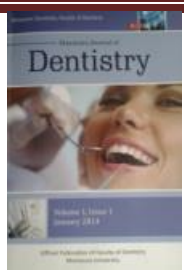

Evaluation of mean vascular density (MVD) and cells adhesion in oral squamous cell carcinoma. An immunohistochemical study.

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Abstract:

Purpose: The aim of this study was to investigate the immunohistochemical expression of CD34 and E-Cadherin among the studied cases of OSCC, Furthermore, the correlation between expression of CD34 and E-Cadherin in OSCC was evaluated to assess the contribution of different grades of OSCC to angiogenesis and loss of epithelial cell adhesion. Also, assess the expression of CD34 and E-Cadherin in OSCC in relation to the available clinical data. This approach may be a basis to determine the prognosis of the OSCC cases.

Methods: Immunoexpression of CD34 and E-cadherin antibody was evaluated in previously diagnosed, paraffin-embedded sections of 40 tissues each of well-differentiated, moderately differentiated OSCC and poorly differentiated OSCC. Three high power fields of tumor were counted for each sample stained by CD34. Moreover, a total mean of three fields was calculated to calculate MVD of the samples. Three high power fields at the invasive front of the tumor were counted for each sample stained by E-Cadherin.

Results: Increased expression of CD34 and decreased expression of E-Cadherin was found to be correlated significantly with clinical factors and a poor prognosis. there is a statistically significant association between OSCC grade and CD34 and E-Cadherin expression.

Conclusion: Increased expression of CD34 and reduced expression of E-cadherin may be a reliable indicator of increase in the invasiveness of OSCC and poor prognosis.

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Introduction

Oral cancer (OC) which is a neoplasm involving the oral cavity considered as a major health problem worldwide [(1)]. Despite the progress in research and therapy, it is one of the 10 most common cancers in the world [(2)], and survival has not improved significantly in the last years, representing a continuing challenge for biomedical science [(3)]. OSCC is the most common pathological type of oral cancer [(4)].Pathologicalprocess controlled by certain biomolecules produced in the body. Angiogenesis is the generation of new microvessels from pre-existing one via the "sprouting" of endothelial cells [(5)]. Physiological angiogenesis processes are critical during embryo development, wound healing, and collateral formation for enhance organ perfusion. However, pathological angiogenesis that's mean abnormally accelerated angiogenesis processes to provide nourishment to proliferated tumor cell. It has been known for long time that growing tumors require increased blood supply in order to obtain sufficient oxygen and nutrients and to discard waste products [(6)].

Immunohistochemical staining with CD34 has been used to measure angiogenesis). CD34 has been used for categorizing newly formed vessels in tumor tissues and the results are evaluated by measuring the microvascular density (MVD) as a result of tumor-associated angiogenesis [(7)]. CD34 is selectively expressed in human hematopoietic progenitor cells and in the vascular endothelium. It may play a role as a mediator of attachment of bone marrow stem cells to bone marrow extracellular matrix or directly to stromal cells glycoprotein which is expressed selectively on hematopoietic progenitor cells, it has been commonly used as a marker to identify and isolate hematopoietic stem cells (HSCs) and progenitor cells in preparation for transplantation of bone marrow and express angiogenesis in tissue [(8)] [(9)].

E-cadherin is a calcium ion-dependent I-form adhesive transmembrane glycoprotein that is situated on the epithelial cell surface to mediate intercellular adhesion in epithelial cells through Ca++ dependent homophilic interactions and mediates the adhesion between epithelial cells stromal cells. It belongs to the cadherin superfamily and is the major cadherin molecule expressed in epithelial cells [(10)]. It plays an important role in maintaining normal epithelial cell morphology, cell polarity, and tissue structural integrity forms a powerful molecular barrier that inhibits the proliferation, shedding, invasion and metastasis of cancer cells [(11)]. It also has important role in signal transduction from the outer cell surface to the cytoskeleton [(12)]. Besides its role in normal cells, it can play a major role in malignant cell transformation, and especially in tumor development and progression.Loss of E-cadherin results in loss and poor intercellular adhesion of tumor cell to each other, loss of the differentiated epithelial morphology, increased cellular motility, and then make the tumor occurred infiltrate, spread, and metastasis, so E-cadherin is considered as an important anti metastatic gene [(13)]

The aim of the present work: Was to assess theexpression of CD34 and E-Cadherin in OSCC in relation to the

available clinical data and determine any correlation between them.

Material and Methods: In this work, the study samples included 40 paraffin blocks containing OSCC that will be collected at the duration of 2017-2020 from archival files of Oral and General Pathology Departments Faculties of Dentistry, and Medicine, Mansoura University.Four micrometer thick tissue sections were stained through standard immunohistochemical (CD 34) and (E-Cadherin) according to the instructions of the manufacturer.

Immunohistochemical procedure:

The slides were deparaffinized in xylene and then hydrated in graded alcohol series. The endogenous peroxidase activity was blocked by incubating the slides with 3% hydrogen peroxide in methanol for 30 minutes.The antigen retrieval was carried out by pressure cooker and EDTA buffer ((PH8)). To prevent nonspecific reactions, sections were washed in phosphate buffer saline (PBS) 3 times for 1 minutes before proceeding. The primary antibody (CD34, E-Cadherin) was applied in optimized dilution (1:25) for CD34 and (1:200) for E-Cadherin. The slides were incubated with the primary antibody for one hour at room temperature. followed by incubation with secondary antibody 1.0 poly HRP (horseradish peroxidase), conjugate for mouse/rabbit, for twenty minutes in room temperature, and then were washed in PSB 3 times for 3 minutes. For the negative control, the primary antibody was eliminated and replaced with PBS.

Immunohistochemical analysis:

Each histological section was examined with light microscope at low magnification (x100), three areas of tumor with the highest number of distinctly highlighted microvessels ("hot spots") were selected. Then, they independently evaluated the slides by microvessel counting using a 400x magnification. The final result was expressed as a number of vessels in the field of view under 400× magnification. The immunohistochemically positive cells were counted using the Image J free software package.For immunohistochemical counting of E-Cadherin. Only epithelial expression was analyzed for all the parameters at the invasive front of all the tumors.In E-cadherin, the membranous staining was considered as positive.The proportion of cells stained, as well as the intensity of E-cadherin immunoexpression, was assessed using image j software.The percentage and intinsity of positive tumor cells were calculated from at least three representative high-power fields per slide and, then, the mean percentage and intinsity graded per field was noted. The score of immunoreactivities of E-cadherin in all the groups was assessed by calculating the immunoreactive score (IRS). as follows: IRS = percentage of immunopositive cells (A) × intensity of immunostaining (B)

S=percentage of immunopositive cells (A) × intensity of immunostaining (B)

0-1: Negative, 2-3: Mild, 4-8: Moderate, 9-12: Strongly positive

A-Percentage of E-Cadherin immunopositive cells was estimated and graded, in three random fields, on a scale of 0-4 as follows:

0 points: No immunopositive cells, 1 point: <10% immunopositive cells, 2 points: 10%-29% immunopositive cells, 3 points: 30-59% immunopositive cells, 4 points:60%-100% immunopositive cells.

B-The intensity of E-Cadherin immunostaining was graded on a scale of 0-3 points as follows

0 points: No staining, 1 point: low staining intensity, 2 points: Moderate staining intensity, 3 points: Strong staining intensity.

Result:

Clinical result: The present study was carried out on 40 cases.

Characteristics	OSCC patient N=	(%)
SEX		
Male	10	25%
Female	30	75%
AGE		
<50	10	25%
50-70	16	40%
>70	14	35%
Tumor site		
Tongue	18	45%
Cheek	10	25%
Lip	6	15%
Mandible	6	15%
Tumor shape		
Ulcer	24	60%
Mass	8	20%
Erythroleukoplakic	8	20%
Tumor size		
T1	16	40%
T2	10	25%
T3	8	20%
T4	6	15%
Nodal involvement		
N0	22	55%
N1	10	25%
N2	8	20%
Distant metastasis		
M0	36	90%
M1	4	10%
Stage		
I	8	20%
II	12	30%

II Histopathological results of cases:

According to the traditional histopathological grading system (who) of OSCC, the most common types (50%) of the current studied cases were moderately followed by well

differentiated (32.5%). The least common type (17.5%) was the poorly differentiated cases.

Distribution of age and sex in relation to the histologic grade: As regard to age and sex of OSCC cases, no significant statistical differences were observed between different histological grades.

III- immunohistochemical result:

1-CD34 expression distribution among studied OSCC cases:

All the studiedcases showedimmuno-reactivity forCD34 antibody.The endothelial lining of the blood vesselswas strongly stained.

CD34 expression showed moderate expression in 20 case (50%) followed by weak and strong expression in 8 cases (25%) for each.

Relation between CD34 expression and histological grades of studied OSCC cases:

There was a statistically significant difference between OSCC grades and CD34 expression among studied OSCC cases (p=0.0039). Advanced histological grades of OSCC were associated with strong reaction. Poorly differentiated carcinoma was associated with strong reaction while 50% of weak expression was found in well differentiated grade.

Microvessels density (MVD) assessment by CD34 in different histological grades of studied OSCC cases*:*

The highlighted CD34 positive endothelial cells or a cell cluster was regarded as a distinct countable microvessel and hence of microvessel density (MVD) was assessed in all studied OSCC. It was found that, well differentiated OSCC demonstrate a mean value of (23±8.09), Moderately differentiated OSCC (27±10.2)and poorly differentiated OSCC demonstrate a mean value of (40±13.7).Poorly differentiated demonstrate the high value followed by moderately differentiated followed by well differentiated OSCC cases.On comparing of the three groups by one-way analysis of variance, a statisticallysignificantdifference was found (P=0.0035).

Table 1: Comparison of Microvessel Density (MVD) between the studied OSCC cases.

	Well differentiated	Moderately differentiated	Poorly differentiated	p-value	
CD34	23±8.09	27±10.2	40±13.7	0.0035	
N	13	20	7	40	

One-way ANOVA, P<0.05 is Significant*

2-E-Cadherin expression distribution among studied OSCC cases:

Immunohistochemical reaction for E-Cadherin in OSCC cases was found in the membranes of surface epithelial cells and membranes of OSCC dysplastic epithelial cells that invade connective tissue and all of the cases show positive expression of E-Cadherin. E-Cadherin expression showed moderate expression in 24 OSCC cases (60%) followed by mild and strong expression in 8 OSCC cases (20%) for each (Table 16& Diagram 12).

Relation between E-Cadherin expression and histological grades of studied OSCC cases:

There was a statistically significant difference between different grades as regard E-Cadherin expression among studied OSCC cases (p=0.00024). Advanced histological grades of OSCC cases were associated with mild reaction for E-Cadherin (62.5%). On the other hand, 87.5% of well differentiated OSCC cases revealed strong expression for E-Cadherin. It was found that the study cases, well differentiated OSCC cases demonstrated a mean value of (7.84±1.51), moderately differentiated OSCC cases (5.4±1.09) and poorly differentiated OSCC demonstrated a mean value of (2.28±0.48). Well differentiated OSCC cases demonstrated the highest value followed by moderately differentiated. The lowest value demonstrated by poorly differentiated OSCC cases.

However, on comparing of the three groups by one-way analysis of variance, a statistically significant difference was found (P= 0.00001) (Table 25).

Table 2: Comparison of E-Cadherin between the studied groups.

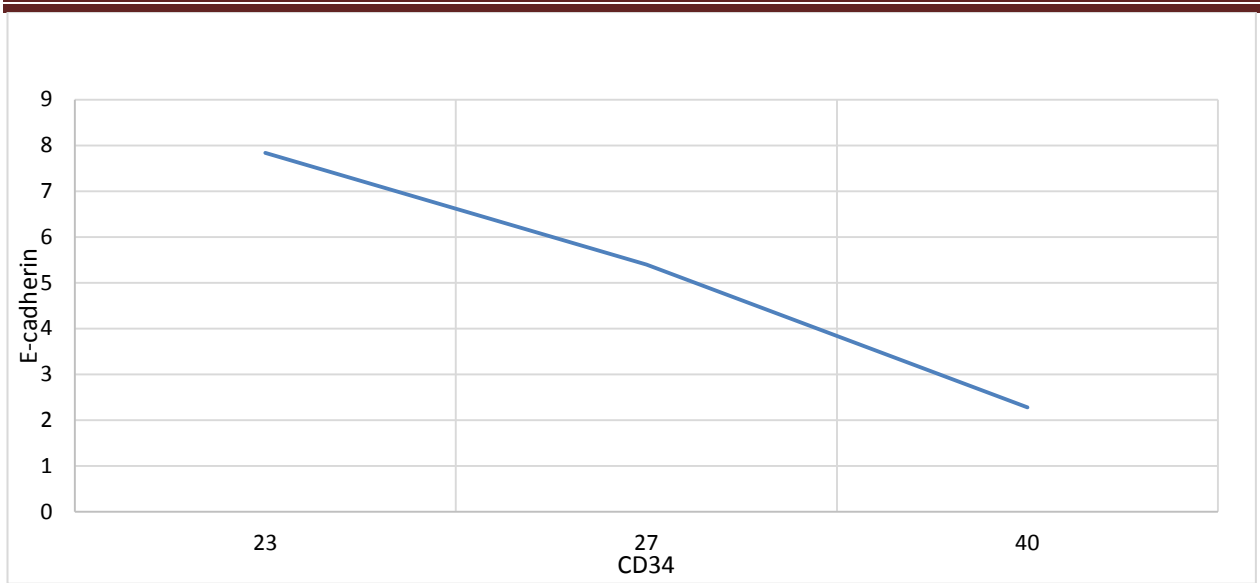
	Well differentiated	Moderately differentiated	Poorly differentiated	p-value	
E-Cadherin	7.84±1.51	5.4±1.09	2.28±0.48	0.00001	
N	13	20	7	40	

One-way ANOVA, P<0.05 is Significant*

Relation between-Cadherin and CD34:

Spearman's correlation coefficient test showed negative correlation between two MVD and E-Cadherin in OSCC cases and the association considered statistically significant.

r_s = -0.5231, *p* (2-tailed) = 0.00053.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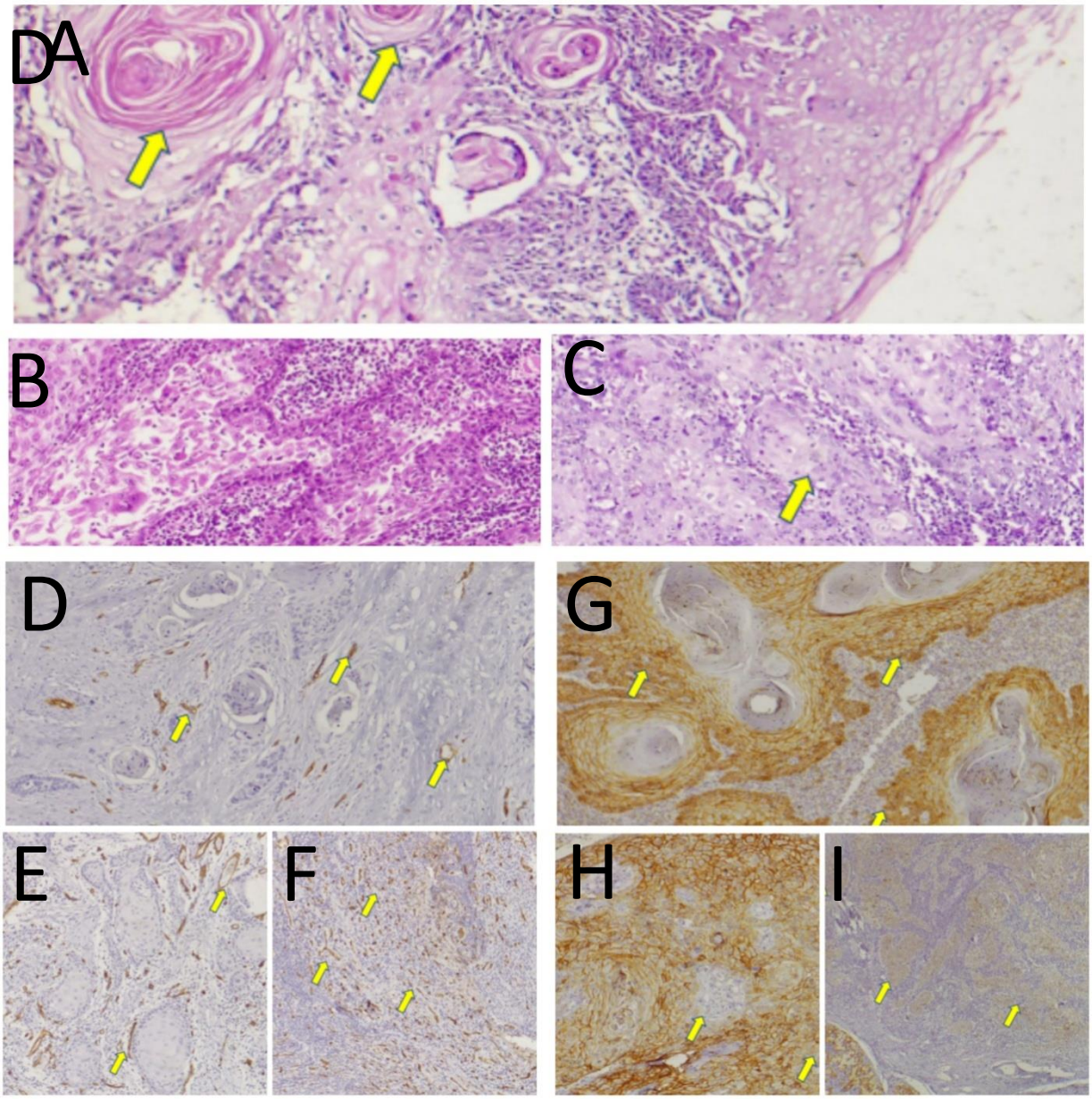
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Fig1: A photomicrograph showingA) Well differentiated OSCC **B)**Moderately differentiated OSCC **C)**Poorly differentiated OSCC (H&E X 200)**D)**week immunoreactivity for CD34 in a case of well differentiated OSCC**E)**moderate reaction for CD34 in a case of moderately differentiated OSCC**F)** poorly differentiated OSCC with strong reactivity for CD34 **G)**Well-differentiated OSCC island exhibit strong intensity **H)**moderately differentiated OSCC exhibit moderate intensity **I)**poorly differentiated squamous cell carcinoma (PDSCC) showed weak membranous immunostaining(PAP-DABx200).

Discussion:

Oral squamous cell carcinoma is the most frequently occurring cancer in the head and neck region. It was rank amongst the 10th most common cancers worldwide. Major risk factors for carcinoma are smokeless tobacco and betel nut, which consumed by 20% of the world population. Early diagnosis, assessment of behavior and prognostic estimates are vital to reduce morbidity and mortality rates of OSCC cases [14].The present study revealed that most of OSCC cases of presented at the age between 50-70 years, with a mean of 59.75±14.12 years. This is within the reported range globally [15]. This result agrees with study by El sherbiny et al., 2018, Kapila et al., and Shenoi et al., 2012found the age between 50-70 years [16, 17, 18]. In contrary, Yosefof et al., 2020 and Satgunaseelan et al, 2020 found OSCC in young age in developing countries without any risk factor [19, 20]. Our population-based data from Egypt are consistent with global epidemiological studies; as it demonstrates that the incidence of OSCC is rising significantly in females where they represent 75% while in males represent only 25%. This similar to the researches in US, Canada and Europe, in addition to India, China and South Korea [21, 22] and also is in agreement with the studies of Satgunaseelan et al.,2020 that have been shown that OSCC affects women over 60 years, and without those classical risk factors [20] as well as study in tongue OSCC in southern Iran [23]. As regard to the site, the tongue was the most common accounting for near 45% of OSCC in this research followed by cheek. This also reported in other investigations of Emerick et al., 2020 in Brazil that showed tongue was the common site and represented almost half of the cases [24], also other researches of Sharma et al and Mehta et al., 2019 that found the increase in tongue SCC has been observed in many regions in India and in Egypt where no such risk factor has yet been identified [25, 26]. According to the findings of the current study at the time of diagnosis, most of the studied OSCC cases presented as non-healing ulcers this finding is similar to previous studies by Almokhtar et al., 2019[27] and other studies [24, 28, 29]. The clinical presentation of OSCC as an ulcer is nearly the same across the world. This may be due to delayed diagnosis of OSCC that lead to a progression to more advanced stage. Therefore, it is scientifically to increase the knowledge and awareness concerning the early symptoms of OSCC among general public and dental health professionals that can have a great impact of the disease [30]. According to the TNM clinical categorization of the current study, most of cases were denoted as T1 and T2 thatwere represented by (40%-25%) then T3 and T4 were represented by (20%-15%). This result agrees withKyzas et al.,2004 study which showed that 66% of the tumors were T1 and 34% were T2[31], other study in Egypt observed similar result that the majority of the primary tumors 52% were measured ≤ 4 cm [32,33]. In the present study, 45% of OSCC cases showed palpable lymph node of N1 and N2. Distant metastasis reported in only 10% of the cases. These results are consistent with Elmorsy et al., 2019 who found palpable lymph nodes in 50% of the cases [29]. While, the results of Fathy et al., 2018 are in disagreement with our study where Lymph node metastasis detected in 35% (19/54) of the cases [33].

Regarding to the clinical staging of OSCC cases, in the present study, the majority of cases were diagnosed as (Stage II and Stage III) 30% for each followed by (Stage I and stage IV) 20% for each. These findings are in agreement with previous research by Asif et al., 2020 who found majority of the OSCC cases were classified as stage III followed by Stage IV, Stage II and stage 1 respectively [16]. All of them need early intervention, as most of the patients had OSCC diagnosed in advanced stages III and IV.The results of the current study contradict study that showed Stage I was the predominant stage (57%) [32] and also contradict other finding by Feng et al., 2020 that revealed majority of cases were diagnosed as stage III and IV (54.05%) [33]. This might be due to variation of the environmental factors. Concerning the histologic grades, most of the current cases were moderately differentiated OSCC 20 cases (50%), followed by well differentiated OSCC 13 cases (32.5%) while the least common type was the poorly differentiated OSCC 7 cases (17.5%). This is in accordance with study by Elmorsy et al., 2019 that revealed that 82% of the cases were moderately differentiated. Each of the well and poorly differentiated OSCC accounted for 9% of the cases [29]. And also, in agreement with Zhang et al., 2017 who reported well and moderately differentiated tumors in 83 cases (71%) and poorly differentiated tumors in 34 cases (29%) [190]. Contradicting our results, other study showed that 60% of the tumors were well differentiated [33]. This might be explained by variable environmental carcinogenic factors.In this work, it was found that the highest mean MVD in the studied cases was observed in poorly differentiated OSCC followed by moderately differentiated followed by well differentiated tumors. There was a direct relation between advancement of tumor gradeand increase MVD. This result is in agreement with research in Egypt that observed increased MVD values when OSSC was less differentiated [29]. Also, other work by Ansari et al., 2020 that showed increased MVD with advanced of tumor grade [34]. Study by Mostafa et al., 2019 in other lesion found that level of CD34 increased with aggressiveness of central giant cell granuloma (CGCG) [35].The present study showed that there was a statistically significant difference association between OSCC grades and E-Cadherin expression among studied OSCC cases (p=0.00024). Advanced histological grades of OSCC were associated with mild reaction. Poorly differentiated carcinomas were associated with mild E-Cadherin reaction. While, 87.5% of strong reaction was found in well differentiated grade. This result is consistent with finding of Zhou et al., 2015 who reported that E-cadherin has 83.3% (10/12), 61.9% (13/21) and 33.3% (3/9) positive expression

rate in well, moderately and poorly differentiation of OSCCs respectively [36]. Angadi et al., 2016 reported the same result that E-cadherin expression showed a progressive reduction from WDSCC to MDSCC to PDSCC [37]. Micro vascular density and E-cadherin expression at the site of deepest tumor invasion all correlated significantly with prognosis in significant relationships to the clinicopathologic findings related to the metastasis of advanced OSCC.

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