

Application of Tissue Microarray Technology to detect expression of Ki-67 in breast carcinoma patients

*Wafaa Fadhil Hamad, **Radhia hussain fadel, ***Saeed hilal khudhair

*College of Health & Medical Technology, Baghdad.

** College of Health & Medical Technology, Kuffa.

***University of Al-Qadisiyah, veterinary medicine college.

Summary:

Breast cancer is the most common cancer of women worldwide. It is a heterogeneous disease. The incidence rate of this disease increased in the last years in Iraq. The current study was conducted to study the immunohistochemical for the breast tumors by using tissue microarray (TMA) technique. The mean age of the Iraqi breast cancer patients in our study was (50.32 ± 11.85) years old; ranging from 23-80 years old. Formalin-fixed, paraffin-embedded (FFPE) samples were used in this study. Two TMAs recipient blocks were designed and constructed for samples. Both two TMAs contain 30 samples of Iraqi breast cancer patients and normal kidney tissue for control. Each donor sample were represented in duplicate, cores were transferred from each donor patient sample to the recipient block, sections from the sample slides cutting and potted on charge slide then stained these sections with hematoxylin and Eosin (H and E) stained then immunohistochemically (IHC) studied for Ki-67 for the TMAs.

The results showed that 35 out of 60 cases was positive expression for Ki-67, and the most cases were grade II for expression all of Ki-67. While results of positive lymph node status were found in 19 cases out of 35 was positive expression for Ki-67. The study revealed that there was a significant relationship between the Ki-67 and histological grade and lymph node status. The results indicated that the Iraqi breast cancer cases used in this study were phenotypical more aggressive. We utilized TMA sections for the validation of the Ki-67. In conclusion, TMA was successfully used as a rapid, economic and effective method for IHC studies.

Introduction

Breast cancer is one of the most common malignancies and a leading cause of death among women. Breast cancer has become one of the most important health problems for women in many Arab countries, and it is the most frequent cancer in Arab women constituting 14% to 42% of all women cancers (1). In Iraq, breast cancer became the commonest type of cancers among Iraqi women since the last two decades and it comprises 31.3% of all female malignant cases. Analyses of fresh frozen or paraffin wax embedded tissues for gene and gene clusters or protein markers using routine techniques is cumbersome, time consuming, requires large amount of costly reagents, is affected by laboratory conditions and person-to-person handling and is scientifically less informative and reliable (2). These limitations can be overcome using tissue microarray (TMA) technology (3). The recent development of tissue microarray technology has potentiated large scale retrospective cohort studies using archival formalin-fixed, paraffin-embedded tissues and its application in biomedical sciences (4;5). TMA technology is a great improvement over conventional procedures of doing tests like immunohistochemistry, FISH and mRNA ISH on each tissue sample separately. Immunohistochemistry (IHC) is the most routinely practiced regimen for analyses of various histological samples including tumor identification. One of the major problems routinely encountered in IHC is 'quality control' (6;7;8). Tissue microarrays overcome these factors and promises highly reliable quality assurance for immunohistochemistry. They can be used for large-scale analysis of gene expression (protein) level using immunohistochemical staining on hundreds of tumor specimens at a time. Ki67 is a

labile, nonhistone nuclear protein that is tightly linked to the cell cycle. It is expressed in proliferating cells during mid G1 phase, increasing in level through S and G2, and peaking in the M phase of the cell cycle (9;10). It is rapidly catabolized at the end of the M phase, and is undetectable in resting (G0 and early G1) cells.² Ki67 expression shows a good relationship with growth fraction in several model systems (11;12) and does not appear to be expressed during DNA repair processes. Hence, it is regarded as a marker of cell proliferation, and in invasive breast cancer, has been used to stratify patients into good and poor prognostic categories. It has also been reported to correlate with clinical response to chemotherapy (13;14). In this research the Tissue microarray technology was used to determine the expression of Ki67 in breast cancer and its relationship with type of breast cancer, grade and auxiliary lymph node involvement.

Materials and Methods

Case Collection and Array Construction For TMA construction, a hematoxylin and eosin-stained (H&E) slide from each block was used to define the representative regions. Tissue cylinders (core) of 0.1 mm were punched from the previously defined areas and brought into a recipient paraffin block using a precision instrument (Beecher Instruments, Silver Springs, MD, USA). To increase the number of evaluable cases, all recipient blocks contained at least two donor tissue cylinders, kidney tissue was included in array as control (figure 1).

Immunohistochemistry

The most representative tumor tissue block was chosen from each case and 3 μ m sections were taken to poly-L-lysine coated slides for immunohistochemical staining. Standard streptavidin-biotin immunoperoxidase method was used for immunostaining with Ki-67 Clone; DakoCytomation, Denmark, dilution: 1:50). The tissue sections were deparaffinized in xylene, rehydrated in alcohol series, immersed in distillate water. The sections were then boiled in citrate buffer solution (10 mmol/L, pH=6.0) in a microwave oven, 3 times for ten minutes for epitope retrieval in staining with Ki-67. Endogenous peroxidase activity was blocked using a 0.3% solution of hydrogen peroxide in tris-buffered saline (TBS) at room temperature for 30 minutes and rinsed with TRIS buffer. Primary antibody was applied for 1hour at room temperature and washed in phosphate buffer saline (PBS) followed by incubation with secondary antibody for 30 minutes using labeled streptavidin-biotin (LSAB) was added for 30 minutes at room temperature and washed in PBS. Peroxidase activity was visualized with 0.03% 3, 3-diaminobenzidine tetra hydrochloride (DAB) applied for 5 minutes. The sections were than washed in deionizer water, counterstained with Harris Hematoxylin and mounted.

Scoring of Immunohistochemistry:

Ki-67 was scored semiquantitatively by evaluating the intensity of stained tumor nuclei staining. immunostains was scored according to pathology department in university of Valencia, and a decision on divergent scores was made using a microscope. Staining intensity (Negative, score 0; weak or mild staining (1-25% score 1); moderate staining (26-50 % score 2); strong staining (51-75 % score 3) and highly strong staining (76-100 % score 4) (15).

Statistic analysis

Analysis of data was carried out using the available statistical package of SPSS-18 (Statistical Packages for Social Sciences- version 18 "PASW"). The significance of difference of different percentages was tested using Pearson Chi-square test (χ^2 -test). Statistical significance was considered whenever the P value was less than 0.05.

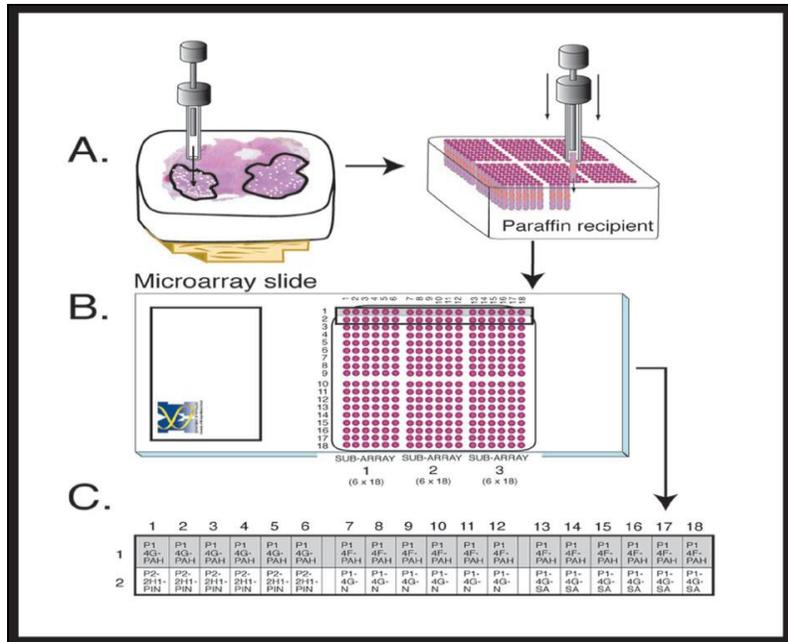


Figure 1. TMA construction and sample identity.

A: Tissue construction showing transfer of sample cores from donor block to recipient block—TMA block 01. **B:** H&E-stained TMA slide of block TMA 01. **C:** Detail of specimen, diagnosis, and array location information associated with each sample for the first two rows

Results.

Clinicopathological analysis

Sixty Iraqi breast cancer samples were included in this study to design and construct a TMA. All samples were from females. The mean age of the patients (50.32 ± 11.85) years old; ranging from (23-80) years old. Ductal carcinoma was found in 56 patients (93.3%), while lobular carcinomas were reported in 4 (6.7%) patients. Grading of these malignancies were assessed according to the Nottingham Grading system, revealing that grade I was reported in 13.3% of cases, grade II 70.0%, while grade III 16.7%. Auxiliary lymph node involvement was found in 43 out of 60 patients presented with mastectomy 35 (81.4%) cases had positive lymph node involvement and 8 (18.6%) cases had negative involvement.

Immunohistochemical detection of Ki67.

Positive expression of Ki67 by immunohistochemistry was detected as a brownish precipitate in the nucleus, This study showed that in ductal carcinoma there 34 (60.7%) cases were positively stained; 16 case (47.1%) show (+) expression, 13 case (38.2%) show (++) moderate expression and 5 case (+++) high expression while within lobular carcinoma only one case (25.0%) show (+) low expression, relation between Ki67 expression and type of tumor was statistically not significant. We found that within grade I there were 6 (75.0%) cases was positively stained; 4 case (66.7%) show (+) expression and 2 case (33.3%) show (++) moderate expression, while we found that within Grade II all 20 (47.6%) cases was positively stained, in grade III 9 (90.0%) cases were positively stained; 2 case (22.2%) show (+) low expression, 4 case (44.4%) show(++) expression, while there were 3 cases (33.4%) gave high (+++) expression these relation between Ki67 expression and grade was statistically significant. From 35 case of positive lymph node there were 19 (54.3%) was positively stained; 8 cases (42.1%) showing (+) low expression, 5 cases (26.3%) showing (++) expression and 6 case (31.6%) showing high expression. In negative lymph node there were no cases showing

(+, ++, +++) expression. There was a significant statistical association between Ki-67 and lymph node status

(Figure 2,3).

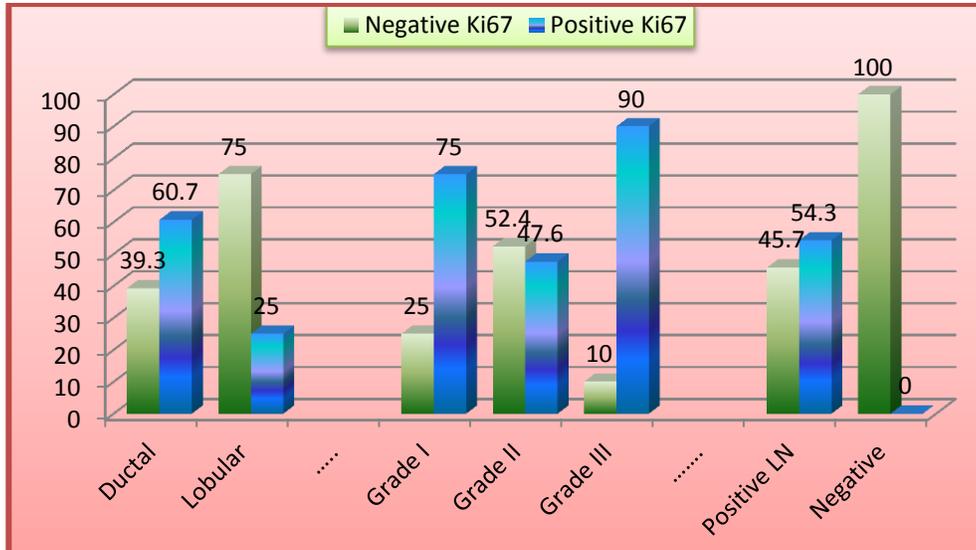


Figure 2: The relationship of Ki67 overexpression and histological characteristics.

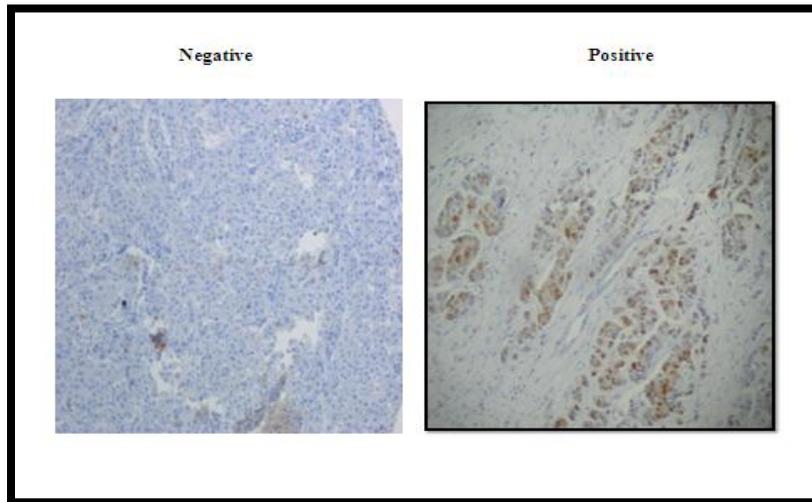


Figure 3: Immunohistochemical positive Expression of Ki-67.

Discussion

Assessment of Ki-67 expression in breast cancer.

Ki-67 belongs to the group of most frequently estimated proliferation markers but its value as a prognostic marker has not been sufficiently proven and studies which dealt with the relationship between cell proliferation and clinical course of a disease fre-interphase Ki-67 co-localizes with perinuclear located DNA and DNA located near the nuclear envelope what suggests that it is involved in perinuclear chromatin organization (16). Expression of Ki-67 may develop also in

situations of an inhibited DNA synthesis or when the cell undergoes the process of apoptosis (17). Ackland and his colleagues study in 2005 showed that the reduction in cell proliferation was associated with decreased expression of nuclear proliferation antigen Ki-67. In research that Surowiak and his colleagues in Poland, in 2005, did, the relationship of the expression of proliferation-related antigen Ki-67 in the cell of ductal breast cancer was evaluated.(18) show a significant correlation between Ki-67 expression and the clinicopathological variables (Grade of tumor, Lymph node status). Ki-67 negative tumors frequently displayed a low tumor grade whereas Ki-67 positive tumors were more likely to exhibit a high tumor grade. These findings suggest that Ki-67 might be involved indistinct pathological and molecular features during breast cancer development (19).

In accordance with other studies (20; 21; 22; 23) this study showed that there was significant correlation between the Ki-67 expression and histological grade ($P < 0.05$) which means the higher grade, the higher positivity of Ki-67, while (24; 25) showed that there was no significant correlation between the Ki-67 expression and histological grade ($P < 0.07$) . On the other hand there was no correlation between Ki-67 expressions in ductal breast cancers of grade 2 of differentiation (26) which is similar to our findings.

Axillary lymphnode(s) is known as an effective and independent factor in breast cancer prognosis, its relation with Ki-67, can help to determinate prognosis of breast cancer. The study showed, lymph node(s) involvement have been seen in 69.6% and correlation found between Ki-67 and axillary lymphnode(s) and also correlation found between grade and Ki-67 and between stage and Ki-67 can be used as an effective factor in determination of breast cancer prognosis (20). In the majority of studies (20; 27; 28) a significant correlation was found between the Ki-67 overexpression and positive lymph node status was comparable to our results, however studies done by(21; 22; 25; 23; 29; 30) showed that there was no correlation between Ki-67 expression and positive lymph node status. From this study concluded that Ki-67 is a biological marker for estimating the occurrence and progression of breast cancer (27).

Conclusions

Tissue microarray is a practical and effective tool for high-throughput molecular analysis of tissues that is helping identify new diagnostic and prognostic markers and targets in human cancers. It has varying degrees of research use and offers a range of potential applications in basic research, prognostic oncology and drug discovery. It has potential to become a widely used tool in tissue related research and its fast applicability will accelerate and push forward the transition of basic research findings into clinical applications. The results of the present study demonstrated that TMAs successfully used to test IHC for the Iraqi samples. Furthermore, this technique was applied and validated, for Iraqi samples, samples of the TMA paraffin blocks. TMA is a rapid and effective method for IHC. There was a significant relationship between the Ki67 expression and Lymph node status and there was significant relationship between Ki67 expression with the histological grade (P value < 0.05). The percentage of positive expression for Ki67 was higher in moderate grade.

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الخلاصة

يعد سرطان الثدي من أكثر أنواع السرطان انتشاراً في العالم، وهو مرض متباين الأصل . لقد ازداد معدل انتشار هذا المرض في العراق في السنوات الأخيرة . أجريت الدراسة الحالية لدراسة كيميائية مناعية نسيجية (IHC) لمرضى سرطان الثدي وذلك باستخدام تقنية المصفوفة الدقيقة للنسيج (Tissue Microarray). والبيانات التي نشرت في المرضية للمرضى أشارت إلى أن متوسط العمر لمرضى سرطان الثدي العراقيين (50.32 ± 11.85) سنة تترافق مع ما بين (23-80) سنة . وأن مؤشر عامل العمر في المرضى العراقيين يدل على أن ظهور هذا المرض في المجتمع أصبح عموماً يظهر بوقت مبكر . وقد تم استخدام عينات من أنسجة مثبتة بالفورمالين، مغمورة فيش مع البارافين . نموذج من مصفوفة النسيج الدقيقة تم تصنيعها وتركيبها ؛ كـلا النموذجين يحتوي على ثلاثين نموذج من سرطان الثدي العراقيين نسيج طبيعي للكلى للسيطرة . كل نموذج تمثله وذلك بقلم كراتين النموذج الواسع تم تحويلها بالنموذج المستقل . مقاطع هذه النماذج تم تقطيعها ووضعها على شرائح موجبة الشحنة وتم تصبغها بالنماذج بصيغة أل- (H&E) ومن ثم دراسة الكيمياء المناعية النسيجية لـ Ki-67 . أظهرت النتائج أن (37) نموذج من أصل 60 قد وجدت موجبة لتعبير Ki-67, وان اغلب الحالات كانت من الدرجة الثانية لتعبير كل من Ki-67 . أما نتائج حالة الخلايا اللمفاوية الموجبة فقد وجدت أن 19 حالة من أصل 35 أعطت موجبة لتعبير Ki-67 ولقد وجدت علاقة معنوية بين Ki-67 والدرجة الثانية للورم وعلاقة معنوية بين Ki-67 والخلايا اللمفاوية . تستنتج هذه الدراسة من متوسط العمر للمرضى العراقيين بان سرطان الثدي يظهر مبكراً في النساء العراقيات . وان المصفوفة الدقيقة للنسيج استخدمت وبناجح كطريقة سريعة واقتصادية وفعالة في دراسة أل IHC .