer's Hematoxylin and bluing reagent. IHC staining of β-catenin (14- mouse monoclonal antibodies, Cell Marque, Dilution 1:150) was performed using the Dako Auto Stainer Link 48 platform.

### *Image acquisition*

Images were captured using binocular light microscope (Olympus BX43) at bright field. Images were captured at x10, x40 and x100 magnification using CCD color video camera (Olympus DP7) attached to a computer system. The tumor invasive fronts were selected with a good contrast of DAB chromogen and hematoxylin as the region of interest. All the images were acquired using Cell Sense application software version 3.5.0 (Germany) which was installed within the computer system. Before capturing the images, the color density and white balance were standardized for all images. All the acquired images were saved as JPEG image format.

### *Image analysis*

After IHC staining for E-cadherin, β-catenin and cyclin D1 by a standard recommended protocol and image acquisition, image analysis was performed by Image J 1.52a version (NIH, Bethesda, Maryland) (Java 1.8.0_112 64-bit) [http://imagej.nih.gov/ij] (Schneider et al., 2012). Validation of Image J analysis for the three above mentioned markers was performed by two observers who are experienced pathologists. Quantification of immuno-score of all the images was blinded for histopathological diagnosis. Prior to start up with the analysis, both observers gained knowledge in the use of Image J with IHC profiler plugin and the controlling of threshold level was skilled and maintained without much adjustment (Varghese et al., 2014).

In the tool bar, "plugins" was opened and clicked for IHC profiler plugin. The cytoplasmic mode selects only DAB cytoplasmic stain. After clicking the cytoplasmic mode, the dialog box for color deconvolution opens with the vector H DAB, and then the image installed gets deconvoluted into three red, green, blue channels of images with separation of only hematoxylin, only DAB immunoreaction image and another image with only highlighted DAB immunoreaction. The threshold was maintained standard, without any adjustment. In the plugin option, IHC macro was clicked to get the quantification of immunoreaction as a log score of high positive, positive, low positive, negative. Final score was shown in a semi-quantitative way (high positive, positive, low positive, or negative). High positive and positive scores were considered as high expression, whereas low positive score is considered low expression (Fig. 1 Right). The following modified algebraic formula was used to measure the mean positive IHC optical density score for the IHC images in order to obtain the same optical density of positive stain (Seyed Jafari and Hunger, 2017).

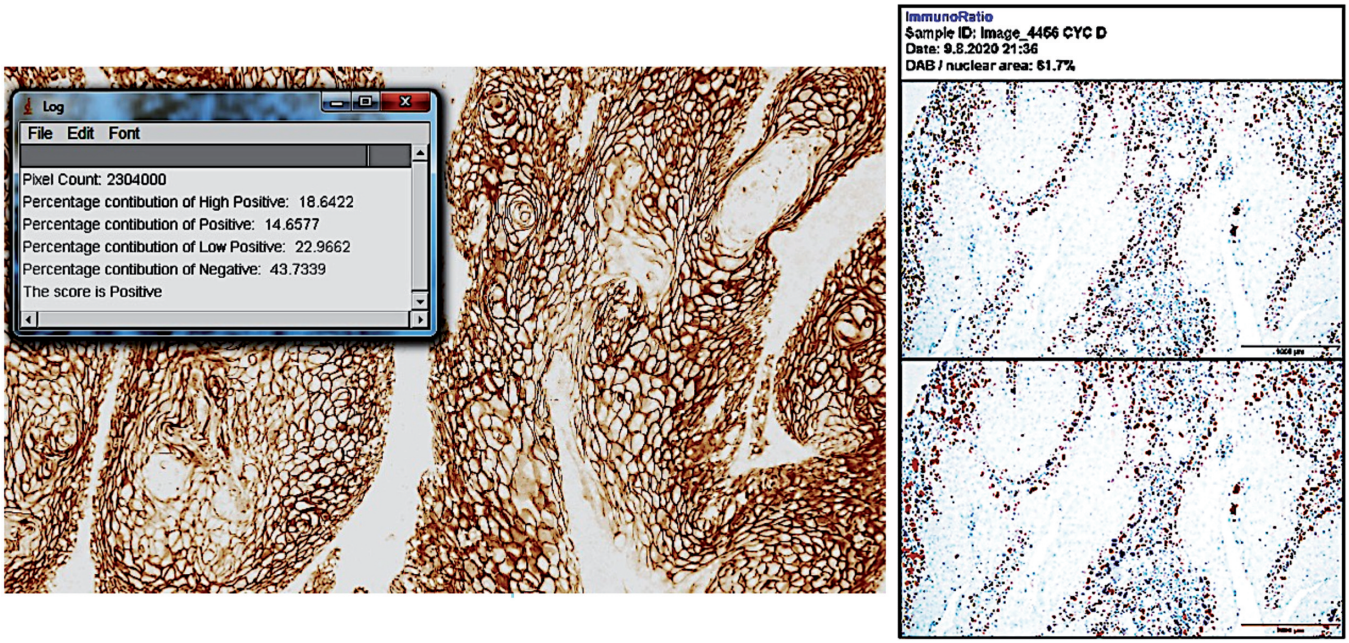


**Fig. 1. Left.** Immune ratio plugin for quantification of nuclear stain using Fiji software. **Right.** Quantification of immunoreaction as a log score using IHC plugin of Image J software.

IHC optical density score = (Percentage contribution of high positive X4+ Percentage contribution of positive X3+ Percentage contribution of low positive X2+ Percentage of negative /100

To determine the relative nuclear stain of cyclin D the proportion of 3,3'-diaminobenzidine (DAB)-stained nuclear area to overall nuclear area was calculated using the Immuno-Ratio method using Image J/Fiji plugin (Schindelin et al., 2012) (Fig. 1 Left). The necrotic region was not taken into account.

### *Statistical analysis*

Patient's age, gender, smoking habits, site, E-cadherin, β-catenin, and cyclin D1 expressions were analyzed for their relationship with survival or risk of recurrence during the follow up. As a multivariate survival analysis, the Cox proportional hazards approach with forward selection was used. The collected data were analyzed using SPSS-23 (IBM), P value less than 0.05 was considered significant.

## Results

### *Clinico-pathological characteristics*

Table 1 summarizes the characteristics of the 65 patients who were analyzed in this report. Males made up 70.8% of the survey, while females made up just 29.2%.The participants ranged in age from 18 to 78 years old, with a median age of 57. More than 60% of the patients in the study were under the age of 60. The most common site of OSCC was the tongue (63.1%), followed by cheek (13.8%) and Jaw bones (9.2%). A total of 13.8% of the sample had cancer in more than one place. More than half of the cases (64.6%) were at late stage (stage III and IV). Recurrent tumors were observed

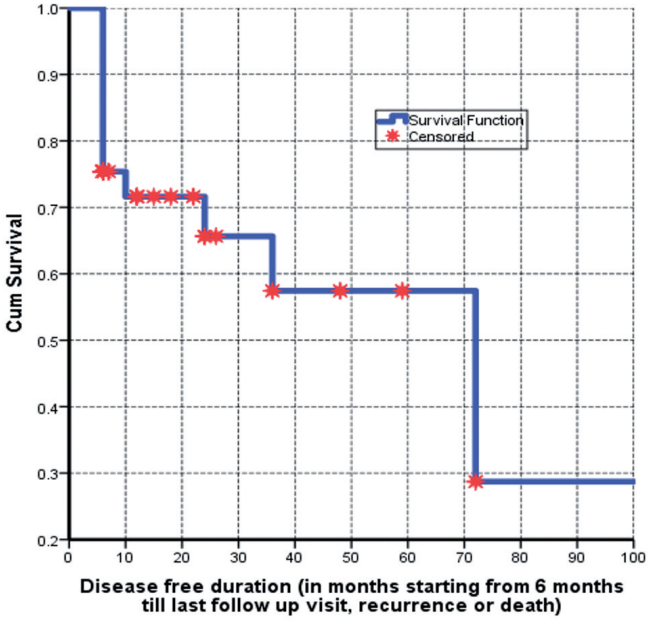


**Fig. 2.** Disease free duration (in months starting from 6 months till last follow up visit, recurrence or death.

**Table 1.** Clinical and histological data of 65 patients with OSCC.

| | Variables | No | % |
|---|---|---|---|
| Age (yrs) | <60 | 40 | 61.5 |
| | 60+ | 25 | 38.5 |
| | Total | 65 | 100.0 |
| Gender | Males | 46 | 70.8 |
| | Females | 19 | 29.2 |
| | Total | 65 | 100.0 |
| Site: | Tongue | 41 | 63.1 |
| | Cheek | 9 | 13.8 |
| | Jaws | 6 | 9.2 |
| | Multiple | 9 | 13.8 |
| | Total | 65 | 100.0 |
| T stage | T1 | 22 | 33.8 |
| | T2 | 15 | 23.1 |
| | T3 | 8 | 12.3 |
| | T4 | 20 | 30.8 |
| | Total | 65 | 100.0 |
| T stage (Tumor size) | Early stage (T1-T2) | 37 | 56.9 |
| | Advanced stage (T3-4) | 28 | 43.1 |
| | Total | 65 | 100.0 |
| N Stage (Cervical LN metastasis) | N0 | 42 | 64.6 |
| | N1 | 6 | 9.2 |
| | N2 | 13 | 20.0 |
| | N3 | 4 | 6.2 |
| | Total | 65 | 100.0 |
| Cervical LN metastasis | Negative | 42 | 64.6 |
| | Positive | 23 | 35.4 |
| | Total | 65 | 100.0 |
| Distant Metastasis | Negative | 32 | 88.9 |
| | Positive | 4 | 11.1 |
| | Total | 36 | 100.0 |
| TNM stage | Stage I | 15 | 23.1 |
| | Stage II | 8 | 12.3 |
| | Stage III | 12 | 18.5 |
| | Stage IV | 30 | 46.2 |
| | Total | 65 | 100.0 |
| Cancer stage | Early stage (stage I&II) | 23 | 35.4 |
| | Late stage (stage III&IV) | 42 | 64.6 |
| | Total | 65 | 100.0 |
| Type of tumor (Primary vs Recurrent) | Primary | 51 | 78.5 |
| | Recurrent | 14 | 21.5 |
| | Total | 65 | 100.0 |
| Tumor differentiation | G1: Well differentiated | 33 | 50.8 |
| | G2: Moderately differentiated | 29 | 44.6 |
| | G3: Poorly differentiated | 3 | 4.6 |
| | Total | 65 | 100.0 |
| Lymphovascular invasion | Negative | 46 | 82.1 |
| | Positive | 10 | 17.9 |
| | Total | 56 | 100.0 |
| Perineural invasion | Negative | 40 | 71.4 |
| | Positive | 16 | 28.6 |
| | Total | 56 | 100.0 |
| Depth of invasion | < 4 mm | 18 | 27.7 |
| | +4 mm | 47 | 72.3 |

in 14 (21.5%) cases only. Lymph node metastasis was found in 23 (35.4%) of the cases, with single or multiple ipsilateral spread.

The use of death itself as an end point is impossible due to the limited number of deaths, which makes any meaningful measure of survival impossible. As a result, we decided to use every end point (local or lymph node recurrence, or cancer-related death) in our survival study as shown in Fig. 2 . The mean survival time for having any end point (recurrence whether local or lymph node or cancer related death was 58.5 months (95% CI (41-76)). As shown in table 3, the survival time in months at each time station was also determined. The majority of the patients had well and moderately distinguished SCC in terms of histological characteristics (50.8, 44.6% respectively). Perineural invasion was seen in 28.6% of the cases, while lympho-vascular invasion was seen in just 17.9% of the cases. More than two third of cases had depth of invasion of more than 4 mm (Table 1).

*Immunohistochemical analysis*

Table 2 shows the expression of E-cadherin, β-catenin, and cyclin D1 and their relationship to clinicopathological characteristics. At invasive fronts, E-cadherin was mostly expressed on the cell membrane of malignant epithelial cells (Fig. 3). E-cadherin expression differed greatly between tumor grades, with the highest in G1 and the lowest in G3 (2.00±0.41 vs. 1.47±0.43). E-cadherin expression was shown to be significantly lower in tumor specimens with remote metastasis (1.37±0.07 vs.1.86±0.44).

β-catenin, on the other hand, showed membranous, cytoplasmic, and limited detection in epithelial cells' nuclei. Just nine patients (13%) had nuclear expression of more than 25% of tumor cells; three had primary tumors, three had chronic tumors, and the other three had metastatic tumors (Fig. 4). As seen in Table 2, β-catenin expression was slightly lower in patients who

**Table 2.** Correlation between E-cadherin, β-catenin, and cyclin D1expression and clinicopathological variables.

| Variables | No (%) | E-cad. expression | | P value | β-cat. expression | | P value | Cyclin expression | | P value |
|---|---|---|---|---|---|---|---|---|---|---|
| | | mean | SD | | mean | SD | | mean | SD | |
| Gender | | | | | | | | | | |
| Males | 46 | 1.78 | 0.46 | 0.12 | 1.58 | 0.54 | 0.44 | 71.14 | 27.23 | 0.13 |
| Females | 19 | 1.98 | 0.43 | | 1.69 | 0.43 | | 81.71 | 24.58 | |
| Age group (years) | | | | | | | | | | |
| <60 | 40 | 1.84 | 0.38 | 0.99 | 1.56 | 0.42 | 0.32 | 66.9 | 28.7 | 0.002* |
| 60+ | 25 | 1.84 | 0.54 | | 1.69 | 0.63 | | 86 | 18.3 | |
| Smoking status | | | | | | | | | | |
| Smoker | 16 | 1.75 | 0.43 | 0.33 | 1.83 | 0.76 | 0.04* | 67.36 | 20.84 | 0.23 |
| Non smoker | 49 | 1.87 | 0.45 | | 1.54 | 0.38 | | 76.48 | 28.22 | |
| T stage | | | | | | | | | | |
| T1 | 22 | 1.98 | 0.42 | 0.63 | 1.56 | 0.42 | 0.94 | 81.06 | 20.17 | 0.16 |
| T2 | 15 | 1.64 | 0.41 | | 1.65 | 0.71 | | 71.39 | 22.37 | |
| T3 | 8 | 1.81 | 0.51 | | 1.60 | 0.53 | | 92.57 | 11.14 | |
| T4 | 20 | 1.85 | 0.45 | | 1.64 | 0.45 | | 61.54 | 34.25 | |
| N Stage | | | | | | | | | | |
| N0 | 42 | 1.85 | 0.45 | 0.80 | 1.57 | 0.53 | 0.82 | 74.08 | 26.43 | 0.31 |
| N1 | 6 | 1.83 | 0.49 | | 1.60 | 0.43 | | 81.25 | 20.72 | |
| N2 | 13 | 1.77 | 0.42 | | 1.67 | 0.52 | | 78.45 | 24.80 | |
| N3 | 4 | 2.04 | 0.58 | | 1.80 | 0.45 | | 51.75 | 40.97 | |
| Cervical LN metastasis (N1-3) | | | | | | | | | | |
| Absent | 43 | 1.83 | 0.45 | 0.85 | 1.58 | 0.53 | 0.51 | 74.60 | 26.33 | 0.88 |
| Present | 22 | 1.86 | 0.45 | | 1.67 | 0.48 | | 73.54 | 28.12 | |
| Distant Metastasis | | | | | | | | | | |
| Absent | 32 | 1.86 | 0.44 | 0.00* | 1.71 | 0.44 | 0.14 | 73.2 | 26.7 | 0.89 |
| Present | 4 | 1.37 | 0.07 | | 2.16 | 1.25 | | 74.7 | 19.4 | |
| TNM stage | | | | | | | | | | |
| Early (II&II) | 23 | 1.84 | 0.44 | 0.98 | 1.60 | 0.34 | 0.92 | 77.10 | 19.77 | 0.527 |
| Late (III&IV) | 42 | 184 | 0.46 | | 1.61 | 0.59 | | 72.67 | 29.98 | |
| Tumor differentiation | | | | | | | | | | |
| G1 | 33 | 2.00 | 0.41 | 0.04* | 1.63 | 0.42 | 0.27 | 69.62 | 30.20 | 0.36 |
| G2 | 29 | 1.71 | 0.43 | | 1.62 | 0.61 | | 78.66 | 22.62 | |
| G3 | 3 | 1.47 | 0.43 | | 1.29 | 0.35 | | 82.26 | 19.86 | |
| Depth of invasion | | | | | | | | | | |
| <4mm | 18 | 1.88 | 0.46 | 0.71 | 1.65 | 0.50 | 0.68 | 85.3 | 15.4 | 0.01* |
| >4mm | 47 | 1.83 | 0.44 | | 1.59 | 0.52 | | 70.6 | 28.7 | |
| Primary vs Recurrent | | | | | | | | | | |
| Primary | 51 | 1.88 | 0.45 | 0.22 | 1.69 | 0.54 | 0.01* | 69.66 | 27.54 | <0.007* |
| Recurrent | | 14 | 1.71 | 0.41 | | 1.32 | 0.27 | | 90.92 | 14.84 |

smoked (1.75±0.43) or had recurrent tumors (1.71±0.41).

Cyclin D1, on the other hand, was found in the nuclei of malignant epithelial cells' basal and spinous cells at the invasive front (Fig. 5). Cyclin D1 expression was significantly higher in subjects over 60 years old (86.00±18.3) and significantly higher in subjects with recurrent tumors (90.92±14.84) according to our findings. The expression pattern and intensity of these three markers does not significantly affected with TNM stage (Table 2).

After correcting for age and gender, the cox-regression model was used to estimate the probability of developing an end point result (local or lymph node recurrence, or cancer-related death) per unit of time as a function of the magnitude of the three markers (Cyclin D1, β-catenin, and E-cadherin). The unadjusted and adjusted hazard ratio figures were estimated.Except for age, none of the predictors were statistically significant, despite the fact that the model was statistically significant. None of the markers contributed towards predicting the risk of an adverse outcome. When compared to younger ages, older age groups (60+ years) were correlated with a statistically significant 4.6 times rise in the probability of having the designated result per unit time. For age, the adjusted and unadjusted HR figures were almost identical (Table 4).

With the exception of age, none of the risk factors evaluated for estimation of the end point result had a statistically meaningful contribution in multivariate modeling. The multivariate model was statistically significant. After controlling for age, gender, and other independent variables used in the model, an advanced T stage (T3-T4) was associated with an apparent increase in the probability of the recurrence or cancer related death per unit time by 2 times as compared to someone with an early-stage tumor (T1-T2). This adjusted risk estimate failed to reach the level of statistical significance. In both univariate and multivariate models, moderate to poorly differentiated tumors were associated with a more than 2-fold rise in the chance of the recurrence or cancer related death per unit time relative to those with well differentiated tumors (T1-T2). Old age was shown to be a strong indicator of having recurrence or cancer related death. As applied to the patient's age and histological classification, the depth of invasion was

**Table 3.** The cumulative survival rate at each time station in months for having any end point (recurrence whether local or lymph node or cancer related death).

| Time (months) | | Cumulative Survival rate (%) at each time station (months) |
|---|---|---|
| 6 | | 75.4 |
| 10 | | 71.6 |
| 24 | | 65.6 |
| 36 | | 57.4 |
| 72 | | 28.7 |
| Mean Survival Time | SE | 95% Confidence Interval |
| 58.5 | 8.9 | (41-76) |

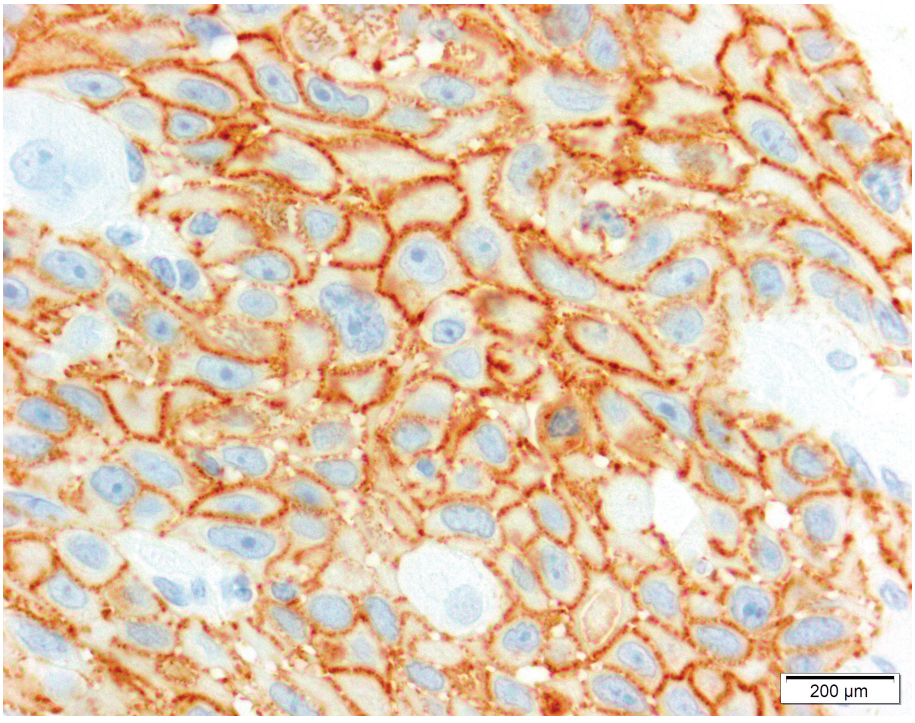


**Fig. 3.** E-cadherin membrane expression. x 100.

also a major indicator of the likelihood of having tumor recurrence or cancer related death , although to a lesser degree (HR 0.47). Cervical metastasis was not associated with significant reduction in risk of having the outcome (Table 5).

## Discussion

The outcome of any treatment and the prognosis of cancer are normally determined by a variety of clinicopathological results. These results, however, are not always accurate, prompting researchers to look for more objective and reliable diagnostic and prognostic markers (Valagussa et al., 1978). The loss of cell adhesion is one of the most critical stages in the process of metastasis, which is a cascade of connected sequential steps. In solid tumors, adhesion molecules are considered to be significant prognostic factors for tumor invasion and metastasis (Wheelock and Jensen, 1992; Pukkila et al., 2001; Qiao et al., 2001).

Cell adhesion molecules are essential for epithelial cell differentiation and parenchymal cell cytoskeleton maintenance during this process. The adherens junction, which is made up of E-cadherin and catenin, allows normal cells to stick together (Kemler, 1993).

E-cadherin is a calcium-dependent glycoprotein with a molecular weight of 120 kDa that forms homotypic relationships between epithelial cells (Takeichi, 1991). The E-cadherin cytoplasmic terminus has been shown to be related to the actin cytoskeleton via α-catenin and β -catenin (Andl et al., 2014).

In human OSCC cases, low E-cadherin expression

**Table 4.** Cox regression model with the risk of having any end point (recurrence whether local or lymph node or cancer related death) per unit of time and the three tested markers (adjusted for age and gender) as predictors.

| | Unadjusted estimates | | | Adjusted estimates (Multivariate modelling) | | |
|---|---|---|---|---|---|---|
| | P | HR | 95% Confidence Interval HR | P | HR | 95% Confidence Interval HR |
| Cyclin D1 (Nuclear marker of cell cycle/proliferation)-mean percent positive cells | 0.36[NS] | 1.01 | (0.99 to 1.028) | 0.96[NS] | 1 | (0.977 to 1.023) |
| β-catenin (cell adhesion molecule cytoplasmic stain)-score method 1 | 0.52[NS] | 1.01 | (0.987 to 1.027) | 0.55[NS] | 1.01 | (0.986 to 1.026) |
| E-cadherin (cell adhesion molecule cytoplasmic stain)-score method 1 | 0.39[NS] | 0.99 | (0.974 to 1.01) | 0.43[NS] | 0.99 | (0.975 to 1.011) |
| Older age group (60+ years) compared to those younger than 60 years | 0.005 | 4.52 | (1.596 to 12.798) | 0.012 | 4.63 | (1.403 to 15.25) |
| Male gender compared to female | 0.34[NS] | 1.69 | (0.569 to 5.031) | 0.28[NS] | 1.86 | (0.603 to 5.75) |

P(Model)=0.030

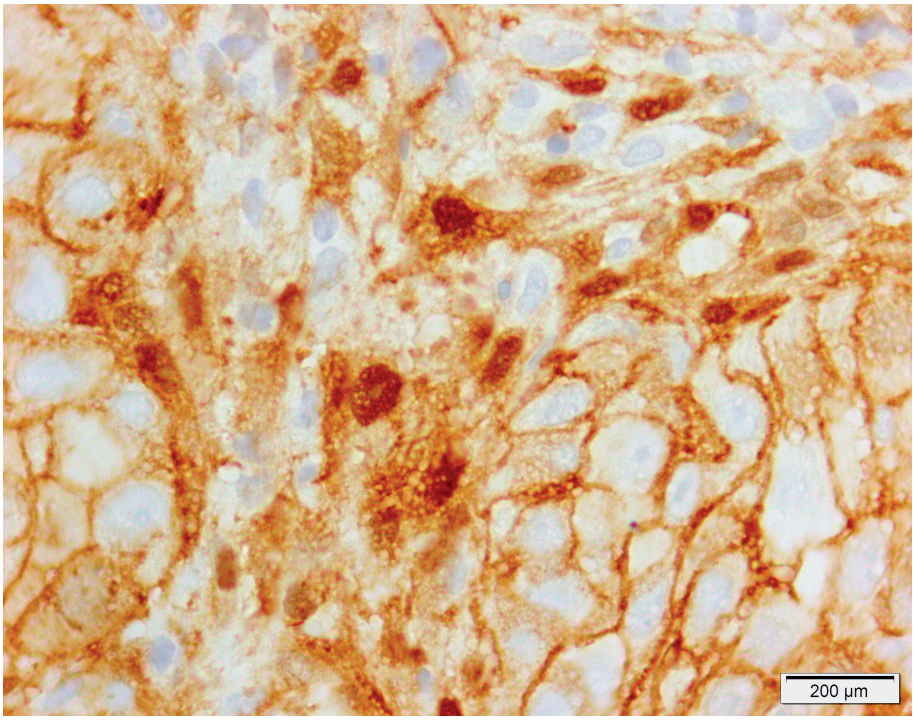


**Fig. 4.** β-catenin membrane and nuclear expression. x 100.

can predict lymph node metastasis and is considered an independent predictor for OSCC patient survival (Huber et al., 2011).

E-cadherin was shown to be strongly inversely associated with tumor differentiation and the occurrence of distant metastasis in this research, implying that E-cadherin may play a role in preventing tumor invasion or metastasis. This result is consistent with the findings of other studies (Downner and Speight, 1993; Blankesteijn et al., 2000; Rosado et al., 2013) implying that when E-cadherin expression is diminished, tumor differentiation is reduced and the risk of distant metastasis increases. While some studies (Krishnadath et al., 1997; Blankesteijn et al., 2000) found a connection between E-cadherin expression and the occurrence of regional lymph node metastasis and/or overall survival, the current study found no such link. This finding is in accordance with the results of other studies (Liu et al., 2010; Ukpo et al., 2012).

The preservation of natural intercellular adhesion requires an intact E-cadherin/-catenin complex. Intercellular adhesion is lost as these complexes' expression or function is disrupted. The loss/reduction of cell-cell junctions, as seen in E-cadherin, may be due to mutations/abnormalities in E-cadherin and/or its related molecular matrix involving catenin and actin, causing disruptions in the epithelium's normal architecture. Disturbances in the cadherin/catenin complex, on the other hand, may be caused by a variety of other factors/mutations (Bánkfalvi et al., 2002).

β-catenin is a multifunctional protein that plays a role in two seemingly unrelated processes: cell-cell adhesion and signal transduction. In addition to its role in regulating E-cadherin-mediated cell adhesion, β-catenin is a transcription cofactor in the wingless (Wnt) signaling pathway and a target of the adenomatosus polyposis coil (APC) gene product, which has been linked to the development of various human cancers (Blankesteijn et al., 2000; Bánkfalvi et al., 2002).

In tumors, reduced or absent expression of β-catenin causes cell proliferation, migration, and invasion, and is linked to a poor prognosis (Krishnadath et al., 1997; Bremnes et al., 2002; Zhao et al., 2003). Reduced expression of catenin, according to Jawhari et al, may be a useful prognostic marker, regardless of tumor form, grade, or level (Jawhari et al., 1997).

In this study, β-catenin levels were significantly lower in smokers and patients with residual tumors than in nonsmokers and patients with primary tumors. The E-cadherin/β-catenin complex can be diturbed during oncogenesis. As a result, the E-cadherin/β-catenin complex promotes cell adhesion by acting as a part of adherent cell-cell junctions. Liu et al. (2010) found no significant association of E-cadherin and β-catenin with smoking status and tumor recurrence (Liu et al., 2010). β-catenin is an anchoring protein present in the cytoplasm and is essential in maintaining normal functions of E-cadherin (Tanaka et al., 2003; Rosado et al., 2013). β-catenin binds to the E-cadherin molecule's cytoplasmic domain directly. This binding is needed for stable cell-cell adhesion and is controlled in part by β-

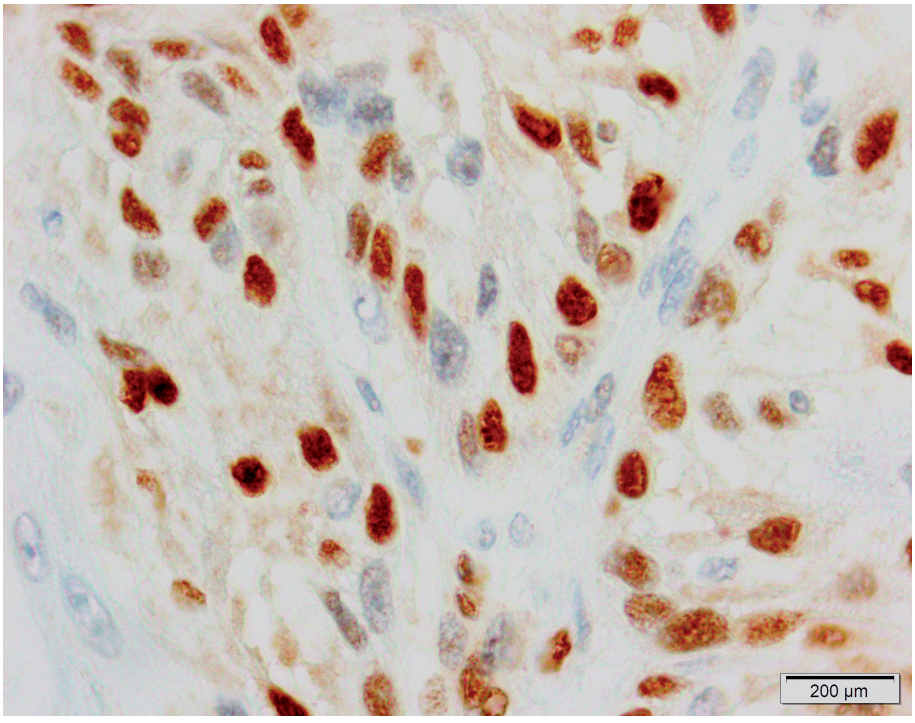


**Fig. 5.** Cyclin D1 nuclear expression. x 100.

catenin.

The pattern of β-catenin expression was found to be very similar to the pattern of E-cadherin expression. This result indicates a connection between HNSCC metastasis and direct downregulation of the cadherin/catenin complex (Do et al., 2004).

When β-catenin is expressed inside the nucleus in squamous cell carcinoma of the pharynx and hepatoblastoma, the prognosis is poor, according to some reports, and expression of β-catenin in the cytoplasm is an indicator of hematogenous metastasis in colorectal carcinoma, according to another study. In (Zhang et al., 2016) study just 9 cases of nuclear β-catenin expression were observed in this study. Three were in patients with primary tumors, three were in patients with recurring tumors, and the other three were in patients with metastatic tumors. As a result, the results of this study's nuclear expression are inconclusive.

Cyclin D1 has been linked to ectopic β-catenin expression in the nucleus and cytoplasm, according to researchers (Qiao et al., 2001). In the canonical Wnt pathway, cytoplasmic-catenin serves as a transcription factor for the nucleus, initiating the transcription of different genes.In this study, people with tumors with a depth of invasion greater than 4 mm had slightly lower cyclin D1 expression. Recurrent tumors, on the other hand, have a slightly higher level of this nuclear marker. Overexpression of cyclin D1 in squamous cell carcinoma of the larynx and hypopharynx has been linked to a poor prognosis in several reports (Volavšek et al., 2003). Other studies reported that overexpression of cyclin D1was associated with good prognosis in breast cancer, and does not have a prognostic value in non-small cell lung cancer. Cyclin D1 overexpression was shown to be linked to clinical stage and nodal status by van Diest et al. (1997) and Do et al. (2004), and this marker was considered a prognostic marker for HNSCC (Do et al., 2004; van Diest et al., 1997).

Upregulation of cyclin D1 is caused by a variety of upstream oncogenic events of varying significance, such as CCND1 gene duplication, polymorphism, and mutation, aberrant activation of pathways linked to physiological mitogenic signaling (PI3K, MAPK), activation of the Wnt/Canonical or NF-κB pathways, and oncogenic alterations of the RB pathway (Ramos-García et al., 2017). These pathways trigger unregulated cell proliferation, and may result in greater oral squamous cell carcinoma and an increased likelihood of lymph node involvement (Ramos-García et al., 2017, 2018).

Garcia et al. (2016) have discovered cyclin D1 overexpression in epithelia adjacent to OSCC, implying that gene amplification and subsequent overexpression of this protein was an early event in oral carcinogenesis (Ramakrishna et al., 2013).

Because of their superior proliferative ability, clonal accumulation of cyclin D1 overexpressing cells in premalignant epithelium, especially in the basal and parabasal layers, can gradually replace normal cells, resulting in premalignant fields that extend into the oral mucosa. This lends credence to the idea that nuclear cyclin D1 overexpression is a key oncogenic factor in the emergence and spread of premalignant tumors (Ramos-Garcia et al., 2017).

The various methods of IHC stain evaluation, which were often subjective and manual, may explain the inconsistencies in the results of different studies. Different antibodies were used, and different rating schemes were used, which may explain the discrepancies in performance. The cytoplasmic expression of E-cadherin in the invasive front was quantified in this analysis using Image J with the IHC profiler plugin, which accurately measures the optical density of DAB staining. Other studies, on the other hand, used a subjective and less sensitive semi-quantitative scoring scheme than Image J analysis scoring. The loss of E-cadherin increases the presence of soluble β-catenin in the cytosol (Rosado et al., 2013).

Using backward selection algorithm, it was found in this study that advanced (T3&4) stage increases the risk by 2.07 times that of early stage (T1&2). This makes depth of invasion and tumor stage significant indicators of poor prognosis in OSCC. To the best of our knowledge, this is the first report using IHC plugin to quantify the exact optical densities of the three markers using TMA with Image J software. However, the number of cases was limited in our study and further studies with larger sample size with multiple cores from invasive fronts are recommended.

## *Conclusion*

High expression of β-catenin and cyclin D1 are significantly correlated with tumor recurrence and old age, advanced tumor stage and low histological grade were a significant predictor for the risk of having tumor recurrence and cancer related death. Depth of invasion and histological grade are surrogate markers for tumor recurrence and lymph node metastasis.

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*Consent for publication.* A permission was obtained from hospital authority to publish the data.

*Availability of data and materials.* The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

*Competing interests.* The authors declare that they have no competing interests.

*Authors' contributions.* N.H: design of the work; M.A: the acquisition of the data; N.H & A.Q: Interpretation of data; A. A: TMA construction; S.A: Reviewing the draft; ASA: Statistical analysis the All authors read and approved the final manuscript.

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