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Cancer Stem Cell Marker NANOG in Gallbladder Cancer and Inflammatory Gallbladder Disease:A Clinicopathological Correlation

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ABSTRACT

Background: Gallbladder cancer (GBC) is an aggressive malignancy associated with poor prognosis and high mortality. Chronic inflammatory conditions of the gallbladder, including cholelithiasis and cholecystitis, may contribute to malignant transformation. Cancer stem cells (CSCs) are considered important in tumor initiation, progression, therapeutic resistance, recurrence, and metastasis. NANOG, a key transcription factor involved in stemness and pluripotency, has been implicated in several solid tumors; however, its role in gallbladder carcinogenesis remains unclear. The present study evaluated NANOG expression in GBC and GID and investigated its association with clinicopathological characteristics.

Methods: The study included 50 subjects comprising 20 histopathologically confirmed GBC cases and 30 GID cases serving as controls. Formalin-fixed paraffin-embedded tissue sections were evaluated histopathologically and subjected to immunohistochemical staining using an anti-NANOG antibody. NANOG expression was assessed based on staining proportion and intensity and correlated with clinicopathological parameters of GBC.

Results: NANOG expression was observed in 15⁄20 (75%) GBC cases compared with 11⁄30 (36.6%) GID cases, with a statistically significant difference ($\chi^2 = 7.065$, $p = 0.0079$). NANOG positivity was significantly higher in moderately/poorly differentiated tumors (11⁄12, 91.7%) than in well-differentiated tumors (4⁄8, 50%; $\chi^2 = 4.44$, $p = 0.03$). No significant association was observed between NANOG expression and age, sex, gallstone status, or pathological stage.

Conclusion: NANOG expression is significantly increased in GBC compared with GID and is associated with poorer tumor differentiation. NANOG may serve as a potential biomarker of tumor aggressiveness and warrants further investigation in gallbladder carcinogenesis.

Key-words: NANOG; Cancer stem cells; Gallbladder cancer; Gallbladder inflammatory disease; Immunohistochemistry

INTRODUCTION

Gallbladder cancer (GBC) is one of the most common malignancies of the biliary tract and is characterized by an aggressive clinical course and poor prognosis. The incidence of GBC is particularly high in northern India and other geographically defined high-risk regions [1,2].

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Cholelithiasis is considered one of the major risk factors for GBC, with chronic inflammation associated with gallstones and cholecystitis contributing to malignant transformation [1,3]. The prognosis of GBC remains extremely poor because of its aggressive biological behavior, late presentation, and limited treatment options, with the overall 5-year survival remaining approximately 5% in patients with advanced disease [4]. GID, particularly cholelithiasis and chronic cholecystitis, have been associated with a sequence of pathological alterations, including epithelial hyperplasia, atypical hyperplasia, adenomatous hyperplasia, dysplasia, carcinoma in situ, and ultimately invasive carcinoma. These observations support the concept that chronic

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inflammation and progressive epithelial alterations may contribute to the development of GBC [3,5].

Advances in the CSCs have provided a new perspective on tumor initiation and progression. CSCs are a subpopulation of tumor cells characterized by self-renewal and differentiation capacity and are thought to contribute to tumor initiation, progression, metastasis, therapeutic resistance, and disease recurrence [6]. Emerging evidence supports the presence and biological importance of CSC populations expressing stemness-associated markers, including NANOG**,** in several solid tumors such as brain, prostate, pancreatic, and ovarian cancers [7–10].

However, the role and clinical significance of NANOG-positive CSCs in GBC remain insufficiently characterized. NANOG is a master transcription factor involved in the maintenance of embryonic stem-cell pluripotency and self-renewal. It regulates stem-cell identity and cell-fate specification through interactions with other pluripotency-associated factors, including OCT4, SOX2, and KLF4 [11]. Aberrant NANOG expression has also been reported in several human cancers and has been associated with tumor cell proliferation, stemness, invasion, metastasis, and resistance to therapy [6,12].

In a study of 100 breast cancer patients, strong NANOG expression was associated with significantly poorer disease-free and overall survival compared with weak NANOG expression [13]. Similarly, in a study of 163 patients with lung cancer, NANOG protein expression was increased in tumor tissues compared with normal lung tissues and was positively associated with clinical stage [14]. Hepatocellular carcinoma also exhibits cellular heterogeneity, with stemness-related genes preferentially expressed in NANOG-positive cancer stem-like cells, supporting a role for NANOG in maintaining tumor stemness [15,16].

Although these findings suggest an important role for NANOG in cancer stemness and tumor aggressiveness**,** its expression and clinicopathological significance in gallbladder cancer remain unclear. Therefore, the present study was undertaken to investigate the expression of NANOG in gallbladder inflammatory disease and gallbladder cancer and to evaluate its association with selected clinicopathological characteristics, particularly tumor differentiation.

MATERIALS AND METHODS

Study Population and Sample Size- This study was conducted over a period of four years. A total of 104 subjects were included, comprising 48 histopathologically confirmed cases of GBC and 54 cases of GID with or without cholelithiasis, which served as controls. Consecutive patients diagnosed with gallbladder cancer for the first time and who had not received any prior treatment were enrolled. Patients with inflammatory gallbladder disease undergoing cholecystectomy were included as controls. Written informed consent was obtained from all participants before inclusion in the study. Patients with gallbladder cancer who had a current or previous history of any other malignancy, those suffering from AIDS or any other known immunodeficiency disorder, and patients in the terminal stage of any disease rendering them unsuitable for surgical intervention were excluded from the study.

Sample Size Calculation- The sample size was calculated based on the study objectives and the expected prevalence of gallbladder cancer, with an appropriate level of confidence and precision. A total of 104 subjects were ultimately included in the study, comprising 48 cases of gallbladder cancer and 54 controls with inflammatory gallbladder disease.

Clinical Evaluation and Data Collection- All enrolled patients underwent detailed clinical assessment by the treating clinician. Demographic information, present and past medical history, and findings from physical examination were recorded on a structured case record form. Routine hematological investigations and liver function tests were also documented.

Tissue Collection and Histopathological Examination: Gallbladder tissue specimens obtained either through biopsy or surgical resection were collected or fixed in 10% neutral buffered formalin. The tissues were processed routinely and embedded in paraffin wax. Histopathological examination was performed on hematoxylin and eosin (H&E)-stained sections for confirmation of diagnosis, tumor grading, staging, and assessment of invasiveness.

Formalin-fixed paraffin-embedded (FFPE) tissue blocks from 20 gallbladder cancer cases and 30 inflammatory gallbladder disease cases were prepared in the

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Department of Histology. Serial sections of 4 µm thickness were cut from each block and stained with H&E for microscopic evaluation.

Immunohistochemical Analysis of NANOG Expression- Immunohistochemical staining for the cancer stem cell marker NANOG was performed on FFPE tissue sections using a commercially available anti-NANOG polyclonal antibody (Proteintech, USA) according to the manufacturer's protocol. Stained slides were independently evaluated by the observer. For quantitative assessment, ten representative hotspot areas showing maximum immunoreactivity were selected on each slide. Positively stained cells were counted at 400× magnification, and the mean count was calculated for each case.

NANOG Immunohistochemical Scoring- NANOG expression was evaluated using a semi-quantitative scoring system based on the proportion and intensity of staining. The proportion score was assigned as follows: 0 = no positive cells, 1 = <10% positive cells, 2 = 10–25% positive cells, 3 = 26–50% positive cells, 4 = 51–75% positive cells, and 5 = >75% positive cells. Staining intensity was graded as 0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining. The immunohistochemical findings were subsequently correlated with the clinicopathological characteristics of gallbladder cancer cases.

Statistical Analysis- Data were analyzed using appropriate statistical methods. Categorical variables were expressed as frequencies and percentages, while continuous variables were summarized as mean ± standard error. The association between NANOG expression and clinicopathological variables was assessed using the Chi-square test. A p-value of <0.05 was considered statistically significant.

Ethical Clearance- According to the Helsinki declaration, the study was approved by the institutional ethics committee (ELMC/R_Cell/EC/2014, August 11, 2014). All the patients who were enrolled have given their written informed consent in both English and Hindi languages.

RESULTS

A total of 50 subjects were enrolled in the study, including 20 histopathologically confirmed cases of GBC and 30 cases of GID. The study was conducted to evaluate and correlate the expression of the cancer stem cell marker NANOG in GBC and GID. Among the GBC cases, 8 (40%) were well-differentiated adeno-carcinomas, while 12 (60%) were moderately to poorly differentiated adenocarcinomas. The mean age of patients with GBC was higher than that of GID patients (50.43 ± 1.43 years vs. 40.65 ± 1.60 years). Females constituted the majority of cases in both groups, accounting for 65% of GBC patients and 53.3% of GID patients. Gallstones were present in 55% of GBC cases and 73.3% of GID cases. Among GBC patients, 70% were diagnosed at pathological stages I–II, while 30% belonged to stages III–IV (Table 1).

Table 1: The demographic and clinical characteristics of GID and GBC subjects

Parameters		GID n (%)	GBC n (%)
Habitat	Urban	18 (60)	4 (20)
	Rural	12 (40)	16 (80)
Food habit	Vegetarian	19 (63.6)	8(40)
	Non-Vegetarian	11 (36.6)	12 (60)
Water habit	Hand pump	11 (36.6)	11 (55)
	Tube well	19 (63.3)	9 (45)
Smoking status	Yes	10 (33.3)	6 (30)
	No	20 (66.6)	14(70)
Tobacco intake	Yes	16 (53.3)	8 (40)
	No	14 (46.6)	12 (60)
Alcohol consumption	Yes	4 (13.3)	5 (25)
	Never	26 (86.6)	15 (75)
Chilli consumption	Low	6(20)	5 (25)
	Moderate	13(43.3)	12 (60)
	High	11 (36.6)	3 (15)
Hypertension	Yes	12 (40)	13 (65)
	No	18 (60)	7 (35)
Pain in abdomen	Yes	28 (93.3)	15 (75)
	No	2(6.66)	5 (25)

Most GBC patients belonged to rural areas (80%), whereas 60% of GID patients were from urban areas. Non-vegetarian dietary habits were more common among GBC patients (60%) compared with GID patients (36.6%). Tobacco consumption was reported in 40% of GBC patients and 53.3% of GID patients. Alcohol consumption was observed in 25% of GBC patients and

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13.3% of GID patients. Hypertension was more frequent among GBC patients (65%) than GID patients (40%). Abdominal pain was the most common presenting symptom and was reported by 75% of GBC patients and 93.3% of GID patients.

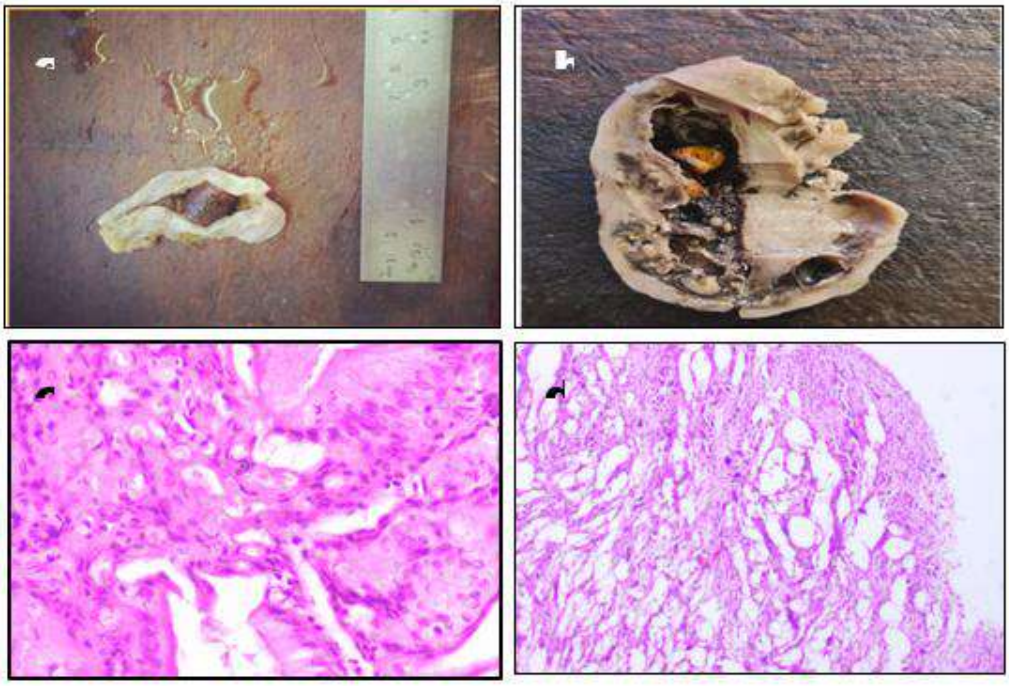

Fig. 1 (a): Gross appearance of gall bladder showing cholelithiasis. **(b)** Gross appearance of gallbladder showing malignant growth with two stones in cancerous lesion. **(c)** Hematoxylin-eosin staining (400X) of gallbladder inflammatory disease. (d) Hematoxylin-eosin staining (400X) of gallbladder cancer cases.

Immunohistochemical analysis demonstrated that NANOG expression was localized predominantly in the nucleus and cytoplasm of epithelial cells. Positive NANOG expression was observed in 15 of 20 (75%) GBC cases and 11 of 30 (36.6%) GID cases. Conversely, negative expression was detected in 5 (25%) GBC cases and 19 (63.3%) GID cases. Strong immunoreactivity for NANOG was predominantly observed in invasive areas of gallbladder carcinoma tissues. The staining intensity ranged from mild to strong, with positive staining involving approximately 10%–90% of tumor cells in NANOG-positive GBC cases. The frequency of NANOG positivity was significantly higher in GBC compared with GID, suggesting a potential role of NANOG in gallbladder carcinogenesis (Table 2, 3 and Fig. 2).

Table: 2: Characteristics of study subjects

Characteristics	**Gallbladder inflammatory Disease (n=30)**	**Gallbladder Cancer (n=20)**
Age (Mean ±S.E) Range (Years)	40.65±1.60	50.43±1.43
Gender: Male Female	 14(46.6) 16 (53.03)	 7 (35) 13 (65)
Gall stones: Present Absent	 22 (73.3) 8 (26.6)	 11 (55) 9 (45)

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Tumor differentiation Well differentiated Moderately/Poorly differentiated	-	 8 (40) 12 (60)
Pathological Staging I-II III-IV	-	 14 (70) 6 (30)
NANOG Positive Negative	 11(36.66%) 19 (63.3%)	 15(75%) 5 (25%)

Table 3: Expression level of NANOG in GBC and GID lesions of gallbladder

Tissue Type Total number	Total Number	NANOG Positive numbers (%)	NANOG Negative numbers (%)	p-value
Gallbladder inflammatory disease	30	11 (36.6)	19 (63.3)	χ^2 = 7.065 df = 1 p = 0.0079
Gallbladder cancer	20	15 (75%)	5 (25)	

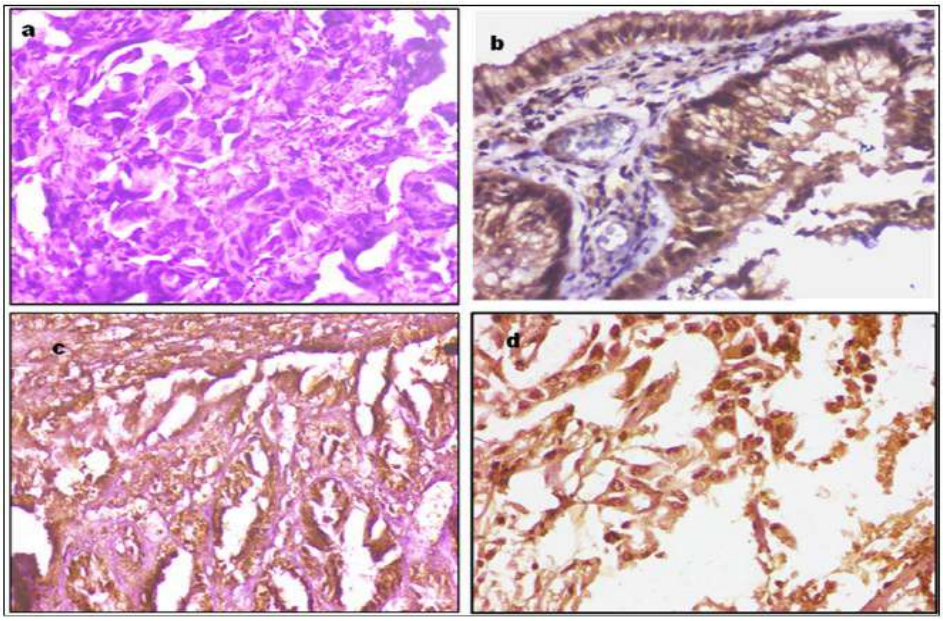

Fig. 2: Evaluation of cancer stem cell marker NANOG in gallbladder inflammatory disease and gallbladder cancer. **(a)** Negative control for NANOG in gallbladder cancer (IHC 400X). **(b)** Low expression of NANOG showing cytoplasmic staining in gallbladder inflammatory disease (IHC 400X) **(c)** Low expression of NANOG showing cytoplasmic staining in gallbladder cancer (IHC, 400X. **(d)** High expression of NANOG showing cytoplasmic and nuclear staining in gallbladder cancer (IHC 400X)

The association between NANOG expression and clinicopathological characteristics of GBC patients is summarized in Table 4. No significant association was observed between NANOG expression and age (χ^2 = 0.08, p = 0.765), sex (χ^2 = 0.07, p = 0.780), gallstone status (χ^2 = 0.606, p = 0.436), or pathological stage (χ^2 = 1.66, p = 0.196). However, a statistically significant association was found between NANOG expression and tumor differentiation (χ^2 = 4.44, p = 0.03). Positive NANOG expression was significantly more frequent in moderately to poorly differentiated tumors (11/12, 91.7%) compared with well-differentiated tumors (4/8, 50%). Furthermore, four of five NANOG-negative cases were well-differentiated tumors. These findings indicate that increased NANOG expression is associated with poor tumor differentiation and may reflect a more aggressive biological phenotype in gallbladder cancer.

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Table 4: Association of NANOG positivity with Clinicohistopathological profile of gallbladder cancer patients.

Variable	No. of cases	NANOG			
		Negative (n=5)		Positive (n=15)	
		No.	%	No.	%
Age					
<40 Yr	5	1	20	4	26.66
>40 Yr	15	4	80	11	73.33
Statistical significance		χ^2 = 0.08; p=0.765			
Sex					
Male	7	2	40	5	33.33
Female	13	3	60	10	66.66
Statistical significance		χ^2 = 0.07; p=0.78			
Gallstones					
Present	11	2	40	9	60
Absent	9	3	60	6	40
Statistical significance		χ^2 = 0.606; p=0.436			
Tumor differentiation					
Well differentiated	8	4	80	4	26.66
Moderately/Poorly differentiated	12	1	20	11	73.33
Statistical significance		χ^2 = 4.44; p=0.03			
Pathological Stage					
I-II	16	3	60	13	86.66
III-IV	4	2	40	2	13.33
Statistical significance		χ^2 = 1.66; p=0.196			

DISCUSSION

The present study demonstrated significantly higher NANOG expression in GBC compared with GID, with NANOG positivity observed in 75% of GBC cases compared with 36.6% of GID cases. This significant difference suggests that NANOG may be involved in the biological processes underlying gallbladder carcinogenesis. NANOG is a key transcription factor associated with maintenance of stemness and pluripotency and has been implicated in cancer stem cell (CSC) self-renewal, tumor initiation, progression, invasion, and therapeutic resistance [17-19]. The increased expression observed in GBC therefore supports the possibility that NANOG-positive cells contribute to the aggressive characteristics of gallbladder tumors.

Importantly, NANOG expression showed a significant association with tumor differentiation. Positive expression was observed in 91.7% of moderately/poorly differentiated tumors compared with 50% of well-differentiated tumors. This finding indicates that increased NANOG expression may be associated with a less differentiated and potentially more aggressive tumor phenotype.

Similar observations regarding the relationship between NANOG expression and tumor aggressiveness have been reported in other malignancies, including breast and lung cancers [20].

In contrast, no significant association was observed between NANOG expression and age, sex, gallstone status, or pathological stage in the present cohort. The absence of association with pathological stage may be related to the relatively small number of patients and limited representation of advanced-stage disease. Interestingly, NANOG expression was also detected in 36.6% of GID cases, suggesting that inflammatory or regenerative processes may involve alterations in stemness-related pathways [21,22].

Overall, these findings suggest that NANOG may represent a potential biomarker of tumor differentiation and aggressiveness in GBC. Larger studies incorporating additional CSC markers, molecular analyses, and survival follow-up are required to establish its prognostic and therapeutic significance. Further studies are required with a larger sample size with other cancer stem cell markers in combination with NANOG to validate the results with more specificity. This would lead to

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development of a novel prognostic and therapeutic biomarker for GBC.

CONCLUSIONS

The present study demonstrates significantly higher NANOG expression in gallbladder cancer compared with gallbladder inflammatory disease. NANOG expression was observed in 75% of gallbladder cancer cases compared with 36.6% of inflammatory gallbladder disease cases. Increased NANOG expression was also significantly associated with poorer tumor differentiation, being more frequently observed in moderately to poorly differentiated tumors than in well-differentiated tumors. However, no significant association was observed between NANOG expression and age, sex, gallstone status, or pathological stage. These findings suggest that NANOG may have a potential role in gallbladder carcinogenesis and may be associated with tumor differentiation and aggressive biological behavior. NANOG may therefore serve as a candidate biomarker of tumor aggressiveness in gallbladder cancer. Further studies with larger sample sizes and evaluation of other cancer stem cell markers in combination with NANOG are required to validate these findings and establish its prognostic and therapeutic significance.

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CONTRIBUTION OF AUTHORS

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