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Review Article

Assessment of immunohistochemical expression of claudin-1 in oral squamous cell carcinoma and its clinicopathological correlation

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ABSTRACT

Oral carcinogenesis is complex and multi-step process, which results from various deleterious habits, multiple environmental factors and genetic susceptibility. CLDN-1 expression is regulated oncogenic Wnt/B-catenin transduction pathway. They recruit matrix metalloproteinases (MMPs) on the cell surface to achieve elevated focal concentrations and eventual activations of proMMP2. These collagenases are responsible for the breakdown of extracellular matrix proteins and thus facilitate invasion and spread of malignant cells. Reduced cell-cell adhesion is associated with loss of contact inhibition of proliferation. This allows escape from growth control signals and triggers carcinogenesis. Significant correlation was observed between histopathological grade of the tumor with the localization and immunostaining intensity of CLDN-1. Determination of localization of CLDN-1 for a particular patient may be important to decide site (cytoplasmic or nuclear) for targeting CLDN-1. This targeted drug therapy for CLDN-1 may prevent worsening of the disease in the patient, resulting in better prognosis.

1. Introduction

The global burden of oral squamous cell carcinoma (OSCC) continues to increase because of an increasing adoption of habits causing cancer, particularly in economically developing countries.[1] In spite of receiving the standard treatment strategies, the 5-year survival rate of patients has remained relatively low. It is because most patients are diagnosed when the disease has reached advanced stages.[2]

Oral carcinogenesis is complex and multi-step process, which results from various deleterious habits, multiple environmental factors and genetic susceptibility. OSCC accounts for 24% of all head and neck cancers.[3] It may arise de-novo or may be preceded by potentially malignant disorders, like Oral submucous fibrosis (OSMF), Oral leukoplakia (OLK), and Oral lichen planus (OLP).[4]

While undergoing malignant transformation, the tissue undergoes various structural, molecular and functional changes i.e. impaired DNA replication, uncontrolled proliferation of cells, epithelial– mesenchymal transition, loss of cell adhesion, increased cell motility etc. Early in OSCC, molecular changes are evident at cellular level even in the absence of phenotypic changes in the tissue. Biomarkers may serve as a useful tool to detect these changes at a molecular level which have diagnostic, prognostic or therapeutic significance.

Intercellular junctional complexes are important structures for maintenance of tissue architecture and physiologic functions. There are three different types of junctions i.e. Adhesive junctions, Gap junction and Tight junction (TJs).[5] The Adherens junctions and desmosomes are primarily involved in cell-to-cell adhesion and gap junctions in cell-to-cell communication. Whereas the TJs

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forms the main structures regulating cell-to-cell interactions in epithelial and endothelial cell layers. They provide a seal between the apical portions of adjacent basolateral cell membranes by preventing diffusion of membrane proteins from the apical to the basolateral cell membrane and also maintain the cell polarity.[6]

Tight junctions are involved in the intracellular signalling and regulation of the epithelial cell proliferation, polarity and differentiation.[7] The main protein of the TJs is claudin. The name claudin comes from the latin word 'claudere', which means 'to close'. Their expression and distribution may vary with tissue types and sites.[8] Claudin-1 (CLDN-1) is a prototype molecule at this crossroad, functioning as adhesion between adjacent epithelial cells and with extracellular matrix plays a significant role in tissue morphogenesis in embryos and also in the maintenance of complex differentiated tissues in adults.[9]

CLDN-1 expression is regulated oncogenic Wnt/B-catenin transduction pathway. They recruit matrix metalloproteinases (MMPs) on the cell surface to achieve elevated focal concentrations and eventual activations of proMMP2. These collagenases are responsible for the breakdown of extracellular matrix proteins and thus facilitate invasion and spread of malignant cells.[10] Reduced cell-cell adhesion is associated with loss of contact inhibition of proliferation. This allows escape from growth control signals and triggers carcinogenesis.[9,11]

CLDN-1 expression is frequently altered in several cancers. Few studies have revealed that CLDN-1 is overexpressed in oral and cervical squamous cell carcinomas.[4] Overexpression of CLDN-1 is correlated with increase in invasive potential, with angiolymphatic and perineural invasion in OSCC. few authors also observed that inverse correlation between expression of CLDN-1 with histopathological grades of OSCC i.e., decreasing in concurrence with the increasing grades of OSCC.[12] CLDN-1 is one of the biomarkers may serve as a useful tool to detect these changes at a molecular level which have diagnostic, prognostic or therapeutic significance.[13]

In the present study, expression of CLDN-1 in OSCC cells was observed for its localization (membrane, cytoplasm, nucleus) and immunostaining intensity (weak, moderate, strong) were analysed among the clinical stages (I-IV) and histopathological grades (I-III) of OSCC.[14]

2. Materials and Methods

The present study involves the analysis of tissue samples from government dental college and cancer Hospital comprising 50 participants of OSCC. Adjacent noncancerous oral gingival mucosal tissues were served as control. The work has been approved by the competent Institutional Ethics Committee, Aurangabad. Informed consents were taken from patients and demographic data were recorded (Table 1). Clinical staging of lesions was done on the basis of tumor-node-metastasis (TNM) classification in accordance with the guidelines published by the American Joint Committee on Cancer.[15] After preliminary examination an incisional biopsy was performed to confirm the diagnosis and histopathological grading was done into three grades according to Anneroth (2005) (Tables 4, 5 and 6).

2.1. Immunohistochemical staining

Sections (4 μm) of formalin-fixed paraffin-embedded tissues were taken onto poly-L-lysine coated adhesive slides and incubated for 2 hours at 52 to 58°C. The immunohistochemical procedure was performed using the IHC Detection Kit (Cell Marque, Rocklin, CA), a three-step indirect immunoperoxidase technique resulting in an antibody- enzyme complex. Antigen retrieval was performed using AR2 solution in a microwave oven at pH 9. Endogenous peroxidase was blocked unnecessary antigen. Sections were incubated with primary antibodies against CLDN-1 (Mouse CLDN-1; Santa Cruz Biotechnology) prepared in 3% bovine serum albumin (BSA; 1:100) at room temperature in a humidifying chamber for 60 minutes. Negative control sections were incubated only with 3% BSA. The slides were then washed three times with wash buffer and subsequently incubated with the secondary antibody. It was followed by incubation with polymer HRP Label (Cell Marque) for 30 minutes. Thereafter, 3,3′-diaminobenzidine tetrahydrochloride (DAB Substrate Kit, Cell Marque) was added in the slides as a substrate chromogen solution and counterstained with hematoxylin for 10 minutes. Finally, sections were washed, dehydrated, and mounted with DPX and observed under the light microscope Leica DMLS (Leica, Wetzlar, Germany).

2.2. Evaluation of immunohistochemical staining, and scoring

Images were acquired using a light microscope equipped with a camera interfaced with a computer. Slides were scored in a blinded fashion by two independent examiners. CLDN-1 expression was evaluated semi quantitatively considering staining intensity (1 = weak; 2 = moderate; 3 = high) and percentage of positively stained cells (0 = <10% positive cells; +1 = 10 – 25%; +2 = 26 – 50%; +3 = 51 – 75%; and +4 = >76%). The final score was calculated by multiplying the intensity and percentage scores. Cases with final score less than or equal to 6 were considered to be low expressed and greater than 6 were considered to be highly expressed for both CLDN-1.

2.3. Score and statistical analysis

Scores of IHC expression were expressed as mean ± SEM. Analyses were performed using IBM SPSS statistics version 22 (IBM Corporation) for Windows. One-way analysis

of variance was used to compare the expression levels of CLDN-1 in control and OSCC. Multiple comparisons among groups were performed using Tukey's multiple comparison tests. A correlation between potential markers and categorical analysis of the patient's clinicopathological parameters were analysed by Pearson's correlation $\chi 2$ test. Survival analysis was performed using the Kaplan-Meier method, and the log-rank test was used to determine the significant differences. The level of significance was set at $P < 0.05$.

3. Result

The present study included total 50 subjects of Clinically and Histopathologically proven cases of OSCC. The peak incidence of OSCC was noted in the 5th decade. The group comprised 8 females and 42 males. In the present study, 18 (36%) patients were ≤ 45 years and the remaining 32 (64%) were above 45 years. The clinical staging was done according to the TNM system (AJCC 2006 & 2009).[16] The OSCC cases were mostly in stage IV 17(34%) and stage III 17(34%), followed by stage II 14(28%) and stage I 02(4%) respectively. The histopathological grading was done according to the anneroth grading system.[14] Most patients had grade I tumor 25(50%) followed by grade II 16(32%) and 09(18%) patients of grade III cases OSCC.

In the present study, showing anatomical site wise CLDN-1 expression in OSCC cases. There buccal mucosa was the most common site 11(22%) to be involved in OSCC patients. Within anatomical site, maximum membranous expression seen at GBC followed by floor of mouth and tongue.

Among positive cases of OSCC, strong expression of CLDN-1 was seen in 20 (43.47%) cases, whereas moderate and weak expression was evident in 18 (39.13%) and 08 (17.39%) cases respectively. Among overall cases of OSCC, 04(8%) cases showed negative Immunostaining intensity. On comparing between CLDN-1 expression and clinical staging, Chi square test revealed that the difference in localisation and Immunostaining intensity for CLDN-1 in all stage I, stage II, stage III and stage IV cases was not statistically (p=0.624) significant.

In 25(32%) cases of grade I, localisation of CLDN-1 expression varied from cytoplasmic in 14(56%) and membranous in 11(44%) cases. In grade II 100% cases showed membranous expression. 05(55.6%) cases out of 09 of grade III showed membranous CLDN-1 expression and remaining 04(44.4%) cases showed negative staining intensity.). In grade I CLDN-1 expressed strong and moderate staining intensity was observed in 19(76%) and 6(24%) cases respectively. In 16(32%) cases of grade II, intensity of CLDN-1 expression varied from strong in 1(6.2%), moderate in 12(75%) cases and weak in 3(18.8%) cases. 05(55.6%) cases out of 09 of grade III showed weak Immunostaining intensity and remaining 04(44.4%) cases showed negative staining intensity. It was seen that, as histopathological grade increased Immunostaining intensity for CLDN-1 decreased.

On comparing by Chi square test, the difference in localisation and staining intensities for CLDN-1 between all the grades (I, II, III) was found statistically highly significant (p=0. 002).

Table 1: Demographic details of OSCC patients

Variables	No of cases (%)
Age	
< 50	22(44%)
> 50	28(56%)
Gender	
Male	42 (84%)
Female	08 (16%)
Anatomical site	
Floor of mouth (FM)	8 (16%)
Buccal mucosa (BM)	11 (22%)
Alveolar mucosa (AM)	08 (16%)
Tongue	10 (20%)
Gingivobuccal complex (GBC)	10 (20%)
Palate	02 (4%)
Lip	01 (2%)
TNM staging	
I	02 (4%)
II	14 (28%)
III	17 (34%)
IV	17 (34%)
Histological grades	
I	25 (50%)
II	16 (32%)
III	09 (18%)

4. Discussion

Oral cancer is predominantly a disease of middle-aged men. Risk of oral cancer increases with age. Mean age of diagnosis of oral cancer varies from 57.1 years in males and 52.5 years in females with highest number of cases occurring in 6th decade of life.[17] In the present study, the age of the OSCC patients ranged from 27-65 years with a mean value of 48.24 ± 11.61 years. Peak incidence was seen in 5th decade.

In the present study, 18 (36%) patients were ≤ 45 years and the remaining 32 (64%) were above 45 years. Also 11 out of 18 such patients were in stage III & IV correlating with the aggressive presentation at the time of reporting. This is in accordance with previous studies where younger patients were seen to harbour disease with advanced stage.[18] In India OSCC is the most common cancer in males and third most common cancer in females, with incidence rates per 100,000 people as 12.8 and 7.5 in males and females respectively.[19] The male to female ratio varies from 1.2:1 to 9.2:1.[20] Similarly, a male preponderance was noted

Table 2: Correlation between cellular localization of theimmunohistochemical expression of claudin-1 and clinicopathological features of OSCC.

Variables	Total cases n = 50	Total Positive cases n=46	Intensity of CLDN-1		
			M n(%)	C n(%)	N n(%)
Age					
< 50	22(44%)	21(42%)	15	6	0
> 50	28(56%)	25(50%)	17	8	0
Gender					
Male	42 (84%)	38 (90.5%)	25	13	00
Female	08 (16%)	08 (100%)	7	1	00
Anatomical site					
Floor of mouth (FM)	8 (16%)	07 (87.5%)	7	0	0
Buccal mucosa (BM)	11 (22%)	09 (81.9%)	4	5	0
Alveolar mucosa (AM)	08 (16%)	08 (100%)	5	3	0
Tongue	10 (20%)	09 (90%)	7	2	0
GB complex (GBC)	10 (20%)	10 (100%)	8	2	0
Palate	02 (4%)	02 (100%)	1	1	0
Lip	01 (2%)	01 (100%)	0	1	0

Table 3: Correlation between intensity ofimmunohistochemical expression of claudin-1 and demographic features of OSCC

Variables	Total cases n = 50	Total Positive cases n=46	Intensity of CLDN-1		
			Weak n(%)	Moderate n(%)	Strong n(%)
Age					
< 50	22(44%)	21(42%)	5	8	7
> 50	28(56%)	25(50%)	3	10	13
Gender					
Male	42 (84%)	38 (90.5%)	6	13	19
Female	08 (16%)	08 (100%)	2	5	1
Anatomical site					
Floor of mouth (FM)	8 (16%)	07 (87.5%)	2	2	3
Buccal mucosa (BM)	11 (22%)	09 (81.9%)	2	1	6
Alveolar mucosa (AM)	08 (16%)	08 (100%)	0	6	2
Tongue	10 (20%)	09 (90%)	1	4	4
GB complex (GBC)	10 (20%)	10 (100%)	3	4	3
Palate	02 (4%)	02 (100%)	0	1	1
Lip	01 (2%)	01 (100%)	0	0	1

Table 4: Distribution and comparison ofImmunostaining intensity for CLDN-1 among various clinical stages of OSCC

Clinical stages of OSCC * Immunostaining intensity of CLDN-1								
Clinical Stage	**Total cases n = 50**	**Total Positive cases n=46**	**Immunostaining intensity of CLDN-1**			**p value**	**X^2**	**df**
			Weak	**Moderate**	**Strong**			
I	02 (4%)	02 (100%)	00	02	00	0.645	13.63	9
II	14 (28%)	12 (85.7%)	02	03	07			
III	17 (34%)	15 (88.2%)	02	05	08			
IV	17 (34%)	17 (100%)	04	08	05			

Table 5: Distribution and comparison of Immunostaining intensity for CLDN-1 among various histopathological grades of OSCC

Histological Stage of OSCC * Immunostaining intensity of CLDN-1								
Histological Stage	**Total cases n = 50**	**Total Positive cases n=46**	**Immunostaining intensity of CLDN-1**			**p value**	**X^2**	**df**
			Weak	**Moderate**	**Strong**			
I	25 (50%)	25 (100%)	00	06	19	0.002*	15.18	6
II	16 (32%)	16 (100%)	03	12	01			
III	09 (18%)	05 (55.6%)	05	00	00			

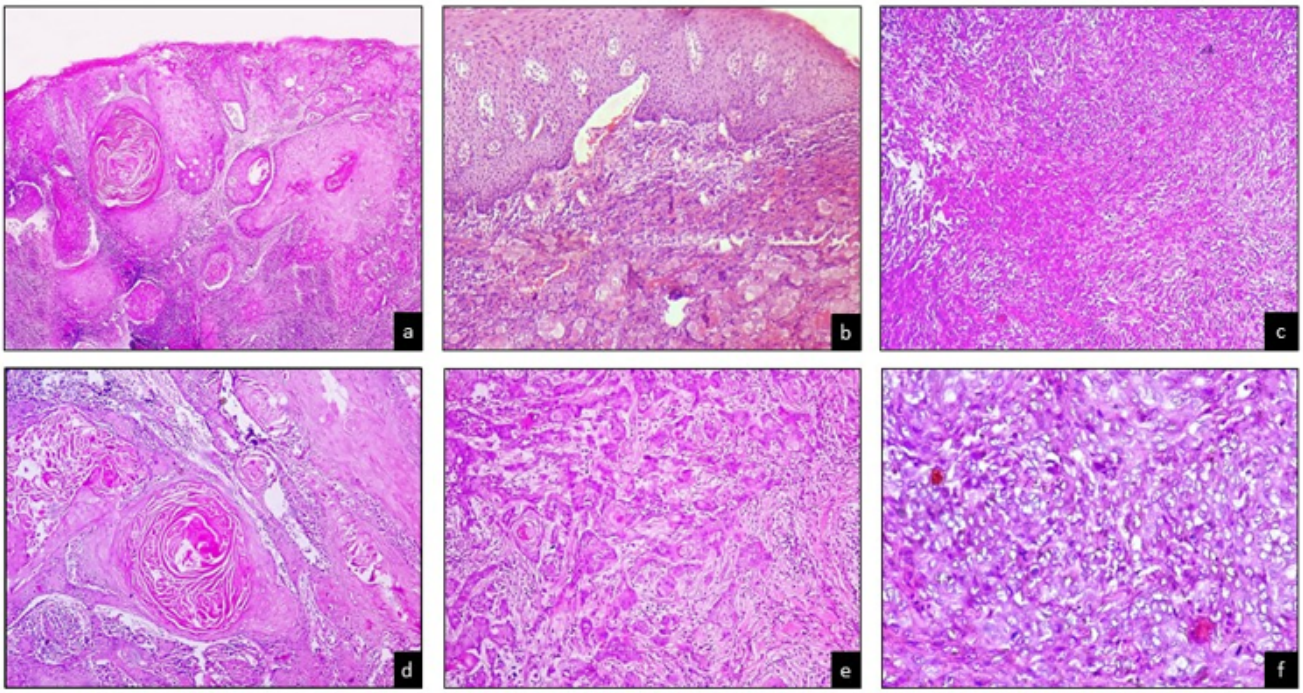

Fig. 1: Hematoxylin and eosin section of oral squamous cell carcinoma. **a** & **c** grade I. **b** & **d** grade II. **c** & **e** grade III.

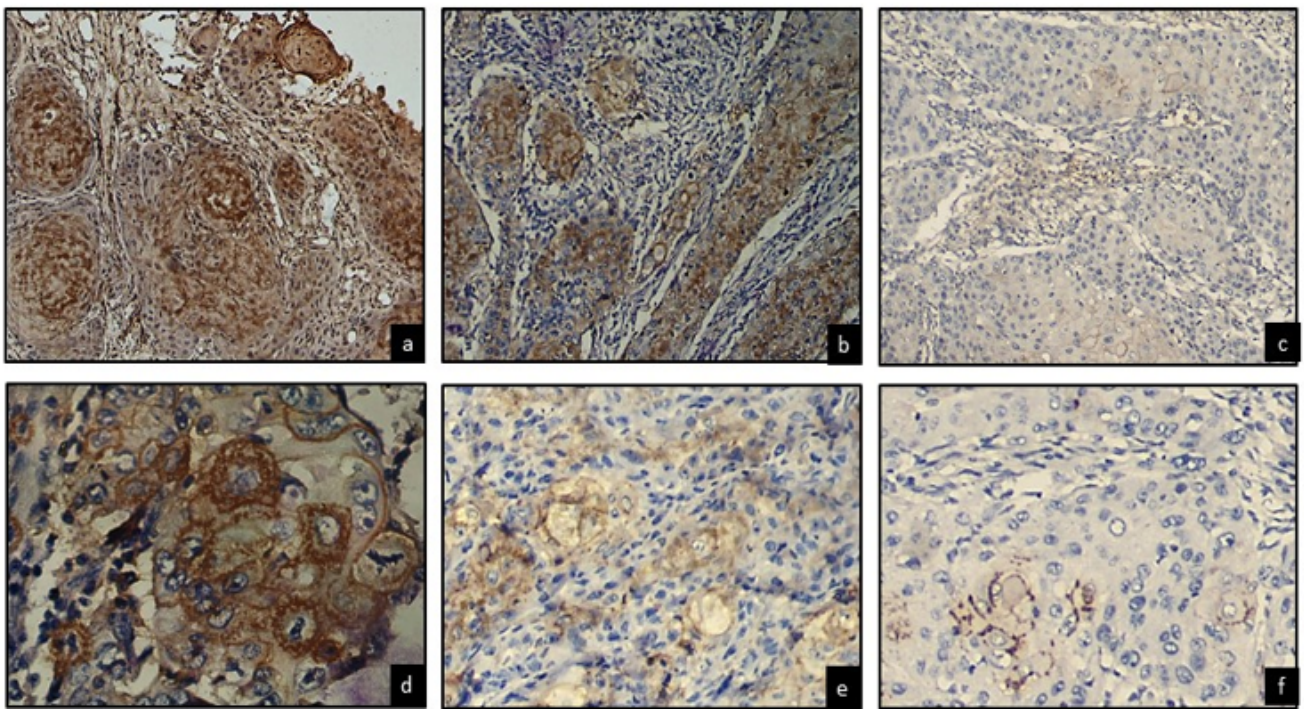

Fig. 2: Immunohistochemical expression of CLDN-1 in section of OSCC. Immuonostaining intensity of CLDN-1, strong expression in grade I (a, 100X), Moderate expression in grade III (b, 100X) and weak to negative expression in grade III (c, 1000X). Membranous and cytoplasmic localisation of CLDN-1 in grade 1 (d, 400x), predominantly membranous localisation of CLDN-1 in grade II (e, 400x) & weak membranous expression of CLDN-1 in grade III (f, 400X)

Table 6: Distribution and comparison of localization of CLDN-1 among various clinical stages of OSCC.

Clinical stages of OSCC * Localization of CLDN-1

Clinical Stage	Total cases n = 50	Total Positive cases n=46	Localization of CLDN-1			p value	X^2	df
			M n(%)	C n(%)	N n(%)			
I	02(4%)	02(100%)	02	00	00	0.624	4.45	11
II	14(28%)	12(85.7%)	07	05	00			
III	17(34%)	15(88.2%)	11	04	00			
IV	17(34%)	17(100%)	12	05	00			

Table 7: Distribution and comparison of localization of CLDN-1 among various histopathological grades of OSCC.

Histological Stage of OSCC * Immunostaining intensity of CLDN-1

Histological grades	Total cases n = 50	Total Positive cases n=46	Immunostaining intensity of CLDN-1			p value	X^2	df
			M n(%)	C n(%)	N n(%)			
I	25 (50%)	25 (100%)	11	14	00	0.000*	37.12	4
II	16 (32%)	16 (100%)	16	00	00			
III	09 (18%)	05 (55.6%)	05	00	00			

in the present study (5.25: 1). However, in gender wise distribution, peak incidence was seen in the 7th decade in females while the peak incidence was seen in the 5th decade in males in the present study. This male predilection and early occurrence may be attributable to heavier indulgence in risk habits and exposure to sunlight (as a part of outdoor occupations in case of lip cancer).[21]

According to Shenoi R, et al (2012) 25% oral cancers are attributable to tobacco usage (smoking and/or chewing), 7–19% to alcohol drinking, 10–15% to micronutrient deficiency, and more than 50% to betel quid chewing in areas of high chewing prevalence.[20] In the present study, 70% of patients had smokeless tobacco habits, 22.5% patients had smoking habits and 7.5% had both smokeless tobacco and alcohol drinking habit. These findings may suggest that, in developing countries like India, where there are high incidences of alcoholism and tobacco misuse from an early age, the incidence of OSCC may follow that trend and also affect younger individuals.

The present study comprised of 2 (4%) cases of stage I, 14 (28%) cases of stage II, 17 (34%) cases of stage III and 17 (34%) cases of stage IV emphasizing that most patients reported with advanced disease at the time of diagnosis. Histopathologically, 25 (50%) cases were of grade I, 16 (32%) cases were of grade II and 09 (18%) cases with grade III tumor.

About 92% cases of OSCC were positive for CLDN-1 in the present study, which is in accordance with high percentage of positivity reported by Bello IO, et al; (2008) 100%, Ouban A, et al; (2012) 94.73% and Jaun C. Vincente et al; (2015) 96.92%.[3,10,22] This high percentage of positivity for CLDN-1 is in contrast with studies done by Patricia P et al; (2008) and Silvia V Lourenco et al; (2010) who reported positivity of CLDN-1 in OSCC cases as 37.5% and 68.57%, respectively.[12,23]

Among positive cases of OSCC, membranous staining was evident in 64% cases which is in dissimilarity with study by Silvia V Lourenco et al; (2010) and Jaun C Vicente et al; (2015) who found membranous expression in 56% and 93.9% cases respectively.[3,12] Reason for such variation could be differences in criteria for membranous and cytoplasmic localization of CLDN-1. In study by Silvia V Lourenco et al; (2010) and Jaun C Vicente et al; (2015) two categories regarding localization of CLDN-1 were considered i.e. membranous expression and cytoplasmic expression without taking into consideration of immunoreactivity score.[3,12]

Furthermore, in the positive cases, Strong and moderate staining intensity of CLDN-1 was seen in 40% and 36% cases of OSCC respectively. These findings appeared to be in accord with Jaun C Vicente et al; (2015) who found strong staining in 52.3% cases and moderate staining in 32.3% cases.[3] In present study, weak staining intensity of CLDN-1 was evident only in 16% cases of OSCC which was contradictory to the results obtained by Silvia V Lourenco et al; (2010) who found weak staining in 50% cases of OSCC.[12]

Oku N, et al; (2006) stated that claudin-1 upregulates cancer cell invasion activity through activation of MT1-MMP and MMP-2, which results in enhanced cleavage of laminin-5 gamma2 chains by CLDN-1 necessary for invasion of cancer cells.[24] CLDN-1 overexpression was associated with advanced stage of OSCC In the current study, did not find any significant correlation (p=0.624) regarding Immunostaining intensity and immunolocalization of CLDN-1 were found among clinical stages, which is in congruence with studies by Patricia P et al; (2008), Bello IO et al; (2008), Silvia V Lourenco et al; (2010), Juan C. De Vicente et al; (2015) and Upadhaya P, et al; (2018).[3,4,12,22,23]

Expression of CLDN-1 in different grades of oral epithelial dysplasia has also been studied and found to be increasing in accordance with the increasing severity of epithelial dysplasia.[25]

In the present study, significant correlation was observed between histopathological grade of the tumor with the localization and immunostaining intensity of CLDN-1. Membrane accumulation of CLDN-1 associated with increased cellular proliferation supports the role of CLDN-1 as a prognostic marker. However, there was a direct correlation among the degree of differentiation and CLDN-1 expression based on histopathologic grades. These finding was supported with the Silvia V Lourenco et al; (2010) and Bello IO et al; (2008).[12,22] Molecular characterization of the localization of CLDN-1 clarifies the role of CLDN-1 in cancer cell survival and proliferation. This provides insight into its utility as a diagnostic and prognostic marker and its further exploitation as a target for cancer therapies. Thus, the present study showed that significant association between degree of differentiation with immunohistochemical expression of CLDN-1.

Marianne D et al. (2010) reported that the altered expression of CLDN-1 proteins in various types of cancer, indicated their involvement in tumorigenesis. Thus, these proteins might be a promising target in studies investigating both the diagnosis and prognosis of cancer and cancer therapy.[13]

5. Conclusion

Activation of this Wnt signaling pathway inhibits degradation of the pivotal component CLDN-1 and decreases its cytoplasmic and membranous accumulation and stabilization. This in turn stimulates transcription of downstream target genes leading to continuous cell proliferation.[26] Hence, determination of localization of CLDN-1 for a particular patient may be important to decide site (cytoplasmic or nuclear) for targeting CLDN-1. This targeted drug therapy for CLDN-1 may prevent worsening of the disease in the patient, resulting in better prognosis.

6. Conflicts of Interest

There are no conflicts of interest.

7. Source of Funding

None.

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