The Role of AgNOR in Improving the Efficacy of FNA in the Detection of Breast Lumps.

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Abstract

Background:

Breast cancer is prevalent and common across the globe. One in every nine women in developed countries and one in every 20 in less developed areas may have the risk of breast cancer. This study aimed to evaluate the role of Argyrophilic Nucleolar Organizer Regions (AgNORs) stain in improving the efficacy of the fine needle aspiration (FNA) technique in the detection of cytological changes in breast lumps among Sudanese women.

Methods:

This prospective cross-sectional study was carried out in Khartoum state (Sudan), among Sudanese women who presented with breast lumps to governmental and private cytology clinics.

FNA samples were collected from each patient. Wet fixed smears were stained with AgNOR and air-dried smears with Pap staining techniques.

For the evaluation the numeric results (The mean and standard deviation ($mAgNOR \pm SD$) of AgNOR dots in 100 tumor nuclei and the Proliferative Index ($pAgNOR$), the percentage of cells having 5 or more AgNOR dots per nucleus in 100 nuclei). Pearson correlation coefficient (p-value) and ANOVA tests were used through the Statistical Package for Social Sciences (IBM SPSS Statistics 21) to compare the values observed in each cytological category and the values observed for counting 50 and 100 tumor nuclei.

Results:

The mean values and SD of AgNOR ($mAgNOR$) and proliferative Index ($pAgNOR$) were 3.66 ± 2.8 and 2.22 ± 2.1 , respectively. On the other hand, **ANOVA** test showed a significant increase in the mean values of the malignant findings in both parameters. The correlation between cytological changes categories and the means of AgNOR ($mAgNOR$) showed a statistically significant test ($P = 0.001$). Also the correlation between cytological assessment categories and the means of proliferative Index ($pAgNOR$) showed a statistically significant test.

Conclusions:

The estimate breast cellular proliferative activity by means of AgNORs per nucleus quantification and cytological atypia improve the accuracy of FNA in comparison with standard Pap stains.

Introduction

Breast cancer is prevalent and common across the globe [1]. One in every nine women in developed countries and one in every 20 in less developed areas may have the risk of breast cancer [2].

Breast cancer is the commonest of human female cancers worldwide. Its incidence is rising at approximately 2% per year in all populations [3].

In Sudan, breast cancer was the most frequent hospital-treated malignancy, accounting for about 16% (4005/25,064) of all reported cancer cases [4].

Fine needle aspiration (FNA) is perhaps the most powerful cytological technique used to study cells that are pulled from their source. Since FNA was introduced, diagnostic cytology has expanded and covered different organ and system diseases. It is a rapid, safe and cost-effective technique. Such advantages placed cytopathology as a good diagnostic choice [5].

The accuracy of diagnosing breast tumors has been improved by fine needle aspiration (FNA) cytology. When specimens are prepared correctly, the cytological results have provided a basis for good diagnoses. However, diagnosis can be difficult because some problems, such as scanty cellularity, air-drying artifact and excessive erythrocytes, can arise [6].

The main goal of diagnostic cytology is the recognition of cells derived from malignant tissues. The smear interpretation is difficult, it depends on the experience of the pathologist and the site of collection of fine needle aspirate. The sampling and quality of stain affect the interpretation of FNA smears. So the selection of the ideal stain is considered as the basic entry to obtain good results.

Nucleolar organizer regions (NORs) are located in the cell nucleoli during interphase. They are loops of DNA in which ribosomal RNA is encoded [7].

Nucleolar organizer regions can be demonstrated with the use of Argyrophilic Nucleolar Organizer Regions (AgNOR) technique which is the silver staining technique for showing Nucleolar organizer regions NORs as black dots inside the nucleus when examined under a light microscope. It is used to differentiate between benign and malignant lesions [8].

The choice to select an appropriate technique for FNA smears is the basis of obtaining reliable and good results. Furthermore, it decreases the rates of false negative and false positive diagnoses. There are two basic factors affect the interpretation of FNA smears. The first one is sampling, and the second is the quality of technique.

In a country like Sudan, where resources are matter, it is crucial to adopt low-cost techniques. The diagnostic power of fine needle aspiration cytology particularly in breast samples can be improved by following sound procedures and scientific standardization of simple low cost techniques like AgNOR. Running low-cost techniques alone is not enough. It should be accompanied by strict evaluation and reliability of these techniques.

This study aimed to evaluate the role of AgNORs in improving the efficacy of FNA in detecting cytological changes in breast lumps among Sudanese women.

Materials And Methods

Study design:

This was a prospective cross-sectional study.

Two hundred and one Sudanese females with breast lumps, who were attended the Governmental hospitals (Ribat University Hospital, Soba University Hospital, and Omdurman Military Hospital) and a private clinic (Almobark Laboratory) in the City of Khartoum, have been included in this study. All patients were referred to the laboratory for breast FNA. The clinic was resuming twice a week. Patients having previous reports or already diagnosed were excluded.

Ethical considerations:

All participants were fully informed about the aims and outcomes of the study, and were asked to sign a written consent before taking the specimen by the pathologist in-charge. The results have been shown to and discussed with the patients. Ethical approval was obtained from the National Ribat University Ethical Committee. The patient's information was highly secured and not used for other purposes than scientific inquiry. Risk and benefits for the patients from outcomes of the research insured.

Cytological samples preparation:

Each patient was prepared in a sitting position. The pathologist, wearing gloves, collected samples using conventional plastic disposable syringes (22 gauges) without using anesthesia. Content was spread on a clean, free grease glass labeled frosted slides. Then, the smears were made by applying gentle pressure with another glass slide [9].

The obtained materials from the FNA were used for the preparation of two direct smears; one of them was immediately fixed in 95% ethyl alcohol, while it is wet for subsequent Pap Stain, while the other direct smears was allowed to air dried and stained according to AgNOR staining method as described by Ploton *et al.*, [10].

Papanicolaou staining method:

For the smears which were stained using the Papanicolaou method, ethyl alcohol fixed smears were hydrated in descending concentrations of 95% alcohol through 70% alcohol to distilled water for 2 minutes in each stage. Then smears treated with Harris' Haematoxylin for 5 minutes, to stain the nuclei, rinsed in distilled water and differentiated in 0.5% aqueous hydrochloric acid for a few seconds to remove the excess stain. They were then immediately rinsed in distilled water to stop the action of discoloration. Then the smears blued in alkaline water for a few seconds and dehydrated in ascending alcoholic concentrations from 70% through two changes of 95% alcohol for 2 minutes for each change. The smears were next treated with eosin Azure 50 for 4 min. For cytoplasmic staining they were treated with Papanicolaou Orange G6 for 2 minutes, rinsed in 95% alcohol and then the smears were dehydrated in

absolute alcohol. The smears were then cleared in Xylene and mounted in DPX (Distrene polystyrene Xylene) mount. All reagents used are from Thermo Electron Corporation, UK [11].

AgNOR staining method:

The air dried smears were stained according to the AgNOR staining method. The working solution was freshly prepared by mixing one volume of 2% gelatin in 1% formic acid solution and two volumes of 50% aqueous silver nitrate solution. All smears incubated with this silver solution for 30 minutes at room temperature in a dark area and they were protected in the dark until each slide analyzed.

Two cytopathologists examined and interpreted the silver-stained cells under light microscope (Olympus BX-51, Japan) at 10x and 40x magnification. All smears screened horizontally from left to right and AgNORs counted in the nuclei of the first 50 non-overlapping, inner layers, nucleated epithelial cells. Superficial cells with pyknotic nuclei were not counted. The AgNOR count was made adopting the method described by Crocker *et al.*, [12]. AgNORs, which are visible as black-dark brown dots located within the nuclei of the cells, were counted; overlapped black dots were counted as one structure [13].

Statistics:

The mean and standard deviation ($mAgNOR \pm SD$) of AgNOR dots in 100 tumor nuclei and the Proliferative Index ($pAgNOR$) i.e. the percentage of cells having 5 or more AgNOR dots per nucleus in 100 nuclei were estimated. A similar thing was done for the 50 tumor nuclei. The data collected were statistically analyzed to generate Pearson correlation coefficient (p-value) using Statistical Package for Social Sciences (IBM SPSS Statistics 21) to compare the values observed in each cytologic category and the values observed for counting 50 and 100 tumor nuclei [14].

Results

This was a prospective cross-sectional study employing cytological methods for the assessment of the accuracy of FNA smears from two hundred and one Sudanese women with breast lumps. AgNORs was applied to each patient sample in addition to the Pap stain.

Laboratory results found that the mean values and SD of AgNOR ($mAgNOR$) dots in 100 tumor nuclei and the proliferative Index ($pAgNOR$) percentage of cells having 5 or more AgNOR dots per nucleus in 100 nuclei were 3.66 ± 2.8 and 2.22 ± 2.1 , respectively (**Table 1**). On the other hand, **ANOVA** test showed a significant increase in the mean values of the malignant findings in both parameters (Mean= 8.09), (Mean= 5.38) respectively (**Tables 2, 3**). The correlation between cytological changes categories and the means of AgNOR ($mAgNOR$) dots in 100 tumor nuclei showed a statistically significant test ($P = 0.001$). Also the correlation between cytological assessment categories and the means of proliferative Index ($pAgNOR$) percentage of cells having 5 or more AgNOR dots per nucleus in 100 nuclei showed a statistically significant test ($P = 0.003$) (**Tables 4 and 5**).

Discussion

In this prospective cross-sectional study, two hundred and one women attended selected governmental hospitals (Ribat University Hospital, Soba University Hospital, and Omdurman Military Hospital) and private clinics (Almobark Laboratory) in the city of Khartoum. Breast FNA samples were investigated to assess the role of AgNORs; in improving the efficacy of FNA in the detection of cytological changes in breast lumps among Sudanese women.

Laboratory results showed the mean values and SD of AgNOR (mAgNOR) dots in 100 tumor nuclei and the proliferative Index (pAgNOR) percentage of cells having 5 or more AgNOR dots per nucleus in 100 nuclei were 3.66 ± 2.8 and 2.22 ± 2.1 respectively. On the other hand ANOVA test showed significant increase in the mean values of the malignant findings in both parameters (Mean= 8.09), (Mean= 5.38) respectively. The correlation between cytological changes categories and the means of AgNOR (mAgNOR) dots in 100 tumor nuclei showed statistically significant test ($P = 0.001$), also the correlation between cytological assessment categories and the means of proliferative Index (pAgNOR) percentage of cells having 5 or more AgNOR dots per nucleus in 100 nuclei showed statistically significant test ($P = 0.003$).

Darkwaha, *et al.*, [14] mentioned that the mAgNOR and pAgNOR were low in normal breast, (at p-value less than 0.05), mAgNOR and pAgOR increases with increase in grade of invasive ductal carcinoma and hence can be used to assess the proliferative index of invasive ductal carcinoma [14].

Mahajan, *et al.*, [15] found that the AgNORs in benign lesions were fewer, small, uniform, and mostly centrally placed where as those in malignant lesions were irregular, generally large and scattered. The mAgNOR scores for the malignant breast lesions were significantly higher than those for benign lesions on both cytologic smears and histologic sections ($p < 0.001$) [15].

Our findings agreed with the above studies, it showed significant increase in the mean values of the malignant findings in both mAgNOR and pAgNOR, while they were low in benign and inflammation cytological results. The correlation between cytological categories and both the means of AgNOR (mAgNOR) and the proliferative Index (pAgNOR) showed statistically significant test ($P \text{ value} < 0.05$).

So, it is clear that the estimated breast cellular proliferative activity by means of AgNORs per nucleus quantification and cytological atypia improves the accuracy of FNA in comparison with standard Pap stain.

Since FNA plays a major role in the first detection of breast cancer, so the improved accuracy of FNA will reduce the cost of other expensive confirmatory tests, and reduce the need of hard currency.

The study recommends that AgNORs as a simple and inexpensive method for analysis of cell proliferation, and can replace the more expensive and complicated procedures like flow cytometry and immunohistochemistry. Further implementation of this study on larger sample size with skilled professionals in cytology may help in overcoming the drawbacks obtained in such techniques.

Limitation of the study:

- -The study ignores women having breast cancer in previous years ago.
- - All study population was from residential area in Khartoum State, the study neglects the other Sudan States.

Conclusion

AgNOR gives good indicator and marker for breast cancer using FNA methods in comparison with conventional PAP cytology. Estimation of mAgNOR and pAgNOR is, therefore, an effective technique for the assessment of the proliferative index and hence could be used as a rapid easier adjunctive tool for categorizing breast lumps.

Abbreviations

FNA

Fine needle aspiration

mAgNOR

Mean and SD of AgNOR

NORs

Nucleolar organizer regions

pAgNOR

Proliferative Index

PAP stain

Papanicolaou staining

Declarations

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

All participants were fully informed about the aims and outcomes of the study, and were asked to sign a written consent before taking the specimen by the pathologist in-charge. The results have been shown to and discussed with the patients. Ethical approval was obtained from the National Ribat University Ethical Research Committee in accordance with the Declaration of Helsinki Principles, and the agreement was taken from all patients before sample and data collection. The patient's information were highly secured

and not used for other purposes than scientific inquiry. Risk and benefits for the patients from outcomes of the research insured.

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Consent for publication:

Not applicable

Availability of data and materials

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AAI and AAM conceived the design and carried out the experiments. NAO obtained, analyzed and interpreted the data. NAO and EAA wrote and revised the manuscript. AAI provides financial support for all experiments. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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Tables

Table (1) The mean and SD (mAgNOR) dots in 100 tumor nuclei and the proliferative Index (pAgNOR) percentage of cells having 5 or more AgNOR dots per nucleus in 100 nuclei from the studied breast lumps.

	N	Mean	Std. Deviation
AgNOR dots in 100 tumour nuclei	201	3.66	2.8
Proliferative Index (pAgNOR)	201	2.22	2.1

Table (2) The Mean $\pm$ SD of cytological assessment categories of breast lumps from Sudanese patients versus AgNOR dots in 100 tumor nuclei.

Cytological assessment categories	Mean	N	Std. Deviation
Benign lump	1.44	27	0.70
Inflammation	2.13	112	1.12
Suspicious of malignancy	5.59	17	1.18
Malignant	8.09	45	1.52
Total		201	

Table (3) The correlation between cytological changes categories of breast lumps from Sudanese patients and the means of AgNOR dots in 100 tumour nuclei.

AgNOR dots in 100 tumor nuclei	Sum of Squares	df	Mean Square	F	Sig.
Between Groups (Combined)	1342.3	3	447.4	320.9	0.001
Within Groups	274.7	197	1.4		
Total	1617.0	200			

*ANOVA test was used.

Table (4) Cytological assessment categories of breast lumps from Sudanese patients versus the means of Proliferative Index (pAgNOR).

Cytological assessment	Mean $\pm$ SD	N
Benign lump	0.26 $\pm$ 0.45	27
Inflammation	1.21 $\pm$ 0.70	112
Suspicious of malignancy	3.65 $\pm$ 1.37	17
Malignant	5.38 $\pm$ 1.37	45
Total		201

Table (5) The correlation between cytological assessment categories of breast lumps from Sudanese patients and the means of the Proliferative Index (pAgNOR).

Proliferative Index (pAgNOR)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups (Combined)	702.4	3	234.1	268.3	0.003
Within Groups	171.9	197	0.9		
Total	874.4	200			

*ANOVA test was used.