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RESEARCH ARTICLE

ALTERED TRANSCRIPTIONAL AND TRANSLATIONAL LEVELS OF Nm23H1 IN NORTH INDIAN BREAST CANCER FEMALES AND IT'S PROGNOSTIC RELEVANCE

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ABSTRACT

Background: Breast cancer is the commonest female cancer worldwide and metastasis deteriorates it's therapeutic outcome. Several clinicopathological parameters have been associated with metastatic suppressor gene (MSG) expression levels that decipher it's prognostic/predictive significance. The role of MSG Nm23H1 in breast cancer is inconclusive. Our goal was to investigate the possible clinical significance and correlation of Nm23H1 with metastatic breast cancer.

Materials & methods: The study was conducted on 178 histologically proven cases of breast cancer and similar number of matched controls. Semi quantitative reverse transcriptase polymerase chain reaction (RT PCR) and immunohistochemistry (IHC) were used to investigate KiSS1 at gene and protein level, respectively. The Nm23H1 levels were correlated with several patient characteristics including age, family history, hormonal receptor status, stage, tumor size, nodal involvement and metastatic manifestation. Statistical analysis was done to evaluate correlation between expression of Nm23H1 and clinicopathological parameters.

Results: Our study revealed (i) Diminished Nm23H1 levels in normal vs breast cancer ($p < 0.05$). (ii) Likewise, a statistically significant down-regulation of NM23H1 was observed in metastatic cases vs non metastatic in breast cancer ($P = 0.04$). (iii) NM23H1 levels strongly correlated with T,N,M category, histological grade and advanced stage ($p<0.001$) and not associated with any other studied parameter.

Conclusion: Conclusively, reduced Nm23H1 expression is a negative prognostic factor for OS, advancing tumor stage, axillary lymph node status, metastatic propensity and advancing grade of the breast cancer patient. Patients with negative Nm23H1 expression may require a more intensive therapeutic strategy.

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INTRODUCTION

Breast cancer (BC) is the second most common cancer among women in the world and, by far 232,340 new cases of invasive breast cancer and 39,620 breast cancer deaths have affected US women in 2013 (DeSantis *et al.,* 2013). In India, breast cancer ranks first leaving cervical cancer behind in terms of annual incidence (Asthana *et al.,* 2014). BC remains a significant health problem especially among females owing it's increasing incidence, histopathological heterogeneity and the development of resistance to the existing therapeutic agents. Therefore, the search for novel prognostic and predictive biomarkers is highly warranted. Metastasis represents a grave therapeutic challenge and contributes to increased fatality associated with breast cancer making it a clinically and socially important issue. The first Nm23 gene was isolated by Steeg *et al.,* in 1988 and on the basis of its reduced expression in highly metastatic murine meloma cell lines, as compared with their nonmetastatic counterparts it was proposed as

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a MSG (Steeg *et al.,* 1988) Since the discovery of Nm23H1, a variety of mechanisms have been attributed to its activity, including a histidine kinase activity, binding of other proteins to regulate metastatic formation, and altered gene expression downstream of Nm23H1. Differential colony hybridization method was adopted to analyze seven cell lines derived from a murine K-1735 melanoma with varying metastatic potential by Steeg and his coworkers (Steeg *et al.,* 1988). Clone 23 exhibited the highest RNA levels and was associated with lower metastatic potential, hence they named it nonmetastatic clone 23, which was abbreviated to Nm23. The second Nm23 gene of mouse was subsequently found by Urano *et al.,* and termed nm23-M2 (Urano *et al.,* 1992). Till now, eight genes in humans (nm23-H1 to nm23-H8) have been documented (Lacombe *et al.,* 1992)**.** There are eight known Nm23 genes in the human genome, and two of them, Nm23-H1 and Nm23-H2. Nm23-H1 and -H2 are small proteins consisted with 152 amino acids, and form homohexamers or heterohexamers (Heo *et al.,* 1997). Although they are highly homologous (88% amino acid identity), their cellular functions and localizations are different. Nm23-H1 is a putative metastasis suppressor of some tumor types (de la Rosa *et al.,* 1995). In vivo, Nm23-NDPKs regulate myriad cellular events including growth and development and have also been implicated in the pathogenesis and metastasis of tumors Both Nm23-H1 and -H2 proteins are found in the cytoplasm, but Nm23-H2 has also been detected in the nucleus (Kraeft *et al.,* 1996; Pinon *et al.,* 1999).

Nm23H1 structurally comprises of 4-6 identically folded subunits of approximately 16-20 kDa that belongs to a large family of structurally and functionally conserved proteins (de la Rosa *et al.,* 1997). These oligomeric proteins exhibit nucleoside diphosphate kinase (NDPK) activity that catalyzes non substrate specific conversions of nucleoside diphosphates to nucleoside triphosphates. NDPK was first discovered in yeast (Berg *et al.,* 1953) and in pigeon breast muscle (Krebs *et al.,* 1953). The first primary structures for NDPK was reported in 1990 for Myxoccus xanthus (Munoz-Dorado *et al.,* 1990). Several clinicopathological parameters have been implicated in prognosis, recurrence and survival of breast cancer. Tumor size, axillary lymph node involvement and extent of metastasis are important prognostic determinants for breast cancer patients (Gur *et al.,* 2010). Estrogen receptor (ER) is long known as a prognostic and a predictive factor for breast cancer (Bevilacqua *et al.,* 2007).

Progesterone receptor (PR) status is also correlated with axillary lymph node involvement and hormone receptor status remains the most significant predictive and prognostic biomarker (Mickey *et al.,* 1989; Harvey *et al.,* 1999). Her2 neu also serves as prognosticator according to earlier reports (Mansel *et al.,* 2006). Late onset of menarche, breast feeding for 1-2 years, and age of first childbirth between 20-30 years were thought to be protective factors (Das *et al.,* 2012). These data together indicate that many clinicopathological parameters may play a key role in breast cancer prognosis and prediction of its risk. The current investigation aims to establish a correlation, if any of the transcriptomic and protein expression of KiSS1 gene with the clinicopathological parameters and prognosis of breast cancer in North Indian patients.

MATERIALS AND METHODS

The study group comprised of 178 histologically proven cases of breast cancer and corresponding normal breast tissue from the same breast resection specimen. The samples were collected from Department of Surgical Oncology, King George's Medical University, Lucknow between November 2011 and December 2012. Breast cancer tissue from tumor mass was obtained for the study. Adjacent normal tissue from the mastectomy specimen served as the control tissue. None of the patients received preoperative chemotherapy or radiation therapy. The Institutional Ethics Committee at King George's Medical University, Lucknow approved the study protocol. Written voluntary informed consent was obtained from all patients before recruitment. Nm23H1 expression at gene and protein level was studied by RT PCR and immunohistochemistry, respectively. The tissue biopsies were collected in 10x buffered formalin at room temperature for immunohistochemical diagnosis and in RNA later at -80 C until further use for RT PCR.

Quantitative Real Time PCR

Total mRNA was isolated following single step mRNA isolation method using RNA isolation kit (Invitrogen, USA). Total mRNA (2 µg) was reverse transcribed to cDNA using RT-PCR kit (Applied Biosystems, USA) following manufacturer's instructions. Real time analysis for Nm23H1 and normalizing gene GAPDH was performed using SYBR GREEN MASTER mix as per the manufacturer's instructions (Applied Biosytems, USA). Analysis were done on Light-cycler 480 (Roche, USA) and fold changes in gene expression was calculated using 2-ΔΔCT method. The q RT PCR primer sequences were F: 5'-ACCTGAAGGACCGTCCATTCTTTG C-3' and 5'-GGGTGAAACCACAAGCCGATCT CCT-3' for Nm23H1; and 5'-AAATCAAGTGGGGCGATGCTG-3' and 5'-GCAGAGATGATGACCCTTTTG-3' for GAPDH.

Immunohistochemistry

Formalin□fixed, paraffin□embedded tissue sections were cut into 4 µm□thick sequential sections. After deparaffinization and rehydration, sections were boiled in citrate buffer (0.01 M, pH 6.0) for antigen retrieval. Sections were then incubated with 3% H_2O_2 and 5% serum to block endogenous peroxidase activity and non□specific binding. For NM23H1 protein, sections were incubated with mouse anti-human NM23H1 monoclonal antibody (sc-465). The sections were then incubated with biotinylated secondary antibodies and visualized by DAB. Counterstaining was carried out with hematoxylin. The sections were dehydrated in alcohol and mounted with DPX. For the negative controls, PBS replaced the primary antibody.

Immunohistochemical Scoring for Nm23H1

IHC evaluation was performed under a microscope by an observer unbiased without the knowledge of clinical outcome. Cytoplasmic staining was considered positive for Nm23H1 expression. The patterns of staining were applied into scales on % of cells with positive immunostaining as 0=complete

absence or negative staining, 1= less than 10 % positive cells, 2=greater than 10% and less than 50 % cells and 3= more than 50% cells positive. In general staining in less than 10% was considered as negative staining and more than 10% was considered positive for Nm23H1.

Statistical analysis

Continuous data were summarized as mean ± SE, while discrete (categorical) in %. Qualitative variables were expressed as numbers and percentages. Comparisons were made between categorical groups by chi-square (χ^2) test. Comparisons were made between two independent groups by independent Student's t-test. A two tailed $p < 0.05$ was considered statistically significant. Kaplan Meier survival curve was made and survival time was compared using Log rank test. All analysis was performed on SPSS (Windows version 21.0) software.

RESULTS

The study included 178 histologically proven cases of breast cancer and similar number of age matched control tissues. The median age of the patients was 49 years (range, 18-70 years).

Quantitative RT PCR

Quantitative mRNA expression was analysed using RT-PCR in 178 breast cancer tumors and same number of controls. The mean fold expression of gene indicated that it was over expressed 11.43±3.7 fold in breast cancer as compared to controls, whilst in non metastatic cases it was over expressed 4.2±2.07 fold as compared to metastatic cases. Moreover, Nm23H1 expression was higher in 55.2% (48/87) patients who exhibited high (>5) Nm23H1 expression and 31.0% (27/87) had low (≤5) breast cancer cases, and undetectable in 13.8% (12/87) breast cancer cases. The relationship between Nm23H1 mRNA and clinicopathological features of breast cancer is summarized (Table 1).

Immunohistochemistry

Since, Nm23H1 gene was detectable in (127/150) 84.6% cases in mRNA expression analysis; hence we proceeded with IHC for 127 cases and discarded the rest (23/150) 15.3%. Cytoplasmic expression was analysed using IHC in 127 breast cancer tumors and similar number of control tissue. Nm23H1 expression was 1 positive in 26.7% (34/127) breast cancer cases, 2 positive in 14.9% (19/127) breast cases, 3 positive in 48.1% (62/127) cases and negative in 9.4% (12/127) breast cases. Representative images have been shown in figure 1. The relationship between Nm23H1 mRNA and clinicopathological features of breast cancer is summarized (Table 2).

Table 1. Correlation of NM23H1 gene expression with patient clinical & histopathological characteristics of breast cancer patients

Variables	NM23H1 high (>4) (n=56) (84.6%)	NM23H1 low (≤4) (n=19) (15.4%)	p value
Age	35 (62.5%)	06 (31.5%)	0.473
≤45	21 (37.5%)	13 (68.5%)	
>45			
Parity	31 (55.3%)	08 (42.1%)	0.167
≤2	25 (44.7%)	11 (57.9%)	
>2			
Oral contraceptives	34 (60.7%)	11 (57.9%)	0.658
No	22 (39.3%)	08 (42.1%)	
Yes			
Family history	38 (67.8%)	08 (42.1%)	0.416
No	18 (32.2%)	11 (57.9%)	
Yes			
Menopause	32 (57.1%)	06 (31.5%)	0.875
No	24 (42.9%)	13 (68.5%)	
Yes			
Node	33 (58.9%)	10 (52.6%)	0.0387
≤2	23 (41.1%)	09 (47.4%)	
>2			
Node	05 (8.9%)	02 (10.5%)	
N0	51 (91.1%)	17 (89.5%)	<0.001
N1+N2+N3			
Tumor	53 (94.6%)	06 (31.5%)	0.001
≤3	03 (5.3%)	13 (68.5%)	
>3			
Metastasis	50 (89.2%)	09 (47.4%)	0.001
M0	06 (10.8%)	10 (52.6%)	
M1			
ER	30 (53.5%)	10 (52.6%)	0.985
-ve	26 (46.5%)	09 (47.4%)	
+ve			
PR	30 (53.5%)	11 (57.9%)	0.739
-ve	26 (46.5%)	08 (42.1%)	
+ve			
Her2 neu	34 (60.7%)	04 (21.0%)	0.15
-ve	22 (39.3%)	15 (79.0%)	
+ve			
Histological grade	11 (19.6%)	03 (15.7%)	0.001
Well differentiated	17 (30.3%)	07 (36.8%)	
Moderately differentiated	28 (50 %)	09 (47.4%)	
Poorly differentiated			
Stage	21 (37.5%)	12 (63.1%)	0.004
Early (T1&T2)	35 (62.5%)	07(36.9%)	
Advanced (T3&T4)			

Table 2. Correlation of NM23H1 protein expression with patient clinical & histopathological characteristics of breast cancer patients

Variables	NM23H1 Positive (n=54) (85.8%)	NM23H1 Negative (n=09) (14.2%)	p value
Age	31 (57.4%)	06 (66.6%)	0.791
≤45	23 (42.5%)	03 (33.4%)	
>45			
Parity	36 (66.6%)	04 (44.5%)	0.189
≤2	18 (33.3%)	05 (55.5%)	
>2			
Oral contraceptives	34 (62.9%)	06 (66.6%)	0.783
No	20 (37.1%)	03 (33.4%)	
Yes			
Family history	29 (53.7%)	07 (77.8%)	0.051
No	25 (46.3%)	02 (22.2%)	
Yes			
Menopause	29 (53.7%)	03 (39.2%)	0.118
No	25 (46.3%)	06(60.8%)	
Yes			
Node	45 (83.4%)	02 (33.4%)	<0.001
≤2	09 (16.6%)	07 (66.6%)	
>2			
Node	10 (18.5%)	01 (11.1%)	0.025
N0	44 (81.5%)	08 (88.9%)	
N1+N2+N3			
Tumor	44 (81.4%)	08 (88.9%)	0.041
≤3	11 (20.6%)	01 (11.1%)	
>3			
Metastasis	46 (85.1%)	05 (55.6%)	<0.001
M0	08 (14.9%)	04 (44.4%)	
M1			
ER	26 (48.1%)	04 (44.4%)	0.79
-ve	28 (51.9%)	05 (55.6%)	
+ve			
PR	22 (40.7%)	07 (77.8%)	0.53
-ve	32 (59.3%)	02(22.2%)	
+ve			
Her2 neu	34 (62.9%)	05 (55.6%)	0.85
-ve	20 (37.1%)	04 (44.4%)	
+ve			
Histological grade	13 (24.1%)	01 (11.1%)	<0.001
Well differentiated	36 (66.6%)	04 (44.4%)	
Moderately differentiated	06 (11.1 %)	04 (44.4%)	
Poorly differentiated			
Stage	07 (12.7%)	02 (22.2%)	0.018
Early (T1& T2)	47 (87.3%)	07 (77.8%)	
Advance (T3 & T4)			

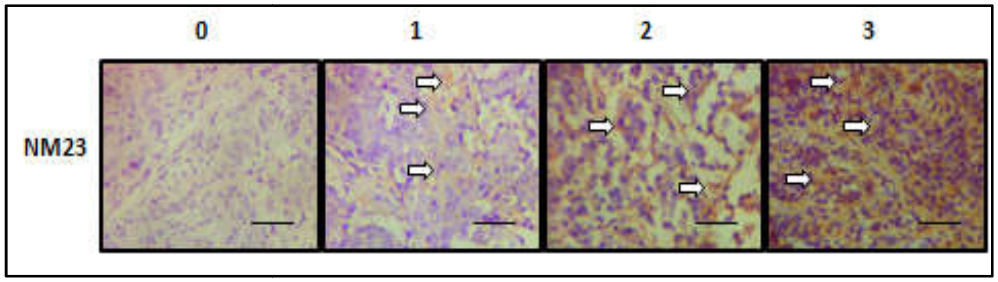

Figure 1. Immunohistochemistry of Nm23H1 in BC cells showing cytoplasmic staining

Clinico pathological characteristics with reference to Nm23H1 genes

The present study showed that down regulation of Nm23H1 at the gene and protein level is significantly correlated with advanced T,N,M categories and higher grade. No significant correlation was observed between Nm23H1 expression and age, ER, PR, Her2neu and menopausal status (Tables 1 & 2).

Nm23H1 expression in different stages and histological grade

The mRNA analysis showed that Nm23H1 expression was low in advanced vs early stage (3.94 ± 0.87, $p<0.05$). Similar results were found in protein analysis wherein Nm23H1 expression levels were lower in advanced as compared to early stage of the disease (13.25 ± 4.49, $p<0.05$). We were unable to identify any significant correlation of Nm23H1 transcript level between grade 1 (well differentiated) and grade 2 (moderately differentiated) and also between grade 3 (poorly differentiated) and grade 2 breast cancer tissues. The mRNA analysis showed that Nm23H1 expression was low in poor vs well differentiated tissues (3.56 ± 1.75, $p<0.05$). Protein analysis also corroborated similar findings wherein Nm23H1 expression levels were lower in poorly differentiated tumors as compared to well differentiated tumors (11.66 ± 3.32, $p<0.05$) (Tables 1 & 2).

DISCUSSION

Nm23H1 has been evaluated in a number of cancers like colon, gastric, prostate, larynx, lung (Goncharuk *et al.,* 2004; Su *et al.,* 2004; Yang *et al.,* 2005; Yang *et al.,* 2008; Yang *et al.,* 2014), not much literature is available on Indian population. The clinicopathological parameters have not been studied in detail in a single population in context to associating with metastatic markers and metastatic propensity. In this cohort study, individuals from North Indian patients with breast cancer were considered. We found low expression of Nm23H1 in non cancerous as compared to breast cancer tissue ($p < 0.05$). Yet another study demonstrated that there was a significant increase in Nm23H1 expression levels in cancerous tissues compared with benign and normal tissue in breast cancer (Li *et al.,* 2000; Srinivas *et al.,* 2002) Nm23H1 expression was higher in non metastatic breast tissue vs metastatic breast tissue ($p < 0.05$). Low Nm23H1 expression was also seen in 7 of 7 lymph nodes with metastasis but only in 5 of 13 (38.5%) nonmetastatic lymph nodes ($P = 0.0102$) in ovarian cancer (Huang *et al.,* 2001). Nm23H1 expression has been shown to be significantly lower in oral malignant melanomas with lymphoid metastasis than in tumors with no lymphoid metastasis. (Korabiowska *et al.,* 2005). Nm23H1 levels were attenuated in advanced T category vs lower T category in our study ($p < 0.05$). Nearly 70.5% of stage I-II ovarian tumors express nm23H1 in sharp contrast to only 25% of stage III-IV ovarian tumors (Kapoor 2009). The immunohistochemical expression of lung cancer proteins was significantly higher in stages 1 and 2 compared with stages 3 and 4 for Nm23H1 ($P=.039$). When all stages were considered together, loss of immunoreactivity for Nm23H1, $P=.004$ for nm23-H1 which is highly suggestive of co-downregulation of the protein in the process of tumor progression. In TNM staging, MSI (43.75%) and nm23H(1) protein (81.25%) in stages I+II were detected more easily than the corresponding indexes (MSI: 7.14%, $P<0.05$ and nm23H(1): 21.43%, $P<0.01$) in stages III+IV (Su *et al.,* 2004). The expression of nm23H1 protein was lower in TNM stage III + IV than in stage I + II of these tumors and in patients with lymphatic metastasis (Yang *et al.,* 2008). We also found lower Nm23H1 expression in poorly differentiated in comparison to well differentiated ones ($p<0.05$). In tumor pathology grading, different differentiation, the difference of nm23H1 mRNA positive expression was statistically significant in prostate cancer ($P < 0.05$) (Ding *et al.,* 2006). The expression rate of Nm23H1 was statistically different between grade I and II ($p=0.016$) respectively and between grade I and grade III ($p = 0.020$), but not statistically different between grade II and III ($p = 0.943$) respectively in esophageal cancer cases as in our study (Liu *et al.,* 2005).

In conclusion, we found strong correlations between decreased NM23H1 expression, aggressive tumor pathology, and poor prognosis of breast cancer in North Indian patients. Further studies including larger sample size in female populations to validate Nm23H1 as a reliable prognostic biomarker for assessing breast cancer behaviour and metastatic propensity is warranted. Our results suggest that measuring Nm23H1 expression will help to identify those breast cancer patients with metastatic propensity and hence facilitate risk stratification of the concerned subset of patients and different therapeutic strategy. Further functional studies are needed to elucidate the mechanism of metastasis suppression by Nm23H1 and to confirm its metastasis suppression function in other tumor types and models. The coming few years will hopefully visualize the development of new strategies by virtue of which may target alter Nm23H1 expression for better management of metastatic BC. Representative staining results of Nm23H1 expressed are shown at 20× original magnification. Sections of formalin-fixed paraffin-embedded specimens were stained for Nm23H1 antibodies. Positive staining for the Nm23H1 antibody is shown, illustrating 0 to 3+ staining scale. Arrows point to the stained cells.

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