THYMIDINE LABELING INDEX AND Ki-67 STAINING IN BREAST CANCER AND THEIR RELATIONSHIP TO CLINICAL AND PATHOLOGICAL VARIABLES

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SUMMARY

In this study, two proliferation markers; i.e. thymidine labeling index (TLI) and Ki-67 scores of 32 breast cancer patients were evaluated by assessing the relationship with other prognostic parameters including lymph node status, nuclear-histopathologic grades, tumor size, estrogen-progesterone status and menopausal status. Both TLI values and Ki67 scores were correlated with nuclear and histologic grades. While indices of both proliferation markers were significantly lower in estrogen receptor positive patients than hormone negative patients, only TLI values were found to be significantly lower in progesterone receptor positive patients. No other correlations or associations between variables reached statistical significance. In conclusion, TLI and Ki-67 scores may be independent prognostic factors as nuclear grade and hormonal status.

Key words: Breast tumour, Cell proliferation, Thymidine labeling index, Ki-67

INTRODUCTION

Cancer is characterized with proliferation, invasion and metastasis. Various kinetic indicators have been used to measure the cellular proliferation of tumors for a long time [1,2]. A number of methods for detecting cellular proliferation assess mitotic cell rates, DNA content and the ratio of cells in certain phases of cell cycle. Thymidine labeling index (TLI) is reported for many human neoplasms to be a popular prognostic marker. It has been used to label the S phase fraction in a tumor cell population using autoradiography [3]. Ki-67 score assessment is another method to measure cellular proliferation by immunohistochemistry. Ki-67 antibody recognizes a nuclear antigen, which is expressed in all phases of the cell cycle, except G0 phase. It is used as a prognostic and histopathologic diagnostic marker in various human neoplastic tissues [4,5].

Cellular kinetics of breast cancer has been studied widely. The contradicting results obtained in these studies concerning correlations with other prognostic factors, stress the need to design large scaled studies involving various clinical and histopathologic prognostic parameters [6,7,8]. The aim of this study was to investigate the relationship between proliferation indices including TLI and Ki-67 scores and other prognostic parameters, namely lymph node status, nuclear-histopathologic grades, tumor size, estrogen-progesterone content and menopausal status in a population of Turkish breast cancer patients.

MATERIALS and METHODS

Histologic grading was done according to Fisher's system [9] and nuclear grading was done using Fisher's modification for Black's nuclear grading [9,10].

FThymidine labeling

The TLI was determined immediately after the surgery on 32 tumor specimens from

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breast cancer operation. After removal of fat and other contaminating tissues, tumors were minced into 8-10 fragments of about $1mm^3$, and the fragments were placed in 2 ml of 199 medium [Gibco Laboratory (Cat no: 1800-027)] with 20% fetal calf serum [Biological Industries (Israel Cat No: 04-121-1A)], streptomycin 100µg/ml, penicillin 100U/ml, 6 µCi/ml ^{3}H-Thymidine [Radiochemical Center (Amersham, United Kingdom TRA 120 specific activity 5Ci/mmol)]. They were incubated for 1 hour in agitation at 37°C in shaker water bath. After the incubation period, the tumor fragments were briefly washed three times in phosphate buffered solution and fixed in buffered 10% formalin solution, dehydrated in alcohol, and embedded in paraffin. Paraffin sections were cut at 5 micron. Slides were coated with emulsion film (Ilford K2) in a dark room and exposed at 4°C for 3-5 days. Autoradiographies were then developed in D 19b 5 minutes at 18°C and fixed in a standard fixer. The slides were stained with hematoxylin and eosin at 4°C.

Ki-67 staining

Same paraffin blocks of tumor specimens that were prepared for TLI determination were cut at 3 micron for Ki-67 staining. Sections were placed on poly-L Lysine coated glass slides. Sections were deparaffinized in toluol and hydrated in ethanol series. For antigen retrieval the slides were pressure-cooked in 0.01M-citrate buffer (pH 6). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol. Sections were covered with normal serum for 20 min to block non-specific binding sites. They were then incubated with an anti Ki-67 antibody (7B11 monoclonal antibody, Zymed Laboratories, USA) 1:50 dilution for one hour. Slides were incubated for 20 min with biotinylated secondary antibody, and then incubated with the streptavidin-peroxidase conjugate for 20 min. The enzyme activity was developed using aminoethylcarbazole (AEC) for 15 min. Negative control sections were prepared by substituting the Ki-67 antibody with phosphate buffer saline.

Estrogen and Progesterone Receptor

Estrogen receptor (ER) and progesterone receptor (PR) status of patients reported as positive or negative were obtained from the archives of Pathology Department of Istanbul Medical Faculty.

Quantification Procedure

The TLI was determined by counting a total of 1000-3000 cells for different specimens of the same tumor under light microscopy with X1000 magnification. When the specimens were small enough to allow precursors to penetrate the counting was done through the entire section. In the other cases, the counting was limited to the periphery of the section. In all cases, 20 grains overlying the nucleus was necessary for a positive count. TLI is expressed as percentage epithelial cells labeled with thymidine. The same observer scored all microscopic slides.

Ki-67 scores were determined similarly by counting the same number of cells with positively stained nuclei under light microscopy with X400 magnification.

Statistics

Ki-67 score and TLI values were evaluated by examining the relationship with seven clinical and pathological variables: menopausal status (premenopausal women aged younger than 50), nodal status, tumor size, histologic grade, nuclear grade, and estrogen and progesterone receptor status. Analysis involving estrogen-progesterone receptors and nodal status were repeated treating them as dichotomous (positive-negative) variables.

The statistical correlations among variables were evaluated by Mann-Whitney U and Spearman rank correlation tests using SPSS 9.0 packet program.

RESULTS

Typical illustrations of thymidine labeled and Ki-67 stained sections are shown in Figures 1 and 2 respectively.

Figure 1. Comparison of TLI and Ki-67 scores of the patients according to histologic grades.

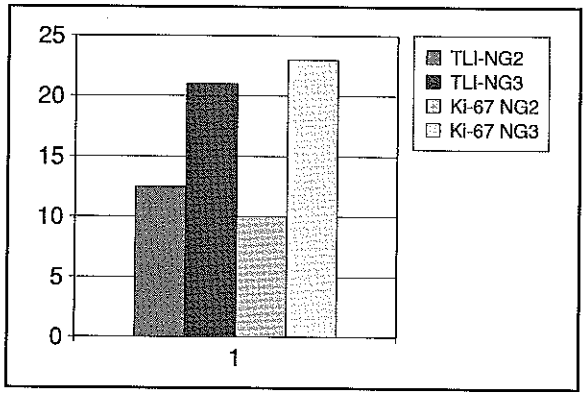
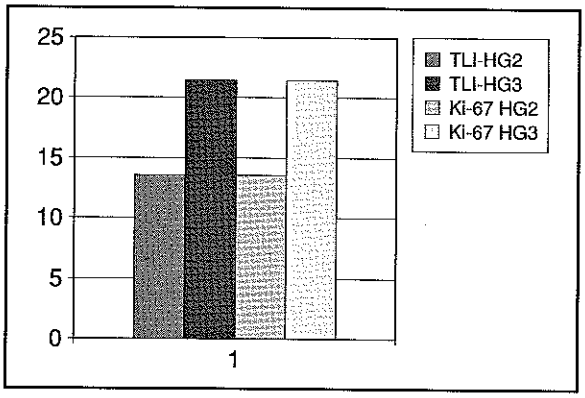


Figure 2. Comparison of TLIs and Ki-67 scores of the patients according to nuclear grades



TLI values showed a range of 3.04-25.46% with a mean of 7.21% and standard deviation of 4.77%, while Ki-67 scores ranged between 3.50% and 32.00% with a mean of 12.48 % and standard deviation of 8.03% (Table 1). There was also a significant association between both proliferation markers and histologic grades as shown in Figure II ($p<0.05$). In evaluation of significance between proliferation markers and nuclear grades, Ki-67 scores showed a stronger association than TLI values ($p<0.001$ and $p<0.05$, respectively) as demonstrated in Figure III..

Proliferation indices of both markers were higher in hormone negative patients than estrogen and/or progesterone receptor positive patients. While this difference reached statistical significance with both proliferation markers for estrogen status [$p<0.05$], only TLI values were found to be significantly lower in progesterone receptor positive patients [$p<0.05$]. No significant relationship was found between both proliferation indices and pre and postmenopausal status. Tumor size and lymph node status was not associated with TLI and Ki-67 indices.

DISCUSSION

Loss of cell proliferation control is one of the most fundamental issues in cancer biology. Cellular proliferation is an important parameter along with other clinical and histopathologic measures such as tumor size, hormonal and nodal status, and histopathologic type in the assessment of correlation with tumor behavior [11].

Among the cellular proliferation markers, thymidine-labeling index is a widely preferred method as an S phase indicator since 1950. There are several reports that provide information about TLI in non-Hodgkin's lymphomas [12,13]. TLI correlations were also reported with several prognostic indicators in breast cancer [14,15,6]. Although immunohistochemical markers of cellular proliferation are still under development, Ki-67 antibody remains as a popular immunohistochemical marker that provides immunoreactions in all phases of cell cycle, except G_0 [17,18].

Table 1. Clinical and histopathologic data of patient populations. HG: histologic grade, NG: nuclear grade, LNS: lymph node status, ER: estrogen receptor, PR: progesterone receptor, HT: histologic type

Case No	Ki-67	TLI	AGE	HG	NG	LNS	ER	PR	SIZE	HT
1	21,2	16,19	53	3	3	Positive	Negative	Negative	2,2	IDC
2	12,5	6,94	44	3	3	Positive	Positive	Positive	4	IDC
3	27	25,46	64	3	3	Negative	Negative	Negative	1,1	IDC
4	32	18,37	49	3	3	Positive	Positive	Positive	2	IDC
5	12,5	5,48	37	3	3	Negative	Positive	Negative	1,5	IDC+ILC
6	16,3	7,44	62	3	3	Positive	Positive	Positive	3,5	IL
7	4,1	5,79	62	3	2	Negative	Negative	Negative	1	IDC
8	4,5	3,87	65	3	2	Negative	Negative	Negative	1	IDC
9	12,4	10,9	56	3	3	Negative	Negative	Negative	4	IDC
10	3,5	4,74	39	2	2	Positive	Positive	Positive	2	IDC
11	3,9	3,2	61	2	2	Negative	Positive	Positive	2	IDC
12	4	3,7	46	2	2	Positive	Positive	Positive	4	IDC
13	6,8	4,74	65	2	2	Positive	Positive	Positive	1,5	IDC
14	19,3	6,3	42	2	3	Negative	Negative	Negative	2,1	IDC
15	20,5	9,27	28	2	3	Negative	Positive	Positive	8,5	IDC
16	23	5,62	66	2	3	Positive	Negative	Negative	3	IDC
17	8,4	7,11	46	2	2	Negative	Positive	Negative	2	IDC+ILC
18	7,91	9,2	63	2	2	Positive	Positive	Negative	1,5	IDC
19	7,8	6,95	67	2	2	Positive	Positive	Negative	3	IDC+ILC
20	3,8	3,39	60	2	3	Positive	Positive	Positive	5	IDC
21	9,1	3,85	44	2	2	Negative	Positive	Negative	2	IDC+ILC
22	8,4	6,4	32	2	2	Positive	Positive	Negative	1,7	IDC
23	6,8	4,74	65	2	2	Positive	Positive	positive	1,5	IDC
24	9,1	7,4	50	2	2	Negative	Negative	Negative	3,5	IDC
25	11	3,63	54	2	2	Negative	Positive	Positive	1,5	ILC
26	18,6	7,11	50	3	3	Positive	Negative	Negative	2	IDC
27	9,3	3,7	52	3	3	Positive	Positive	Positive	4,5	IDC
28	16	5,68	63	2	3	Negative	Positive	Positive	1,3	IDC
29	7,8	3,04	68	2	2	Positive	Positive	Positive	2	IDC+ILC
30	13	6,55	33	2	2	Negative	Positive	Negative	1,5	IDC
31	32	9,19	32	3	3	Negative	Negative	Negative	1,5	IDC
32	6,9	4,9	41	2	2	Negative	Positive	Negative	1,2	IDC

In this study we aimed to investigate the relationship of these two methods with other prognostic markers. Histologic and nuclear grading of tumor differentiation provides valuable information about the prognosis of breast cancer [19,20]. Some authors reported a correlation between proliferation index and nuclear grade [21,22]. Histologic grade is also reported to be associated with proliferation rates in several studies [23,24]. In our study,

Figure 1. Photomicrograph of thymidine labeling in breast carcinoma, counterstained with haematoxylin and eosin X 1000

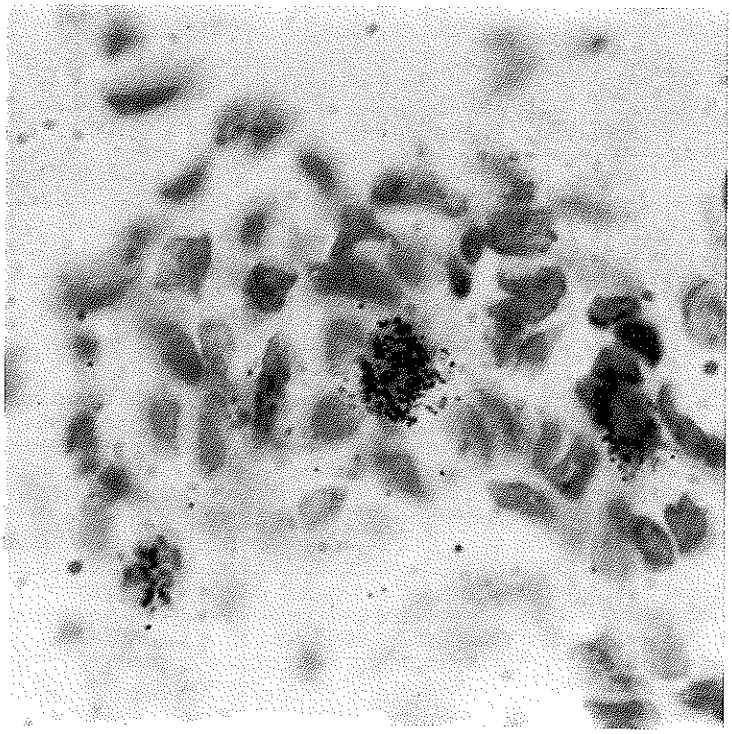
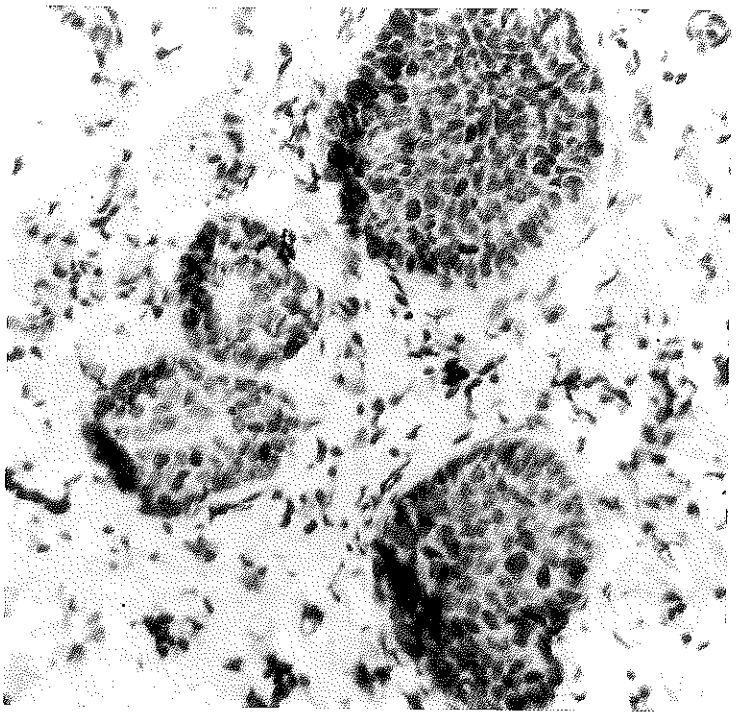


Figure 2. Photomicrograph of immunohistochemical staining for Ki-67 in breast carcinoma, counterstained with haematoxylin and cosin X 520



we found that histologic grades were associated with both TLI and Ki-67 scores. Besides, similar correlation was found in evaluations of relationship between TLI and Ki-67 scores and nuclear grades, although the correlation with the latter proliferation marker was stronger.

Hormone receptor status is also considered to be an important prognostic factor in breast cancer [25,26]. Inverse correlation between Ki-67 index and estrogen-progesterone receptor status was reported in other studies [24,27,28]. In our study, both proliferation markers showed a tendency to be higher in hormone negative patients, but only the relationship between Ki-67 indices and progesterone status failed to reach statistical significance. These findings may result from the differences in the targeted phases of cell cycle of the two methods. The thymidine labeling method involves the incubation of fresh tumor tissue with tritium-labeled thymidine. It provides an estimate of the fraction of tumor cells that are in the S (DNA synthesis) phase of the cell cycle. On the other hand, Ki-67 antibody specifically recognizes a human nuclear epitope present in cells in the S, G2, M, and postmitotic G1 phases of the cell cycle. The antigen is not expressed in the resting G0 cells.

Tumor size is also a well-known prognostic factor in breast cancer [19,29]. In literature there are various studies aimed at verifying the relationship between cell proliferation and tumor size [30,31]. Although these re-

ports show association between large size and increased proliferation indices, our results indicated that there is no link between both proliferation markers and tumor size.

One of the most important independent prognostic factors in breast cancer is lymph node status. In node negative breast cancer, S-phase fraction and cell proliferation index were reported as possible prognostic indicators [32,33]. Neither TLI indices nor Ki-67 scores showed association with node positive-negative status in our study. These findings are consistent with previous studies that reported no correlation between cell cycle kinetics and overall extent of lymph node metastases [24,7].

Breast carcinoma tends to have a poor prognosis in young patients [34]. In this study, Ki-67 and TL indices were found slightly increased at postmenopausal cases and not found significantly associated with menopausal status. However, negative correlation was reported between the age of the patient and Ki-67 indices [7]. In node negative and postmenopausal cases the life expectancies of patients with fast-growing tumors were reported to be very different from that of the patients with slow-growing tumors [35].

In conclusion, given that cell kinetics has an impact on tumor behavior, Ki-67 scores may provide substantial information on the prognosis of cancer patients like TLI, which is a well-established method of determination of tumor prognosis. These parameters may provide valuable information on the prediction of the outcome and hence management of the disease.

REFERENCES

1. Quinn MC, Wright AN. The clinical assessment of proliferation and growth in human tumours: Evaluation of methods and applications as prognostic variables. J Pathol 1990; 160:93.
2. Scott RJ, Hall A, Haldane JS et al. Comparison of immunohistochemical markers of cell proliferation with experimentally determined growth fraction. J Pathol 1991; 165:173.
3. Amadori D and Silvestrini R. Prognostic and predictive value of thymidine labelling index in breast cancer. Breast Cancer Res Treat 1998; 51: 267.
4. Burger PC, Shibata T, Kleihues P. The use of the monoclonal antibody Ki-67 in the identification of proliferating cells: Application to surgical neuropathology. Am J Sur Pathol 1986; 10: 611.
5. Brown DC, Gatter KC. Monoclonal antibody Ki-67: its use in histopathology. Histopathology 1990; 17: 489.
6. Midulla C, Cenci M, De Iorio P et al. DNA ploidy and TLI in association with other prognostic parameters in breast cancer. Anticancer Res 1999; 19: 381.
7. Barnard NJ, Hall PA, Lemoine NR, Kadar N. Proliferative index in breast carcinomas determined in-situ by Ki-67 immunostaining and its relationship to clinical and pathological variables. J Pathol 1987; 152: 287.
8. Hasebe T, Mukai K, Tsuda H, Ochiai A. New prognostic histological parameter of invasive ductal carcinoma of the breast: clinicopathological significance of fibrotic focus. Pathol Int 2000; 50: 263.
9. Fisher ER, Redmond C, Fisher B. Histologic grading of breast cancer. Pathol Annu 1980; 15: 239.
10. Black MM, Speer FD, Nuclear structure in cancer tissues. Surg Gynecol Obstet 1957; 105: 97.
11. Daidone MG and Silvestrini R. Prognostic and predictive role of proliferation indices in adjuvant therapy of breast cancer. J Natl Cancer Inst Monographs 2001; 2001: 27.
12. Del Bino G, Silvestrini R, Costa A, S et al. Morphological and clinical significance of cell kinetics in non-Hodgkin's lymphomas. Basic Appl Histochem 1986; 30:197.
13. Akerman M, Brandt L, Johnson A, Olsson H. Mitotic activity in non-Hodgkin's lymphoma. Relation to the Kiel classification and to prognosis. Br J Cancer 1987; 55: 219.
14. Ozmen V, Cabioglu N, Dolay K et al. Biological considerations in locally advanced breast cancer treated with anthracycline-based neoadjuvant chemotherapy: thymidine labelling index is an independent indicator of clinical outcome. Breast Cancer Res Treat 2001; 68: 147.
15. Pronzato P, Queirolo P, Vecchio S et al. Thymidine labeling index analysis in early breast cancer patients randomized to receive perioperative chemotherapy. Oncology 2001; 60: 88.
16. Araki K, Kimura M, Sakamoto K, Nishimura R, and Akagi MI. Significance of the tritiated thymidine labeling index in breast cancer. Jpn J Surg 15: 144-9 (1985).
17. Hall PA. Rewiew: Assessment of cell proliferation in histological material. J Clin Pathol 1990; 43: 184-92.
18. Gerdes J, Li L, Schlueter C et al. Immunobiochemical and molecular biologic characterization of the cell proliferation associated nuclear antigen that is defined by monoclonal antibody Ki-67. Am J Pathol 1991;138: 867.
19. Wolberg WH, Street WN, Mangasarian OL. Importance of nuclear morphology in breast cancer prognosis. Clin Cancer Res 1999; 5: 3542 .
20. Picnta KJ and Coffey DS. Correlation of nuclear morphometry with progression of breast cancer. Cancer 1991; 68: 2012 .
21. McGurrin JF, Doria MI Jr, Dawson PJ et al. Assessment of tumor cell kinetics by immunohistochemistry in carcinoma of breast. Cancer 1987; 59: 1744.

22. Yang Q, Mori I, Sakurai T, Yoshimura G et al. Correlation between nuclear grade and biological prognostic variables in invasive breast cancer. Breast Cancer 2001; 8: 105.

23. Gerdes J, Lelle RJ, Pickartz H et al. Growth fraction in breast cancers determined in situ with monoclonal antibody Ki-67. J Clin Pathol 1986; 39: 977.

24. Raymond WA, Leong AS-Y. The relationship between growth fractions and oestrogen receptors in human breast carcinoma, as determined by immunohistochemical staining. J Pathol 1989; 158: 203.

25. Correale M, Abbate I, Paradiso A et al. pS2- a new cytosolic protein recognized by monoclonal antibodies as a marker of hormone sensitivity in breast cancer. Cell Biophysics 1993; 22: 101.

26. Pichon MF, Pallud C, Brunet M, Milgrom E. Relationship of presence of progesterone receptors to prognosis in early breast cancer. Cancer Res 1980; 40: 3357.

27. Cattoretti G, Rilke F, Andreola S et al. P53 expression in breast cancer. Int J Cancer 1988; 48: 4368.

28. Wrba F, Reiner A, Markis-Ritzinger E et al. Prognostic significance of immunohistochemical parameters in breast carcinomas. Pathol Res Pract. 1988; 183: 277 .

29. Gebauer G, Fehm T, Lang N, Jöger W. Tumor size, axillary lymph node status and steroid receptor expression in breast cancer: prognostic relevance 5 years after surgery. Breast Cancer Res Treat 2002; 75:167.

30. Silvestrini R, Daidone MG, Luisi A et al. Cell proliferation in 3,800 node-negative breast cancers: consistency over time of biological and clinical information provided by 3H-thymidine labeling index. Int J Cancer 1997; 74: 122.

31. Meyer JS, Province MA. S-phase fraction and nuclear size in long term prognosis of patients with breast cancer. Cancer 1994; 74: 2287 .

32. Sigurdsson H, Baldetorp B, Borg A et al. Indicators of prognosis in node-negative breast cancer. N Engl J Med 1990; 322: 1045.

33. Rudolph P, Alm P, Heidebrecht HJ et al. Immunologic proliferation marker Ki-S2 as prognostic indicator for lymph node-negative breast cancer. J Natl Cancer Inst 1999; 91: 271.

34. Kroman N, Jensen MB, Wohlfahrt J et al. Factors influencing the effect of age on prognosis in breast cancer: population based study. Commentary: much still to learn about relations between tumour biology, prognosis, and treatment outcome in early breast cancer. BMJ 2000; 320: 474.

35. Veronese SM, Gambacorta M, Gottardi O et al. Proliferation index as a prognostic marker in breast cancer. Cancer 1993; 71: 3926 .