Original Article

Elucidating the immunohistochemistry of Nanog: A transcription marker in the oral squamous cell carcinoma with emphasis on its origin as embryonic stem cell

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Abstract

Background: Nanog is a key transcription factor regulating pluripotency in mammalian early embryos and pluripotent stem cells. Nanog plays a central role in pluripotency and forms autoregulatory loops to maintain ESC (embryonic stem cell) identity. 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 OSCC.

Objective: The present study was done to evaluate Nanog role in OSCC.

Materials and Methods: Thirty normal subjects and 30 patients of oral squamous cell carcinoma (OSCC) were included in study. The cases were staged clinically based on tumour node metastasis (TNM) staging and graded histopathologically using modified Broder's grading system. Thirty tissue sections of 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 a statistically significant difference between normal subjects and in patients with OSCC with respect to mean of IHC score ($P = 0.0001^{*}$). High mean values for Nanog in tissue with OSCC in both histopathological ($P = 0.0001^{*}$) and clinical grading ($P = 0.0276^{*}$) with statistically significant result were observed.

Conclusion: The increased expression of Nanog in patients with OSCC was statistically significant, suggesting its role as diagnostic biomarker. Statistically significant result with respect to clinical staging and histopathological grading of Nanog expression in patients with OSCC suggests its role as prognostic biomarker also.

Keywords: Biomarker, cancer stem cell, embryonic stem cell, oral squamous cell carcinoma, pleuripotency

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Head and neck squamous cell carcinoma, including oral squamous cell carcinoma (OSCC), is the sixth most

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prevalent malignancy worldwide and the third most common cancer in developing nations.[1] The prognosis of OSCC remains dismal because more than 50% of patients die of this disease or complications within 5 years under current therapies.[2] Since the traditional histological grading systems are limited in their ability to accurately predict the tumour aggressiveness and prognosis, various studies have been undertaken for the detection of more effective prognostic markers in OSCC patients.[3]

Embryonic stem cells (ESC), derived from the inner cell mass of the early embryo, are a vital tool for studying early developmental processes and cell-fate decisions. They have the ability to propagate indefinitely (self-renewal) in an undifferentiated state and have the potential to specify cell types of all three germ layers (pluripotency).[4]

Recent findings show that transcription factors form the core regulatory machinery components involved in gene expression maintenance and epigenetic regulation of pluripotency.[5] Core pluripotency transcription factors that include Nanog,[6] Oct4[7] and Sox2[8] collaboratively form a strong self-reinforcing regulatory network that serves to govern the stable expression of self-renewal factors and repression of genes that promote differentiation. The generation of induced pluripotent stem cells from somatic cells using various mixtures of pluripotency factors like Oct4, Sox2 and Nanog coupled with accessory components such as c-Myc and Lin28[9,10] has created promise for the eventual use of induced pluripotent stem cells in cell therapy.[11]

Nanog (from Irish mythology Tír na nÓg, Land of Eternal Youth) is a key transcription factor regulating pluripotency in mammalian early embryos and pluripotent stem cells.[12] Nanog protein, coded by *Nanog1* gene, consists of 305 amino acids and contains conserved homeodomain that binds to DNA. Human *Nanog1* gene (gi 13376297) is localized on chromosome 12 and consists of 4 exons and 3 introns.[12] Cooperating with other master regulators of pluripotency, Nanog plays a central role in pluripotency[12] and forms autoregulatory loops to maintain ESC identity.[13] NANOG was initially identified from its ability to confer mouse (m) ESC self-renewal without dependence on leukaemia inhibitory factor (LIF) when overexpressed in mESCs.[6] Disruption of the Nanog gene in mESCs compromises their pluripotency[6]; however, mESCs can maintain their self-renewal without Nanog.

Similar to ESCs, cancer stem cells (CSCs) are cancer cells that possess characteristics associated with normal stem cells, including self-renewal and differentiation into multiple cell types. These features also characterize embryonic stem cells, thus suggesting common molecules might exist between CSCs and ESCs.[14] However, several studies seem to support the theory that CSCs arise from differentiated tumour cells that have undergone a process of dedifferentiation to become more stem-like. Evidence from other studies also indicated that the CSC-like cells might be generated with processes that are related to the activation of the epithelial–mesenchymal transition (EMT), which impacts cell differentiation and tumour metastatic potential. Thus, CSC biology and the EMT are thought to be mechanistically correlated and may be key components of cancer progression and metastasis.[15] It is hypothesized that CSCs are one of the major causes of tumour relapse and metastasis by developing new tumour.

Therefore, understanding the Nanog-involved mechanism underlying CSC self-renewal and differentiation is essential for developing specific therapy against cancers, especially metastatic cancers. It has been reported that Nanog family members are critical for CSCs: (1) expression of Nanog proteins is increased in many types of cancer, (2) enhanced levels of Nanog proteins are related with CSC-like phenotype,[16] and (3) knockdown or knockout of *Nanog* gene could reduce cancer malignancy. Altogether, Nanog family proteins are pivotal to maintain the function of ESCs under physiological conditions, as well as CSC phenotype under pathological conditions.[17] Nanog is highly expressed in cancer stem cells and may thus function as an oncogene to promote carcinogenesis. High expression of Nanog correlates with poor survival in cancer patients.[17]

Aims and objectives

Hence, the present study was undertaken with the following purpose

1. To compare the expression of Nanog in normal subjects and in patients with OSCC.
2. To correlate the expression of Nanog with respect to the clinical staging of OSCC.
3. To evaluate the expression of Nanog with respect to different histopathological grades of OSCC.

MATERIALS AND METHODS

Thirty patients of oral squamous cell carcinoma (OSCC) visiting the outpatient department of Mamata Dental College, Khammam, were included for the purpose of this study. The content and purpose of this retrospective study have been approved by the Institutional Ethical Committee with IEC number MDC_T_D158803022. The history and clinical findings of each patient were recorded in a prescribed proforma. These cases were staged clinically

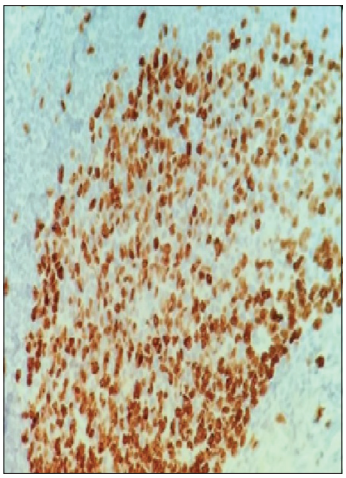

Figure 1: Photomicrograph of breast cancer tissue as a positive control for Nanog expression (10×)

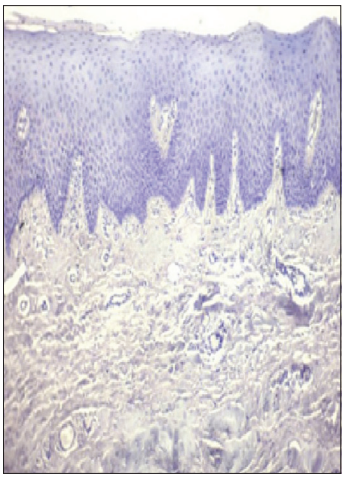

Figure 2: Photomicrograph of normal oral mucous tissue as a positive control for Nanog expression (10×)

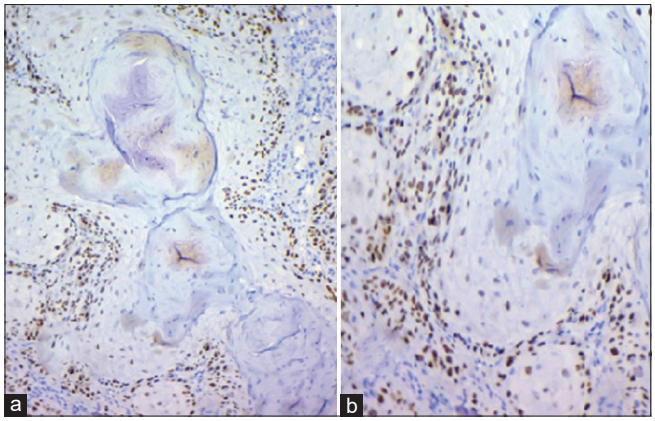

Figure 3: Photomicrograph showing Nanog expression in well-differentiated OSCC (a: 10×, b: 40×)

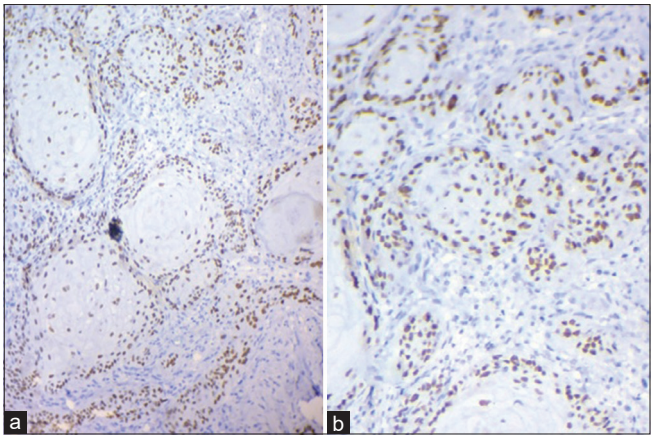

Figure 4: Photomicrograph showing Nanog expression in moderately differentiated OSCC (a: 10×, b: 40×)

based on tumour node metastasis (TNM) staging[18] and graded histopathologically using modified Broder's grading system.[19] Thirty apparently normal subjects were included as controls for the study.

Immunohistochemical study: Three—four μm thickness of paraffin-embedded tissue sections from each block was taken onto silanized slides and subjected to immunohistochemistry using Nanog monoclonal antibody (Bionova system). The sections were deparaffinised by keeping the slides on the slide warmer at 60°C for 15–20 min. Rehydration was done by taking the tissue sections through two changes of xylene, absolute alcohol, 95% alcohol, 70% alcohol for 5 min, respectively. Then, the slides were kept immersed in distilled water for 30 s.

Antigen retrieval was done by placing the slides were placed in a plastic container containing a metal slide rack which in turn was kept in a microwave oven containing boiling Tris-buffered saline. The slides were heated four times at 100°C for 5 min. All the slides were allowed to cool to room temperature. All the reagents stored in the refrigerator were brought to room temperature (24–28°C) prior to immunostaining. All the incubations were performed at room temperature using a humidifying chamber. At no time the tissue sections were allowed to dry during the staining procedure. Sections were washed gently with PBS three times for 2 min each. After tapping off the excess buffer from the slide, the sections were covered with 3% hydrogen peroxide for 15–20 min. They were then washed gently with PBS three times for 2 min each. After tapping off the excess buffer from the slide, the sections were covered with Power Block for 15–20 min. After Power Block was tapped off, the sections were covered completely with pre-diluted Nanog primary antibody except for the negative control. The slides were incubated for 1 h at 21°C in a humidifying chamber and then washed gently with PBS three times for 2 min each. Then, Super Enhancer was applied and left for 30 min and then washed gently with PBS three times for

2 min each. After tapping off the excess buffer, the sections were then incubated with secondary antibody for 30 min and then washed gently with PBS three times for 2 min each. Excess buffer was tapped off and tissue sections were completely covered with freshly prepared substrate chromogen solution using Pasteur pipette for 10 min. Then, the sections were washed gently with distilled water for 2 min. The sections were counterstained by immersing them in Mayer's haematoxylin for 2 min and then washed gently under running tap water for bluing. Dehydration was done by taking the tissue sections through absolute alcohol, 95% alcohol, 70% alcohol for 5 min, respectively. The sections were kept immersed in xylene bath and later were mounted using dibutyl phthalate in xylene.

Interpretation of staining

The presence of brown-coloured end product at the site of target antigen was indicative of positive immunoreactivity for Nanog. Breast cancer tissue was taken as positive control for the antibody [Figure 1], while normal mucosal tissue was taken as negative control by omitting the primary antibody [Figure 2].

To enumerate Nanog-stained slides, random fields were chosen and 300 cells were counted manually in five areas, and the mean percentage of immunopositive cells were calculated for Nanog in each histopathological section. All these observations were carried out by two observers to eliminate inter-observer bias. The results were analysed statistically using ANOVA test.

RESULTS

Thirty tissues of OSCC (n = 30) were evaluated for the IHC expression of Nanog and compared with normal tissue (n = 30). Clinically, the OSCC cases were graded based on TNM staging into four stages: stage I (n = 10), stage II (n = 8), stage III (n = 7) and stage IV (n = 5) [Table 1].

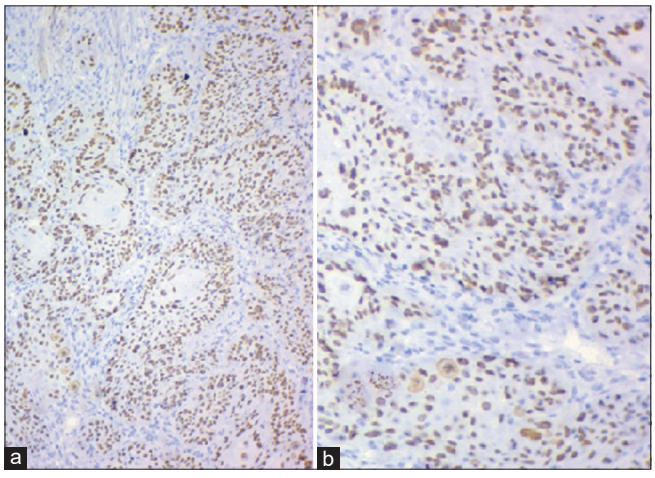

Figure 5: Photomicrograph showing Nanog expression in poorly differentiated OSCC (a: 10×, b: 40×)

Based on the histopathological grading, the OSCC cases were divided into three stages: well differentiated (n = 12), moderately differentiated (n = 11) and poorly differentiated (n = 7) [Table 2].

A comparison of the expression of Nanog in normal mucosa and in patients with OSCC was done. The mean of IHC score with respect to normal subjects and in patients with OSCC was found to be 2.00 and 0.27, respectively, using Mann–Whitney U test. A statistically significant difference was observed between normal subjects and in patients with OSCC with respect to the mean of IHC score (P = 0.0001*) [Table 3].

A comparison of Nanog expression with respect to different clinical stages of OSCC was done. The mean of IHC score in case of stage I (n = 10), stage II (n = 8), stage III (n = 7) and stage IV OSCC (n = 5) was 1.40, 1.50, 2.29 and 3.60, respectively, using Kruskal–Wallis ANOVA test. A statistically significant difference was observed between all the clinical stages of OSCC with respect to the mean of IHC score counted in random fields (P = 0.0276*) [Table 4].

A comparison of the expression of Nanog with respect to different histopathological grades of OSCC was done. The mean number of IHC score in well-differentiated OSCC (n = 12) [Figure 3], moderately differentiated OSCC (n = 11) [Figure 4] and poorly differentiated (n = 7)

Table 1: Number of cases in each clinical stage of OSCC

Clinical stage	No. of cases
Stage I	10
Stage II	8
Stage III	7
Stage IV	5

Table 2: Number of cases in each histological grade of OSCC

Histopathological grade	No. of cases
Grade I	12
Grade II	11
Grade III	7

Table 3: Comparison of Nanog expression in normal subjects and in patients with OSCC with respect to IHC score obtained by the number of positive cells per 300 cells counted in random fields

Groups	*n*	Mean	SD	*U*	*P*
OSCC	30	2.00	1.44	145.00	0.00001*
Normal subjects	30	0.27	0.45		

Mann-Whitney U test *P<0.05—significant

[Figure 5] OSCC was found to be 0.30, 2.25 and 3.75, respectively, using Kruskal–Wallis ANOVA test. A statistically significant difference was observed between all the grades of OSCC with respect to the number of IHC score counted in random fields ($P = 0.0001$*) [Table 5].

DISCUSSION

Embryonic stem cells (ESC) have several distinctive features that cumulatively set them apart from all known cell types. First, they are immortal and, like cancer cells, exhibit the ability to undergo limitless self-activity. Second, ESC possesses the ability to form teratomas when transferred subcutaneously to syngeneic or immunocompromised mice. The final and most important characteristic of embryonic stem cells is their ability to contribute to all three major germ layers (ectoderm, mesoderm and endoderm) either *in vitro* or *in vivo*. The exact mechanisms controlling stemness have not been determined, but the research has revealed key extracellular and intracellular players.[20]

Efforts in understanding the uniqueness 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. Nanog is to some extent very different from the other two core transcription factors. Heterogeneous expression of Nanog is observed in ESC and overexpression of Nanog is enough to keep ESC maintenance in the absence of LIF. Nanog has also recently been implicated in G1 to S transition, where Nanog overexpression results in quicker cell cycle progression through accelerated S-phase entry by direct binding and regulation of two proteins important for this process.[21]

Table 4: Comparison of Nanog expression with respect to different clinical stages of OSCC by ANOVA test

TNM stage	Mean	Std. Deviation	Median	Sum of ranks
Stage I	1.40	1.17	1.50	119.00
Stage II	1.50	1.51	1.50	100.50
Stage III	2.29	1.25	2.00	120.50
Stage IV	3.60	0.89	4.00	125.00
H			9.1282	
P			0.0276*	

ANOVA test P=0.0276—significant

Table 5: Comparison of Nanog expression with respect to different histopathological grades of OSCC by ANOVA test

Histopathological grades	Mean	Std. deviation	Std. error	Sum of ranks
Grade I	34.00	8.58	2.48	55.00
Grade II	59.80	15.00	4.74	201.00
Grade III	76.13	14.48	5.12	209.00
F			26.1663	
P			0.0001*	

ANOVA test $P<0.05$*—significant

Cancer stem cells (CSCs), defined by a small fraction of cells within the bulk of tumour have the ability of self-renewal and generating new tumours, are being the hot spots in recent cancer research. It has been considered that CSCs might be responsible for cancers' relapse and metastasis. These features also characterize embryonic stem cells (ESCs), thus suggesting common molecules might exist between CSCs and ESCs.[14,22]

CSCs are thought to contribute not only to tumour initiation and maintenance but also to aggressive tumour behaviours such as chemoresistance, anti-apoptosis and metastasis; thus, they may be responsible for tumour persistence and recurrence after the treatment.[23] On the basis of the cancer stem cell (CSC) hypothesis, a tumour may be sustained by a subset of cancer cells with stem cell-like features that have the ability for self-renewal and pluripotency. These CSCs have tumorigenic potential and proliferate indistinctly. The same molecular pathway that manages self-renewal in normal stem cells also seems to manage CSCs in tumours. Nanog is believed to function in conjunction with other factors such as Oct4 and Sox 2 to form an embryonic stem cell identity.[24]

Shi W *et al*.[25] identified the protein network in which Nanog operates in mouse ES cells. Using affinity purification of Nanog under native conditions followed by mass spectrometry, they identified physically associated proteins. This tight protein network seems to function as a cellular module dedicated to pluripotency.

Jeter *et al*.[26] suggested that Nanog possesses protumorigenic attributes and NANOG-mediated oncogenic reprogramming may underlie clinical manifestations of malignant diseases that Nanog potentiates the molecular circuitry of tumorigenesis, and thus may represent a novel therapeutic target or biomarker for the diagnosis, prognosis and treatment outcome of cancer.

Gawlik-Rzemieniewska *et al*.[27] reviewed the role of NANOG in cancer cell proliferation, epithelial–mesenchymal transition (EMT), apoptosis and metastasis. In addition, they also described a correlation between NANOG and signal transducer and activator of transcription 3 (STAT3) in the maintenance of cancer stem cell properties and multidrug resistance. They also demonstrated that NANOG is strictly involved in the process of carcinogenesis and is a potential prognostic marker of malignant tumours.

Pitrone *et al*.[28] analysed stem cell transcription factors NANOG, SOX2 and OCT4 by immunoblotting and

real-time PCR. *NANOG*, *SOX2* and *OCT4* interplay was explored by gene silencing. *NANOG* silencing induced a significant *OCT4* and *SOX2* downregulation, whereas *SOX2* silencing did not affect *NANOG* gene expression. Adipose tissue is an important source of MSC, and siRNA experiments endorse a hierarchical role of *NANOG* in the complex transcription network that regulates pluripotency.

Yin *et al*.[29] concluded that Oct4 and Nanog initiate stem cell characteristics in hepatocellular carcinoma and promote epithelial–mesenchymal transition through the activation of Stat3/Snail signalling. The findings propose Stat3/Snail pathway as a novel therapeutic target for the treatment of progression and metastasis of HCC with CSC-like signatures and epithelial–mesenchymal transition phenotype.

Many recent studies have reported that high expression of NANOG in OSCC specimen is directly associated with histologically poor differentiation status, clinically late-stage tumours and frequently with neck node metastasis, resulting in poor overall survival rates.[30] In the present study, an attempt was made to evaluate the expression of Nanog immunohistochemically in various clinical and histopathological grades of 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, the results were found to be statistically significant with a *P* value of 0.001 with respect to the expression between normal oral mucosa and OSCC. Fu *et al*.[31] observed increased expression of Nanog in cancer cells and corresponding tumour-associated normal tissue (CTAN) of OSCC patients when compared to normal mucosa which was in accordance with the present study.

The results of the present study were found to be statistically significant with a *P* value of 0.0001 with respect to the expression in various clinical stages of OSCC. We also observed that there is an increased expression of Nanog in clinical stage IV which was in accordance with studies conducted by Chiou *et al*.[32] and Kim *et al*.[31] where they observed increased expression of Nanog in the late clinical stages, thereby predicting poor prognosis of OSCC patients.

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 in accordance with the previous studies conducted by Kim *et al*.[30] and Watanabe *et al*.[33] where they demonstrated 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 may be a useful strategy for the treatment of OSCC metastasis.

CONCLUSION

The increased expression of Nanog in patients with OSCC cases in comparison with normal subjects was statistically significant, suggesting its role as a diagnostic biomarker. A statistically significant result with respect to the clinical staging and histopathological grading of Nanog expression in patients with OSCC suggests its role as a prognostic biomarker as well. However, further studies involving larger samples may be undertaken for more definitive and conclusive results.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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