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Association of NANOG Expression with Tumor Differentiation, Progression, and Survival in Oral Squamous Cell Carcinoma (OSCC)

Rida Fatima Khan
rida.fatima@duhs.edu.pk
Dow University of Health Sciences
Shazia Akbar
Dow University of Health Sciences
Uzma Bukhari
Dow International Medical College, Dow University of Health Sciences
Ammar Ali Muhammad
Interactive Research and Development
Sadia Farooqi
Sindh Government Hospital
Amber Saeed
Dow University of Health Sciences

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Authors and Affiliations:

Rida Fatima Khan[1], Shazia Akbar[1], Uzma Bukhari [2], Ammar Ali Muhammad[3], Sadia Farooqi[4], Amber Saeed[5]

Affiliations:

[1] Department of Oral Pathology, Dow Dental College, Dow University of Health Sciences, Pakistan

[2] Department of Pathology, Dow International Medical College, Dow Diagnostic Research and Reference Laboratory (DDRRL), Dow University of Health Sciences, Karachi, Pakistan

[3] Department of Global Surgery, Interactive Research Department, Karachi, Pakistan

[4] Department of Dentistry, Sindh Government Hospital, Karachi, Pakistan

[5] Department of Medical Education, Dow Dental College, Dow University of Health Sciences, **Karachi, Pakistan**

Corresponding Author:

Dr. Rida Fatima Khan

Department of Oral Pathology, Dow Dental College, Dow University of Health Sciences, Karachi, Pakistan

Telephone: +92321-8626929

Email: rida.fatima@duhs.edu.pk

Abstract

Objective: To evaluate the association of NANOG expression with clinicopathological characteristics, including tumor differentiation, pathological stage, lymph node metastasis, and tumor thickness, and to explore its relationship with overall survival in patients with oral squamous cell carcinoma (OSCC).

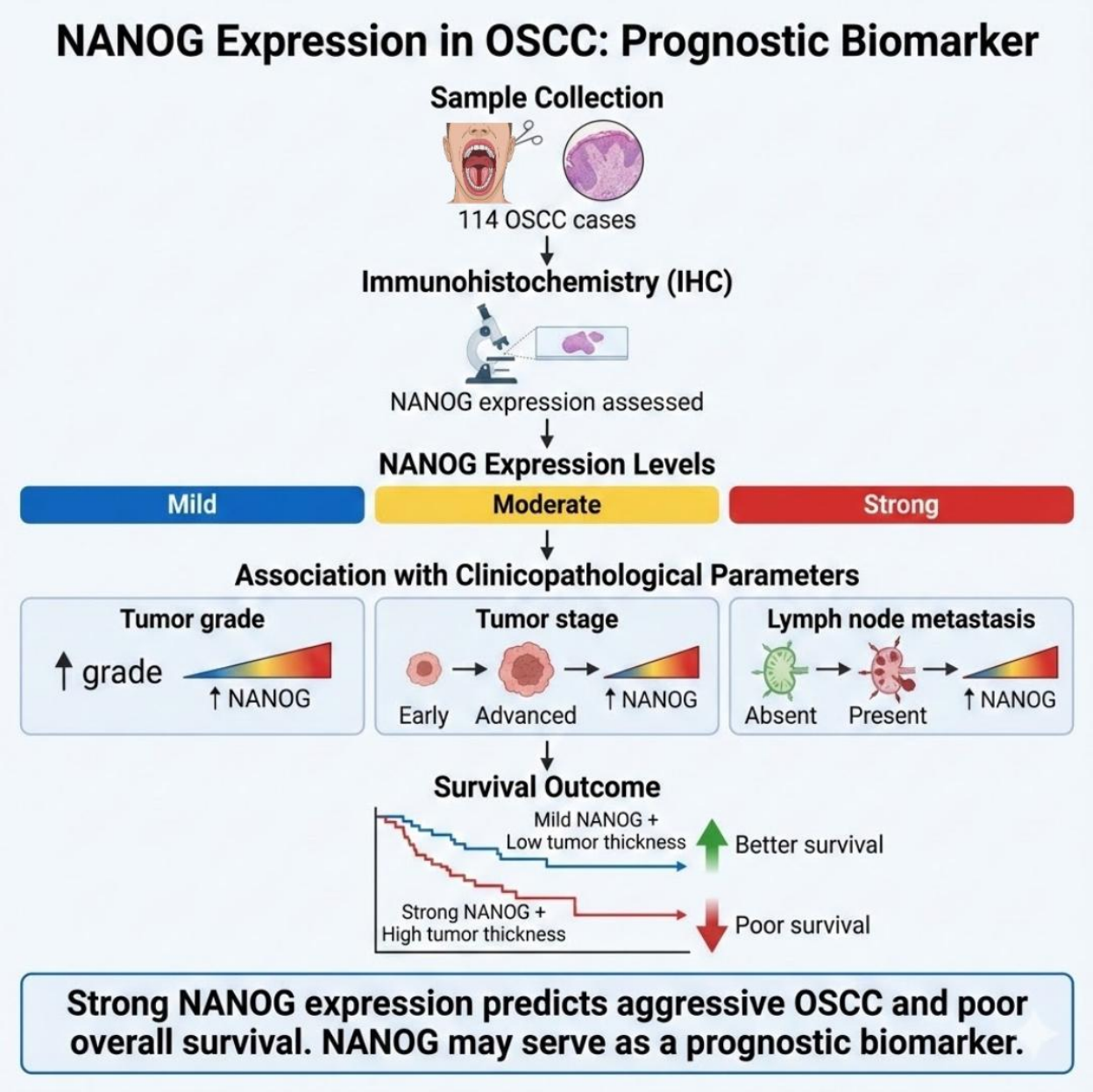
Methodology: A retrospective cohort study was conducted on 114 biopsy-proven OSCC cases collected during 2021–2022. NANOG expression was assessed by IHC using an IRS system. Clinicopathological associations were analysed using Chi-square/Fisher's exact tests, while Kaplan–Meier and Cox regression analyses evaluated overall survival.

Results: NANOG expression was significantly associated with gender ($p = 0.001$), tumor grade ($p < 0.001$), tumor stage ($p = 0.008$), and lymph node metastasis ($p = 0.003$). No significant correlations were found between the site of tumor, tumor thickness, perivascular invasion, perineural invasion, and extranodal extension ($p > 0.05$). Multivariable Cox regression identified strong NANOG expression (HR = 5.94; 95% CI: 1.02–34.63; $p = 0.048$) and tumor thickness >1 cm (HR = 3.16; 95% CI: 1.49–6.71; $p = 0.003$) as predictors of poor overall survival.

Conclusion: NANOG expression was correlated with adverse clinicopathological features of OSCC, especially poor histological differentiation, advanced pathological stage and lymph node metastasis. Tumor thickness was an independent predictor of overall survival. The prognostic impact of NANOG is still uncertain and should be validated in larger prospective trials with adequate follow-up.

Keywords: Oral squamous cell carcinoma, NANOG, Immunohistochemistry, Tumor Biomarkers, Survival.

Graphic Abstract:



INTRODUCTION

Lip and oral cavity cancer constitute a significant global health burden. [1] Globally, lip and oral cavity cancers accounted for approximately 389,846 new cases and 188,438 deaths in 2022 according to GLOBOCAN estimates, with Asia reporting the highest burden, contributing 258,440 cases (66.3%) of the total global incidence. [2] Oral squamous cell carcinoma (OSCC) is the most common and aggressive malignancy of the head and neck. [3] Despite advancements in diagnostic and therapeutic strategies, the overall five-year survival rate for OSCC remains approximately 50%, largely attributable to late-stage diagnosis, cervical lymph node metastasis, and high recurrence rates [4].

OSCC arises from the keratinocytes of the oral epithelium, most commonly affecting the tongue, buccal mucosa, gingiva, palate, and lips. [5] Its aggressive clinical course is driven in part by cancer stem cells (CSCs), a small subpopulation of tumor cells capable of self-renewal, tumor initiation, invasion, and resistance to therapy. [6, 7] CSCs contribute to tumor progression, recurrence, and treatment resistance and may therefore have prognostic relevance. [8]

Among transcription factors regulating cancer stem cell (CSC) properties, NANOG is essential for maintaining pluripotency and self-renewal in embryonic stem cells by preventing differentiation. Aberrant NANOG expression in OSCC has been implicated in maintenance of stemness, cellular proliferation, epithelial–mesenchymal transition, invasion, metastasis, and treatment resistance.[9,10-17] Although several immunohistochemical studies have associated increased NANOG expression with aggressive clinicopathological features and poorer outcomes, other studies have reported weak or non-significant associations.. [18] However, the prognostic significance of NANOG in Oral Squamous Cell Carcinoma remains controversial. These

discrepancies may reflect differences in tumor subsite, disease stage, sample size, follow-up duration, immunohistochemical methods, and cutoff values. [19, 20]

Conventional clinicopathological parameters, including T stage, lymph node status, histological grade, depth of invasion, and margin status, remain central to prognostic assessment in OSCC. However, these parameters may not fully capture the biological heterogeneity underlying differences in patient outcomes

Importantly, the independent prognostic value of NANOG beyond established pathological factors remains insufficiently defined, particularly in studies incorporating multivariable survival models. Therefore, the present study evaluates NANOG expression in OSCC in relation to key clinicopathological characteristics and overall survival, using Kaplan–Meier analysis and multivariable Cox regression to determine whether NANOG provides independent prognostic information.

MATERIAL AND METHODS:

2.1 Study Design and Ethical Approval

This was a retrospective cohort study conducted at the Histopathology Section of the Dow Diagnostic Research and Reference Laboratory (DDRRL), Dow University of Health Sciences (DUHS), Karachi, Pakistan. A total of 114 biopsy-proven cases of oral squamous cell carcinoma (OSCC), diagnosed between 2021 and 2022, were included. Archived formalin-fixed, paraffin-embedded (FFPE) tissue specimens from these cases were retrieved, and NANOG immunohistochemical assessment was performed in 2023. Survival outcomes were subsequently ascertained from hospital records and telephone follow-up until August 2025. Institutional Review

Board (IRB) of DUHS (IRB Number: IRB-2566/DUHS/Approval/2022/1007) provided ethical approval. The study was carried out in compliance with Helsinki Declaration and its amendments.

2.2 Inclusion and Exclusion Criteria

Inclusion criteria comprised all histopathological confirmed cases of Oral Squamous Cell Carcinoma, regardless of age or gender, with adequate excisional biopsy and neck dissection specimens containing at least 70% full-thickness viable tumor tissue. Exclusion criteria included inconclusive biopsies, poorly fixed tissue, recurrent tumors, prior chemotherapy, and patients with chronic illnesses, autoimmune disorders, or congenital syndromes.

2.3 Tissue Processing and Clinicopathological Assessment

Demographic and clinicopathological data were recorded for all patients. Formalin-fixed, paraffin-embedded (FFPE) oral squamous cell carcinoma (OSCC) tissue specimens obtained from routine diagnostic surgical resections and biopsy samples received at the Dow Diagnostic Research and Reference Laboratory (DDRRL), Dow University of Health Sciences, Karachi, received at study period. All specimens were routinely fixed in 10% neutral-buffered formalin as part of standard histopathological processing and subsequently processed and embedded in paraffin for further analysis. Two adjacent non-tumorous oral epithelium samples were used as controls. However, there was no expression of NANOG in non-tumorous tissue. Hematoxylin and eosin (H&E) stained slides were examined to confirm the diagnosis, determine histological grade, and evaluate tumor stage and lymph node metastasis. Histological grading was performed according to Broder's classification: Grade 1 (G1, well-differentiated), Grade 2 (G2, moderately differentiated), and Grade 3 (G3, poorly differentiated). Tumor pathological staging (pT) and nodal involvement (pN) were assessed based on the AJCC/UICC TNM 8th edition (2017) guidelines.

Tumor thickness (TT) was measured from the tumor surface to the deepest point of invasion in full-thickness sections. For statistical analysis, TT was categorized into low (<0.4 cm), moderate (0.4–1.0 cm), and high (>1.0 cm) risk groups, as previously established in prognostic OSCC studies. TT was used instead of depth of invasion (DOI) because it provides a reproducible measure in full-thickness specimens and correlates with nodal metastasis and survival outcomes.

2.4 Immunohistochemistry staining for NANOG

Oral squamous cell carcinoma (OSCC) tissue blocks were cut into 4-µm-thick sections and stained with immunohistochemistry. The sections were cut with a rotary microtome (Leica Biosystems, Wetzlar, Germany) and mounted on positively charged Super frost Plus glass slides (Thermo Fisher Scientific, Waltham, MA, USA) and dried at 70°C for 60 min.

Sections were deparaffinized in three changes of xylene for 5 min each and rehydrated through graded ethanol (100%, two changes for 3 min each; 95% for 3 min; and 70% for 3 min), followed by rinsing in distilled water for 2 min. Heat-induced epitope retrieval was performed using Tris-EDTA buffer (pH 9.0). After retrieval the slides were slowly cooled to room temperature for 30 min and washed three times in PBST (pH 7.4) for 5 min each.

3% hydrogen peroxide in distilled water for 10 min at room temperature was used to block endogenous peroxidase activity. Three washes were performed with PBS for 5 min each. The nonspecific binding was blocked by incubation with 5% normal goat serum in PBST for 20 min at room temperature. Excess blocking solution was removed without washing.

Sections were stained for the presence of Rabbit polyclonal anti-NANOG antibody (Thermo Fisher Scientific, Waltham, MA, USA; Cat. It was diluted in PBS with 1% bovine serum albumin (BSA) (No. PA1-097) at working dilution of 1:100. The antibody dilution was determined by preliminary

titration to achieve the maximum specific staining with minimal background staining. Diluted antibody (about 100 µL) was dispensed on each section and incubated for 60 min at room temperature in a humidified chamber.

Following primary antibody incubation, sections were washed three times in phosphate buffered saline (PBS) for 5 min each. An HRP-labelled polymer-based anti-rabbit detection system (EnVision+ Single Reagent, Agilent Dako, Santa Clara, CA, USA; Cat. was used for detection. No. K400311-2) for 30 min at room temperature. Then, three washes were performed on the sections using PBS, each for 5 min.

The immunoreactivity was detected by 3,3′-diaminobenzidine (DAB) as substrate-chromogen (Vector Laboratories, Newark, CA, USA, Cat. No. SK-4100). Working DAB solution was prepared following the manufacturer's instructions and applied for 5 min at room temp. Rinsing with distilled water was used to stop the reaction.

The sections were counterstained in Mayer's hematoxylin (Sigma-Aldrich, St. Louis, MO, USA; Cat No. MHS 20) The samples were rinsed and placed in No. MHS16 for 90 s and then under running tap water for 5 min. Each section was then dehydrated in graded ethanol (70%, 95%, and two changes of 100%), cleared in xylene and permanently mounted in DPX mounting medium (Sigma-Aldrich; Cat. No. 06522).

2.5 Immunohistochemical controls

As a positive control for NANOG immunoreactivity, human seminoma FFPE tissue was processed at the same time. All staining conditions were identical, except that the primary antibody was replaced by antibody diluent (1% BSA in PBS) in the negative control. Both positive and negative

controls were used to evaluate the performance and specificity of the immunohistochemical staining procedure.

2.6 Immunoreactivity Scoring (IRS)

NANOG expression was assessed using a semi-quantitative immunoreactivity scoring (IRS) system [21], based on the percentage of NANOG-positive tumour cells and staining intensity. Immunohistochemically stained sections were independently evaluated by two experienced histopathologists who were blinded to the clinicopathological characteristics and survival data of the patients. Each histopathologist independently assessed the proportion of positive tumour cells and staining intensity according to predefined scoring criteria.

Manual microscopic assessment was used to determine the percentage of NANOG-positive tumour cells. The proportion of positive tumour cells was categorized into five scores: 0 = no positive cells, 1 = less than 10%, 2 = 10–50%, 3 = 51–80%, and 4 = greater than 80%. Staining intensity was graded on a four-point scale: 0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining.

The immunoreactive score (IRS) was calculated by multiplying the percentage positivity score by the staining intensity score:

IRS = Percentage positivity score × Staining intensity score.

The resulting IRS values were categorized as follows: 0–1 = negative expression, 2–3 = mild expression, 4–8 = moderate expression, and 9–12 = strong expression, according to the scoring system described by Salam et al. (2023) [21].

2.7 Inter-examiner agreement

Both histopathologists independently assessed all 114 cases. Concordant assessments were obtained in 111 cases (97.4%), while discrepancies or uncertainty in scoring were observed in three cases (2.6%). These three cases were referred to a third experienced histopathologist for independent review and expert opinion. The final scores following the third expert's assessment were used for statistical analysis.

2.8 Digital validation using Fiji ImageJ

The manually assessed percentage of NANOG-positive tumour cells was additionally evaluated using Fiji ImageJ software (National Institutes of Health, Bethesda, MD, USA). Representative areas of immunohistochemically stained sections were photographed using a Motic BA310E LED trinocular compound microscope (Motic, China) fitted with a digital camera. Images were acquired under standardized magnification and illumination conditions and saved in TIFF format without adjustments to colour, brightness or contrast.

The tumour areas were manually outlined, excluding necrotic areas, haemorrhage, tissue folds, staining artefacts and non-tumour tissue. Colour deconvolution was performed using the haematoxylin-DAB (H-DAB) stain vector to separate the brown DAB reaction product from the blue haematoxylin counterstain. The DAB channel was converted into an 8-bit grayscale image, and a predefined threshold was applied consistently to distinguish positive staining from background. The thresholded image was converted into a binary image, and the Analyze Particles function was used to identify and count individual DAB-positive nuclear objects. Watershed segmentation was applied where necessary to separate adjacent positive nuclei. Detected objects were visually reviewed against the original images to verify accurate identification.

The total number of tumour nuclei was determined separately, and the percentage of NANOG-positive tumour cells was calculated as follows:

Percentage of positive cells = (Number of NANOG-positive tumour cells / Total number of tumour cells) × 100.

The digitally obtained percentages were compared with the manual histopathological assessments to evaluate the consistency of the scoring.

2.9 Survival Assessment

Patients were followed up until August 2025, and survival status (alive, lost to follow-up, or deceased) was recorded. Overall survival was defined as the time from diagnosis to death from any cause or last follow-up.

2.10 Statistical Analysis

All data were analysed using STATA version 17. Descriptive statistics were expressed as frequencies and percentages for categorical variables and mean ± standard deviation (SD) for continuous variables. The association between NANOG expression (IRS score and intensity) and clinicopathological parameters (tumor grade, stage, lymph node metastasis, tumor thickness, perineural invasion, lymphovascular invasion, extranodal extension, and tumor margin) was evaluated using Chi-square test or Fisher's exact test, as appropriate.

We used log rank test and then univariable and multivariable Cox proportional hazards regression models to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) for overall survival. NANOG expression was the primary exposure variable in the multivariable Cox regression model irrespective of its statistical significance in the univariable analysis, based on the a priori study hypothesis and its biological plausibility as a potential marker of tumor progression. Other relevant

clinicopathological covariates were incorporated into the model to evaluate the association of NANOG expression with overall survival after adjustment for other covariates. Proportional hazards assumption was checked using Global test. Statistical significance was defined as $p < 0.05$.

Data Availability

The datasets generated during and/or analysed during the current study are not publicly available due to patient confidentiality but are available from the corresponding author upon reasonable request.

3. Results

3.1 Association of NANOG Expression with Clinicopathological Characteristics

A total of 114 OSCC tissue specimens has been analysed for NANOG expression. Mild, moderate, and strong NANOG expression based IRS score was observed in 45 (39.5%), 47 (41.2%), and 22 (19.3%) cases, respectively.

The expression of NANOG was significantly associated with gender ($p = 0.001$), histological grade ($p < 0.001$), tumor stage ($p = 0.008$) and lymph node metastasis ($p = 0.003$) (Table 1). Male patients had a higher rate of moderate expression of NANOG compared to females. Concerning histological grades, mild expression was more common in well-differentiated tumors while moderate to strong expression was more common in poorly differentiated tumors (Figure 1). Strong expression was also more common in pT3 than other stages of tumors. Likewise, high expression of NANOG was more frequent in the tumors with LN involvement than in those without LN involvement.

There was no significant correlation between NANOG expression and the site of the tumor ($p = 0.921$), tumor thickness ($p = 0.273$), perivascular invasion ($p = 0.168$), perineural invasion ($p = 0.183$), or extranodal extension ($p = 0.211$).

3.2 Comparison of Clinicopathological Characteristics According to Survival Status

According to Table 2, a total of 114 patients were studied out of which 18 (16%) were alive, 48 (42%) were lost to follow up (LTF) and 48 (42%) died during the study. The log rank test analysis with LTF patients included as well as excluded, showed that the intensity of the NANOG expression did not show any significance amongst the survival groups. Similarly, no significant differences were observed for age group, gender, laterality, tumor grade, tumor stage, lymph node metastasis, percentage of positive cells, IRS category, tumor margin status, lymphovascular invasion, extranodal extension and treatment status. However, thickness of the tumor and perineural invasion were found to be significantly different. The perineural invasion did not meet the proportional hazards assumption, therefore was not included in Cox regression analysis.

3.3 Cox Regression Analysis of Overall Survival

The Univariate and Multivariable Cox regression was run by including LTF patients (shown in Table 3(a) and by excluding LTF patients in Table 3(b)

Univariate Cox regression analysis in Table 3(a) showed that there was a statistically significant relationship between tumor thickness and overall survival. Patients with tumor thickness >1 cm had a higher risk of death compared with those with tumor thickness <0.4 cm (HR = 3.07; 95% CI: 1.46–6.47; $p = 0.003$).

The age, gender, tumor grade, tumor stage, presence of lymph node metastasis, surgical margin status, extranodal extension, treatment modality, intensity of expression of NANOG and IRS score did not significantly correlate with overall survival ($p > 0.05$).

In the multivariable Cox regression model in Table 3(a), death was positively associated with strong NANOG expression (HR = 5.94; 95% CI: 1.02–34.63; p = 0.048). Tumor thickness >1 cm also remained an important predictor of poor overall survival (HR = 3.16; 95% CI: 1.49–6.71; p = 0.003). None of the other variables in the adjusted model were statistically significant (Table 3a).

Univariate Cox regression analysis in Table 3(b) showed that there was a statistically significant relationship between tumor thickness and overall survival. Patients with tumor thickness >1 cm had a higher risk of death compared with those with tumor thickness <0.4 cm (HR = 2.44; 95% CI: 1.15–5.15; p = 0.02).

The age, gender, tumor grade, tumor stage, presence of lymph node metastasis, surgical margin status, extranodal extension, treatment modality, intensity of expression of NANOG and IRS score did not significantly correlate with overall survival ($p > 0.05$).

In the multivariable Cox regression model in Table 3(b), death was positively associated with tumor thickness >1 cm that remained an important predictor of poor overall survival (HR = 2.55; 95% CI: 1.20–5.43; p = 0.015). The NANOG expression as a primary exposure of interest showed medium NANOG expression (HR = 1.81; 95% CI: 0.95–3.45; p = 0.07) and strong NANOG expression (HR = 1.91; 95% CI: 0.84–4.30; p = 0.120).

3.4 Kaplan–Meier Survival Analysis

Consulting with the clinical team, the researchers used the Kaplan–Meier method to evaluate overall survival based on the intensity of NANOG expression and the thickness of the tumors.

Patients with low expression of NANOG had the worst survival probability at follow up, while patients with mild expression had the best (Figure 2).

There were also differences in survival probability based on tumor thickness (Figure 3). Patients with tumors thickness >1 cm had the lowest chance of survival, and those with tumors thickness <0.4 cm had the highest chance of survival. Those who had a tumor thickness level between 0.4 and 1.0 cm had intermediate survival. The Cox regression analysis showed similar results, with the thickness of the tumor being the independent factor associated with mortality (tumor thickness >1 cm).

Discussion:

OSCC is the sixth most common human malignancy and accounts for many new cases worldwide annually. It has a high mortality rate because of poor prognosis as it is associated with late-stage diagnosis, rapid growth and metastasis, resistance to adjuvant chemo and radiotherapy. [22]

Our study is the first study conducted in Pakistan that aimed to investigate about the role of NANOG in OSCC tissue and finds its correlation with tumor grade, pathological stage, lymph node metastasis, and patient survival, thereby evaluating its potential as a prognostic biomarker in OSCC. Hence, we suggested from our results that NANOG has a positive correlation with tumor grade, stage and lymph node metastasis. The expression of NANOG increases with increase in tumor grade, stage and lymph node metastasis. Moreover, we also observed that patients with tumor thickness >1cm had less chances of survival.

To prove this association, the result of our study coincides with the results of previous literature. In a study done by Chiou SH et al in the role of Oct-4 and NANOG in oral cancer stem-like cells (OC-SLC) were studied using OSCC cell lines and patient tissues. Their findings revealed that

elevated levels of NANOG/Oct-4/CD133 in CSCs (cancer stem cells) were correlated with higher tumor stage and poor survival. Hence, their study demonstrated that NANOG-positive CSCs were responsible for OSCC progression. However, due to small sample size (n=52) the generalizability was limited, and mechanistic insights remain incomplete. [10] Similarly, in a cohort study of 60 OSCC patients done by Watanabe M et al NANOG expression was assessed using IHC technique. It also correlated with differentiation, metastasis, and therapy response. It was found that NANOG was upregulated in poorly differentiated tumors and metastatic foci. It also persisted after preoperative adjuvant therapy, which suggested its role in resistance. Their results also support NANOG as a biomarker for aggressive variant of OSCC. But the results showed limitation due to lack of molecular exploration of pathways. [11] In 2017, Kim HM et al also analyzed IHC expression of NANOG and Midkine (MK) in 62 OSCC patients. Both markers showed similar expression patterns as they were upregulated in later-stage and high-grade OSCC. It was also observed that both markers were significantly associated with poor survival and their combined expression strongly predicted unfavorable outcomes. Their study provided a valuable prognostic insight into the pool of previous literature, but due to small sample size and absence of functional assays the mechanistic conclusions were limited. [14] Similarly, Lee HJ et al also observed high expression of NANOG and mutant p53 correlated with late stage, high grade, lymph node metastasis and reduced survival. Hence, co-expression of NANOG and mutant p53 predicted the poorest prognosis. [15] Moreover, Kashyap T et al studied 110 human OSCC tissues, cisplatin-resistant OSCC cell lines, and siRNA knockdown models to evaluate NANOG's role in tumor progression and chemo resistance. The overexpression of NANOG was correlated with invasion, stemness, and cisplatin resistance, while its knockdown reduced these effects. It was observed that NANOG directly

regulated genes like Slug, c-Myc, and E-cadherin. But larger clinical cohorts are needed for this validation. [13]

In another laboratory investigation conducted in 2011 by Tsai LL et al NANOG and Oct-4 expression were examined in cisplatin-sensitive and resistant OSCC specimens. They used OSCC cell lines (OC2) and IHC technique on resistant tumors and found that cisplatin-resistant cells displayed enhanced self-renewal, stemness and migration. Hence, they observed that NANOG/Oct-4 overexpression was significantly associated with chemo resistance. [23] Therefore, the study agreed on the fact that NANOG was a driver of therapy resistance. Therefore, according to the reviewed literature, NANOG is consistently upregulated in OSCC tissues and cell lines exhibiting strong associations with poorly differentiation cases, advanced tumor stage, lymph node metastasis, and reduced survival. [10, 11, 13-15] Whereas, some studies also validate its role in cisplatin resistance and recurrence, reinforcing NANOG's significance in therapy response. [13, 23]

Grubelnik G et al investigated the expression of NANOG and its regulation in OSCC. They observed an upregulation of NANOG and OCT4 in OSCC samples with lymph node metastasis. [16] Moreover, Kashyap and Grubelnik, provided detailed insights that implicate NANOG-regulated transcriptional networks and miRNAs in OSCC aggressiveness [13, 16]. De Vicente JC et al in 2019 evaluated emerging role of NANOG as an early cancer risk biomarker in patients with oral potentially malignant disorders. They evaluated 55 oral epithelial and 125 OSCC patients. They used IHC technique to evaluate NANOG as a biomarker of cancer risk. It was also observed that cytoplasmic NANOG expression was associated with higher dysplasia grade and risk of malignant transformation and was more common in early-stage OSCC. [17]

Likewise, Fu TY et al conducted a study in 2016 in which they studied 436 OSCC cases. The expressions of OCT4, SOX2, and NANOG were assessed by IHC on tissue microarrays. The

observed that NANOG was significantly higher in tumor and adjacent tissue when compared to normal mucosa. But the prognostic role of NANOG was less pronounced. [19] In 2020, Pedregal-Mallo D et al carried out a multicenter study and analyzed NANOG, SOX2, and OCT4 expressions in 348 HNSCC patients. NANOG was expressed in 72% of cases and was correlated with better survival rate particularly in cases of pharyngeal tumors with lymph node metastasis. But the study showed limitations of heterogeneity of HNSCC subsites, complicating OSCC-specific conclusions. [20]

However, findings are not entirely consistent. Some reports which were contrary to our study suggested that the expression of NANOG expression may not always predict poor outcomes. [19, 20] As in a study of pharyngeal tumors, it was associated with better prognosis. These inconsistencies likely arise from differences in tumor sub site, sample size, stage distribution, and methods of detection (IHC vs. qPCR). Furthermore, contrary results were also found in a study done by Nataša Kenda Šuster et al in 2017. They evaluated the expression of NANOG in 159 cases of ovarian serous tumors and found that NANOG expression was only positive in high grade tumors whereas, in low grade borderline and benign tumor the expression of NANOG was completely negative. Whereas in our study NANOG was expressed in all cases of OSCC but the expression increased with the increased in tumor grade and stage. [24] Whereas, in our study NANOG was expressed in all cases of OSCC but the expression increased with the increased in tumor grade and stage.

Overall, the previous literature emphasized that NANOG is a key regulator of OSCC progression, invasion, and chemo resistance, but its role in prognosis still needs further investigations. Nevertheless, NANOG expression through immunohistochemistry (IHC) alongside histological grade, pathological stage, and lymph node metastasis, and further validating these results through survival analysis. Hence, this study aims to establish a comprehensive understanding of NANOG's

clinical importance in OSCC. While histological grading exhibits tumor differentiation, staging indicates the magnitude of disease spread, and nodal status remains the most powerful independent factor to determine prognosis. Whereas survival analysis directly reveals the impact of these variables on patient outcomes. Using NANOG expression with these established clinicopathological markers not only has the possibility to identify high-risk patients more promptly but may also establish NANOG as a reliable biomarker for detecting disease progression, response of treatment and survival. Furthermore, this approach fills the gap between molecular markers and conventional pathology.

Limitations, Future insights and Recommendations:

This study has several limitations. As a single-center retrospective cohort study, the findings may have limited generalizability. The relatively small sample size and potential loss to follow-up may also have affected the survival analysis. Although the prospective design allowed assessment of survival outcomes, its observational nature limits the ability to establish a causal relationship between NANOG expression and OSCC progression. Additionally, tumor thickness was used instead of depth of invasion, which is considered a more accurate prognostic parameter in OSCC.

Future multicenter studies with larger, more diverse populations are suggested to confirm these results. Further studies using complementary molecular methods, such as RT-PCR or western blotting, or RNA sequencing, could provide additional insights into the biological function of NANOG. Future research should also assess the relationship between NANOG expression and treatment response to explore its potential as a predictive biomarker and therapeutic target in OSCC.

Conclusion:

NANOG expression was significantly correlated with histological grade, tumor stage, lymph node metastasis and gender in oral squamous cell carcinoma patients. Tumor thickness was an independent predictor of overall survival. The prognostic role of NANOG was uncertain as survival analyses yielded inconsistent results. Large prospective studies with adequate follow-up are needed to determine the prognostic significance of NANOG in OSCC.

Declarations

Funding

No external funding was received for this study. All research expenses, including procurement of immunohistochemistry (IHC) antibodies and other laboratory materials, were personally borne by the principal investigator. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical Approval

Ethical approval for this study was obtained from the Institutional Review Board of Dow University of Health Sciences (IRB No: IRB-2566/DUHS/Approval/2022/1007). The study was conducted in accordance with the Declaration of Helsinki and its later amendments. Informed consent was obtained from all participants prior to inclusion in the study. Participation was voluntary, and confidentiality of participant information was strictly maintained throughout the research process. No ethical issues were identified during the conduct of the study.

Consent

Verbal informed consent was obtained from all participants as approved by the Institutional Review Board of Dow University of Health Sciences.

Author Contributions (CRediT Taxonomy)

Rida Fatima Khan: Conceptualization; Methodology; Data curation; Formal analysis; Investigation; Writing – original draft; Writing – review & editing.
Shazia Akbar Ansari: Methodology; Validation; Supervision; Writing – review & editing.
Uzma Bukhari: Validation; Supervision; Interpretation of results.
Ammar Ali Muhammad: Formal analysis; Statistical analysis; Data interpretation.
Sadia Farooqi: Data curation; Investigation; Writing – original draft.
Amber Saeed: Data curation; Follow-up data collection.

All authors made substantial contributions to the conception and design of the study, acquisition, analysis, and interpretation of data; participated in drafting or critically revising the manuscript for important intellectual content; approved the final version to be published; and agree to be accountable for all aspects of the work. All authors have checked the manuscript for plagiarism and accept equal responsibility in case of any plagiarism-related issues identified by the journal.

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Data Availability

The datasets generated and/or analysed during the current study are not publicly available due to patient confidentiality but are available from the corresponding author on reasonable request.

Use of Artificial Intelligence

Artificial intelligence tools (ChatGPT, OpenAI) were used to assist in language editing and improving clarity of the manuscript. The authors take full responsibility for the scientific content, data interpretation, and conclusions of the study.

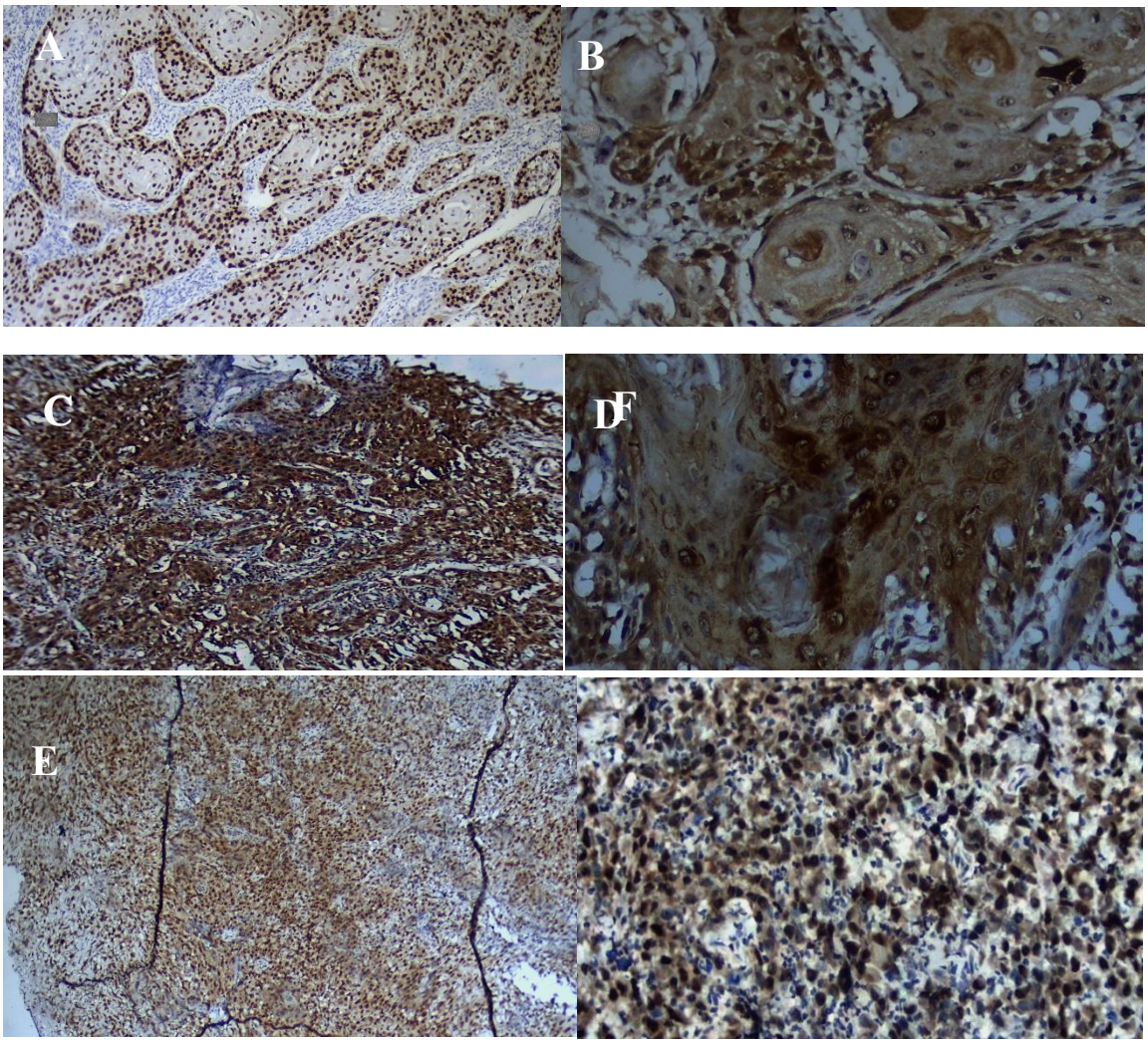

Figure 1: Immunohistochemical staining of NANOG in OSCC tissue. (A & B) Well-differentiated tumor (G1) showing mild nuclear staining; (C & D) Moderately differentiated

tumor (G2) showing moderate staining in nucleus and cytoplasm; (E & F) Poorly differentiated tumor (G3) showing moderate to strong nuclear and cytoplasmic staining. Magnification: 10× and 40×.

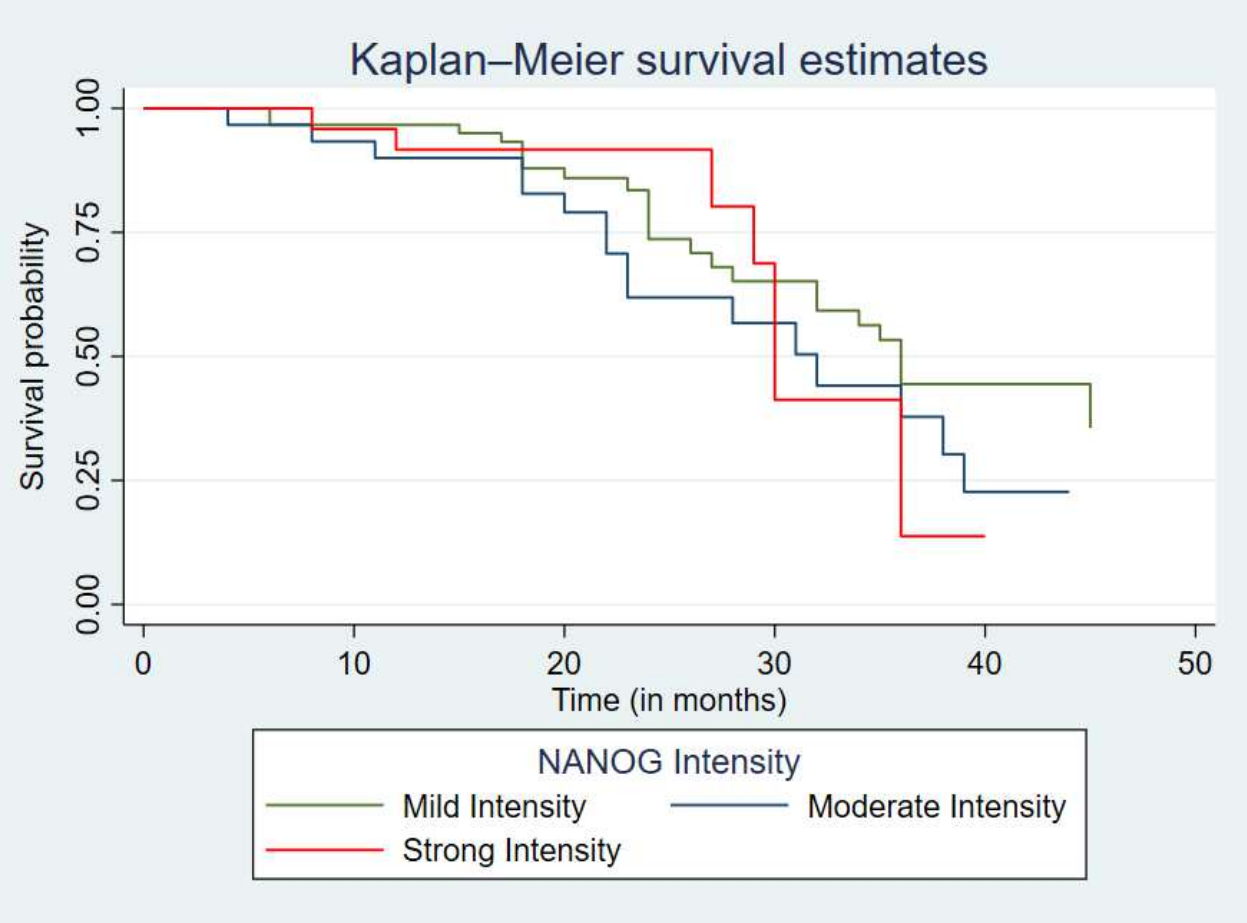

Figure 2: Kaplan- Meier survival curve showing Survival probability over time, by NANOG expression intensity levels

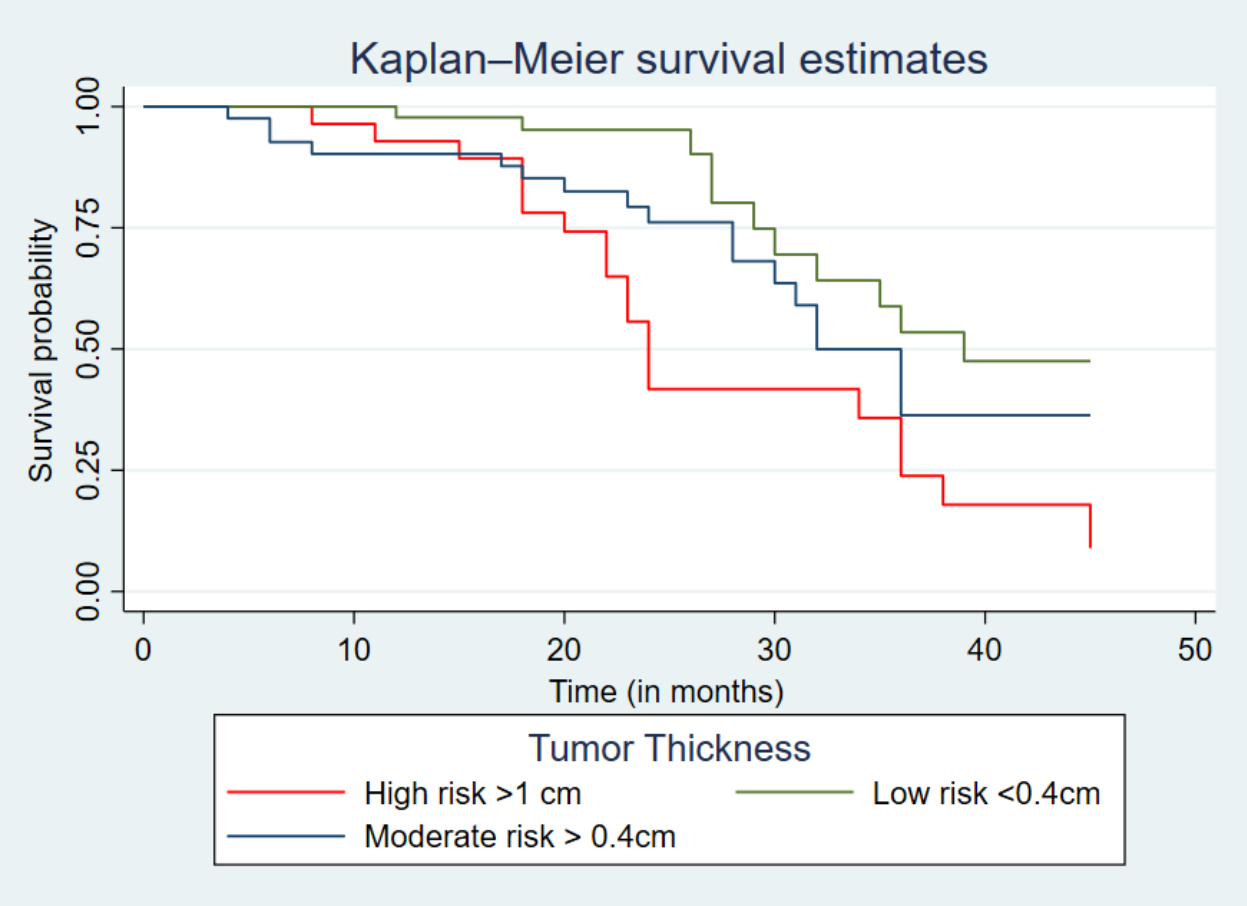

Figure 3: Kaplan- Meier survival curve showing Survival probability over time, by Tumor thickness

Table 1: Association of clinicopathological characteristics with NANOG Immunoreactivity score in oral squamous cell carcinoma tissue specimens (n=114).

Clinicopathological Parameter		NANOG staining based on IRS score				p-value
		Mild (n (%)	Moderate n (%)	Strong n (%)	Total n (%)	
Total Cases		45 (39.5)	47 (41.2)	22 (19.3)	114 (100)	
Gender	Male	27 (30.7)	43 (48.9)	18 (20.5)	88 (77.2)	
	Female	18 (69.2)	4 (15.4)	4 (15.4)	26 (22.8)	0.001*
Histological Grade	Well differentiated tumor (G1)	23 (65.7)	12 (34.3)	0 (0.0)	35 (30.7)	
	Moderate differentiated tumor (G2)	15 (34.1)	26 (59.1)	3 (6.8)	44 (38.6)	
	Poor differentiated tumor (G3)	7 (20.0)	9 (25.7)	19 (54.3)	35 (30.7)	<0.001*
Tumor Stage	pT1	19 (61.3)	9 (29.0)	3 (9.7)	31 (27.2)	
	pT2	7 (23.3)	19 (63.3)	4 (13.4)	30 (26.3)	
	pT3	7 (27.0)	9 (34.6)	10 (38.4)	26 (22.8)	
	pT4	12 (44.4)	10 (37.0)	5 (18.6)	27 (23.7)	0.008*
Lymph Node Metastasis	Absent	35 (50.7)	26 (37.7)	8 (11.6)	69 (60.5)	
	Present	10 (22.2)	21 (46.7)	14 (31.1)	45 (39.5)	0.003*
Tumor Site	Buccal Mucosa	25 (41.0)	26 (42.6)	10 (16.4)	61 (53.5)	
	Tongue	8 (40.0)	8 (40.0)	4 (20.0)	20 (17.5)	
	Lip	5 (35.7)	6 (42.9)	3 (21.4)	14 (12.3)	
	Alveolus	3 (25.0)	5 (41.7)	4 (33.3)	12 (10.5)	
	Others	4 (57.1)	2 (28.6)	1 (14.3)	7 (6.1)	0.921
Tumor Thickness	Low risk(<0.4cm)	15 (33.3)	18 (40.0)	12 (26.7)	45 (39.5)	
	Moderate risk (0.4–1cm)	20 (48.8)	14 (34.1)	7 (17.1)	41 (36.0)	
	High risk (>1cm)	10 (35.7)	15 (53.6)	3 (10.7)	28 (24.6)	0.273

	Identified	16 (51.6)	12 (38.7)	3 (9.7)	31 (27.2)	
Perivascular invasion	**Not identified**	29 (34.9)	35 (42.2)	19 (22.9)	83 (72.8)	0.168
	Identified	16 (48.5)	14 (42.4)	3 (9.1)	33 (29)	
Perineural Invasion	**Not identified**	29 (35.8)	33 (40.7)	19 (23.5)	81 (71.1)	0.183
	Positive	6 (35.3)	10 (58.8)	1 (5.9)	17 (14.9)	
Extranodal extension	**Negative**	39 (40.2)	37 (38.1)	21 (21.6)	97 (85.1)	0.211

Chi square test and fisher exact test is applied. p value <0.05 is significant.

Table 2: Comparison of clinical and demographic characteristics amongst patients throughout the study period. (n=114)									
Independent variables		**Lost to follow up (LTF) (n=48)**		**Alive (n=18)**		**Dead (n=48)**		**p value of Log Rank test with LTF included**	**p value of Log Rank test with LTF excluded**
Time (in months)	Mean (SD)	21.2	4.4	42.6	1.7	24.7	9.8		
	Median (IQR)	20.0	18-24.5	42.0	42-44	24.0	18-32		
		n	%	n	%	n	%		
NANOG intensity	Mild Intensity	22	46%	14	78%	24	50%	0.33	0.12
	Moderate Intensity	11	23%	3	17%	16	33%		
	Strong Intensity	15	31%	1	6%	8	17%		
Age group (in years)	22-36	12	25%	5	28%	15	31%	0.45	0.37
	37-45	11	23%	3	17%	13	27%		
	46-55	12	25%	5	28%	11	23%		
	56-84	13	27%	5	28%	9	19%		
Gender	Female	12	25%	4	22%	10	21%	0.99	0.75
	Male	36	75%	14	78%	38	79%		
Laterality	Left	26	54%	11	61%	23	48%	0.38	0.33
	Right	11	23%	6	33%	15	31%		
	Midline	5	10%	0	0%	3	6%		
	Not specified	6	13%	1	6%	7	15%		
Tumor grade	Well-Differentiated Tumor	15	31%	5	28%	15	31%	0.79	0.76
	Moderately Differentiated Tumor	11	23%	10	56%	23	48%		
	Poorly Differentiated Tumor	22	46%	3	17%	10	21%		
Tumor stage	T1	15	31%	6	33%	10	21%	0.40	0.46
	T2	13	27%	4	22%	13	27%		
	T3	10	21%	3	17%	13	27%		
	T4	10	21%	5	28%	12	25%		
LNM	Tumor without Lymph node involvement	29	60%	12	67%	28	58%	0.64	0.84
	Tumor with Lymph node involvement	19	40%	6	33%	20	42%		
No. of positive cells	10-50% positive cells	16	33%	10	56%	19	40%	0.69	0.62
	51-80% positive cells	17	35%	7	39%	21	44%		

	>80% positive cells	15	31%	1	6%	8	17%		
IRS score	2-3=Mild	13	27%	13	72%	19	40%	0.31	0.14
	4-8=Moderate	20	42%	4	22%	23	48%		
	9-12=Strong	15	31%	1	6%	6	13%		
Tumor margin	Clear margin >0.5cm	35	73%	5	28%	15	31%	0.76	0.81
	Close margin 0.1-0.5cm	5	10%	9	50%	24	50%		
	Positive margin <0.1cm	8	17%	4	22%	9	19%		
Tumor thickness	Low risk <0.4cm	26	54%	8	44%	11	23%	0.01	0.04
	Moderate risk > 0.4 cm – < 1.0 cm	15	31%	8	44%	18	38%		
	High risk >1 cm	7	15%	2	11%	19	40%		
Perineural invasion^	Not identified	42	88%	10	56%	31	65%	0.003	0.03
	Identified	6	13%	8	44%	17	35%		
Lymphovascular invasion	Not identified	41	85%	13	72%	27	56%	0.23	0.69
	Identified	7	15%	5	28%	21	44%		
ENE	Negative	44	92%	15	83%	38	79%	0.41	0.59
	Positive	4	8%	3	17%	10	21%		
Treatment	Surgery only	35	73%	11	61%	27	56%	0.72	0.80
	Radiotherapy before surgery	6	13%	4	22%	6	13%		
	Radiotherapy after surgery	7	15%	3	17%	15	31%		

^Perineural invasion's proportional hazard (Global) test p value (with LTF included) = 0.0032 (assumption not met, therefore not included in Cox regression)
^Perineural invasion's proportional hazard (Global) test p value (with LTF excluded) = 0.0163 (assumption not met, therefore not included in Cox regression)

Table 3 (a): Univariate and multivariable Survival (Cox) regression analysis to determine the strength and direction of association of demographic and clinical characteristics with survival probability and survival time among alive/lost to follow up and dead patients

Independent variables		Univariate survival (Cox) regression				Multivariable survival (Cox) regression			
		Hazard Ratio	p-value	[95% Conf	Interval]	Hazard ratio	p-value	[95% Conf	Interval]
NANOG staining intensity	Mild Intensity (Reference)		1				1		
	Moderate Intensity	1.604	0.148	0.846	3.04	1.901	0.223	0.677	5.342
	Strong Intensity	1.223	0.628	0.542	2.758	5.94	0.048	1.019	34.628
Age group (in years)	22-36 (Reference)		1						
	37-45	1.43	0.348	0.677	3.022				
	46-55	0.838	0.657	0.384	1.829				
	56-84	0.791	0.579	0.345	1.813				
Gender	Female (Reference)		1						
	Male	0.994	0.987	0.495	1.999				
Laterality	Left (Reference)		1						
	Right	0.984	0.962	0.513	1.89				
	Midline	1.266	0.703	0.376	4.255				
	Not specified	2.003	0.111	0.852	4.709				
Tumor grade	Well-Differentiated Tumor (Reference)		1						
	Moderately Differentiated Tumor	0.87	0.675	0.452	1.672				
	Poorly Differentiated Tumor	0.759	0.504	0.338	1.703				
Tumor stage	T1 (Reference)		1						
	T2	1.186	0.689	0.516	2.726				
	T3	1.894	0.13	0.828	4.331				
	T4	1.542	0.312	0.665	3.575				
LNM	Tumor without Lymph node involvement (Reference)		1						
	Tumor with Lymph node involvement	1.143	0.651	0.641	2.038				
IRS score	2-3=Mild (Reference)		1				1		
	4-8=Moderate	1.536	0.171	0.831	2.84	0.842	0.741	0.305	2.329
	9-12=Strong	0.996	0.992	0.392	2.526	0.179	0.09	0.025	1.306
Tumor margin	Clear margin >0.5cm (Reference)		1						
	Close margin 0.1-0.5cm	1.262	0.49	0.652	2.445				
	Positive margin <0.1cm	1.077	0.862	0.468	2.478				
Tumor thickness	Low risk <0.4cm (Reference)		1				1		

	Moderate risk > 0.4 cm & < 1.0 cm	1.654	0.189	0.781	3.504	1.5	0.304	0.692	3.249
	High risk >1 cm	3.071	0.003	1.458	6.468	3.16	0.003	1.488	6.712
ENE	Negative (Reference)	1							
	Positive	1.329	0.427	0.658	2.684				
Treatment	Surgery only (Reference)	1							
	Radiotherapy before surgery	0.806	0.636	0.331	1.965				
	Radiotherapy after surgery	1.168	0.633	0.618	2.204				

Outcome variable: Patients' status (0= Alive/Lost to follow up (Reference), 1=Dead)

Proportional hazards assumption (Global) test p values for Univariate cox

1) NANOG: p=0.414 2)Tumor thickness: p=0.296 3) IRS score: 0.091 (Insignificant p values mean Proportional hazards assumption is met)

Proportional hazards assumption (Global) test p value for Multivariable cox model: p=0.330

Table 3 (b): Univariate and multivariable Survival (Cox) regression analysis to determine the strength and direction of association of demographic and clinical characteristics with survival probability and survival time among alive and dead patients

Independent variables		Univariate survival (Cox) regression				Multivariable survival (Cox) regression			
		Hazard Ratio	p-value	[95% Conf	Interval]	Hazard Ratio	p-value	[95% Conf	Interval]
NANOG intensity	Mild Intensity (Reference)	1				1			
	Moderate Intensity	1.816	0.068	0.958	3.445	1.813	0.070	0.953	3.448
	Strong Intensity	1.705	0.195	0.760	3.827	1.907	0.120	0.845	4.304
Age group (in years)	22-36 (Reference)	1							
	37-45	1.425	0.354	0.673	3.015				
	46-55	0.765	0.500	0.351	1.667				
	56-84	0.777	0.552	0.340	1.780				
Gender	Female (Reference)	1							
	Male	0.894	0.753	0.445	1.797				
Laterality	Left (Reference)	1							
	Right	0.910	0.778	0.475	1.746				
	Midline	1.164	0.806	0.346	3.909				
	Not specified	1.995	0.114	0.848	4.694				
Tumor grade	Well-Differentiated Tumor (Reference)	1							
	Moderately Differentiated Tumor	0.813	0.534	0.424	1.560				
	Poorly Differentiated Tumor	1.013	0.975	0.453	2.265				
Tumor stage	T1 (Reference)	1							
	T2	1.208	0.656	0.525	2.778				
	T3	1.864	0.141	0.814	4.267				

	T4	1.403	0.430	0.605	3.252				
LNM	Tumor without Lymph node involvement (Reference)		1						
	Tumor with Lymph node involvement	1.060	0.845	0.594	1.891				
IRS score	2-3=Mild (Reference)		1						
	4-8=Moderate	1.817	0.056	0.985	3.351				
	9-12=Strong	1.534	0.364	0.609	3.862				
Tumor margin	Clear margin >0.5cm (Reference)		1						
	Close margin 0.1-0.5cm	0.818	0.544	0.427	1.565				
	Positive margin <0.1cm	0.836	0.672	0.364	1.917				
Tumor thickness	Low risk <0.4cm (Reference)		1				1		
	Moderate risk > 0.4 cm & < 1.0 cm	1.456	0.326	0.687	3.085	1.502	0.292	0.705	3.201
	High risk >1 cm	2.437	0.020	1.152	5.153	2.548	0.015	1.196	5.429
ENE	Negative (Reference)		1						
	Positive	1.205	0.603	0.597	2.430				
Treatment	Surgery only (Reference)		1						
	Radiotherapy before surgery	0.746	0.519	0.306	1.818				
	Radiotherapy after surgery	0.929	0.821	0.493	1.751				

Outcome variable: Patients' status (0= Alive(Reference), 1=Dead)

Proportional hazards assumption (Global) test p values for Univariate cox

1) NANOG: p=0.733 2)Tumor thickness: p=0.406 (Insignificant p values mean Proportional hazards assumption is met)

Proportional hazards assumption (Global) test p value for Multivariable cox model: p=0.715

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