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Original Research Article

Immuno histochemical analysis of nanog – A core pleuripotent protein in oral leukoplakia and oral squamous cell carcinoma

Syeda Neelam Afroze[1]*, Surekha Ramulu[1], Purnima Vadla[1], Sowmya Katheramalle[1]

[1]Dept. of oral Pathology, Mamata Institute of Dental Sciences, Hyderabad, Telangana, India.

Abstract

Background: NANOG is an important stem cell transcription factor, with a complex regulation role in human development, determining cell fate, proliferation, and death. After birth, it is expressed at very low levels or is silenced and remains in that state throughout the lifespan. However, NANOG expression is detectable in a proportion of cancer cells that exhibit stem cell-like properties. Cancer stem cells (CSCs) can be the source of malignant transformation, progression and development of metastases.Oral Squamous Cell Carcinoma (OSCC) is an extensively studied malignancy that occurs due to accumulated genetic and epigenetic changes. Hence the current study was done to evaluate role of Nanog in Oral Leukoplakia and OSCC.
Aims and Objective: The present study was done to evaluate Nanog role in Oral Leukoplakia and OSCC.
Materials and Methods: 30 normal subjects and 30 patients of Oral Leukoplakia and Oral Squamous Cell Carcinoma (OSCC) each were included in study. The cases were graded histopathologically using WHO classification for Oral Leukoplakia and Modified Broder's grading system for OSCC respectively. 30 tissue sections of Oral Leukoplakia and OSCC were subjected to ImmunoHistoChemistry (IHC) with Nanog antibody. Random fields were chosen and 300 cells were counted in five areas and mean percentage of immunopositive cells were calculated. The results were analysed using ANOVA test.
Results: The results demonstrated high mean values of Nanog in tissues with OSCC (2.60) compared to Leukoplakia (2.13) and normal tissue (0.00) with a high level of statistical significance (0.0001). There is also an increase in percentage levels of Nanog with increase in the histological grade of differentiation in Leukoplakia as well as OSCC.
Conclusion: The increased expression of Nanog in patients with OSCC was statistically significant suggesting its role as diagnostic biomarker and can be a therapeutic target for OSCC.

Keywords: Biomarker, Cancer Stem Cell, Embryonic stem cell, Oral Squamous Cell Carcinoma, Pleuripotency.

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1. Introduction

Oral squamous cell carcinoma (OSCC) is a type of malignancy that is characterized primarily by genomic instability and dysregulated cell growth caused by oncogene overexpression, suppression of tumor-suppressor genes, and numerous other genetic, epigenetic, and microRNA alterations.[1] These alterations are linked to negative prognosis in patients with OSCC, especially in cases when there is locoregional recurrence or metastasis to regional lymph nodes or distant sites.[2] Despite the recent advances in cancer therapy , the high rate of morbidity and mortality has remain unchanged. The persistent high mortality rate adjoined with local recurrence, systemic metastasis and secondary tumors necessitates for developing strategies focused on early detection.[3-4]

The probable explanations for the aggressive biological behaviour of cancer have been a thriving area of research. One notable description was the existence of cancer stem cells (CSCs) suggested by Francesco Durante in 1874.[5] According to American Association of Cancer Research "CSCs are defined as specific subset of cells with the competency of self-renewal and differentiation into different lineages that make up the tumor mass."[6] However, several studies suggest that CSCs may originate from differentiated tumour cells that have undergone dedifferentiation process obtaining more stem-like characteristics. Supplementary research shows that the CSC-like cells might be formed

*Corresponding author Syeda Neelam Afroze
Email: neelamsyeda@gmail.com

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through mechanisms associated with activation of the epithelial–mesenchymal transition (EMT) that impacts cell differentiation and tumour metastatic potential of tumors. Therefore, biology of CSC and the EMT are assumed to be mechanically related and play decisive roles in cancer progression and metastasis.[7]

Lately, there has been suggesting evidence that supports CSC theory in HNSCC. It has also been proposed that persistence of CSCs may contribute to the aggressiveness and recurrence of HNSCC. Several characteristics that distinguish CSCs from other tumor cells are their self-renewing ability to differentiate into diverse phenotype, their ability to initiate tumors even from very few cells, and improved resistance to chemotherapy.[8] Cancer stem cells normally exist in inactive G0 phase of cell cycle making them unaffected to radiotherapy and chemotherapy thus felicitating tumour progression and relapse even after vigorous conventional management.[9]

Nanog derivative from Irish folklore notion of Tír na nÓg, (Land of Eternal Youth) serves as a essential transcription factor leading pluripotency in early mammalian embryos and pluripotent stem cells. The Nanog protein, programmed by *Nanog1* gene, includes of 305 amino acids and structures a well-maintained homeodomain that co-operates with DNA. The human *Nanog1* gene (gi 13376297) is positioned on chromosome 12 and has 4 exons and 3 introns.[10] In alliance with other principal regulators of pluripotency, Nanog is influential in upholding pluripotency and forms auto regulatory loops to preserve ESC identity.[11] NANOG was first documented for its capability to facilitate mouse (m) ESC self-renewal independent of leukaemia inhibitory factor (LIF) when overexpressed in mESCs. While the disruption of the Nanog gene in mESCs destabilizes their pluripotency; mESCs can withstand their self-renewal in absence of Nanog[12] The development of induced pluripotent stem cells from somatic cells using several combinations of pluripotency factors including Oct4, Sox2 and Nanog along with auxiliary components such as c-Myc and Lin28 has generated potential for prospect applications of induced pluripotent stem cells in cell therapy.[13-14]

Carcinigenesis is a complex multi-stage progression involving genetic alterations that occur before the histopathological changes of tissue. It is pointed that these molecular modifications can be detected even at initial stages when the tissue still appears histologically normal. Consequently, identifying the crucial molecules that undergo changes at the initial stages of carcinogenesis can significantly enhance the early detection of OSCC.[14-15]

Understanding the mechanisms involving Nanog in self-renewal and differentiation is crucial for development of targeted therapy for cancers, particularly metastatic ones. Research suggests that members of Nanog family play significant role in CSCs: (1) expression of Nanog proteins is higher in several types of cancer, (2) higher levels of Nanog proteins are related with CSC-like phenotype,[16] and (3) knockdown or knockout of *Nanog* Gene can reduce malignancy. Collectively, Nanog family proteins are indispensable for maintaining the function of ESCs under normal functional conditions, as well as CSC phenotype in pathological conditions.[17] In light of this objective, the present study aimed to evaluate and compare the expression of Nanog in different histological grades of Oral Leukoplakia (OL) and Oral Squamous Cell Carcinoma (OSCC) tissue samples through immunohistochemical analysis.

2. Materials and Methods

The content and objectives of this retrospective study have received approval from the Institutional ethical committee bearing IEC number MDC_T_D158803022. A total of 30 neutral buffered formalin fixed, paraffin embedded (FFPE) tissues of Oral Leukoplakia(OL) and 30 FFPE tissues of OSCC were obtained from the Department of Oral Pathology and Microbiology, Mamata Dental College, Khammam for the comparative analysis with normal tissues. Hematoxylin and eosin (Hand E) stained slides of each case were reviewed and histologically graded OL using World Health Organization criteria and cases of OSCC were graded histopathologically using Modified Broder's grading system. The categorized slides were illustrated in **Table 1** & **Table 2**.

Two to three serial sections, each with a thickness of 3µm t were prepared from FFPE (Formalin Fixed Paraffin Embedded) tissues and taken onto silanized slides. The sections underwent deparaffinisation by placing the slides on the slide warmer at 60º C for 15-20 min followed by rehydration of the sections through 2 changes of xylene, and subsequently into absolute alcohol, 95% alcohol, 70% alcohol for 5 min respectively. Then the slides were then kept placed in distilled water for 30 seconds. Antigen retrieval was performed by placing the slides in a plastic container containing a metal slide rack which was placed in a microwave oven containing boiling Tris buffered saline (pH 9.0 - 9.2). The slides were heated four times at 100ºC for 5 minutes and were subsequently allowed to cool to room temperature.

2.1. Immunohistochemistry staining procedure

All the reagents stored in the refrigerator were allowed to reach room temperature (24º-28º C) before the immunostaining process commenced. Incubations were conducted at room temperature within a humidifying chamber. The sections underwent washed gently in Phosphate Buffered Saline (PBS) three times each lasting 2 minutes. To inhibit endogenous peroxidase activity, slides were treated with 0.3% H2O2 for 15-20 minutes followed by three gentle washes with PBS, each lasting two minutes. After removing off the excess buffer from the slide, the sections were covered with Power Block (Contains casein and proprietary additives in phosphate buffered saline with 15mM sodium azide) for 15-20 minutes. The primary

antibody was applied after Power Block was removed; the sections were fully covered with pre-diluted Nanog primary antibody except for the negative control which consisted of non- immune serum in Phosphate Buffered Saline with 0.09% sodium azide. The slides were incubated for 1 hour at 21ºC in a humidifying chamber followed by gentle washing with PBS three times for 2 min each. Subsequently, Super Enhancer was applied and left for 30 minutes, and were washed again with PBS three times for 2 minutes each.

A MultiLink secondary antibody which is a pre-diluted biotinylated anti-immunoglobulin solution in phosphate buffered saline containing a carrier protein and 0.09% sodium azide, was applied after removing the excess buffer, the sections were then incubated with secondary antibody for 30 minutes. Following this, the slides were gently washed with Phospate Buffer Saline three times, each wash lasting 2 min each. After tapping off the excess buffer, the tissue sections were entirely covered with a freshly prepared substrate chromogen solution (1 ml DAB buffer with 2 drops DAB chromogen) using Pasteur pipette for 10 minutes. The sections were then washed gently with distilled water for 2 minutes. Counterstaining was performed by immersing the slides in Mayer's hematoxylin to achieve bluing. Dehydration of the tissue sections was carried out by sequentially immersing them in absolute alcohol, 95% alcohol, 70% alcohol for 5 minutes each. The sections were subsequently placed in xylene bath and were finally mounted using DPX (Lendrum Di-n-butyl Phthalate in Xylene).

2.2. Interpretation of staining

The presence of a brown- coloured end product at the site of target antigen indicated positive immunoreactivity. In contrast, the negative control tissue (Normal mucosal tissue omitting the primary antibody) demonstrated absence of staining. Brain tissue served as positive control (**Figure 1**) and normal oral mucosal tissue was utilized as negative control. (**Figure 2**) The evaluation of study cases was conducted in a similar manner, categorizing them as positive or negative. To quantify the Nanog stained slides, 300 cells were manually examined across at least five areas and a mean percentage of positive–stained slides was calculated. Each sample was then assigned a staining scores based on the following criteria: 0 for less than 10%, 1 for 11 to 25%, 2 for 26 to 50%, 3 for 51 to 75%, 4 for 76 to 90% and 5 for 91 to 100% (Fedchenko et al., 2014).Two observers independently examined the slides to mitigate interobserver bias. The intraclass correlation coefficient (ICC) analysis indicated a strong agreement between two observers (ICC = 0.9). The results were expressed in percentages and statistically analysed using ANOVA test analysis with Social Sciences software, version 20.0 (IBM Corp. Released 2011, IBM SPSS Statistics for windows version 20.0, and Armonk, NY: USA). A p-value of 0.0001 was deemed statistically significant.

3. Results

Thiry tissues of Oral Leukoplakia (n=30) and Oral Squamous Cell Carcinoma (n=30) each were analyzed for the immuohistochemical expression of Nanog, with comparisons made to normal tissues (n=30).

In the cohort of 30 cases of Oral Leukoplakia, there were 13 cases (43.33%), classified as mild dysplasia, 10 cases (33.33%) as moderate dysplasia and 7 cases (23.33%) as severe dysplasia. In the carcinoma group, among 30 cases in carcinoma, 12 cases (40%) were categorized as Grade I, 10 cases (33.33%) as Grade II, 8 cases (26.67%) as Grade III and no cases (0%) as Grade IV.

Within the dysplasia group, the scoring distribution was as follows 0 in 3 cases(10%), 1 in 11 cases (36.67%), 2 in 7 cases (23.33%), 3 in 2 cases (6.66%), 4 in 2 cases (6.66%) and 5 in 5 cases (16.66%). In the carcinoma group, the scoring for total 30 cases, revealed to be 0 in 1 case (3.33%), 1 in 7 cases (23.33%), 2 in 7 cases (23.33%), 3 in 7 cases (23.33%), 4 in 2 cases (6.66%) and 5 in 6 cases (20%). In the normal group, all 30 cases (100%) scored 0. A statistical comparison of staining scores among normal, dysplasia and carcinoma groups yielded significant results with a *P* value of 0.00001.(**Table 3** and **Figure 2**)

Among 13 cases of mild dysplasia, the staining scores were 0 in 3 cases (23.07%), 1 in 10 cases (76.9%). In 10 cases of moderate dysplasia, the staining score were found to be 1 in 1 case (10%), 2 in 7 cases (70%) and 3 in 2 cases (20%). In 7 cases of severe dysplasia, the staining scores were 4 in 2 cases (28.57%) and 5 in 5 cases (71.43%). A statistically significant difference was observed between various histopathological grades of dysplasia with respect to immunohistochemistry (IHC) scores with a $P = 0.00001$. (**Table 4** and **Figure 3**)

Of 12 Grade I cases of OSCC, the staining score was observed to be 0 in 1 case (8.33%), 1 in 7 cases (58.33%), 2 in 4 cases (33.33%). In 10 cases of Grade II, the staining score was 2 in 3 cases (30%) and 3 in 7 cases (70%). In 8 cases of Grade III, the staining score was found to be 4 in 2 cases (25%), 5 in 6 cases (75%). A statistically significant difference was noted across various histopathological grades of OSCC with respect to IHC scores with a $P = 0.00001$ (**Table 5** and **Figure 4**).

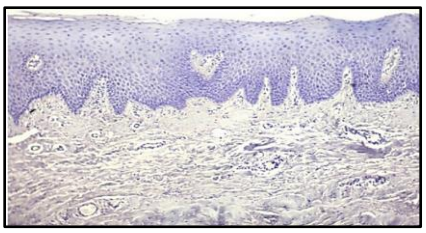

Figure 1: Photomicrograph of Brain as a positive control for Nanog expression (10X).

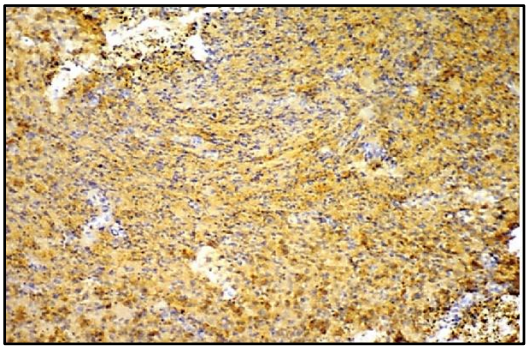

Figure 2: Photomicrograph of Normal Oral Mucosa as a negative control for Nanog expression (10X)

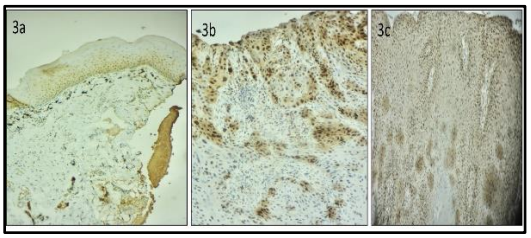

Figure 3: Photomicrograph showing Nanog expression in different grades of Oral Leukoplakia (3a- mild, 3b- moderate and 3c – severe dysplasia, 10X).

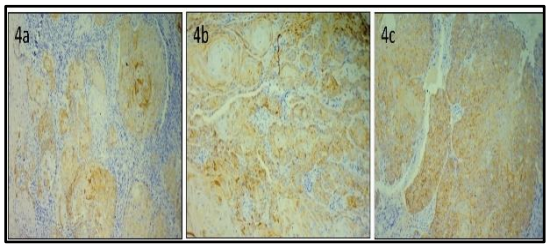

Figure 4: Photomicrograph showing Nanog expression in different grades of Oral Squamous Cell Carcinoma (3a- Well, 3b- Moderate and 3c – Poorly differentiated OSCC 10X).

Table 1: Number of cases in OED based on histological grading

Histological grade	**No. of cases**
Mild Dysplasia	13
Moderate Dysplasia	10
Severe Dysplaisa	7

Table 2: Number of cases in OSCC group based on Histological grading

Histological grade	**No. of cases**
Well Differentiated	12
Moderately Differentiated	10
Poorly Differentiated	8

Table 3: Comparison of normal, oral epithelial dysplasia(OED) and oral squamous cell carcinoma(OSCC) groups for nanog expression with respect to staining intensity scores using ANOVA test.

Groups	**N**	**Mean**	**SD**	**U-value**	**P-value**
OSCC group	30	2.67	1.52	145.00	0.0001*
OED group	30	2.13	1.63		
Normal group	30	0	0		

Anova test *p <0.05 - significant.

Table 4: Comparison of various histological grades of dysplasia (Mild, Moderate, Severe) for Nanog expression with respect to staining intensity score.

Histopathological grading	**Mean**	**SD**	**P-value**
Mild Dysplasia	0.77	0.44	0.0001*
Moderate Dysplasia	2.10	0.57	0.0001*
Severe Dysplasia	4.71	0.49	0.0001*

Kruskal wallis anova test *p <0.05 - significant.

Table 5: Comparison of various histological grades of OSCC (Well, Moderate, Poor) for Nanog expression with respect to staining intensity score.

Histopathological grading	**Mean**	**SD**	**P-value**
Well Differentiated OSCC	1.38	0.91	0.001*
Moderately Differentiated OSCC	2.98	0.56	0.001*
Poorly Differentiated OSCC	4.57	0.54	0.001*

Kruskal wallis anova test *p <0.05 - significant.

4. Discussion

Research into distinct characteristics of embryonic stem cells have led to the identification of three core transcription factors that are essential for the maintenance of ES cells: Oct4, Sox 2 and Nanog.[18] NANOG is a transcription factor that is vital during embryonic development and serves as a principal regulator of pluripotency in both embryonic stem cells,[19,13] adult stratified epithelia, including oral mucosa.[20] The NANOG gene is located on the 12 chromosome specifically at 12p13.31.[21]

NANOG is a significant stem cell transcription factor, playing a multifaceted role in human development, determining cell fate, proliferation, and apoptosis. Following birth, its expression diminishes signicantly or is silenced and remains in that state throughout an individual's life.[22] NANOG is primarily downregulated by OCT4, although its expression can persist even in the absence of OCT4.[19] The discovery of downstream regulatory pathways influenced by NANOG reveals its involvement in several biological processes associated with cancer progression, such as self-renewal, tumor cell proliferation, motility, epithelial-

mesenchymal transition, evasion of immune response, and drug resistance, all of which are characteristic features for CSCs.[23-24]

In view of the current concept of cancer pathogenesis, the cancer stem cell hypothesis, a definite subset cells named as cancer stem cells (CSCs) or Tumor Initiating Cells (TICs) is documented for its substantial involvement not only in in tumor origination and maintenance, but also in tumor aggressiveness, microenvironment variation, evasion of apoptosis, and metastatic dissemination.[25-29] Cancer stem cells (CSCs) are regarded as as a characteristic subpopulation in cancers that possess the capability to initiate neoplasm and sustain tumour self-renewal.[30] Also, self-renewal is the trademark of embryonic stem cells (ESCs), specifying the potential existence of shared molecular mechanism between CSCs and ESCs.[31] The molecular pathway leading self-renewal in normal stem cells appears to modulate CSCs in tumours.[32]

The prime transcription factors NANOG, OCT4, and SOX2 are indispensable to maintain the pluripotency and self-renewal capability in both embryonic and adult stem cells. Additionally, these factors are strategic regulators of CSCs properties and self-renewal in head and neck squamous cell carcinomas (HNSCC).[33] Especially, NANOG expression has increased in different types of cancers including OSCC, and its overexpression has been linked with poor differentiation status, poor prognosis, and resistance to chemotherapeutic drugs,[33-34], suggesting that NANOG may promote aggressive tumor phenotypes.[35] Increased NANOG expression has been recognized in various cancer types (lung, breast, colorectal, etc.), including in OSCC, and it is pointed that it can be a valuable prognostic biomarker.[21,22,36,37,38]

The concept of CSC has been validated in studies related to leukemia[39] and breast cancer[40-41] however research regarding the involvement of CSC in the onset and involvement of oral cancer remains in early stages. Numerous researchers have concentrated on examining the expression of CSC markers and their prognostic value in normal oral mucosa and OSCC.

Gawlik-Rzemieniewska *et al*.[36] conducted a review on the role of NANOG in several cancer related mechanisms such as cancer cell proliferation, epithelial–mesenchymal transition (EMT), apoptosis and metastasis. They also suggested a connexion between NANOG and signal transducer and activator of transcription 3 (STAT3) which plays a key role in the maintenance of cancer stem cell properties and multidrug resistance. They also demonstrated that NANOG is complexly involved in the process of carcinogenesis and is a prospective prognostic marker of malignant tumours.

Moon JH et al[42] described the function of Nanog in cancer stem-like cells. Using primary murine p53-knockout astrocytes (p53(-/-) astrocytes), they established that Nanog expression can escalate the cellular growth rate and transform phenotypes in vitro and in vivo. Further they also suggested that Nanog drives p53 astrocytes toward a dedifferentiated, CSC-like phenotype with distinctive neural stem cell/progenitor marker expression, neurosphere formation, self-renewal activity, and tumor development. These results demonstrated that Nanog enables the dedifferentiation of p53-deficient mouse astrocytes into cancer stem-like cells by changing the cell fate and altering cell properties.

Miyazawa et al[43] studied the immunohistochemical expression of Nanog, octamer 4 (OCT4), cluster of differentiation 133 (CD133) and NESTIN, which are all CSC markers, in relation to prostate carcinogenesis. The CSC markers, in specific OCT4 and Nanog, showed immune histochemical expression in prostate cancers. Furthermore, HIF-1α expression affected Nanog and/or OCT4 expression. The findings suggest that Nanog expression also could be a biomarker for the diagnosis of prostate cancer, and the coexpression of Nanog and HIF-1α may be involved in prostate carcinogenesis.

Many recent studies have suggested a direct correlation between elevated NANOG expression in OSCC specimen and poor histological differentiation, advanced clinical stage tumours and higher incidence of neck node metastasis, resulting in poor overall survival rates.[44] In the present study, an attempt was made to evaluate the expression of Nanog immunohistochemically in various histopathological grades of Oral Leukoplakia and oral squamous cell carcinoma. The results from the present study showed an increased expression of Nanog with respect to various clinical and histopathological grades, respectively.

In the present study, an evaluation of NANOG expression was conducted immunohistochemically across various grades of Oral Leukoplakia and OSCC. The results demonstrated a significant increase in NANOG expression corresponding to different clinical and histopathological grades. Statistical analysis revealed a significant difference in expression between normal oral mucosa and OSCC with a *P* value of 0.001. Fu *et al*.[45] observed increased expression of Nanog in cancer cells and corresponding tumour-associated normal tissue (CTAN) of OSCC patients when compared to normal mucosa which aligns with the findings of the present study.

NANOG protein was detected in 60% of laryngeal dysplasias, with 27% of dysplasias showing strong immunostaining for NANOG. Five years after the initial diagnosis, only 20% of patients with negative to moderate NANOG expression and 55% of patients with strong NANOG expression developed laryngeal cancer.[46] In oral dysplasia, NANOG protein expression was significantly correlated with higher risk of progression to invasive carcinoma and higher cancer incidence with a stronger cytoplasmic reaction.[47] Other studies also showed NANOG protein expression to increase with the grade of dysplasia,

and NANOG protein expression in 31–100% of OSCC samples with immunostaining of various intensity.[22,37] This goes very well in accordance with the present study which evaluated the Nanog expression in Oral Dysplasias.

Habu N et al[27] studied and the expression of the CSC markers was examined by semi-quantitative RT-PCR and immunocytochemistry. *In vitro* proliferation, migration, and invasion assays were conducted to assess cellular behaviors. Clinicopathological factors and immunohistochemical expressions of Oct3/4 and Nanog were assessed using surgical specimens from 50 patients with stage I/II TSCC. The results suggest that Oct3/4 and Nanog represent probable CSC markers in HNSCC, which contribute to the development of DNM in part by increasing cell motility and invasiveness.

In the present study, the results were found to be statistically significant with a *P* value of 0.0001 with respect to the expression in various histological grades of OSCC. The results of our study were consistent with the previous studies conducted by Kim *et al*[44] and Watanabe *et al*.[47] where it was revealed that poorly differentiated OSCC shows increased expression of Nanog when compared to well-differentiated OSCC. Also the undifferentiated cancer cells overexpressing NANOG are important for metastatic OSCC. Therefore, targeting NANOG protein might be a beneficial strategy for the treatment of OSCC metastasis.

Jeter *et al*.[48] proposed that Nanog exhibits protumorigenic characteristics indicating that NANOG-mediated oncogenic reprogramming may contribute to the clinical manifestations of malignant diseases.They suggested that Nanog enhances the molecular mechanisms involved in tumorigenesis, and potentially serving as a new therapeutic target or biomarker for cancer diagnosis, prognosis and treatment outcomes.

5. Conclusion

The study led to following conclusions:

1. The findings suggested statistical significance in the expression of NANOG in normal subjects, patients with OL and patients with OSCC.
2. Expression of NANOG exhibited variation with different histopathological grades of epithelial dysplasia and OSCC.
3. Increased IHC scores correlated with higher grades of dysplasia and OSCC suggesting its role as a prognostic marker.
4. A statistically significant difference was observed in the staining scores between different histological grades of Oral Leukoplakia and OSCC.

6. Source of Funding

None.

7. Conflict of Interest

None.

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