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CORRELATION BETWEEN AgNORs AND KI-67 PROLIFERATIVE MARKERS AND THE DEGREE OF DIFFERENTIATION OF BREAST INVASIVE DUCTAL CARCINOMA IN SUDANESE PATIENT

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ABSTRACT

Introduction: Cell proliferation reflects the nature of tissue whether it's malignant or not. In this study we investigated the expression of (Argyrophilic Nuclear Organizing Regions) and Ki-67 proliferative tumor marker (Immunohistochemistry), and their correlation to the grade of differentiation of invasive ductal carcinoma. **Objectives:** To determines the relationship between AgNORs score and degree of differentiation of invasive ductal carcinoma and the relationship between Ki-67 and grade differentiation of invasive ductal carcinoma. **Methodology:** Retrospective cross-sectional study done in Khartoum state. Fifty females' patients diagnosed with invasive ductal carcinoma. Silver stain for detection of expression of AgNORs and Ki-67 proliferative immunological marker were done in histological section of study population. The data were analyzed using SPSS computer program 20.0. **Results:** Age ranged between 30 and 70 years, varied in tumor grade. Ki-67 showed insignificant expression with tumor grade with maximum of 48% of sample size expression was more than 20% and P.value (P.value=0.234), So as AgNORs expression showed insignificant expression with tumor grade with the maximum of 13% of sample size scored more than 2 dots per cell, and P.value more than 0.005 (P, value=0.737). **Conclusions:** very strong correlation between Ki-67 reading and AgNORs score with (P.value 0.005

INTRODUCTION

Breast cancer is the top cancer in women both in the developed and developing countries. Breast cancer survival rates vary greatly worldwide, ranging from 80% or over in North America, 60% in middle-income countries and below 40% in low income countries.[1] Invasive ductal breast Carcinoma is a malignant tumor is a group of cancer cells that can grow into (invade) surrounding tissues or spread (metastasize) to distant areas of the body. The disease occurs almost entirely in women, but men can get it, too.[3] The proliferative capacity of breast cancer is an important prognostic factor and can be evaluated by a variety of methods such as number of mitoses per 10 high power field (HPF), thymidine labeling index, bromodeoxyuridine labeling, S-phase fraction, and Ki-67 /MIB-1 antigen, Ki-S1 antigen and proliferating cell nuclear antigen proliferative index.[6] One such molecular tumor marker is AgNOR's which stands for silver stained (Ag) nucleolar organizer regions (NORs). NORs are loops of DNA present in nucleus of cell on acrocentric chromosomes 13, 14, 15, 21 and 22. NORs are associated with argyrophilic proteins (having affinity for silver) like polymerase C23 and B23. Simple silver staining technique can recognize these argyrophilic associated proteins.[7] The Ki-67 protein was originally defined by the prototype monoclonal antibody Ki-67 (Gerdes et al., 1983), which was generated by immunizing mice with nuclei of the Hodgkin lymphoma cell line L428. The name is derived from the city of origin (Kiel) and the number of the original clone in the 96-well plate.[8] Ki-67 is a labile, nonhistone nuclear protein that is tightly linked to the cell cycle and is expressed in all continuously cycling cells of mid-G1, S, and G2 phase and in mitosis, but not in quiescent or resting cells in the G0 and early G1 phase.[4], 9]

In 2009, the first National Population-based Cancer Registry (NCR) was established in Sudan. They reported in this study, the first data from the NCR for Khartoum State for the period 2009–2010. The NCR staff used passive and active approaches to collect data on cancer diagnosed by all means in Khartoum State. Rates were age standardized to the 2010 Sudan Standard Population and 1966 and 2000 World Standard Population and expressed per 100,000 populations. During 2009–2010,

6771 new cancer cases were registered. Of those, 3646 (53.8%) cases were in women and 3125 (46.2%) were in men. The most commonly diagnosed cancer among women was breast.[10] Its a discreptive case-control study to correlate between AgNORs and Ki-67 marker with the degree of differentiation of breast invasive ductal carcinoma.

MATERIAL AND METHODS

Study Area and Study Population

This study conducted in Khartoum state at RICK hospital, department of histopathology, it is one of the major hospitals in Khartoum state Sudan. Cases diagnosed as invasive ductal carcinoma in the department of histopathology in study area from January 2014 to December 2014.

The study was conducted in Faculty of Medical Laboratory Science during the period from January 2014 to May 2015.

Permission was taken from the Faculty of Medical Laboratory Science and RICK samples and performs this study.

DATA COLLECTION AND ANALYSIS

Data were collected from patients request forms into predesigned questioner with detailed, personal, clinical and pathological data. The histological paraffin wax embedded blocks were sectioned by rotary microtome 3um sections into three slides; one for routine Haematoxyline and Eosin stain, another one for performing silver impregnation AgNORs, and the last section for using it in immunological proliferative marker Ki-67.

All the slides were reviewed by pathologist for diagnosis of Invasive Ductal Carcinoma and its' grading, AgNORs count, and interpreting the Ki-67 score.

Was analyzed using SPSS program version20.0

METHODOLOGY

Haematoxyline and Eosin:

Sections were taken to distilled water, stained with Haematoxyline and eosin, then slides were mounted in DPX and examined primary by investigator and then results were confirmed by histopathologists for confirmation of cancer grading.

3.7.2 AgNORs method

The test was done by using simple Bancroft method were sections were taken to water and left in freshly prepared silver solution for 30 to 45 minutes, washed carefully with two changes of de-ionized water for one minute then dehydrated in ascending grade of alcohol, cleared in Xylene and mounted in DPX. This old method gave better result, more permanent and clear reaction and by minimizing the gelatin percentage and using adhesive slides, the background staining was minimized but not eliminated.

Ki-67 Proliferative Marker

Nuclear Antigen Immunohistochemistry Method as follows

Sections (3µm) from formalin-fixed, paraffin-embedded tumors were cut and mounted onto salinized slides (Fisher brand). Following de-paraffinization in Xylene, slides were rehydrated though a graded series of alcohol and were placed in running water. Samples were steamed for Antigen retrieval for Ki-67 using PT link. Briefly Slide tank containing enough Sodium Citrate buffer (pH 9.0) to cover the sections, then were boiled in high temperature for 20 minutes then sections were allowed to cool at room temperature. Endogenous peroxidase activity were blocked with3% Hydrogen Peroxidase and Methanol for 10 minutes, after that slides were incubated with 100-200 µL of primary Antibody for 20 minutes at room temperature in a moisture chamber, and then were rinsed in Phosphate Buffer Saline. The primary Antibody Ki-67, (monoclonal) was ready to use (Thermo). After washing with PBS for 3 minutes binding of Antibodies was detected by incubating for 20 minutes with Dextran labeled polymer (Thermo Kit). Finally, the sections were washed in three changes of PBS, followed by adding 3,3diaminobenzidine tetra hydrochloride (DAB) as chromogen to produce the characteristic brown stain for the visualization of the Antibody/enzyme complex for up to 5 minutes. Slides were counterstained with Mayrs' haematoxylin. For each run of staining, positive and negative control slides were also prepared. The positive control slide was containing the Antigen under investigation and the negative control slide was prepared from same tissue block, but was incubated with PBS instead of the primary Antibody. Each slide was evaluated by investigator. Positive Ki-67 staining will be identified in form of dark brown nuclear staining. The obtained results and variables were arranged in standard master entered a computer program SPSS and analyzed.[16]

RESULTS

In this descriptive cross-sectional study using age, site of tumor, Ki-67, Ag NOR, size of tumor, and tumor histological grade as variables to be studied and correlated. The study was done in histological sections of fifty females with invasive ductal carcinoma.

Table (1) shows the age group of patients and the most and the least frequent age group. Only 6% of the patient's age ranged between 60 and 79 years old, while 32% of them lied between 30 and 39 years age group.

Table (2) Along the study we found that 74% of female patients diagnosed with invasive ductal breast carcinoma that the lump was located on their left breast, on the other hand, about 26% the lump was located on their right breast.

Table (3) show the majority of histological sections 42% were diagnosed as carcinoma grade II, where as carcinoma grade I was only 10%, and grade III was 19% of total percentage.

Table (4) show Ki-67 reading was conclusive with a maximum of 48% of reading scores more than 20%, and the minimum of 20% of reading were scored between 10% and 20%.

table (5) On the other hand, AgNORs expression read as 70% scored more than 2 dots per cell, but only 30% scored 2 dotes or less per cell examined under high power field liens.

table (6) The AgNORs score among grade I were; 2 samples scored as ≤2, and 8 samples scored >2dots per cell. While grade II only 7 scored≤2, and 14 scored >2 dots per cell. Finally, grade III invasive breast carcinoma; about 6 samples scored≤2, and 13 scored >2dots per cell. P value was (0.737) insignificant for correlation.

Table (7) Less than 10% Ki-67 score holds 2 samples diagnosed grade I, 6 samples grade II and grade III 8 samples. Ki-67 value between 10%-20%, around 4 samples were grade I, 5 grades II, and only one was grade III. Whereas, only 4 sample of carcinoma grade I, 10 samples grade II and 10 samples grade III lied in the range of more than 20%. P value equal to (0.234) also, insignificant so no correlation was found. Table (7).

Table (8) Show Ki-67 reading were conclusive with a maximum of 30% of reading lies between the scores 5%and 30%, and the minimum of 18% of reading were scored between 61% and 90% with a very strong correlation between Ki-67 reading and AgNORs with (P.value 0.005).

Table (1): The frequency of age groups among the study population

Age group (Years)	Frequency	Percent
30-39	16	32%
40-49	14	28%
50-59	14	28%
60-69	3	6%
70-79	3	6%
Total	50	100%

Table 2: Site of tumor: left or right breast among studied.

Site	Frequency	Percent
left	37	74.0
right	13	26.0
Total	50	100.0

Table (3): Tumor Grade among study population

	Frequency	Percent
grade I	10	20%
grade II	21	42%
grade III	19	38%
Total	50	100%

Table (4): Ki-67 proliferative index among study population

Proliferative index	Frequency	Percent
≤10%	16	32.0
10%-20%	10	20.0
>20%	24	48.0
Total	50	100.0

Table(5)AgNORs count among the study population		
AgNORs score	**Frequency**	**Percent**
≤2	15	30.0
>2	35	70.0
Total	50	100.0

Table (6): show Correlation between AgNORs and grade of cancer

Count		grade			Total
		grade I	grade II	grade III	
AgNORs	≤2	2	7	6	15
	>2	8	14	13	35
Total		10	21	19	50

P value= 0.737

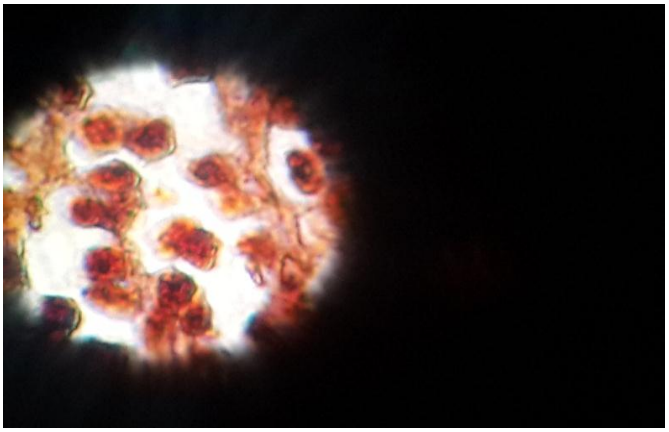

Figure (1): AgNOR score more than 2 dots per cell

Table (7): show Correlation between Ki-67 and grade of cancer

Count		grade			Total
		grade I	grade II	grade III	
Ki67	<10%	2	6	8	16
	10%-20%	4	5	1	10
	>20%	4	10	10	24
Total		10	21	19	50

P value= 0.234

Table (8): show AgNORs as proliferative and count of cancer and Ki-67 as rate of proliferative for cancer

count		AgNORs					Total
		+1	+2	+3	+4	0	
Ki-67	<5%	0	1	2	4	8	15
	5%-30%	5	3	6	1	1	16
	31%-60%	0	3	3	2	2	10
	61%-90%	0	3	3	3	0	9
Total		5	10	14	10	11	50

P. value 0.005

DISCUSSION

Our study revealed that there is no correlation between Ki-67 proliferative index and degree of differentiation of invasive ductal carcinoma (P value =0.234) which is statistically insignificant.

Our study also revealed that there is no correlation between AgNORs count and degree of differentiation of invasive ductal carcinoma (P value =0.737) which is statistically insignificant.

On the other hand, there is perfict correlation between AgNORs count and Ki-67 proliferative index (P value =0.005) In a 2tailed table Correlation is significant at the 0.05 level (2-tailed) where Ki-67, AgNORs, and Tumor Grade are correlate similutanusly.

Our study against studies done by Manisha Sharma, Mridu Manjari, and SK kahlon in 2011 studied and concluded that Ki-67 positivity and AgNORs count increases with the increase in tumor grade of breast carcinoma.[14]

Another study by; Caccrelli C, et al. Micron. 2000 is also against our study; the result demonstrated a highly significant association between AgNORs protein

quantity and tumor prognosis. Moreover, an independent prognostic value together with Ki-67-labelling index (L1), Nucleus state and tumor size.[15] This may be due to our small sample size.

CONCLUSION and RECOMMENDATION

From our study we concluded that there is no correlation between Ki67 proliferative index and degree of differentiation of invasive ductal carcinoma. There is no correlation between AgNORs proliferative index and degree of differentiation of invasive ductal carcinoma.

RECOMMENDATION

Further study with larger sample size is recommended.

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