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# Immunohistochemical Evaluation of Cancer Stem Cells in Oral Potentially Malignant Disorders and Oral Squamous Cell Carcinoma Using Aldh1a1

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**Abstract:** ***The oral mucosal epithelium contains a large reservoir of epithelial stem cells necessary for tissue homeostasis. Practically Oral mucosa is continuously exposed to environmental forces and thus has to be constantly renewed and have higher risk of undergoing mutation and become Cancer stem cells which could be responsible for initiation ofOral squamous cell carcinoma, its relapse, metastasis, chemoresistance and ultimately the death of the patient. However in the present scenario little is known about Cancer stem cells (CSC) in Oral potentially malignant Disorder (OPMD) and Oral squamous cell carcinoma(OSCC). The present study was designed to detect and quantify the CSCs in OPMD and OSCC. Methods: Total of 250 samples were collected, out of which 50 samples each were of normal oral mucosa, OPMD with Moderate oral epithelial dysplasia, Well differentiated, Moderately differentiated and Poorly differentiated Oral squamous cell carcinoma. All were subjected to molecular analysis with stem cell marker ALDH1A1. Results: ALDH1A1 expression was found to increase according to progression, from Moderate oral epithelial dysplasia to higher grades of Oral squamous cell carcinoma. Conclusion: Collectively, the results unveil that ALDH1A1 Cancer stem cells is critical for malignant transformation of Oral potentially malignant lesions and for progression of this disease and could be a good prognostic marker.***

## 1. Introduction

The Oral potentially malignant disorders (OPMDs)/lesions likeleukoplakia, erythroplakia and oral lichen planus (OLP) haveincreasedchanceofconverting to malignancies. Currently it is difficult to predict exactly which lesions could progress to malignancy, although the degree of epithelial dysplasia isfrequently used for assessing the risk of malignant transformation of OPMDs.[1]

Multifactorial conditions are found to underlie the progression of OPMDs to Oral squamous cell carcinoma (OSCC) and there is currently need for better understanding and prediction of malignant transformation. According to hierarchical model, any given tumor consists of a heterogeneous population of cells, with only a small quantity of them being CSCs. [2]These small CSCs, having self-renewing property is thought to be responsible for tumor initiation and growth maintenance. [3]The hypothesized presence of cancer stem cells in dysplastic oral tissues paved way for more informed assessment of progression of potentially malignant oral lesions (PMOL). [4]Despite lot of scientific advances in stem cell and its behaviour in a number of tissues, fewer studies have been devoted to the stem cells in the oral epithelium.

The oral epithelium contains a large reservoir of epithelial stem cells that has the self renewal property. [5]As oral mucosa is continuously exposed to environmental factors, it has to constantly renew itselfand maintain tissue homeostasis. [6]It is found that multistep genetic and epigenetic changes in these basal stem cells, would result in accumulating abnormalities in the otherwise quiescent normal basal stem cells, most likely due to exposure of mucosa to carcinogens and its longer survival rate.[7]

The easiest way to identify and measure CSC within tumors or in the bloodstream is to use CSC specific or associated cell surface marker proteins. Bystaining cells with antibodies against the markers, populations of interest can be easily identified and quantified by either flow cytometry [8, 9] (which requires live cells) or immunohistochemistry (which does not require live cells). The commonly used markers to isolate CSC in normal and tumor tissue are CD44, CD24, and CD133. [10, 11]

Aldehyde dehydrogenase (ALDH) is a valid stem cell marker [12] and is known to play a impotant role in maintaining the self-renewal properties and tumorigenicity inhead and neck squamous cell carcinoma (H&NSCC) derived CSCs. [13]Aldehyde dehydrogenase 1 (ALDH1) is a cytosolic detoxifying isoenzyme which oxidizes intracellular aldehydes and thus contributes to the oxidation of retinol resulting in retinoic acid in early stem cell differentiation. This is required for the maintenance of the self renewal property. It is not only a potential marker of “stemness”, but it also plays a role in the biology of cells initiating tumor. [14]

Identifying and quantifying CSCs in patients tumors could be used to determine the relative aggressiveness of a cancer, and is of greatest importance for discovery and development of anticancer drugs targeting CSCsthat avoid potential significant side effects caused by inhibition of normal stem cell function. In the light of these factors, the aim of this research was to detect and quantify the CSCs in OPMD and different grades of OSCC using CSC marker ALDH1A1.

The study was a Case Control, cross sectional analytical study consisting of 250 old and new samples, obtained randomly from the department of Oral Pathology and Microbiology of G.D.C.R.I, Bangalore, Karnataka. The sample size was determined by setting the Type I error at 5% ($\alpha = 0.05$) and the Power of the study at 80% ($\beta = 0.2$). Eligibility was assessed by case report, history and oral examination. The presence of oral potentially malignant disorder with moderate epithelial dysplasia (MED) and Oral squamous cell carcinoma (OSCC) of grade I, II and III was verified by microscopic examination. Informed consent and Ethical Clearance were obtained.

The samples were selected into 5 groups, by simple random sampling method, using random number tables with 50

samples each in normal group (without tobacco habits, oral epithelial dysplasia or oral cancer), Group II with Moderate epithelial dysplasia (MED), Group III with Well differentiated oral squamous cell carcinoma (WDOSCC), Group IV with Moderately differentiated oral squamous cell carcinoma (MDOSCC) and Group V with Poorly differentiated oral squamous cell carcinoma (PDOSCC).

Case file was thoroughly searched for all the relevant data like age of the patient, sex and tobacco habits and the tissue samples were subjected to immune-histochemical analysis by treating with CSC marker ALDH1A1 antibody procured from Santa Cruz. From 5 groups, each case was allotted with the specific number and the diagnosis was masked and from each case 4 micrometer thick sections were obtained, one section was stained with H and E and the case confirmation was done after which remaining sections of each case were immune-stained with ALDH1A1.

The slides were observed by 2 trained oral pathologists for the features in a blinded fashion without knowledge of any patient's clinico-pathologic information. The observation was calibrated with 20% of the total cases. Each case was observed under the BX50, Olympus microscope.10 hot spots were selected from each case and in 20X all the cells with brown colour from 10 hotspots were counted with the help of progress capture software. The cells exhibiting brown colour was taken as positive. The generalized brown background was not considered as it represents background staining. The average percentage of the positive cells and their staining intensity was recorded and grading was given according to the IRS scoring system as shown in the table. The data were directly entered on the excel sheet.Negative controls included substituting the primary antisera with pre-immune sera from the same species and omitting the primary antibody. For positive control the carcinoma lymph node, carcinoma breast and esophageal carcinoma were used.

The IRS score was calculated by combining the quantity score (percentage of positive stained cells) with the staining intensity score. The quantity score ranges from 0-4 and the staining intensity score ranges from 0-3. The final IRS score was obtained by multiplying quantity score with the intensity score. The scoring method are as follows :(Remmele W, Stegner H E)[15]

**Table 1**

| Quantity score | Staining intensity score | IRS |
|---|---|---|
| 0= no positive cells | 0=No colour | 0-1=Negative |
| 1 = < 10% of positive cells | 1-Mild reaction | 2-3=Mild |
| 2 = 10-50% positive cells | 2-Moderate reaction | 4-8=Moderate |
| 3 = 51-80% positive cells | 3-Intense reaction | 9-12=Strongly positive |
| 4 = > 80% positive cells | | |

The data was analysed using the statistical analysis like Chi square test, Post Hoc Tests, Kruskal-Wallis Test – equivalent to ANOVA.Chi Square test was used to compare the categorical variables like gender and habits across the groups. Kruskal Wallis test /Anova was used to compare the continuous variables like age, and expression of markers (Anova was used for normally distributed and Kruskal Wallis test for non- normally distributed variables)

The inter observer reliability to interpret the score was done by two observers. The finding of both observers were recorded and both the intra observer and inter observer agreement was calculated using Kappa Statistics.

## 2. Results

**Demographic data** [Table.2&3]

The mean age in Normal group was 43.66±9.53, in OPMD Group was 54.46±11.43, in WDOSCC Group - mean age was 54.58±12.59, in MDOSCC Group was 57.94±13.00 and in PDOSCC Group was 57.34±13.05.

**Table 2:** Mean age distribution in designated groups in years

| Groups | Minimum[yrs] | Maximum | Mean | Std dev |
|---|---|---|---|---|
| Normal | 26 | 64 | 43.66 | 9.53 |
| OPMD | 27 | 73 | 54.46 | 11.43 |
| WDOSCC | 32 | 87 | 54.58 | 12.58 |
| MDOSCC | 20 | 82 | 57.94 | 13 |
| PDOSCC | 29 | 88 | 57.34 | 13.05 |

**Table 3:** Post Hoc Tests [Age, Bonferroni]

| (I) group | (J) group | Mean Difference (I-J) | Sig. |
|---|---|---|---|
| Normal | PM | 10.800* | 0.001 |
| | WDOSCC | -10.920* | 0.001 |
| | MDOSCC | -14.280* | 0.001 |
| | PDOSCC | -13.680* | 0.001 |
| PM | WDOSCC | -.120 | 1.00 |
| | MDOSCC | -3.480 | 1.00 |
| | PDOSCC | -2.880 | 1.00 |
| WDOSCC | MDOSCC | -3.360 | 1.00 |
| | PDOSCC | -2.760 | 1.00 |
| MDOSCC | PDOSCC | .600 | 1.00 |

**2. Gender distribution among designated groups [Table. 4& 5]**

Out of 250 cases, 121(48%) were female patients and 129(51.6%) were male patients. In normal group out of total 50 cases, 31(62%) were female and 19(38%) were male patients. In OPMD group 21(42%) were female and 29 (58%) males. In WDOSCC 25(50%) were femaleand 25(50%)were male patients. In MDOSCC 24(48%) were female and 26(52%) were male patients. In PDOSCC group 20(40%) were female and 30(60%) were males.There was no significant difference in sex distribution across the groups since p value was0.200

**Table 4:** Gender Distribution in designated groups [Crosstab]

| | | | Groups | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Normal | MOED | WDOSCC | MDOSCC | PDOSCC | Total |
| **Gender** | Female | Count | 31 | 21 | 25 | 24 | 20 | 121 |
| | | % within Group | 62.0% | 42.0% | 50.0% | 48.0% | 40.0% | 48.0% |
| | Male | Count | 19 | 29 | 25 | 26 | 30 | 129 |
| | | % within Group | 38.0% | 58.0% | 50.0% | 52.0% | 60.0% | 51.6% |
| **Total** | | Count | 50 | 50 | 50 | 50 | 50 | 50 |
| | | % within Group | 100% | 100% | 100% | 100% | 100% | 100% |

*The mean difference is significant at the 0.05 level

**Table 5**

| | Value | Df | p-value |
|---|---|---|---|
| Pearson Chi-Square | 5.990[a] | 4 | .200 |
| No of valid cases | 250 | | |

**Tobacco Habit Distribution among designated groups. [Table. 6 & 7]**

Among 250 patients, 164(65.6%) were found to have tobacco habits and 86(34.4%) did not have any tobacco habits. Out of 50 normal patients all 50(100%) did not have any habits, In OPMD group 37(74%) patients had tobacco habits and 13(26,0%) had no tobacco habits. In WDOSCC 41(82.0%) patients were found to have tobacco habits and 9(18.0%) patientsdid not have tobacco habits. In MDOSCC 43(86.0%) were found to have tobacco habits and 7(14.0%) did not have the tobacco habits. In PDOSCC 43(86.0%) were found to have tobacco habits and 7(14.0%) did not have the tobacco habits. Tobacco consumption among different groups was statistically analysed using Chi Square test. There was highly significant difference among different groups with p value of 0.001. Tobacco consumption was found to be high in subjects with OSCC.

**Table 6:** Tobacco Habit distribution among groups

| Tobacco Habits | | GROUPS | | | | | |
|---|---|---|---|---|---|---|---|
| | | Normal | MOED | WDOSCC | MDOSCC | PDOSCC | Total |
| NIL | Count | 50 | 13 | 09 | 07 | 07 | 86 |
| | % within group | 100.0% | 26.0% | 18.0% | 14.0% | 14.0%34.4% | |
| YES | Count | NIL | 37 | 41 | 43 | 43 | 164 |
| | % within group | NIL | 74.0% | 82.0% | 86.0% | 86.0% | 65.0% |
| Total | Count | 50 | 50 | 50 | 50 | 50 | 250 |
| | % within group | 100.0% | 100.0% | 100.0% | 100.0% | 100.0% | 100.0% |

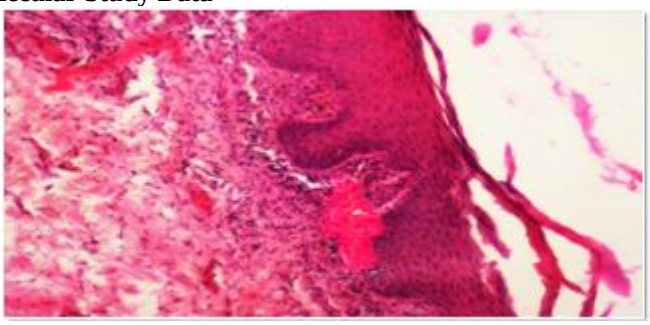
**Table 7**

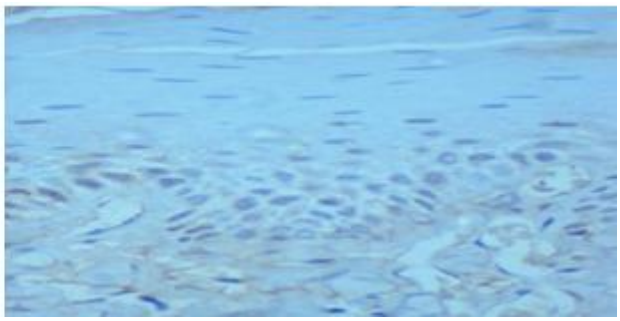
| | Value | Df | p-value |
|---|---|---|---|
| Pearson Chi-Square | 121.313[a] | 4 | 0.001 |
| No of valid cases | 250 | | |

**Aldh1a1 Expression:** [Table. 8]

There was highly significant difference in ALDH1A1 expression between normal samples and all the other groups with p value of 0.001, between MOED and WDOSCC marginal difference was noted with the p value of 0.040 where as between MOED and MDOSCC/PDOSCC highly significant difference of p value 0.001 was found. The expression did not vary between WDOSCC and MDOSCC. Marginal difference was seen between MDOSCC and PDOSCC. Expression varied significantly between WDOSCC and PDOSCC. The Mean with 95% confidence interval for expression of ALDH1A1 in normal samples was 0.66±0.24, for MOED was 1.60±0.30, for WDOSCC was 2.36±0.49, for MDOSCC was 3.18±0.64for PDOSS was 4.58±0.96. ALDH1A1 is negligibly expressed in normal buccal mucosa, and is found to increase as the disease is progressing.

**Molecular Study Data**





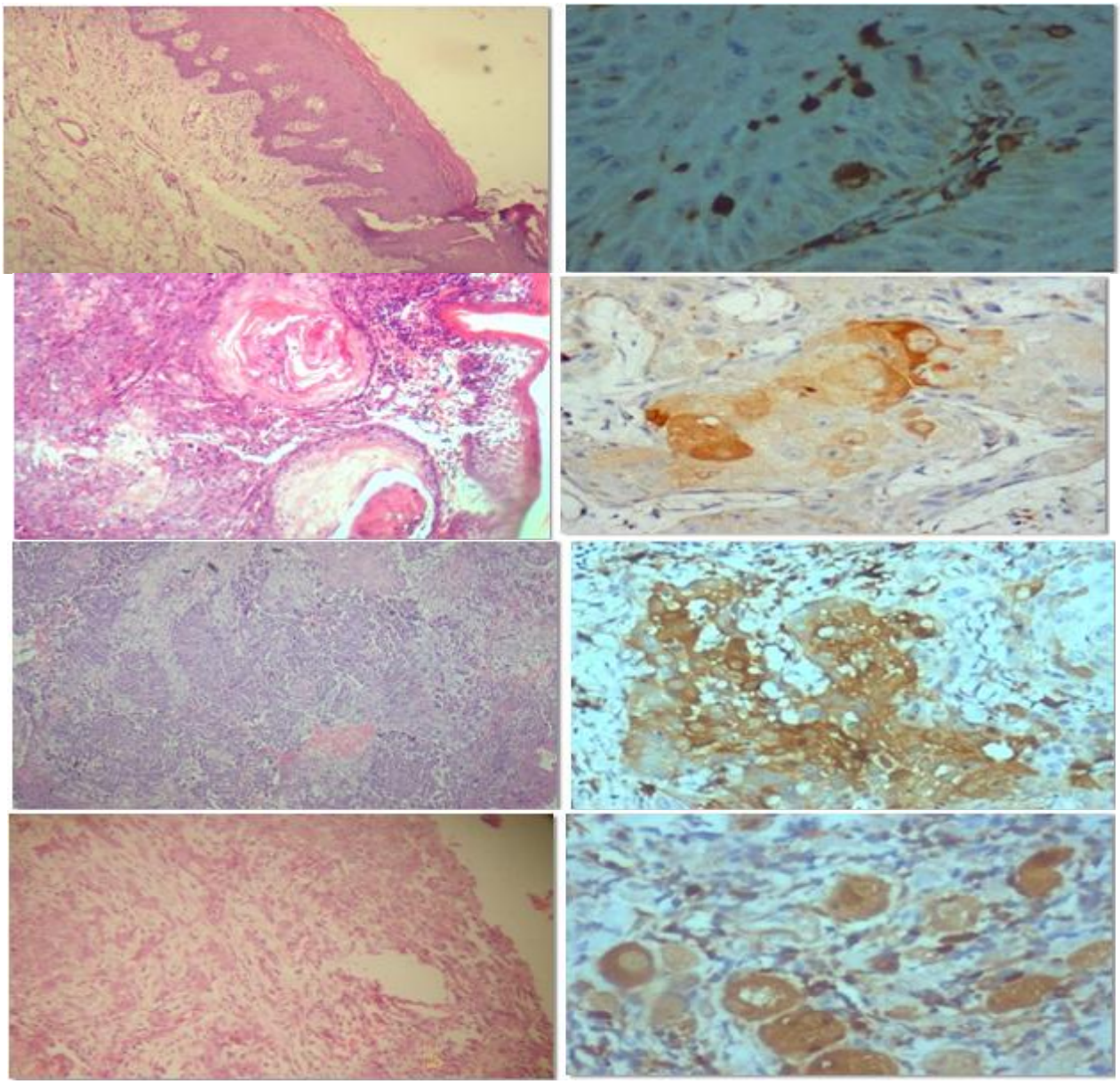


**Figure 1:** Normal epithelium : a) H & E Stained section b) ALDH1A1 Immuno-stained section,2. MOED :a) H & E Stained section,b) ALDH1A1 Immuno-stained section, 3. WDOSCC a) H & E Stained section b) ALDH1A1 Immuno-stained section, 4:a) H & E Stained section b) ALDH1A1 Immuno-stained section of MDOSCC, 5. PDOSCC: a) H & E Stained section b) ALDH1A1 Immuno-stained section.

**Table 8:** ALDH1A1Expression

| Group | No. | Mean | Median | Mode | SD | 95%CI | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| Normal | 50 | .66 | .00 | 0 | .872 | 0.66 ±0.24 | 0 | 3 |
| OPML | 50 | 1.60 | 2.00 | 2 | 1.069 | 1.60±0.30 | 0 | 3 |
| WDOSCC | 50 | 2.36 | 2.00 | 2 | 1.782 | 2.36±0.49 | 0 | 8 |
| MDOSCC | 50 | 3.18 | 2.00 | 2 | 2.327 | 3.18±0.64 | 0 | 8 |
| PDOSCC | 50 | 4.58 | 3.00 | 2 | 3.494 | 4.58±0.96 | 0 | 12 |

ALDH1A1differed significantly between ≤50 and > 50 yrs. ALDH1A1are higher among >50 yrs. ALDH1A1 did not differ significantly between males and females and with respect to habits. The data showed increased expression of ALDH1A1 in higher grades of oral squamous cell carcinoma where as these markers are mildly expressed in dysplasia and not significantly expressed in normal mucosa.

## 3. Discussion

In recent years, it is discovered that many cancers seems to be supported by cells possessing stem-like properties. According to cancer stem cell theory, tumor develops by a distinct subpopulation of tumor cells, named cancer stem cells (CSCs) with the ability to self-renew itself and to resist the to chemotherapy thus preventing the elimination of cancer. These CSCs play a major role in recurrence of cancer and metastatic spread which is a common cause of the high morbidity and death of the patients with HNSCC. Thus,

the identification and the targeted elimination of such cells have been considered as a fundamental task for cancer treatment. [16]Studies at the University of Michigan have identified many CSC markers in HNSCC like e.g. ALDH, CD44, Bmi-1. [17, 18, 19]CD133, Oct-4[7, 13]

The oral mucosa epithelium has large reservoir of epithelial stem cells necessary for tissue homeostasis. Oral mucosa is continuously exposed to environmental forces and thus has to be constantly renewed, [6]During this process multistep genetic and epigenetic changes would result in mutation due to its long survival and constant exposure to carcinogens, in contrast maximum number of cells do not exist so long to accumulate these changes as their survival rate is only 14-24 days.[7, 20, 21]Oral squamous cell carcinoma (OSCC) arise either de novo or from pre-existing leukoplakia, erythroplakia and oral submucous fibrosis.[22]

A major task for producing drugs against CSCs is to distinguish the normal stem cells from the CSCs and to understand the biology of normal stem cell and cancer stem cell along with its pathways and niches. [23]

The consideration of the present study was to find the existence of CSC, in OPMD with MOED and in progressive grades of OSCC and was found a highly significant difference in ALDH1A1 expression between normal samples and all the other groups with p value of 0.001, between MOED and WDOSCC marginal difference was noted with the p value of 0.040 where as between MOED and MDOSCC/PDOSCC highly significant difference of p value 0.001 was found. The expression did not vary between WDOSCC and MDOSCC. Marginal difference was seen between MDOSCC and PDOSCC. Expression varied significantly between WDOSCC and PDOSCC.

The mean value with the 95% confidence interval for expression of ALDH1A1 in normal samples was 0.66±0.24, for MOED was 1.60±0.30, for WDOSCC was 2.36±0.49, for MDOSCC was 3.18±0.64 for PDOSCC was 4.58±0.96. This shows that ALDH1A1 increases as the disease progresses. According to Chen, ALDH1+ cells from HNSCC have greater potential fortumor formation and are highly resistant to radiotherapy than ALDH- cells. [24]Clay et al. found that a small percentage of ALDH high tumor cells can form new tumor when transplanted into mice which is immune suppressed. [17]

Notably, it was seen that ALDH-positive cells are found basically in the basal layer of the normal oral epithelium, where stem cells of the skin and oral mucosa is normally found. In contrast, in the MOED and OSCC the ALDH-positive cells exhibited a more disperse localization [Figure. 1]. The ALDH-positive cells were in close proximity to blood vessels as explained in earlier studies that this close association of cancer stem cells and blood vessels could be due to the requirement of nutrition for the stem cells and cancer stem cells. [25]As ALDH1A1 expression was high in samples of oral dysplasia compared to the normal buccal mucosa, it can be a promising biomarker for malignant transformation of potentially malignant disorders with dysplasia as well as a prognostic marker, however future studies with more samples and advanced technique may further favour this result.

Collectively, can inference that ALDH1A1 levels increases from moderate epithelial dysplasia, through progressive grades of OSCC, ALDH1A1 + CSCs play an important role in tumorigenesis of OSCC and Increase in ALDH1A1 immuno-expression can be a predictive marker for malignant transformation of epithelial dysplasia also a prognostic marker of OSCC.

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