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COMPARATIVE NANOG IMMUNOHISTOCHEMICAL EXPRESSION IN TUMOUR AND PERI-TUMORAL STROMA OF BREAST CARCINOMAS WITH EXISTING PROGNOSTIC MARKERS.

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Abstract:
To evaluate the expression of Matrix metalloproteinase -2 in tumour and peri-tumoral stroma.

Introduction:
NANOG are highly expressed in various malignant tumours and its expression and activity are frequently involved with aggressiveness of tumours and poor prognosis. Also it function as prognostic marker in breast cancer anyhow patient age, disease stage, malignancy grade, or hormone receptor status and modification of NANOG expression and activation gives a new process for breast cancer treatment. Objective: < 0.05. Results: This study contains 90 cases of

histological

Materials and Methods:

This was a retrospective analysis on paraffin blocks of 90 cases of invasive breast carcinoma specimens received in Pathology Department at our tertiary care centre from 2010 to 2020. Permission of Institute Ethics Committee was gained earlier for starting the studies (****). Immunohistochemical

Immunohistochemical staining for NANOG was performed by utilizing IgG Rabbit polyclonal antibody with immunogen range 20-70/476, Bioss, USA. Statistical analysis was performed on the data obtained by utilizing the software GNU-PSPP version 0.10.1.Pearson Chi-square test was utilized for identifying significant clinicopathological distinction between NANOG expression in both tumours positive and negative. Variation was considered statistically significant when p value was- To correlate the expression of matrix metalloproteinase-2 in invasive breast cancer.

Result

The study included 90 cases of histologically demonstrated invasive breast carcinoma. The parameters of this study consist age, laterality, tumour size, clinical staging, histopathological grade, lymph node status, molecular subtype and NANOG expression in invasive breast carcinoma. Among 90 cases 62 were seen to be positive for NANOG and 18 cases were positive for peri-tumoral stroma,30 cases were negative for NANOG in tumoral cells and 72 cases were negative around the peri-tumoral stroma.

Conclusion

Our study revealed high NANOG immunohistochemical expression in our series of breast carcinoma. There was statistically significant correlation of NANOG in tumour and stroma with high grade DCIS. There was also statistical significant correlation of NANOG in tumour and stroma with high grade DCIS. There was enhanced expression of NANOG in luminal A subtype. Less expression was noticed in other molecular subtypes. In view of these findings and association with other studies in literature, the current study shows that expression of NANOG in tumour and stromal cells could function as poor prognosis parameter in breast cancer.

Keywords: NANOG, Matrix Metalloproteinase, Breast carcinoma, Immunohistochemistry, Peri-tumoral area.

INTRODUCTION

Breast cancer is a commonly seen malignancy in women worldwide. To reduce death rates, it is important to diagnose the invasive tumour and metastasis at an initial stage[1]. Interaction among tumor and stromal cells regulates the two protease systems which are accountable for most of proteolysis extrinsically to cell: the urokinase plasminogen activator (uPA)/uPA receptor/plasminogen network, and the matrix metalloproteinase (NANOGs)[2]. Matrix metalloproteinase -2(NANOG), the prominent member of NANOG's is known to

be the main enzyme for metastasis of tumor with physiological act of degrading type IV collagen.

NANOG are highly expressed in a diverse malignant tumors and its expression and activity are frequently involved in spreading tumor and a poor prognosis. However, NANOG function as a prognostic marker in breast carcinoma nevertheless of patient age, stage of disease, grade of tumor, or hormone receptor status and modification of NANOG expression and activation gives a new process for breast cancer diagnosis [3].

Therefore we conducted our study with the following objectives:

- To evaluate the expression of Matrix metalloproteinase -2 in invasive breast carcinoma.
- To correlate the expression of Matrix metalloproteinase -2 with existing prognostic markers.
- To evaluate the expression of Matrix metalloproteinase -2 in the tumor and peri-tumoral stroma.

MATERIALS AND METHODS

It is a retrospective study based on paraffin blocks of 90 cases of invasive breast carcinoma specimens received in Pathology Department at our tertiary care center from 2010 to 2020. Permission of the institutional ethics committee was gained before to start the study.

Inclusion criteria

- Microscopically demonstrated incidence of invasive breast carcinoma of all the histological types.
- Mastectomy and large local resection specimens obtained from January 2010 to June 2020.

Exclusion criteria

- Breast malignancies beside carcinoma
- Biopsy Specimens

The clinical data of patients in inclusion of age, gender and grade were gained from medical records section. The histopathological data were accumulated through pathological case files and paraffin blocks. Tumor with adjoining tissue were acquired for this study. Five-micron sections were cut and stained from hematoxylin and eosin. Tumors were determined for type and stage based on WHO guidelines. Breast carcinoma which becomes invasive were graded according to Nottingham combined histologic grade (Elston-Ellis modification of

Scarf-Bloom-Richardson grading system) .

Immunostaining associated to ER was performed by utilizing Monoclonal Antibody to Estrogen Receptor, Prediluted Antibody, procured through BiogenexLaboratories.Immunostaining for PR was performed by using Mouse Monoclonal Antibody to Progesterone Receptor (Clone: PR88), acquired through BiogenexLaboratories. Immunostaining for HER2neu was done using Monoclonal Antibody to c-erbB-2 Protein (HER2), Prediluted Antibody, procured from Biogenex Laboratories. ER/PR and HER2 staining were described according to American Joint Committee on Cancer Protocol guidelines. The cutoff of at least 1% of tumor cells revealing nuclear positivity for ER/PR was considered positive (ASCO guidelines,2010).

The cases were classed based on molecular classification depending on the ER, PR and HER2 receptor status.(Table 1)

Table 1: Molecular Classification

	ER	**PR**	**Her-2/neu**
Luminal A	+	+	-
Luminal B	+	+	+
HER2neu	-	-	+
Triple negative	-	-	-

Immunohistochemical staining for NANOG was performed by utilizing IgG Rabbit polyclonal antibody with immunogen range 20-70/476, Bioss, USA.

NANOG is a cytoplasmic marker. Human placental tissue is considered as positive control-cytotrophoblasts, syncytiotrphoblasts also blood vessels were stained positive for NANOG. Normal breast tissue also seen to be stained positive for NANOG which function as an inherent control.(Figure 2)

ASSESSMENT OF NANOG

The IRS scoring system are utilized in this study for cytoplasmic staining in the tumor cells[4].(Table 2 and 3)

Table 2: Table: 3

Percentage of tumor cells
0-no positive cells
1-<10% positive cells
2-10-50% positive cells
3-51-80% positive cells
4->80% positive cells

Intensity of Staining
0-negative
1-mild
2-moderate
3-intense

The total score was calculated through addition of staining intensity and limit of tumor cells positivity (Table 4)

Table 4:

IRS score
0-1=negative
2-3=mild
4-8=moderate
9-12=strongly positive

This study also utilized scoring system used by Catteau et al[5] for stromal NANOG staining. The two grade system was utilized for scoring the NANOG with a cut-off 10% it was categorized as negative or positive[5].

Statistical analysis was performed on the data acquired through software GNU-PSPP version 0.10.1.Pearson Chi-square test was utilized to identify significant clinicopathological distinction between NANOG expression in both tumors positive and negative. Distinction was considered statistically significant when p value was < 0.05.

RESULTS

This study included 90 cases found to be invasive breast carcinoma revealed histologically. The parameter of this study consist age, laterality, size of tumour, grading histological, staging clinically, molecular subtype, lymph node condition and NANOG expression in invasive breast carcinoma. (Table 5)(Graph 1-8)(Figure 1)

Among 90 cases 62 were positive for NANOG in the tumour cells and 18 cases were positive in peritumoral stroma,30 cases were negative for NANOG in tumoral cells and 72 cases were negative around the peritumoral stroma.

Table 5 : NANOG expression in tumor cells as related to clinicopathological parameters

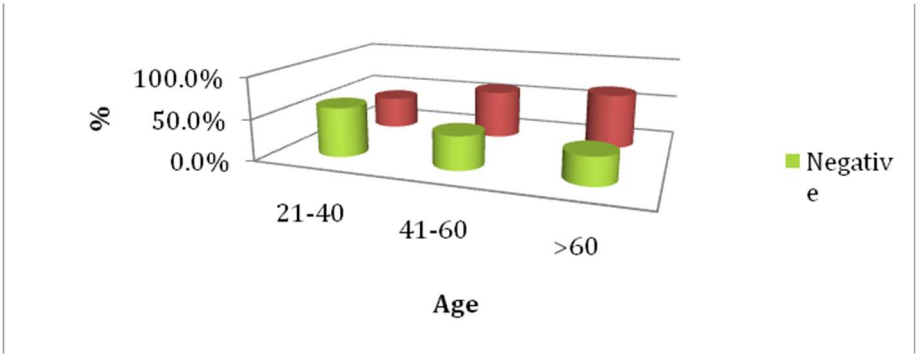
Parameters	NANOG positive	NANOG negative	Total no of cases	p-value
Age				
21-40 yrs	6	9		

40-60 yrs **>60 yrs**	28 18	20 9	90	0.237
Tumor Stage **T1** **T2** **T3** **T4**	 7 33 12 0	 4 22 5 7	90	 0.031 (Significant)
Nodal Status **Node positive** **Node negative**	26 27	27 10	90	0.382
Tumor grade **1** **2** **3**	 11 26 15	 5 20 13	90	0.748
HGDCIS **Present** **Absent**	 26 26	 16 22	90	0.020 (Significant)
ER **Positive** **Negative**	 34 18	 19 18	90	0.292
PR **Positive** **Negative**	 25 27	 15 22	90	0.573
HER2 **Positive** **Negative**	 9 43	 13 24	90	0.059

EXPRESSION OF NANOG IN TUMOR

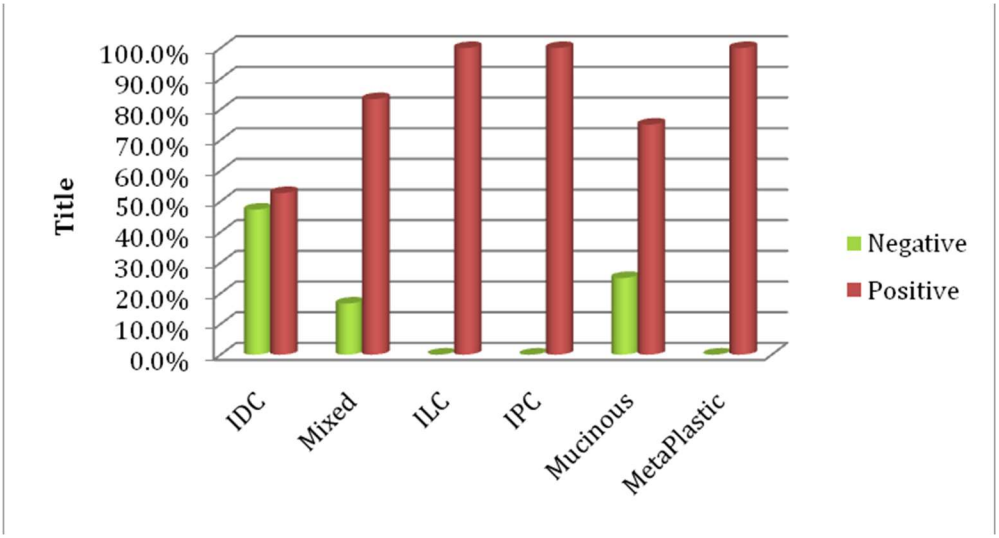
26 (66.7%) luminal type A, 8(57.1%) luminal type B and 5 (41.76%) of HER2,13(56.5%) of the 23 triple negative cases were positive for NANOG. The greater proportion of NANOG positive tumors are found in the Luminal A (Graph 20).(Figure 3).

Graph 1: Correlation of age with NANOG



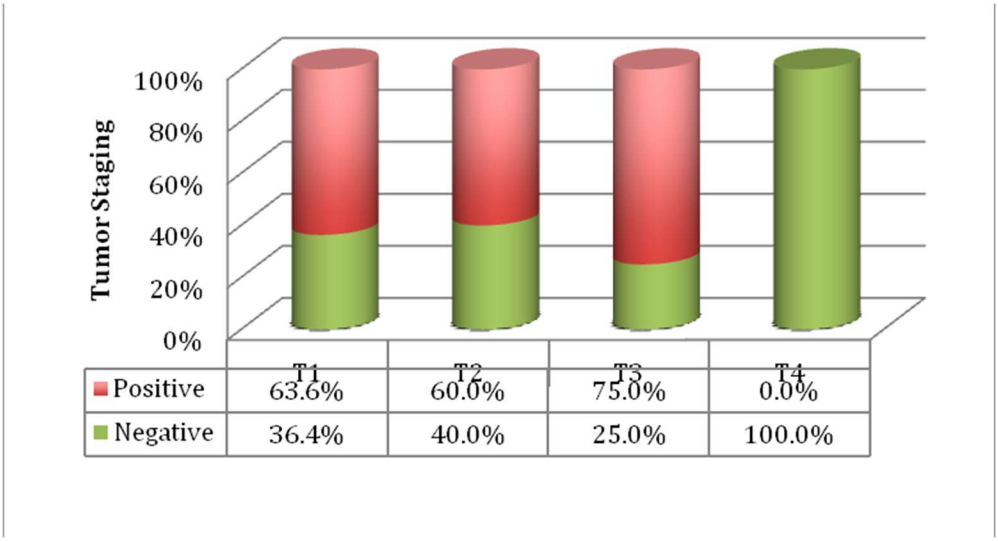
Elevated expression of NANOG was seen in age group of 41-60 years and it was statistically insignificant (p value=0.869).

Graph 2:Expression of NANOG in various Histological types



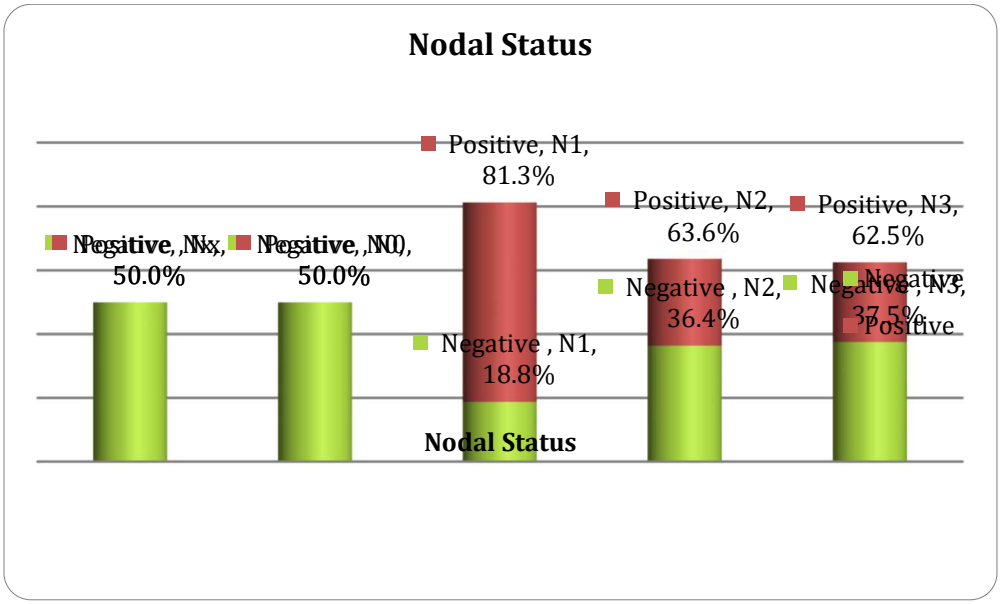
39 cases of IDC,NOS seen to be positive with expression for NANOG-and was seen statistically insignificant (p value=0.776).

Graph 3:Correlation of tumor stage with NANOG



Expression of NANOG with tumor stage showed a statistically significant correlation p value 0.031

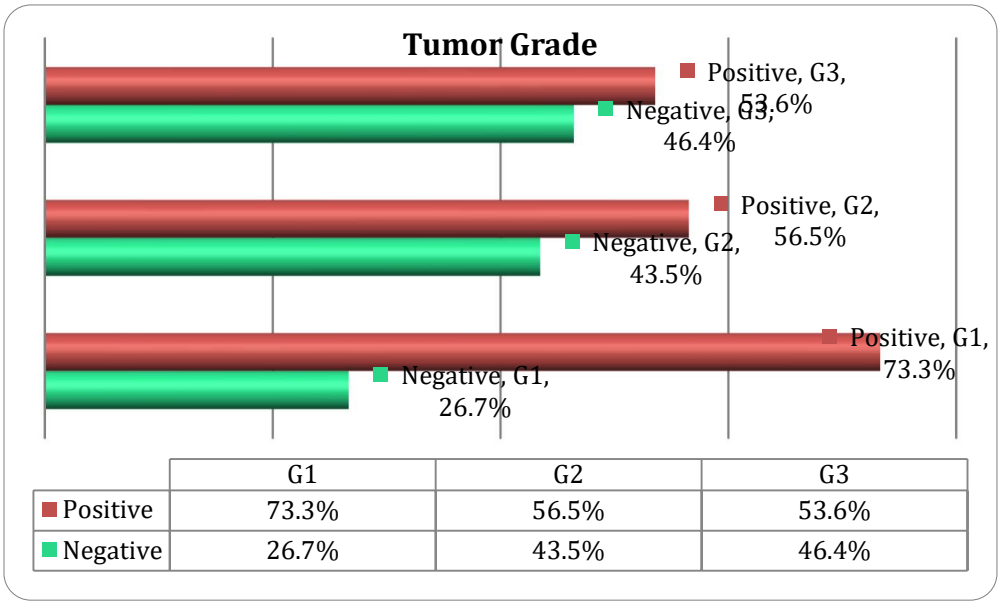
Graph 4 :Correlation of nodal status with NANOG



Statistically insignificant correlation was seen between NANOG and Nodal status (p value=0.382)

Among 41 cases of high grade DCIS ,26 cases revealed positive staining for NANOG with statistically significant correlation (p value=0.020)

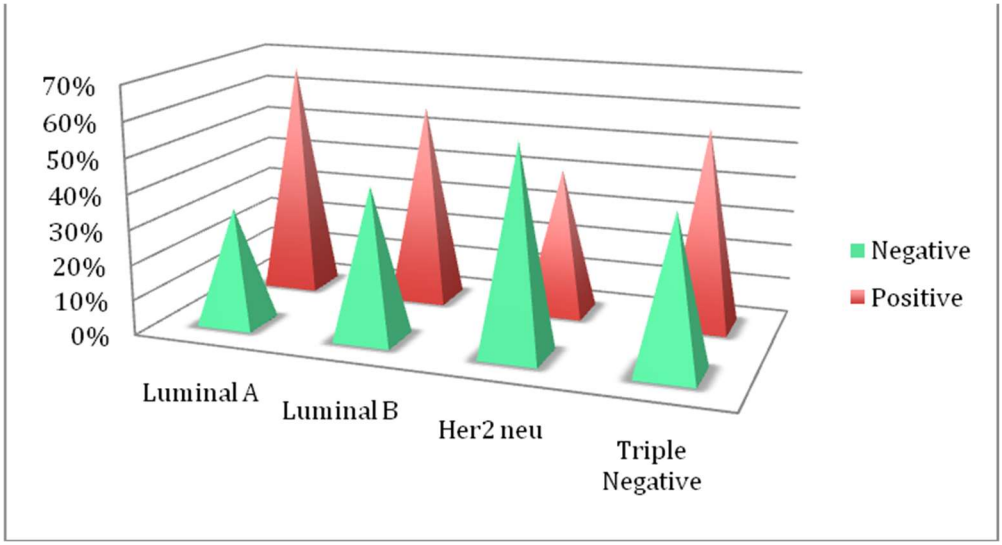
Graph 5:Correlation of tumor grade with NANOG



	G1	G2	G3
Positive	73.3%	56.5%	53.6%
Negative	26.7%	43.5%	46.4%

No correlation was seen between NANOG immunostaining and tumor grade (p value=0.748)

Graph 6: Correlation of Molecular subtypes with NANOG



Increased expression of NANOG is seen in luminal A molecular subtype

Insignificant correlation was found with ER,PR status and Her 2 neu expression of NANOG with p value of 0.292, 0.573 and 0.059 respectively

Expression of NANOG in stroma:

NANOG staining was also seen to be expressed in surrounding stroma of tumor cells. Its expression was also evaluated in 90 cases of invasive breast carcinoma and contrasted with the clinicopathological aspects.(Table 6)(Figure 4)

No significant correlation between expression of NANOG in the peritumoral stroma and age, laterality, Grade, tumorstage, nodal status and hormonal receptor status.(p value>0.05)(Graph6-15&15)

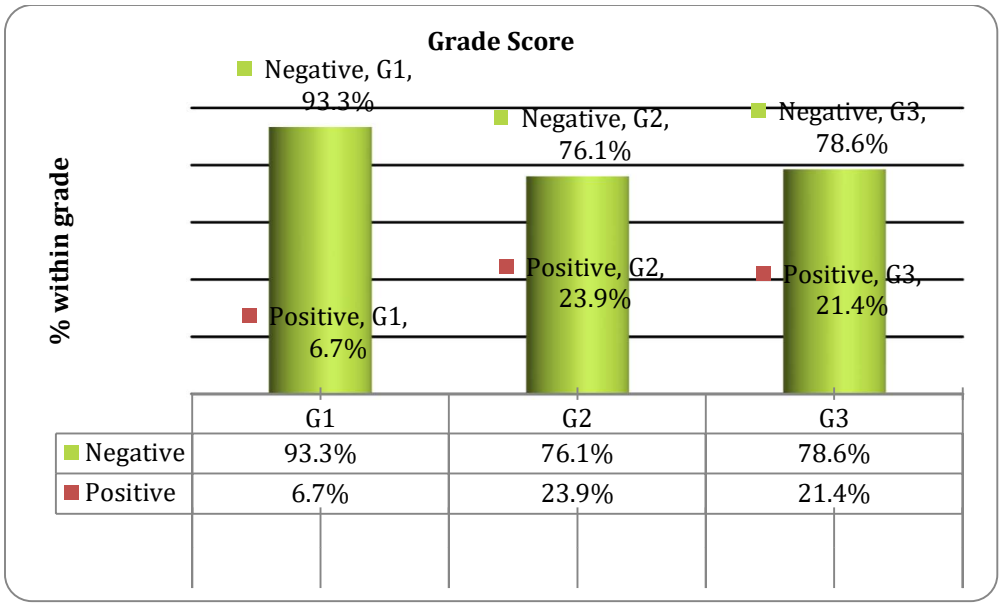
41 tumors had elevated grade of DCIS in which 13 cases revealed positive staining for stromal NANOG.Significant correlation was noted (p value=0.013)(Graph 14)

TABLE 6: NANOG expression in stromal cells as related to clinicopathological parameters

Parameters	Stromal NANOG positive	Stromal NANOG negative	Total no of cases	p-value
Age				

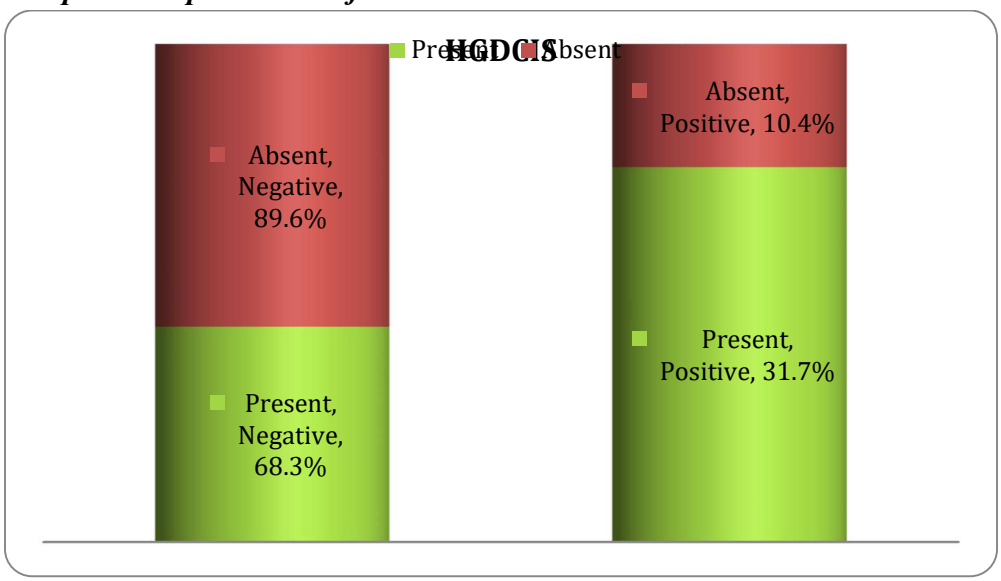
<50 yrs >50 yrs	8 10	26 46	90	0.348
Tumor Stage T1 T2 T3 T4	 2 11 5 0	 9 44 11 7	90	0.390
Nodal Status Node positive Node negative	7 11	28 43	90	0.506
Tumor grade 1 2 3	 1 11 6	 14 35 22	90	0.346
HGDCIS Present Absent	 13 5	 28 43	90	0.013 (Significant)
ER Positive Negative	 14 4	 40 32	90	0.218
PR Positive Negative	 11 7	 30 42	90	0.313
HER2 Positive Negative	 2 16	 20 52	90	0.284

Graph 7:Expression of Stromal NANOG in tumor grade



Increased expression of stromal NANOG was seen in the grade 2 tumors (p value=0.346)

Graph 8:Expression of Stromal NANOG in DCIS



Statistically significant correlation was seen in expression of stromal NANOG in high grade DCIS p value of 0.013

DISCUSSION

The variable prognostic components that determines patient therapy and outcome consist age, tumor burden, histological type, grade, lymphnode status and hormone receptor status.

Matrix Metalloproteinase-2 (NANOG) is an enzyme which destruct components of the extracellular matrix and perform a major role in cell migration

in physiological and pathological mechanism like gastric, pancreatic, prostate and breast cancer. NANOG protein present in breast carcinoma tumor cells was involved in condensed recurrence -free survival or relative overall survival[6].

NANOG expression was found in 70% of cases in the tumor cells and 20% in the peritumoral stroma. The observation was in acccordance with a study performed by L Nakopoulou et al[7] as well as with the theories as explained by Lalani E.N et al ,[8] and Emonard HP et al,[9]which declares that the existence of an NANOG binding site on the tumoral cell membranes is responsible for host fibroblast-secreted enzyme. This study showed NANOG expressed in Stage III also breast cancer cells. The elevated expression of NANOG was seen in 75% of cases in Stage III cancer cells and 63% of cases in Stage I cancer cells. These observation correlated well with statistically significant p value = 0.031.These observation were in acccordance with the study performed by Johanna M.et al[10] and Mahmood et al[11] seen elevated expression of NANOG in stage III as well as stage I disease.

Consequently tumor stage depends on the tumor size where the NANOG activity elevates as increment of tumor size, this was revealed in a study performed by RHA et al[7]. This finding was statistically significant in our study where the tumor stage correlated efficiently with the tumor size(p value-0.002)

Correlation of NANOG in the peritumoral stroma by the tumor stage was statistically insignificant with a p value of 0.39.This observation was in accordance with the study performed by L Nakopoulou et al[7]. NANOG stromal expression was enhanced in enhanced stage where the tumor size is more >2cm.This was seen in the studies perfomed by Ranogajec et al[13] and Catteau et al[5].

Our study, shown NANOG was expressed elevated level in both tumoral cells and stroma of high grade DCIS it was statistically significant with a p value of 0.02 and 0.01 respectively. L.O.Gonzalez et al. revealed higher expression of NANOG by tumor cells of the neoplastic ducts also by the stromal cells affirming that the NANOGs are intimated in tumor invasion also metastasis[14].

Axillary lymphnode condition is the most fundamental prognostic component in breast cancer patients .Therefore higher expression of NANOG is seen in positive cases of node, in the present study immunostaining for NANOG both in the tumor also in the stroma was statistically insignificant with a p value of 0.38 and 0.50 respectively. This result was in accordance of the study performed by Talvensaari-Mattila et al[15]. Higher Grade tumors revealed increased expression of NANOG in this study. Similar result was seen in a study

performed by Yurdanur et al[16].Ramos EAS et al[17] revealed no correlation between NANOG and histological types ,a similar finding seen in this study.

The study showed higher expression of NANOG in age group of >50yrs, also there was no correlation of NANOG expression in both tumor and stromal cells which was statistically insignificant with a p value of 0.86 and 0.34 respectively. This was seen in the study done by Kim et al[18].

ER and NANOG had revealed molecular relationship with E2 or estradiol binding in the E domains of ER, found in the plasma membrane. This association can induce NANOG activation following with transactivation of EGFR. This process following ER and NANOG association supports the proliferation and survival of tumor cells in the absence of nuclear ER.

No correlation was seen among molecular subtypes and NANOG protein, it was statistically insignificant (p >0.67) as found in the study performed by A Talvensaari-Mattila et al[15]. In contrast, of study performed by Abbas et al[16] showed elevated correlation with ER and PR condition (p=0.034).

This present study showed higher expression of NANOG found in Luminal A subtype as compared to Triple Negative and Luminal B subtypes. This study was in contrast to the study performed by Y.Sullu et al, he found elevated expression of NANOG in Triple Negative tumors and less expression was found in Luminal A tumors[16].

There was no significant correlation NANOG between ER, PR and Her2neu status as well (p>0.05).

CONCLUSION

From our study NANOG was found elevated NANOG immunohistochemical expression in our sequence of breast carcinoma. There was statistically significant correlation of elevated NANOG with tumor stage. There was also statistically significant correlation of NANOG in the tumor and stroma with high grade DCIS. Also elevated expression of NANOG in Luminal A subtype. Less expression was seen in the other molecular subtypes. On showing these findings and correlation with additional studies in the literature, current study concludes expression of NANOG in tumor and stromal cells could function as markers of poor prognosis in breast cancer.

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Images:
Figure 1a:Modified Radical Mastectomy specimen showing Infiltrating mammary carcinoma. Fig1b:InfiltratingDuctal carcinoma ,NOS GradevH&E 200x. Fig 1c:Infiltratinglobular carcinoma H&E 200x.Fig 1c:Infiltratingductolobular carcinoma H&E 200x

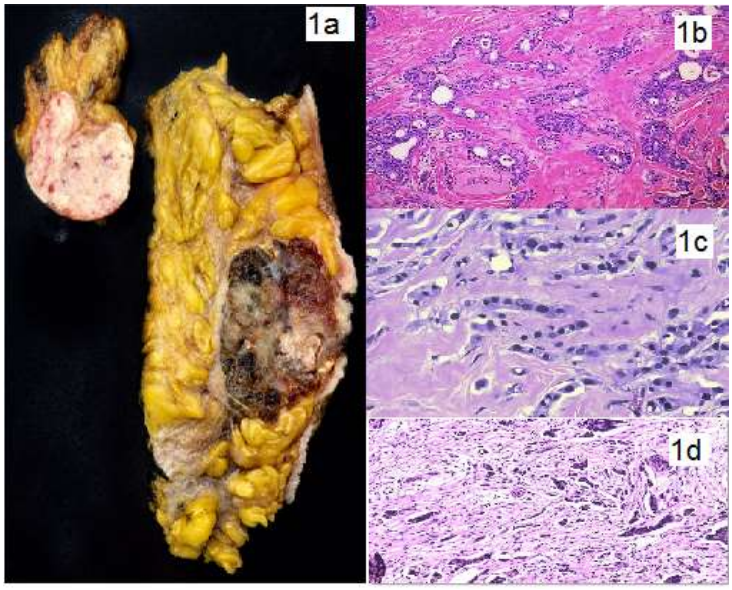

Figure2:2a:Positive control of NANOG in normal placenta-IHC 200x2b:NANOG immunostain in normal ductal epithelium IHC -100x

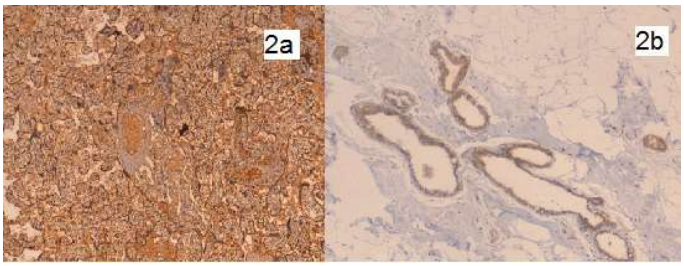

Figure3:3a:NANOG positive staining-1+ (IHC-200x) 3b:NANOG positive staining-2+ (IHC-200x) 3c:NANOG positive staining-3+ (IHC-100x)3d:NANOG positive staining-3+ (IHC-200x)

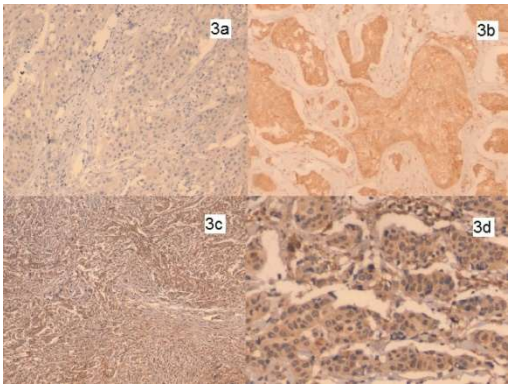

Figure4:4a: Positive 3+expressionof tumour and stromal NANOG –IHC 200x4b:Positive 2+ expression of tumour and stromal NANOG –IHC 200x4c:Positive 2+ expression of tumour and stromal NANOG –IHC 200x