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A prospective immunohistochemical study using E-cadherin and N-cadherin to depict cadherin switch in oral squamous cell carcinoma.

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ABSTRACT

Introduction: A decreased expression of E-cadherin and increased expression of N- cadherin, called 'cadherin switch,' promotes an invasive oral squamous cell carcinoma (OSCC) aggressive behavior. We conducted the following immunohistochemical study to evaluate whether there was a switch in the cadherin expression in OSCC and provide data for its future prognostic predictions.

Methods: Formalin-fixed and paraffin-embedded (FFPE) tissue blocks of 30 diagnosed cases of OSCC and 30 normal tissue were prospectively selected from the Department of Oral Pathology and subjected to immunohistochemical staining of E-cadherin and N-cadherin to study the presence of cadherin switch.

Results: Cadherin switch was seen in 11 oral squamous cell carcinoma cases. All samples of the control group were negative for cadherin switch. There was also a statistically significant difference in the N- cadherin expression in the normal control group and OSCC group.

Conclusion: The findings of our study suggest that a switch from E-cadherin to N-cadherin occurs in OSCC and could play an important role in conferring an invasive and aggressive behavior to OSCC. However, its role in determining the prognosis of OSCC could not be justified.

Keywords: Cadherin, E-cadherin, N-cadherin, Epithelial-Cadherin, Oral Squamous Cell Carcinoma

INTRODUCTION

According to Globocan 2018, lip and oral cavity cancer are among the five most common cancers in India in both genders. Additionally, it ranks first among men and fourth among women [1]. In India, over 90% of the oral cancers are Oral squamous cell carcinomas (OSCCs) [2]. OSCC has emerged today as a significant public health problem in India. It is diagnosed at later stages, which results in low treatment outcomes and considerable costs to the patients [3,4]. It has a 5-year poor survival rate attributed to its high frequency of local recurrence, second primary tumors, and distant metastases. Epithelial-to-mesenchymal transition (EMT) plays a crucial role in metastasis of OSCC. In EMT, there is a development of motile cells from non-motile cells through the aberration in various molecules, like cell-surface proteins,

cytoskeletal proteins, extracellular matrix (ECM) components, and transcription factors [5,6].

Cadherins are a group of cell surface proteins amongst E-cadherin that play a crucial role in developing and maintaining epithelial cell polarity and tissue architecture [7]. Another type of cadherin is the N-Cadherin or Neural Cadherin, which is typically found, as the name suggests, in neural tissues [8].

A decreased expression of E-cadherin and increased expression of N- cadherin, called cadherin switch, is known to contribute to the development of invasive and aggressive behavior of OSCC. The term cadherin switching is also a switch from the expression of E-cadherin to an expression of any other type of cadherin, e.g., including R-cadherin, cadherin 11, T-cadherin, or even P- cadherin [9]. This process of 'Cadherin Switching' occurs via various growth and transcription factors [10].The use of E-cadherin and N-cadherin in OSCC and normal oral mucosa was executed in view to evaluate the presence or absence of a switch in the Cadherin expression and it's correlation with the increasing grades of OSCC. To this end we aimed to analyse whether this phenomenon could aid in prognostication of OSCC. The sample size of each group was determined based on availability of the subjects along with a mathematical formula of sample size determination.

METHODS

The study was conducted over a period of two years, wherein a criteria for inclusion and exclusion was applied to determine the subjects selected for study and control group. All subjects of both groups were also selected through an elimination of confounding factors like age, gender and systemic illnesses.

Study group

The inclusion criteria of this study were histopathologically confirmed cases of OSCC, healthy individuals with no bar for age and gender, and individuals willing to participate in the study procedure with written consent. The exclusion criteria were individuals having systemic illness such as diabetes and hypertension, patients undergoing chemotherapy or radiotherapy and, individuals unwilling to participate in the study.

Control group

The inclusion criteria were individuals who were healthy, with no bar for age and gender, and should not have habit history of tobacco, betel nut, and alcohol. While the exclusion criteria were individuals having any systemic illness such as diabetes and hypertension and those unwilling to participate in the study.

The study required no follow-up hence the chances of drop-outs was absent.

Tissue samples

The study protocol was reviewed and approved by the Ethics Committee of our institute. Thirty randomly selected patients of OSCC were clinically grouped using the tumor, node, and metastasis (TNM) classification [11,12]. Hematoxylin and eosin (H&E)-stained slides were examined, and the histopathologically diagnosed cases of OSCC were scored as per the criterion given by Bryne *et al*. in 1991 [13]. The scores were calculated for each H & E-stained slide, and the prognosis was determined. In the control group, thirty patients were included that comprised healthy volunteers (normal oral mucosa obtained from crown-lengthening procedure and third molar/impacted tooth removal surgery).

Immunohistochemical staining

Formalin-fixed paraffin-embedded sections of three- to four-micrometer thick were dewaxed in xylene and hydrated in graded ethanol. These slides were kept immersed in distilled water for 5 minutes. The slides were placed in a pressure cooker with the help of a metal slide rack. The pressure cooker contained a boiling antigen retrieval solution for antigen retrieval. The pressure cooker was then sealed and brought to full pressure. The pressure cooker was allowed to cool down to room temperature with the slides in the buffer solution for 15–20 mins. Endogenous peroxidase was blocked with 3% H2O2 for 10 min. The slides were gently washed with Tris buffer and placed in the humidity chamber (buffer bath) for 5 min. The excess buffer on the slides was tapped off and covered with a power block for 10 mins. The power block solution was gently tapped off the slides. The sections were covered with primary antibody (Mouse Monoclonal Antibody E-cadherin, Agilent Dako, Catalog. No. M3612, and

Rabbit Polyclonal antibody N-cadherin, Abcam Catalog No. AB18203) for 1 hour. The slides were then washed gently with Tris thoroughly twice, kept in the Tris buffer bath, and covered with a linker for 30 min. Then, sections were incubated with secondary antibody for 30 minutes followed by two consecutive buffer washes, each for 5 minutes. Horseradish peroxidase was added to the sections and incubated for 30 minutes. The chromogen diaminobenzidine (DAB) was prepared fresh, before use, by mixing one drop (approximately 20 ml) of chromogen to 1 ml of buffer and was applied over the sections. After 5 minutes, the sections were washed in buffer and water, then counterstained with haematoxylin, air dried, cleared, and mounted with dibutyl phthalate in xylene (DPX). Normal gingiva was the positive control for E-cadherin, and Colon was the positive control for N-cadherin.

Interpretation of the immunohistochemistry slides

The stained sections were scanned under low power to determine the area that stained (Fig.1). The presence of brown-coloured precipitate at the site of target antigens (cytoplasm & membrane) was indicative of positive immunoreactivity [14].

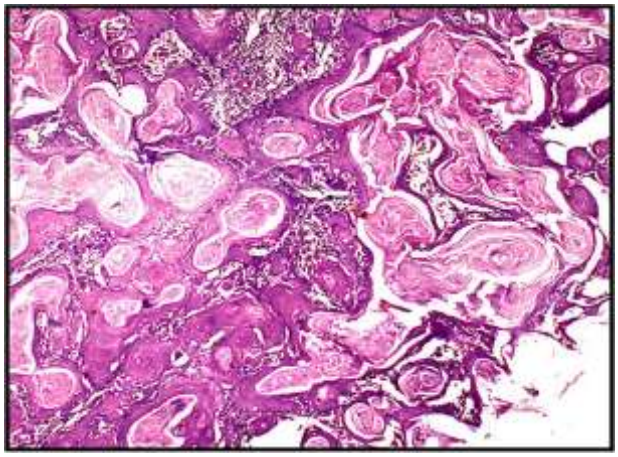

FIGURE 1: H & E-stained section of OSCC(4X)

The E-Cadherin immunohistochemically (IHC) stained positive slides were graded as follows, 0: 1%-25%, 1: 25%-50%, 3: >50% immunopositive cells. Furthermore, a correlation was made with the clinicopathological parameters [15,16]. The N-Cadherin immunohistochemically (IHC) stained slides were analyzed as, Positive: if the cytoplasmic or membranous immunostaining was in 5% or more of the epithelial tumor cells or, Negative: if the cytoplasmic or membranous immunostaining was in less than 5% of the epithelial tumor cells. The immunoexpression was correlated with clinicopathological parameters. Other factors such as staining intensity and localization pattern (cytoplasmic or membranous or both) of IHC-stained slides were also evaluated for E-cadherin and N-cadherin (Fig.2).

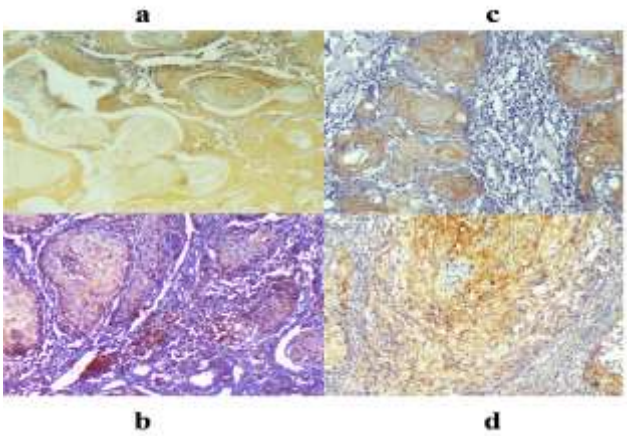

FIGURE 2: Cadherin Switch positive; a) & b) N-cadherin positive at cytoplasmic location, c) & d) E-cadherin positive in 25-50% cells and in membranous and cytoplasmic location,x100

Intensity was scored through an average wherein 0: no cells stained, 1: visible at high power magnification, X40, 2: visible at low power magnification, X10 and 3: visible at scanner view, X4 (Fig.3). Three qualified oral pathologists analyzed all IHC-stained slides along with the corresponding H & E sections. There were continuous variables for E-cadherin evaluation, however it didn't prove as an obstacle since the evaluation was subjective, a mean of the three observer scores was calculated and the final score was assigned.

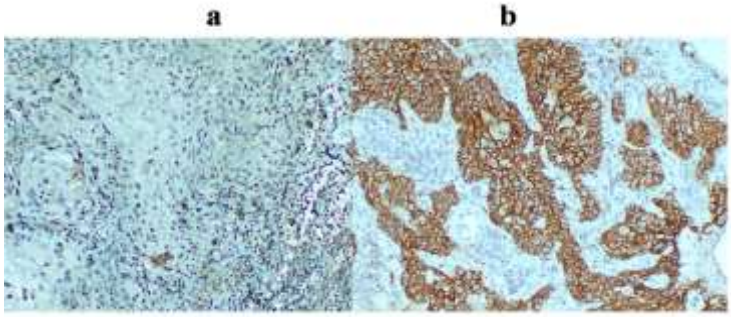

FIGURE 3: Cadherin Switch negative; a) N-cadherin negative; b) E-cadherin positive in >50% cells and membranous location,X100

Statistical Analysis

The data was entered and analyzed using the Statistical Package for Social Sciences (SPSS) for Windows 26.0. (SPSS, Inc. Chicago, Illinois) Confidence intervals were set at 95%, and a p-value ≤ of 0.05 was considered statistically significant. Both the E-cadherin and N-cadherin expressions were correlated to understand whether a switch was taking place in OSCC. Chi-square analysis was used to find the significance of study parameters on a categorical scale.

RESULTS

The gender distribution of the study group showed 20 males (66.7%) and 10 females (33.3%). The study group's age ranged from 24-75 years with a mean of 49.4 years, and the highest number of subjects belonged to 30-60 years age group**.** Among the prevalent habits in the study group, 66.67% (n=20) subjects were habituated to tobacco chewing, while 10% (n=3) subjects had a habit of tobacco chewing with smoking. Mishri usage was prevalent among 6.7% (n=2) of the study group, while one each, i.e., 3.33% each of Betel quid (Paan)(n=1) and Gutka chewing (n=1) was recorded (Table 1).

TABLE 1: Demographic data of the study group

Gender distribution in OSCC study group		
Gender	**Number of cases**	**Percentage (%)**
Male	20	66.7
Female	10	33.3
Age Group	**Number of cases**	**Percentage (%)**
20-40 years	11	36.7
40-60 years	16	53.3
> 60 years	3	10.0
Habit	**Number of cases**	**Percentage (%)**
Tobacco chewing	20	66.67
Tobacco and Paan	1	3.33
Tobacco with guthka	1	3.33
Tobacco with smoking	3	10
Mishri	2	6.7
No habits	3	10

The study group comprised 30 cases of OSCC, of which 24 cases were well-differentiated squamous cell carcinoma (WDSCC) and 6 cases were of moderately differentiated squamous cell carcinoma (MDSCC) (Table 2).

TABLE 2: Histological and immunohistochemical data of study group

HISTOLOGICAL GRADE	Number of cases	Percentage (%)
Well Differentiated	24	80
Moderately Differentiated	6	20
Poorly Differentiated	0	0

In the OSCC study group, all 30 cases showed positive staining for E-cadherin. Out of these, 9 cases showed 1-25% cells positive, 11 cases showed 25-50% cells positive, while the remaining cases showed >50% positive. In the control group, all cases showed >50% cells positive. There was a statistically significant association between the reduction in the E-cadherin expression in the control group & the OSCC study group, as $p<0.001$. Eight cases showed membranous staining, while 22 cases showed both membranous and cytoplasmic staining. Sixteen cases (53.3%) of OSCC showed mild staining, while 14 cases (36.67%) showed intense staining for E-cadherin. All of the control group cases showed intense membranous staining. In the OSCC study group, 11 cases showed positive staining for N-cadherin, while all the control samples were negative for N-cadherin expression. There was also a statistically significant difference in the N- cadherin expression in the normal control group & OSCC group ($p < 0.05$).The immunoexpression of all the 11 cases of the study group showing N-cadherin positivity was localized to the cytoplasm. The intensity of staining was mild in all the 11 N-cadherin positive cases. Hence, the cadherin switch was present in 11 cases of OSCC and was absent in all the control group samples. Cadherin switch was not statistically significant. ($p>0.05$) Statistical significant difference was found between cadherin switch & histopathological grades of OSCC according to Bryne's criteria, as $p = 0.007$. The cadherin switch from E-cadherin to N-cadherin had reached a statistically significant difference between the different clinical stages of OSCC as $p=0.021$ (Table 3).

TABLE 3: Immunohistochemical findings of Cadherin switch.

Number Of N-Cadherin Positive Cases In Different Scores Of E-Cadherin Percent Positive Cells				
E-cadherin		**N-cadherin**		**Cadherin Switch**
Percent positive cells	**Number of cases**	**Positive cases**	**Negative cases**	
0	0	0	0	0
1-25%	9	4	5	4
25-50%	11	4	7	4
>50%	10	3	7	3
Total	30	11	19	11
Comparison Of Cadherin Switch With Bryne'set al Histopathological Grading				
	Cadherin switch			
	Absent	Present		Total
GOOD	**12**	**2**		14
MODERATE	**7**	**5**		12
POOR	**0**	**4**		4
Total	**19**	**11**		30
Comaprison of cadherin switch with TNM staging				
	Absent	**Present**		Total
STAGE I	**5**	**1**		6
STAGE II	**3**	**0**		3
STAGE II	**11**	**6**		17
STAGE IV	**0**	**4**		4
Chi-square test, $p<0.05$ statistically significant.				
LOCALIZATION OF E-CADHERIN AND N-CADHERIN IN OSCC				
SCORE	**E-CADHERIN**		**N-CADHERIN**	
	Control	**Cases**	**Control**	**Cases**
NEGATIVE (SCORE 0)	0	0	30	19
MEMBRANOUS (SCORE 1)	30	8	0	0
CYTOPLASM (SCORE 2)	0	0	0	11
BOTH (SCORE 3)	0	22	0	0
INTENSITY OF E-CADHERIN AND N-CADHERIN IN OSCC				
SCORE	**Control**	**Cases**	**Control**	**Cases**
INTENSE	30	14	0	0
MILD	0	16	0	11
NO STAIN	0	0	30	19

Chi-square test, $p<0.05$ statistically significant.

DISCUSSION

Oral Squamous Cell Carcinoma(OSCC) has emerged today as a significant public health problem. It is diagnosed in later stages, resulting in low treatment outcomes and considerable costs. The 5-year poor survival rate can be attributed to its high frequency of local recurrence, second primary tumors, and distant metastases. During the development and progress of OSCC, several events take place, as the development of a limitless replicative potential, self-sufficiency in growth signals, lack of sensitivity to anti-growth signals, the ability to evade apoptosis, increased angiogenesis, invasion, and metastasis [17]. These

events involve changes in various molecules during the progression of OSCC. IHC uses these molecules as tumor markers to understand the tumor cells' origin and evaluate their distribution, localization, intensity, and quantity expressed within the tissue. These ultimately assist in arriving at an appropriate diagnosis, prognosis, and treatment modality for OSCC [18]. Amongst the various molecules used to diagnose and determine the prognosis of OSCC,cadherins are one of the most frequently used ones.

Amongst cadherins, E-cadherin is a prominent intercellular adhesion molecule and plays a crucial role in developing and maintaining epithelial cell polarity and tissue architecture. Another cadherin in the focus of research in context to tumor invasion is N-cadherin which is expressed in tissues derived from the mesoderm and neuroectoderm and not by normal squamous epithelium. 'Cadherin-switching' usually refers to a switch from the expression of E-cadherin to the expression of N-cadherin and includes situations in which E-cadherin expression levels do not change significantly. Still, the cells turn on (or increase) the expression of N- cadherin. It also includes examples in which other cadherins replace or are co-expressed with E-cadherin, including R-cadherin (Retinal cadherin), cadherin 11, T-cadherin(Truncated cadherin/ Heart cadherin), and even P-cadherin. The expression of the 'inappropriate cadherin' might alter the behaviour of the tumor cells. [19]

Cadherin switching usually occurs during embryogenesis and morphogenesis. There is a constant change of pattern of expression of cadherins during development. Also, it allows a selective population of cells to separate from neighbours due to their property selective cell adhesion. When mesodermal cells migrate into the space between the ectoderm and endoderm, they lose E-Cadherin during the migration and start acquiring N-cadherin [20]. So, based on this example, we could assume that something similar could be occurring to the malignant epithelial cells during invasion and migration into the connective tissue.

In our study, 80% (n=24) of the cases in our study were histologically graded as WDSCC, and 20% (n=6) were MDSCC. Our findings were similar to the findings of Malhotra A *et al.* (2014) [21]. In the study group (n=30), 20 (66.7%) patients were male, and 10 (33%) were female with a male-female ratio of 2:1 [22]. Similar findings were also reported in a study conducted by Singh V *et al.* (2018) [23]. The reported gender difference can be attributed to greater indulgence in the risk habits by men and exposure to sunlight (lip cancer) as a part of outdoor occupations. The patients' age ranged from 28-77 years, with a mean of 49.4 years. Although there were a significant number of patients in the age group of 4th to 6th decades of life, similar to Baruah *et al.* (2017), our findings also indicated an increasing incidence among the 3rd to 4th decades of life [24]. This shows the changing trend in the age of occurrence of OSCC, probably due to the increased usage of tobacco amongst younger age groups.

Prevalence of habits was also studied, and the highest number of subjects in the study group showed an indulgence in smokeless forms of tobacco, which accounted for 24 cases (80%). Three cases (10%) of the study group had tobacco chewing and smoking habits. 3 cases (10%) didn't have any habit history (Figure 4). Similar findings were reported in a study by Shenoi R et al. (2012), in which a maximum of their study group patients (31.86%) had a habit of tobacco chewing alone.

On clinical TNM staging, the lesions were predominantly found in stage III (17, 56.7%), followed by stage I (6, 20%), stage II (3, 10%), and stage IV (4, 13.3%). Similar results were found in a study by Shenoi R *et al.* (2014) wherein 82.37% of patients were in Stage III. 11.53% presented in stage II and 6.1% in stage IV. The reason for a maximum number of patients reporting in later stages could be due to a delay on the patient's side in seeking an opinion from a professional, or on the professional's side in making an early and accurate diagnosis, or due to a combination of both [25].

Of the 30 cases, all cases showed positive staining for E-cadherin of which, 20 cases (66.66%) showed reduced, i.e., <50% (Score 1 & 2) expression. While, (33.33%) 10 showed preserved i.e. >50% (score 3). This reduction in E-cadherin in OSCC compared to the normal mucosa was statistically highly significant ($p < 0.001$). Similar findings were reported by Angadi *et al.* (2016). The difference in localization of E-cadherin between the study group and control group had

reached a statistically significant value as p<0.001. The adhesive function of E-cadherin critically depends on the association with the cytoplasmic adaptor protein β-catenin that links the terminal tail of E- cadherin to the actin cytoskeleton. Loss of this complex predisposes to the cytoplasmic shift, leading to reduced cell adhesion.

The staining intensity of E-cadherin depends on its binding with β-catenin- a cytoplasmic adaptor protein. As the binding reduces in various histologic grades of OSCC, the staining intensity also reduces. In the present study, intense staining was seen in 14 cases, and 16 cases showed mild staining.83 However, intensity is a variable factor as IHC staining is highly technique sensitive; also, fixatives and fixing conditions can alter the expression of IHC markers [26].

N-cadherin showed increased expression in 11 cases (36.7%) in the present study. The increase in N-Cadherin expression as compared to normal mucosa was statistically significant (p=0.025). Oda et al. in 1998 analyzed gastrulation in Drosophila embryo, and it showed that *Twist,* which represses E-cadherin, can also activate N-Cadherin [27].

The N-cadherin status did not statistically significantly correlate with the histological grade (p>0.05). This finding was concurrent with that of Angadi *et al.* (2016), wherein 80% of the OSCC cases showed N-cadherin positivity, but it had not reached a statistically significant value in relation to the histological grades. The staining was localized to the cytoplasm and was mild in intensity in all cases. These findings coincide with the results from studies conducted by Gravdal *et al.* (2007) [28] and Pyo *et al.* (2007) [29], wherein both studies reported cytoplasmic staining of N-cadherin in OSCC cases.

Eight cases (26.7%) of the study group showed cadherin switch, that is, a reduced (<50%) expression of E-cadherin with an increased expression of N- cadherin. This increase in N-cadherin downregulates the expression of E-cadherin, thus resulting in a stromal-oriented adhesion profile increasing the motility and invasiveness of the malignant epithelial cells. The switch in cadherin expression from E-cadherin to N-cadherin in OSCC compared to normal mucosa was present in 11 cases and was statistically significant in our study (p= 0.025). Out of the 11 cases, 8 cases showed reduced E-cadherin while 3 cases showed preserved E-cadherin expression.

Three cases in the present study showed positive N-cadherin but did not show a reduction in E-cadherin. This is concurrent with a study done by Honda et al. (2017), in which they found a co-expression of E-cadherin and N-cadherin, with N-cadherin positive in smaller nests and E-cadherin positive in larger nests. This co-expression is explained through the fact that cadherin switch does not mean only a reduction of E- cadherin and an increase in N-cadherin, but also could be a co-expression of both molecules. Additionally, it could also involve situations where E-cadherin expression is not changed, but cells increase N-cadherin expression. The cadherin switch was correlated with histological grade, but it did not reach a statistically significant value as p>0.05.

The presence of cadherin switch associated with Bryne's histopathological grading had reached statistical significance (p=0.02). This finding is in accordance with a study conducted by Afrem *et al.* (2014). There was no statistical significance in the presence of Cadherin switch in different TNM stages (p>0.05). This is concurrent with the findings of Costa *et al.* (2014).

Many mechanisms repress E-cadherin during the switch, including mutation, DNA methylation, and transcriptional control. At the same time, N-cadherin is upregulated at a transcriptional level. However, the transcriptional regulators still need characterization. Some authors have suggested the role of Twist proteins and SNAI proteins in EMT and cadherin switch from E- cadherin to N-cadherin [30].

Additionally, post-transcriptional control of the cadherin switch is essential for maintaining the cadherin switch in the tissue. p120-catenin plays a critical role in stabilizing cadherins and regulating the quantity of cadherin in a cell. It is shown to bind to the juxta membrane domain of classical cadherins and prevent their degradation. In the absence of p120-catenin, the cadherin synthesis is not altered, but its degradation rate is increased. Certain limitations were unavoidable such as technique sensitivity of immunohistochemistry which needed false negatives to be reassessed. A larger sample size from the tumour front would have helped in a more elaborate understanding of

the role of cadherin switch in prognostication of the lesion.

CONCLUSION

This study shows that the switch from E-cadherin to N-cadherin is an important phenomenon in conferring an invasive and aggressive behavior to OSCC. However, the switch from E-cadherin to N-cadherin could not significantly predict the prognosis of the lesion. This could implicate that it may have a plausible vital role in the initial stages of OSCC. These findings can be exploited in developing and using therapies preventing this switch from E-cadherin to N-cadherin in OSCC to improve treatment outcomes.

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