KI-67 LI Expression in Triple-Negative Breast Cancer Patients and Its Significance

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ABSTRACT

PURPOSE: Triple-negative breast cancer (TNBC) is a subset of breast cancer which is known to carry a poor prognosis because of lack of targets for hormonal therapy. Research efforts have focused in recent years on discovering biomarkers of management in TNBCs. KI-67 Labelling Index (LI) is a nuclear protein which has proven to play diagnostic and prognostic roles in many cancers.

MATERIALS AND METHODS: We analysed the expression of KI-67 LI by immunohistochemistry in TNBC cases from the University hospital. This expression was cross-checked against clinical-pathological criteria of TNBC patients and against Vimentin expression in TNBC patients with significant KI-67 expression.

RESULTS: KI-67 LI was significantly expressed in the majority of TNBC cases. This expression was significantly correlated with lymph node metastases, tumour invasion, high tumour nuclear grade, clinical stage, adverse survival outcome, and failure to achieve pathological complete response. TNBCs' KI-67 LI expression was also correlated with Vimentin expression, the mesenchymal chief marker of the EMT phenomenon.

CONCLUSION: Collectively, our study presents a strong argument for the use of KI-67 LI as a biomarker of aggressive, metastatic TNBC disease with poor outcome. This study, along with mounting evidence in the scientific literature, presents a case for the use of this nuclear protein in diagnosis, prognosis, and follow-up of patients with this difficult diagnosis.

KEYWORDS: Breast cancer, EMT, KI-67 LI, prognosis, Vimentin

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Introduction

Breast cancer is the most common cancer in women worldwide. Despite improvement in diagnosis and treatment, breast cancer is still a leading cause of cancer-related mortality in women worldwide.[1] Most deaths stemming from breast cancer are related to progression and metastases of the disease.[1]

Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer defined by the lack of expression of oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER 2/neu), and the absence of *ERBB2* gene amplification. TNBC accounts for approximately 16% of all breast cancers in the world, amounting to 200 000 cases each year,[2] and has a higher cancer death rate due to its aggressive nature and lack of therapeutic targets.[2,3] The quest is ongoing in the discovery of new biomarkers of invasion, metastases, and resistance of TNBC.[4,5] These targets will hopefully stratify the TNBC into subgroups with different biological behaviours and prognosis; thus, a more tailored therapy could be designed.[4,5]

KI-67 Labelling Index (LI) is a nuclear protein expressed during all phases of the cell cycle, except the G0, and its expression is correlated with the tumour cell proliferation rate.[6] Several studies have analysed the prognostic and predictive role of KI-67 in TNBC.[7,8] More recently, KI-67 LI immunohistochemical (IHC) expression has been investigated as a possible predictive and prognostic biomarker in achieving pathological complete response (pCR) following neo-adjuvant chemotherapy (NAC) in patients with TNBC.[9,10] Pathological complete response is a highly valuable element in breast cancer management because it is associated with longer cancer-free and overall survival rates.[11,12]

TNBC has also recently been linked to the epithelial mesenchymal transition (EMT) phenomenon.[13] The EMT phenomenon is a favourable explanation for distant metastases in breast cancer.[13,14] It is characterized by losing the epithelial characteristics of the cells while gaining a mesenchymal phenotype.[13] Of particular importance among mesenchymal

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markers gained by a tumour cell during the EMT phenomenon is Vimentin (VIM).[14] This protein is regarded as a major and conventional canonical marker of EMT.[14,15]

In this study, we will shed light on the expression of KI-67 LI and VIM as representative biomarkers of the EMT phenomenon in TNBC cases in a cohort of TNBC patients from University hospital. We will analyse the expression of both proteins and cross analyse this expression against a set of clinical/pathological criteria.

Materials and Methods

Tissues samples

The records of the Department of Pathology at the University hospital were examined for cases of TNBC. The search encompassed a 5-year period (May 2015-May 2020). A total of 52 TNBC patients were included. All available clinical and pathological material were retrieved and reviewed by 2 pathologists (AO and MA). We used biopsies for the IHC staining. Patients with positive sentinel (SN) lymph node on frozen section underwent full axillary dissection. Patients with clinically and radiographically suspicious lymph nodes underwent full axillary dissection without frozen section. Patients with negative frozen section underwent only SN lymph node excision. The reviewed histopathological material comprised routinely processed and prepared hematoxylin-eosin–stained slides, as well as routinely prepared IHC preparations, including ER, PR, HER2/neu, and *ERBB2* amplification in HER2/neu equivocal cases. The baseline data included age and tumour characteristics (tumour size, histological type, tumour grade, the presence of ductal carcinoma in situ [DCIS], the presence of lympho-vascular invasion, ER/PR/HER2/neu expression, and lymph node metastases). The presence of residual tumour following NAC and achievement of pCR were recorded for those patients who received this type of management. In total, this latter group amounted to 22 patients. The stage of the tumours was also provided. There were 3 stage I patients, 23 stage II, 18 stage III, and 7 stage IV patients. Relapse-free survival, overall survival, and death information were added. While not all the time data were available, the end point was recorded for every patient. The tumour grade was assigned in accordance with the 4th edition of the *World Health Organization Classification of Tumors of the Breast*[16] and the TNM stage was given according to the American Joint Committee on Cancer, *Cancer Staging Manual, 8th edition*.[17] A total of 10 cases of benign breast lesions were also randomly selected and included as controls. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the University hospital (E-19-4013). The age of the patients ranged between 19 and 84 years, with a mean age of 52 years. This work is a retrospective study, and as such, an informed consent is not required.

IHC stains

A rabbit monoclonal antibody against VIM (ab 92547, abcam, UK) and a rabbit polyclonal antibody against KI-67 (ab 15580, abcam, UK) were each diluted 1:300 in antibody diluent (Agilent, Santa Clara, CA, USA) and applied to 5-mm-thick sections from formalin-fixed, paraffin-embedded tissue blocks, using the avidin-biotin peroxidase method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA), as per the manufacturer's instructions. The IHC stain was performed manually at room temperature. Negative controls were used with omission of primary antibody. Separate positive controls of normal skin were used for test optimization and run validation.

Staining evaluation

The IHC slides were evaluated by 2 pathologists (AO and MA), independently. The evaluating pathologists were blinded to the clinical and pathological data. The KI-67 index was expressed as tumour cells showing positive nuclear staining in at least 500 cells in the proliferative area (hot spot). The staining intensity was not relevant.[18]

In the present study, a cytoplasmic expression of VIM in more than 1% of tumour cells is considered as gain of function and is scored as positive.[14]

Statistical analysis

The threshold for a 'high' KI-67 protein expression level was 30% or above. The χ^2 test was used to compare the expression of a KI-7 LI protein in breast cancer cases (N = 52). The χ^2 test was also used to examine the relationship between high KI-67 protein expression levels (N = 51) and various clinical and pathological criteria (right vs left, tumour size, tumour type, grade, the presence of DCIS, SN lymph node status, survival data, clinical stage, VIM score, and pCR). Statistical analyses were performed with IBM SPSS Statistics software package, version 25.0. A $P < .05$ was considered statistically significant.

Results

KI-67 LI is highly expressed in TNBC tissues

A total of 52 TNBC patients were included in this study, with a median age of 52 (range = 19-84) years. The median result of the KI-67 LI expression was 70%, with a range of 20% to 95%. We performed a receiver operating characteristics (ROC) curve and calculated area under the curve (AUC) to evaluate the value of KI-67 to determine the most appropriate cut-off value of KI-67 in predicting pCR (data not shown). According to the coordinates of our generated curve, the best cut-off value for KI-67 was 30% with a sensitivity of 100% and a specificity of 95%, and an AUC of 0.503. Given the aforementioned

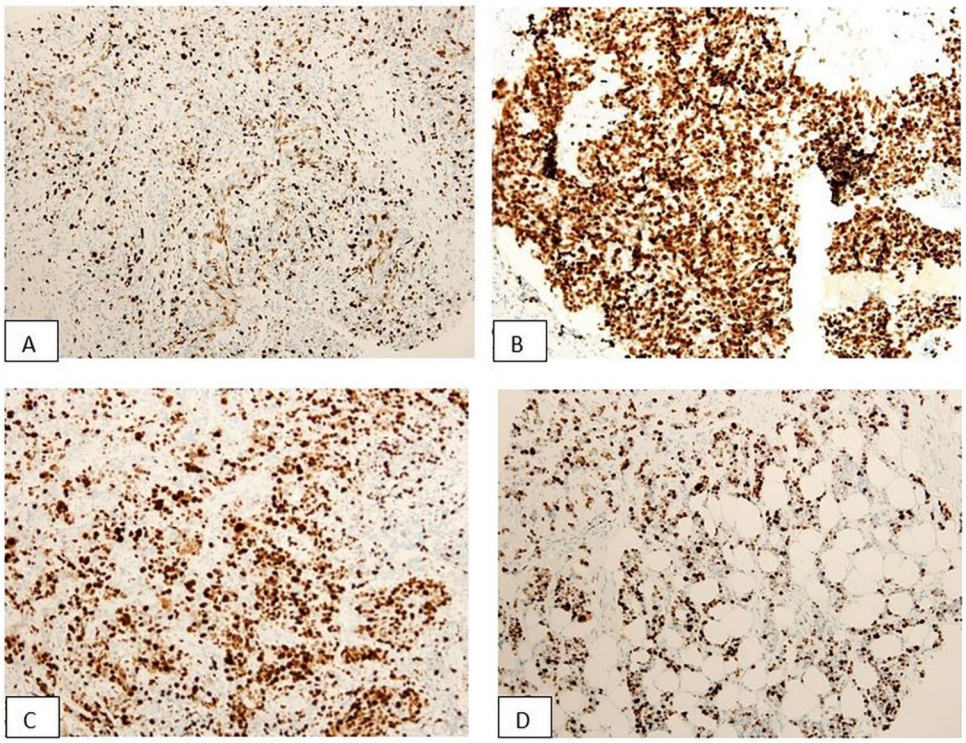

Figure 1. (A to D) Examples of robust proliferation activity in TNBCs as evident by high KI-67 LI, H&E stain, ×20.
TNBC indicates triple-negative breast cancer.

results, our categories were defined as low (<30%) and high (>30%) KI-67 groups. Within our sample, 51 of 52 cases showed high expression for KI-67 LI (Figure 1A to D).

Association between KI-67 LI expression and clinic-pathological parameters of TNBC

The associations between KI-67 expression and clinical-pathological parameters were analysed in Table 1. High expression for KI-67 LI was significantly correlated with DCIS absence, positive SN lymph node status, higher nuclear grade, diagnosis of an invasive tumour, advanced clinical stage, and adverse survival outcome. There was no significant association between high expression of KI-67 and other clinicopathological parameters, including right vs left breast, administration of NAC, and tumour size (all data in Table 1).

Association between KI-67 LI and VIM

The expression of VIM is increased in our TNBC sample (Figure 2A to C). The association between a high expression of KI-67 LI and that of VIM is shown in Table 1. The high expression of KI-67 LI was correlated with an increased expression of VIM ($P < .001$).

Association of KI-67 LI expression and pCR

Pathological complete response, defined as the absence of invasive residual tumours in the breast and corresponding lymph nodes after NAC, was found best to discriminate between patients with favourable and unfavourable outcomes.[19] In this study, we analysed the relationships between pCR and the high expression of KI-67 LI. A statistically significant association was found between failure to achieve pCR and high expression of KI-67 LI protein ($\chi^2 = 4.481$, $P = .034$) (Table 1).

Discussion

While standardized cut-off values for KI-67 have not been established, our calculated cut-off value of 30%, which we derived by performing an ROC curve, showed clinical relevance in several studies in TNBC.[20,21] In a systematic review and meta-analysis of 64 196 patients, a KI-67 value of >25 is associated with greater risk of death compared with lower expression rates.[22] The high expression of KI-67-LI in our sample was found to be significantly correlated with SN lymph node metastases, higher nuclear grade, advanced clinical stage, adverse survival outcome, invasive tumour diagnosis, and failure to achieve pathological remission. We also analysed the expression of VIM, a chief mesenchymal marker gained by tumour cells during the EMT phenomenon, in our TNBC sample.[13,14] A high expression of KI-67 LI was significantly correlated with positive expression of VIM in our TNBC patients (Figure 2A to C, Table 1).

The strong correlation seen in TNBC patients between high levels of KI-67 LI and positive expression of VIM, as an indicator of the EMT phenomenon, may be explained by the weakening of the junctional strands during the EMT phenomenon. These strands pin down normal cells in place. This process will coincide with the gain of VIM expression. Once those

Table 1. KI-67 expression in TNBC.

VARIABLE	KI-67 HIGH (N = 51) N (%)	χ^2	*DF*	*P*
Age (years)		1.923	1	.166
<50	29 (56)			
>50	22 (44)			
R VS L (5 cases are NA)		1.800	1	.180
Left	27 (58)			
Right	19 (42)			
SBR nuclear grade		46.080	2	<.001*
1	0			
2	1 (2)			
3	50 (98)			
Stage (1 case is NA)		18.837	3	<.001*
I	3 (6)			
II	23 (46)			
III	17 (34)			
IV	7 (14)			
Survival data (2 cases are NA)		30.875	2	<.001*
Died	9 (18)			
AWD	4 (8)			
AWOD	35 (74)			
Tumour type (1 case is NA)				
IDC	51			
Sentinel lymph node		13.520	1	<.001*
Positive	39 (76)			
Negative	12 (24)			
NAC administered		0.308	1	.579
Yes	27 (53)			
No	24 (47)			
pCR achieved following NAC		4.481	1	.034*
Yes	8 (29)			
No	19 (71)			
Tumour size (cm) (1 case is NA)		0.510	1	.475
>3	26 (52)			
<3	24 (48)			

(Continued)

Table 1. (Continued)

VARIABLE	KI-67 HIGH (N = 51) N (%)	χ^2	*DF*	*P*
VIM positivity		38.720	1	<.001*
Positive	48 (94)			
Negative	3 (6)			
Ductal carcinoma in situ		6.480	1	.011*
Present	17 (34)			
Absent	34 (66)			

Abbreviations: AWD, alive with disease; AWOD, alive without disease; IDC, invasive ductal carcinoma; NAC, neo-adjuvant chemotherapy; pCR, pathological complete response; SBR, Scarff-Bloom-Richardson grading; TNBC, triple-negative breast cancer; VIM, Vimentin.
Chi-square analysis was used to correlate high expression of KI-67 with clinicopathological criteria of TNBC patients.
*Indicates a statistically significant result, $P < .05$.

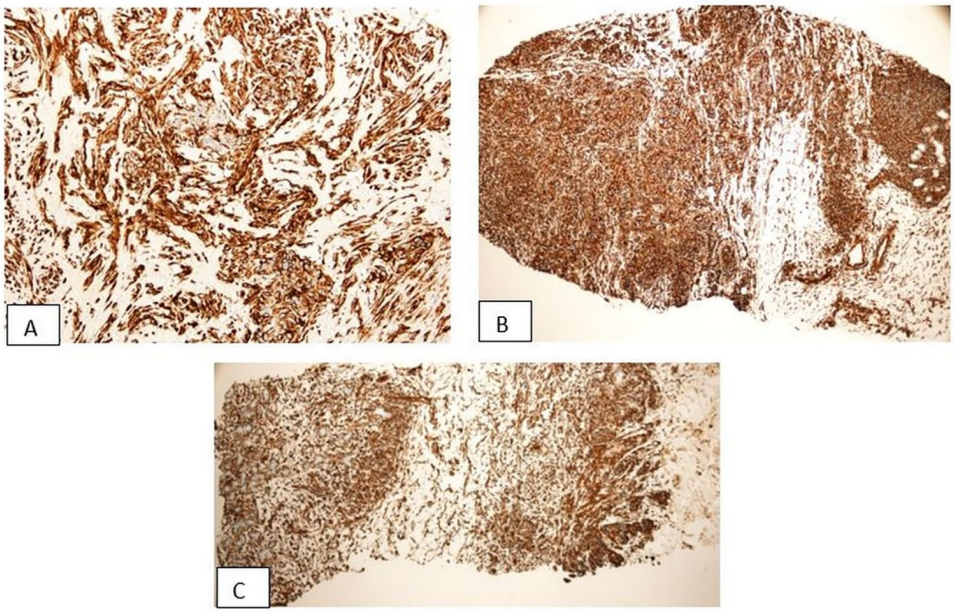

Figure 2. Heavy Vimentin staining characterizes TNBC cases: (A) H&E stain, ×20, (B) and (C) H&E stain, ×10.
TNBC indicates triple-negative breast cancer.

cancer cells find themselves free, and in more favourable conditions, they will resume proliferation, thus the high KI-67 LI seen in TNBC cells.[13-15]

The use of KI-67 LI as a prognostic and predictive marker in breast cancer has been studied extensively. However, only few studies have addressed this expression in TNBCs exclusively.[9,23-25] In our study, we focused on the KI-67 LI as a biological marker of aggressive disease and connected the dots between the pre-therapeutic KI-67 levels and the achievement of pCR. Patients with a high expression of this protein failed to achieve pCR in our study. In line with our results, Miyashita et al[24] reported that in the preoperative setting, a high KI-67 expression of >10% was significantly associated with poor relapse-free survival and overall survival in TNBC patients. In the same light, Wang et al[25] reported that high expression of KI-67 (>40%) is significantly correlated with a worse prognosis in TNBC patients, irrespective of the size of the tumour and lymph node status. Other authors, on the contrary, reported that a higher pCR rate was more significantly seen in patients with higher KI-67 LI levels.[26] Furthermore, the authors of this study found no pathological response in cases with KI-67 < 25%.[26] Similar results were observed with the clinical complete response (CCR), where clinical response was improved in tumours with KI-67 > 40%.[27,28] Some of the explanations of the divergent results between our study results and others[26-28] could be related to heterogeneous patient population, small sample size, and different chemotherapeutic regimens used.[29-35]

Finding a reliable biomarker to help stratify patients with challenging, difficult-to-treat tumours, such as the TNBC is an ongoing quest. In like manner, finding a low-cost method which can be used on a large scale in clinical settings is important, as access to state-of-the-art techniques is limited to large centres in well-funded laboratories. KI-67 fits the bill on both counts. One drawback of its use is the fact that methods used for calculating KI-67 LI vary considerably.[36] To address this considerable interobserver variation among pathologists, an international breast cancer research group dedicated to has made recommendations and guidelines.[18] These guidelines addressed analysis, reporting, and use of this important biomarker based on available evidence. Our study methodology has followed those guidelines accordingly. In previous studies, cut-off points for the KI-67 biomarker have ranged widely from 10% to 65%,[23,37,38] and KI-67 values for TNBC are much higher than those for other subtypes of breast cancer.[23] Over the years, the median of KI-67 values has gradually been adopted in breast cancer research studies (general type).[39] Establishing a more appropriate cut-off value for KI-67 is in order in TNBC patients. Particularly highlighting the importance of this issue is the fact that NAC is 1 of the very few options left for TNBC patients.

In summary, our study sheds more light on KI-67 LI, an increasingly important biomarker of predictive and prognostic value in triple-negative breast cancers. Its value stems from the few options left for patients carrying this difficult diagnosis and from the fact that it is a relatively easy, low-cost method that could be applied on a large scale in a clinical setting. Our study, in concert with other recent investigations, has presented KI-67's role in invasion, metastases, and treatment resistance in TNBCs. We have also highlighted gaps regarding its use, including a more robust testing of the cut-off points of KI-67, when used to manage TNBC patients.

Author Contributions

Maria A Arafah provided the pathology samples with attached data including diagnoses, clinical- pathology attributes and survivors' data. She also provided critical review of the manuscript.

Abderrahman Ouban conceived of the presented idea, developed the theory, planned and carried out the experiments, interpreted the results, wrote the manuscript, and was in charge of overall direction, planning and funding.

Omar Z Ameer performed the numerical and analytical calculations of the provided data. He also provided critical review of the manuscript.

Ko Jin Quek performed the numerical and analytical calculations of the provided data, and assisted in interpretations. She also provided critical review of the manuscript.

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Supplemental Material

Supplemental material for this article is available online.

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