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SIGNIFICANCE OF KI-67 PROTEIN EXPRESSION IN BREAST CANCER

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ABSTRACT

Breast cancer is the most predominant cancer among women in India. The highly heterogeneous nature of breast cancer requires better prognostic markers for treatment decisions. In the present study, we have focussed on the prognostic value of Ki-67 in breast cancer. The protein level expression of Ki-67 was evaluated in both breast tumor and adjacent normal breast samples. Immunohistochemical analysis revealed a high expression of Ki-67 in breast tumor samples while its expression was abrogated in normal breast tissues, suggesting Ki-67 as a potent proliferation marker. Enhanced expression of Ki-67 is a poor prognostic factor for breast cancer. In this study, Ki-67 protein expression also significantly correlated with tumor size and grade of breast cancer patients. The Kaplan-Meier analysis also revealed a significant association between Ki-67 postivity and overall survival of breast cancer patients. The present study showed a decrease in overall survival of breast cancer patients with Ki-67 protein expression. We speculate that Ki-67 can be routinely used as a histopathological parameter of proliferation index, prognostic marker for treatment decisions and as a predictive tool for assessing the overall survival of breast cancer patients.

KEYWORDS: Ki-67, Breast Cancer, Cell Proliferation, Clinicopathological Features, Overall Survival

Breast cancer is the most common cancer among women in India (Malvia et al., 2017). It is a heterogeneous disease with diverse morphological features. Biomarkers for the early detection and risk assessment for breast cancer is therefore essential. One of the important parameters for deciding the treatment regime in breast cancer is by assessing tumor cell proliferation. Ki-67 is a non-histone nuclear protein that is expressed in all active phases of cell cycle (G1, S,G2 and M). Ki-67 expression signature in proliferating tissues and absence in quiescent cells has rendered it as a marker of cell proliferation. It is confirmed as an independent predictive and prognostic factor in early breast cancer. Breast cancer with high expression of Ki-67 is associated with poor prognosis and reflects tumors of an aggressive nature (Kim et al., 2015). Understanding the importance of Ki-67 expression in cancers, we investigated the protein expression of Ki-67 in breast tumor and adjacent normal breast tissues. We further determined its association with clinicopathological parameters of breast cancer patients. We also examined the prognostic significance of Ki-67 in predicting the overall survival of breast cancer patients.

MATERIALS AND METHODS

Breast tumor samples and adjacent normal breast tissues were collected from breast cancer patients who were previously untreated and undergoing primary surgery for breast cancer at Regional Cancer Centre, Thiruvananthapuram, India. An informed consent was obtained from each breast cancer patient and this particular study was recognized by the Regional Cancer Centre Review Board and Human Ethical Committee. The patient details and clinicopathological features were obtained from their medical records maintained by the hospital. 55 breast tumor samples and 10 normal samples were used for protein analysis of Ki-67 expression.

Four µm thick sections of formalin fixed, paraffin-embedded breast tissues and adjacent normal breast samples were first subjected to Haematoxylin and Eosin staining to confirm whether they were tumor and normal samples and to ensure that the number of tumor and normal cells were sufficient for Immunohistochemical (IHC) analysis. For IHC analysis, serial sections of breast tumor tissues and adjacent normal breast tissues were deparaffinized in xylene and hydrated through graded alcohol. Endogenous peroxidase activity was blocked using 0.3% H_2O_2 in methanol for 30 min. These sections were then subjected to antigen retrieval by boiling in 10 mM citrate buffer (pH 6.0)for 15 minutes. The slides were then cooled and non-specific binding sites were blocked by incubating with 3% bovine serum albumin (BSA) for 20 minutes in a humidified chamber at room temperature. The sections were then incubated overnight at 4°C with primary antibodies specific for Ki67 (prediluted; Biogenex). Negative controls were performed by substituting the primary antibody with 1% BSA in PBS. The bounded primary antibody was detected by addition of secondary antibody conjugated with horseradish peroxidase polymer (HRP) and diaminobenzidine (DAB) substrate using Super Sensitive Polymer HRP Detection System (Biogenex). The sections were then counterstained with haematoxylin, dehydrated through graded alcohol, cleared in xylene and were mounted using distyrene,

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plasticizer (tricresyl phosphate) and xylene (DPX; Merck) mountant.

NuclearImmunostaining was evaluated independently by two individuals. The percentage of positive cells was estimated from 0 to 100%. A nuclear score of 0 to 10% was considered negative, 11-25% was designated as mild, 26-60% as moderate and >60% as intense.

The clinicopathological features as listed in table1 were collected from each breast cancer patient and the parameters were correlated to the protein expression of Ki-67. The Overall Survival defined as the length of time from cancer diagnosis until death, was analyzed on the basis of Ki-67 protein expression in breast tumor tissues.

Statistical analysis was carried out using SPSS software 17.0 (SPSS Inc. Chicago, USA). To estimate the correlation between Ki-67andclinicopathological variables of breast cancer patients, Spearman's rho correlation test (two-tailed) was used. Univariate survival analysis was performed using Kaplan-Meier method (Log rank test) to predict the prognostic significance of Ki-67 with the overall survival of breast cancer patients. $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The protein expression levels of Ki-67 were analyzed in 55 breast tumor samples and 10 normal breast tissues using IHC. Ki-67 staining was primarily confined to the nucleus. There was a remarkable increase in the frequency of expression of Ki-67 at the protein level with 96% of tumor cells exhibiting Ki-67 positivity while only 20% of normal samples expressed Ki-67 (Figure 1).Ki-67 is a nuclear protein that is tightly linked to the cell cycle (Tan et al., 2005). It is an important prognostic factor in cancer and the level of Ki-67 expression is closely associated with biologically aggressive behavior of tumor (Azambuja et al., 2007). The nuclear immunolocalization of Ki-67 proteins (Figure 2) revealed a moderate frequency of expression (58%) of these proteins in most of the breast tumor samples (Figure 3). 29% of tumor samples showed intense nuclear immunolocalization for Ki-67 while only 5% of tumor tissues were negative for Ki-67. Contrarily, majority of the normal breast tissues were negative for Ki-67 nuclear positivity. Intense nuclear immunolocalization was not observed for Ki-67 in normal samples. Majority of the normal tissues exhibited mild nuclear positivity for Ki-67 (Figure 4). Few studies have reported that Ki-67 is expressed at a very low level (< 3%) in normal breast or normal epithelium (Urruticoechea et al., 2005). This is consistent with the Ki-67 expression in normal samples observed in this particular study.

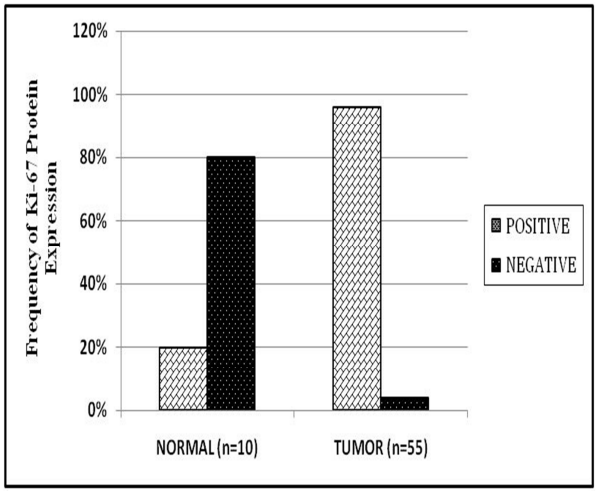

Figure 1: Frequency of protein expression levels of Ki-67 in breast tumor and normal breast samples.

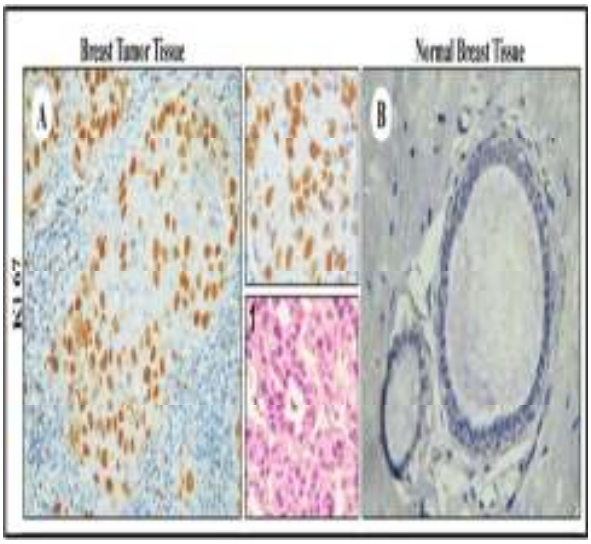

Figure 2: Protein expression levels of Ki-67 were elevated in breast tumor samples compared with normal breast samples. A and B represents immunolocalization of Ki-67 in breast tumor and normal breast samples respectively.1 represents haematoxylin and eosin (H&E) staining of tumor samples. Inset picture demonstrates enlarged image of immunolocalization of Ki-67 in breast tumor tissues. Brown indicates specific immunostaining, purple indicates nuclear haematoxylin staining. Image magnifications are 400x.

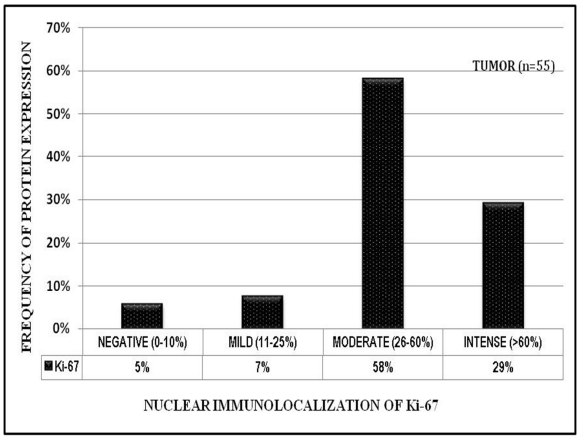

Figure 3: Frequency of protein expression level of Ki-67 in breast tumor samples.

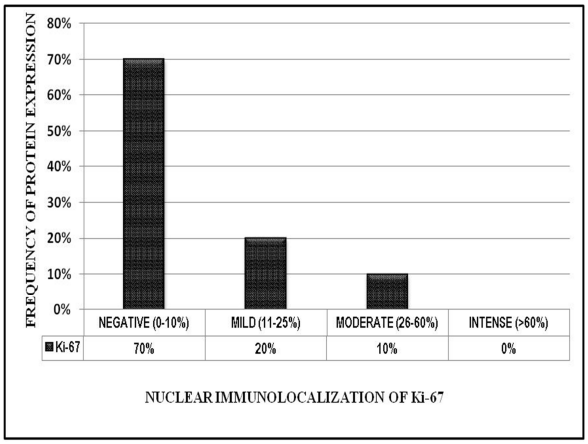

Figure 4: Frequency of protein expression levelof Ki-67 in normal breast samples.

In the present study, we evaluated the association between Ki-67 protein expression and clinicopathological features of breast cancer patients (Table 1). The protein expression of Ki-67 significantly associated with tumor size ($r = 0.427$, $p = 0.001$) and tumor grade ($r = 0.266$, $p = 0.049$) of breast cancer patients. This is in concordance with the study conducted by Gong et al., (2013) in which Ki-67 expression was found to strongly associate with tumor size in invasive breast cancer. In triple-negative breast cancer, elevated expression of Ki-67 significantly correlated with breast tumor size (Han et al., 2011). No other significant associations were found between Ki-67 and clinical features of patients.

Table 1: Clinicopathological features of patients with breast cancer.

VARIABLES	NUMBERS (n=55)	%
Age at diagnosis (years)		
≤ 50	29	52.7%
> 50	26	42.3%
Menopausal status		
Premenopause	24	43.6%
Postmenopause	31	56.4%
Parity		
Nulliparous	2	3.6%
Monoparous	5	9.1%
Multiparous	48	87.3%
Tumor size (cm)		
< 2	3	5.5%
2-5	45	81.8 %
> 5	7	12.7%
Tumor Stage		
Stage I	4	7.3%
Stage II	38	69.1%
Stage III	12	21.8%
Stage IV	1	1.8%
Histological grade		
IDC I	2	3.6%
IDC II	11	20%
IDC III	42	76.4%
Lymph node metastasis		
Negative	26	47.3%
Positive	29	52.7%
Family history of Breast Cancer		
Unknown	3	5.5%
No	46	83.6%
Yes	6	10.9%
Family history of other Cancers		
Unknown	3	5.5%
No	44	80%
Yes	8	14.5%
Therapy		
Surgery alone	4	7.3%
Sur + Chem	13	23.6%
Sur + Rad	0	0%
Sur + End	2	3.6%
Sur + Chem + Rad	20	36.4%
Sur + Chem + End	5	9.1%
Sur + Rad + End	1	1.8%
Sur + Chem + Rad + End	10	18.2%

Overall survival of breast cancer patients was analysed using Kaplan-Meier and log rank test based on the protein expression levels of Ki-67. A significant correlation was observed between Ki-67 positivity and overall survival (p = 0.032). Positive expression ofKi-67 protein decreased the overall survival of breast cancer patients (Figure 5).This finding corroborates to another study in breast cancer wherein Ki-67 was found to be an independent prognostic marker for overall survival of breast cancer patients (Li et al., 2014).

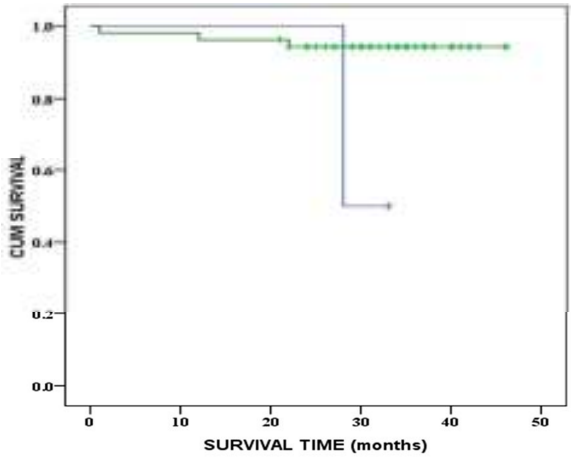

Figure 5: Kaplan-Meier curve for overall survival of breast cancer patients expressing Ki-67 positivity.Positive expression of Ki-67 (blue line) significantly reduced the overall survival of cancer patients (p = 0.032*).

In conclusion, the elevated Ki-67 protein expression in breast tumor tissues supports the fact that it can be used as a proliferation marker in routine pathological examination of tumor tissues. The significant associations between Ki-67 and tumor size and grade envisages it as a prognostic marker and predictive indicator for the overall survival of breast cancer patients.

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