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Comparative predictive value of three prognostic markers--S-phase fraction, PCNA and mitotic count on axillary lymph node metastasis in carcinoma breast

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COMPARATIVE PREDICTIVE VALUE OF THREE PROGNOSTIC MARKERS S-PHASE FRACTION, PCNA AND MITOTIC COUNT ON AXILLARY LYMPH NODE METASTASIS IN CARCINOMA BREAST

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Background: Axillary lymph node metastasis is the single most important prognostic factor in carcinoma of the breast. Therefore, prognostic markers that may reliably predict probability of lymph node (LN) metastases are of great value. This study was conducted to compare the predictive value of two novel prognostic / proliferative markers i.e. S-phase fraction (SPF) and proliferating cell nuclear antigen (PCNA) in parallel with mitotic index. **Methods:** Data of consecutive cases of infiltrating ductal carcinoma (IDC) breast diagnosed from July 2003 to July 2004 at the section of the Histopathology, The Aga Khan University Hospital, Karachi, were reviewed. A total of 112 cases of infiltrating ductal carcinoma (IDC) of the breast with axillary LN sampling were selected. SPF was calculated by flow cytometry while PCNA staining was done by immunohistochemistry. Mitotic count was calculated according to modified Bloom and Richardson's grading guidelines. **Result:** It was observed that the number of axillary LN metastases was increased with higher SPF (p value: 0.008). However no significant difference was found between the results of various categories of PCNA on axillary LN metastases (p value: 0.182) and mitotic count with axillary lymph node metastases (p value: 0.324). **Conclusion:** It was concluded that mitotic count and / PCNA alone cannot be used in predicting axillary LN metastases. SPF was found to be a more reliable marker compared to PCNA reactivity and conventional mitotic count in predicting axillary LN metastases.

Keyword: S-phase fraction, Proliferating cell nuclear antigen, Mitosis, Axillary Lymph node.

INTRODUCTION

Breast Cancer is the most common malignancy in women in Pakistan. It constitutes approximately 33.4% of all cancers in women in Karachi[1]. There are several conventional prognostic markers, which predict the overall survival (OS) in breast cancer patients. These include tumor size, tumor grade and axillary LN status etc. Axillary LN status is considered to be the single most important predictor of OS in breast cancer patients[2-4]. With the passage of time and advancement in medical technology a number of novel prognostic markers have emerged. These include DNA ploidy[5], s-phase fraction (SPF)[5,6], proliferative markers like Proliferating Cell Nuclear Antigen (PCNA)[7], p-53, C-Erb-B2/Her2, Cathepsin D, Epidermal Growth Factor Receptor (EGFR) etc. According to various studies some of these markers are relatively reliable while others appear to be of no significance and thus some of these markers in different studies contradict each other's results [5,6,8,9]. SPF represents the proportion of cells preparing for mitosis by their active replication of DNA content in the S-phase of cell cycle[8]. PCNA (Cyclin) is an auxiliary protein of DNA polymerase and the level of its synthesis correlates directly with rates of cellular proliferation and DNA synthesis. This protein is associated with cell cycle & accumulates in the nuclei of proliferating cell in the late G1 and S-phase [10]. Mitotic index is defined as the number of mitotic figures in any given area of tumor[3]. In this study it was evaluated that among three prognostic markers, mitotic count (conventional), PCNA (novel) & SPF (novel), we can utilize mitotic count and or PCNA alone as an alternate low cost test to predict axillary LN metastasis in our breast cancer patients.

MATERIAL AND METHODS

The study was conducted in the section of the Histopathology at Aga khan university hospital, Karachi. Only those cases were included in the study in which besides the main tumour, axillary lymph node sampling was also done. For mitotic count, modified Bloom and Richardson criteria was used. A score of I was given to less than 10 mitoses/10hpf, II for 11-19 mitoses/10 hpf and III for ≥ 20 mitoses/10hpf.

For PCNA estimation immunohistochemistry was employed using PAP method. Anti mouse PCNA monoclonal antibody (Clone PC 10), a mouse IgG antibody (Dako) was used at a dilution of 1/25. Immunostaining was performed on 4µm thick section on Poly-L-Lysine coated slides. Diamino-benzidine (DAB) was employed as a chromogen. Positive staining for PCNA was seen in the nuclei. PCNA positivity was divided into two categories. Positivity of 25% nuclei or less was considered as low while greater than 25% was regarded as high.

For estimation of SPF, flow cytometric technique was employed using 25 µm thick formalin fixed paraffin embedded tissue sections. Sections were dewaxed in two changes (2 X 10 minutes) of xylene

and rehydrated in 100%, 90%, 70% & 50% of alcohol for 10 minutes each. The sections were then rinsed in PBS X 10 minutes and incubated in 0.5% Pepsin solution at pH 1.5 at 37°C for 30 minutes. Hypodermic needles of 40 & 25 bore were then used to break up the tissue. Released nuclei were then spun, washed and cytopreps made to check their condition. These were then stained with Propidium Iodide in isoton (250µg/ml) containing 1mg/ml RNAase for 30 minutes at 4°C before analysis on FAC Scan (B&D) flow cytometer using the software MODFIT version 1.01 for data acquisition and analysis. Flow cytometric data was acquired and displayed in two standard parameter dot plots using FL2 width and FL2 areas as the axis. This allowed us to draw gates in which debris below the first G0/G1 distribution and particles with extended time in flight (presumed dublets) were excluded from analysis using carefully defined and standardized gating criteria. FL2 area signals were then used to generate single parameter DNA histograms. A total of 10,000 nuclei were counted in each case. Specimens were rejected if the median half peak coefficient of variation (CV) of the diploid peak was more than 5. Peak channels of diploid, aneuploid and G2M were estimated along with fitting a rectangle between two peaks to calculate SPF by MODFIT model. SPF values were divided into two categories i.e. equal to or less than 10 % was considered as low and greater than 10% as high. Axillary LN status was divided into three categories i.e. Negative lymph nodes (I), one to three lymph nodes positive (II), and four or more lymph nodes positive (III).

The statistical analysis of the data was performed using SPSS 13 software. Students't' test and chi square test were employed.

RESULTS

A total of 112 cases of IDC of breast were included in the study. 24 cases had mitotic score I (less than 10 mitoses/10hpf), 63 cases had mitotic score II (10-19 mitoses/10hpf) and 25 cases had mitotic score III (≥20mitoses/10hpf). It was found that relationship between mitotic count and axillary LN metastasis was not significant (p value: 0.324). PCNA positivity was seen in every case (Fig 1), ranging from 5% to 60% (mean 28.25%). In 49 cases, PCNA value was found to be equal to or less than 25%, while in the remaining 63 cases it was greater than 25%. No significant correlation was observed between PCNA and axillary LN metastasis (p value: 0.182). S-phase fraction ranged from 3.26% to 54.3% (mean 22.83%). As for as axillary LN status is concerned, 43 cases had no lymph nodes (I), 30 cases had one to three lymph nodes positivity (II), whereas 39 cases had four or more lymph nodes positivity (III). It was observed that the number of axillary LN metastases was higher with higher SPF (p value=0.008).

Table 1- Relationship of mitosis versus axillary lymph node metastasis

	Metastatic LN (Number)			Total
Mitosis	**0**	**(1-3)**	**(4 and above)**	
I	8	8	8	24
II	27	12	24	63
III	8	10	7	25
Total	43	30	39	112

p value = 0.324

Table 2 - Relationship of PCNA versus axillary lymph node metastasis

	Metastatic LN (Number)			Total
PCNA	**0**	**(1-3)**	**(4 or more)**	
≤ 25	23	13	13	49
> 25	20	17	26	63
Total	43	30	39	112

p value = 0.182

Table 3 - Relationship of SPF versus axillary lymph node metastasis

	Metastatic LN (Number)		
SPF	**(1-3)**	**(4 or more)**	**Total**
≤10	2	1	13
>10	28	38	99
Total	30	39	112

p value = 0.008

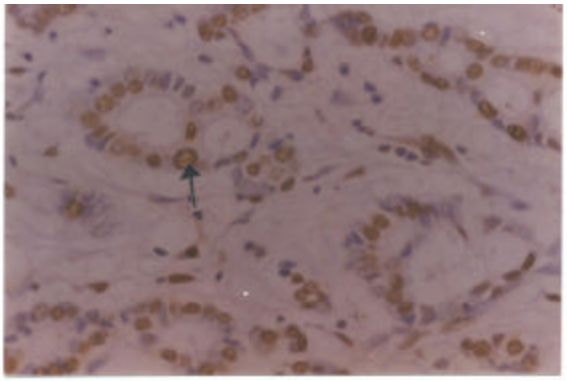

Figure 1 - Photomicrograph of infiltrating ductal carcinoma of breast stained with a monoclonal antibody against PCNA (PC10). Note brown nuclear staining in a large number of tumour cells (Arrow)

DISCUSSION

This study did not reveal any significant correlation between axillary LN metastases and mitotic count, whereas in contrast many other studies did notice significant correlation.[4,5,11] It may be due to a number of factors like variation in selection of the mitotically active areas, personal bias on accepting a mitotic figure, lack of standardization of the size of hpf etc. The authorities recommend that mitotic counts should be calculated at ten consecutive most mitotically active hpf of the tumour. Secondly to avoid personal bias, only clearly identifiable mitotic figures should be

counted (like cells in prophase, metaphase and anaphase). Similarly size of the hpf should be standardized according to modified Bloom and Richardson grading criteria[3].

PCNA is done by a simple immunohistochemical technique. In this study 1000 cell nuclei were counted and then percentage of positively stained nuclei was calculated. It is a simple, cost effective and non-radioactive technique. However, uptill now role of PCNA as a reliable prognostic marker is not well established. Lack of association between PCNA and axillary lymph node status in our study is in agreement with most previous studies[7,12,13]. In studying PCNA mostly PC 10 antibodies were used which is substantially affected by fixation. Recently a new antibody (19a2) has emerged which is not affected much by the duration of fixation. Furthermore PCNA may be expressed in response to injury even in non-proliferating cells[14]. These factors together with the findings of our study suggest that PCNA is not a consistently useful marker of proliferation nor is it a good predictor of axillary LN metastases in breast cancer patients.

This study, however, identified a significant correlation between SPF and axillary lymph node metastases. In our study SPF ranged from 3.26% to 54.30% with a mean of 22.83%. Our mean value was found to be higher than those reported by some other studies[3,15,16]. This difference in SPF could be due to variation in the interpretation of DNA histogram and differences of methodology of flowcytometric techniques, staining and sample preparation in various centers[17]. It is also suggested that the patients in Pakistan have a different and more aggressive disease, thus more cells are in the synthetic phase of the cell cycle[8]. In our patients mean tumor size was 5.04 cm with a standerd deviation of ± 3.35.

CONCLUSION

It was concluded that mitotic count and PCNA alone cannot be used to predict axillary LN metastases in breast cancer patients. SPF was found to be more reliable marker compared to PCNA reactivity and conventional mitotic count in predicting axillary LN metastases. This could be due to subjective interpretation of the latter two markers compared to better standardized SPF estimation.

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