DOI: 10.4149/BLL_2013_138

Bratisl Lek Listy 2013; 114 (11)
645 – 649

CLINICAL STUDY

A comparison of Ki 67 proliferative index in primary tumor and axillary metastatic lymph nodes with length of survival in patients with breast cancer

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Abstract: *Objective:* The aim of the present study was to investigate the possibility to predict the histopathological features of breast and metastatic lymph nodes and survey and prognosis of patients and likelihood of being a predictive factor for treatment by using Ki67 immunohistochemical stain.
Materials and methods: 95 patients who were admitted to Uludag University Medical Faculty, Department of General Surgery with a diagnosis of stage II–III breast cancer between dates May 1997 and December 2002 were retrospectively evaluated with respect to breast cancer related prognostic factors treatments and last-control related data. Ki67 immunohistochemical staining was performed to appropriate specimens using Streptavidin-biotin technique. Ki67 was reported as the proliferation index, and the number of stained nuclei were stated to be / 1000.
Results: In the evaluation of the lymph node by univariate analysis, we ascertained that duration of survival is shorter above the 227 cut-off value for Ki67 proliferative index. Length of survival of patients with tumor Ki67 proliferative index below 141 and with no distant metastasis was established to be better. Ki67 proliferative index in the lymph node was detected to increase more with increasing histological and nuclear grade, estrogen and progesterone receptor negativity and at stage III.
Conclusion: Since numerous factors are effective on breast cancer, each patient and tumor behaves differently. A lot of prognostic factors are taken into account while treatment choice is determined. We may have information on the biological behavior of the tumor in patients who underwent sentinel lymph node biopsy or axillary dissection in staining with Ki67 pattern *(Tab. 5, Fig. 3, Ref. 13)*. Full Text in PDF *www.elis.sk.*
Key words: breast cancer, prognostic factor, Ki67.

Breast Cancer is the most prevalently seen cancer type among women. In the United States 207 090 new cases of breast cancer diagnosis and 39 840 related deaths were estimated in 2010 (1). According to The Ministry of Health of Turkey Cancer Control Department report between 2004–2006, breast cancer detected in 6597 patients through 27 709 cancer case among women. Breast cancer was in the first place of total cancer cases with a rate of 23.8 % (2).

Natural course is very variable in breast cancer. Biological markers are required to estimate the risk of relapse and predict poor-outcome patient groups. All measurements present during diagnosis and surgery, and in relation with health and general survival in the absence of adjuvant treatment are called prognostic factors (demographic features such as age, menopausal status and ethnic structure and biological markers such as tumor suppressor genes, growing factors, proliferation markers). Measurements exhibiting response or no response to a specific treatment in advance are called predictive factors. The estrogen receptor status of the tumor is an example of predictive factor determining response to the hormone therapy (3).

Growth fraction or proliferative capability is important in the development of breast cancer. The investigators' evaluation of some measurements, present in various phases of cell cycle and can reflect capability of tumor cell proliferation has lead to the development of new techniques. Mitotic index thymidine, labeling index, flow cytometry, S-phase fraction, proliferating cell nuclear antigen and Ki67 are among proliferation measurements.

Ki67 is a monocolonal antibody raised against a nuclear antigen which is found only in proliferating cells (late G_1, S, M, G_2). Prognostic factors are associated with life cycle. The utilization of prognostic factors is estimated by measuring the effect of the tumor on proliferation. The Ki67 monoclonal antibody is also one of the agents demonstrating proliferation (4). Working with fresh frozen breast tissue is possible. Nuclear pleomorphism, mitosis and differentiation measurements of tumor cells are determined by grading. The Ki67 staining pattern determines tumor cell proliferation more accurately than grading system. The cell proliferation index Ki-67 was observed to increase by the degree of cell proliferation before tumor cells present cancer symptoms or after they become symptomatic (5). The Ki67 proliferative index and tumor size are

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directly correlated with histological grade, vascular invasion and axillary node involvement and indirectly correlated with involvement of steroid receptor (6). Poor prognostic factor and correlation with clinical response to chemotherapy should be considered in the proliferating cells and in patients with invasive breast cancer (7).

Since numerous factors are effective for breast cancer, each patient and tumor behaves differently. Many prognostic factors should be considered in determining treatment options. The purpose of this study is to be able to predict the histopathological features of breast and metastatic lymph nodes and survival time of patients using Ki67 immunohistochemical stain and to contribute application of the appropriate treatment. Many studies are available regarding the prognostic significance of Ki67 in breast tumors. In this study we aimed to compare the same effect in the axillary lymph node.

Materials and methods

115 patients who were admitted to Uludag University Medical Faculty, Department of General Surgery with a diagnosis of stage II-III breast cancer between dates May 1997and December 2002 were included to the study. 20 patients who were diagnosed with stage II T2N0M0 and T3N0M0 breast cancer according to the TNM staging scheme and without lymph node metastases were excluded from the study. The remaining 95 patients were included to the study. The criteria (histological type, stage-grade, estrogen and Progesterone receptor positivity, presence of lymphovascular invasion, tumor diameter, number and location of metastatic lymph node and the presence of distant metastases), affecting the prognosis of patients and used for staging, treatments and their last control dates were recorded retrospectively by utilizing the data obtained from the archives of Departments of Breast Surgery and Medical Oncology and Central archives of Uludag University Medical Faculty. Phone connection was established with patients who were undergoing follow-up in other centers.

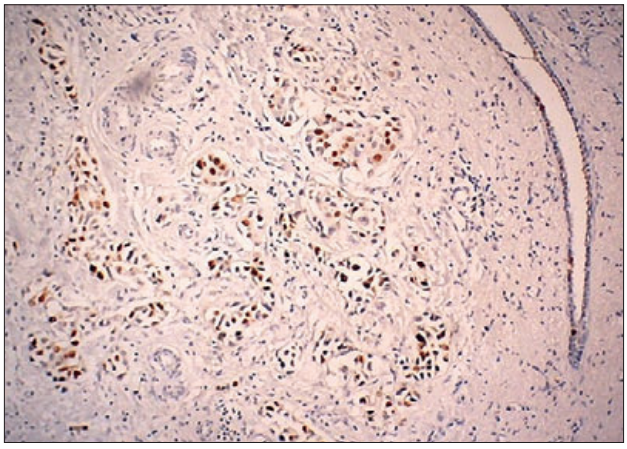

Fig.1. Ki67 proliferation index 420/1000 (x100) in the breast tissue.

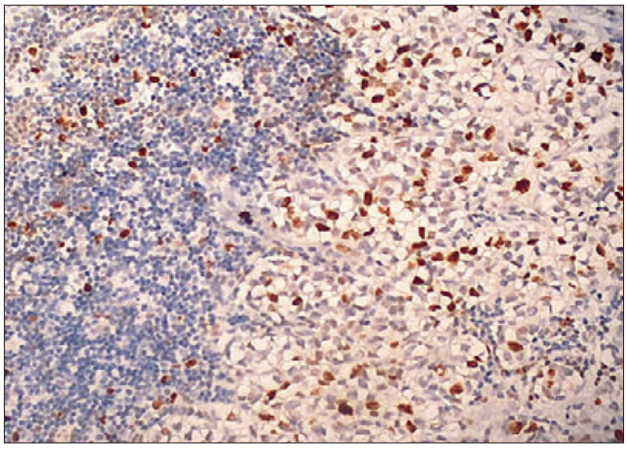

Fig. 2. Ki67 proliferation index 370/1000 (x200) in the lymph node.

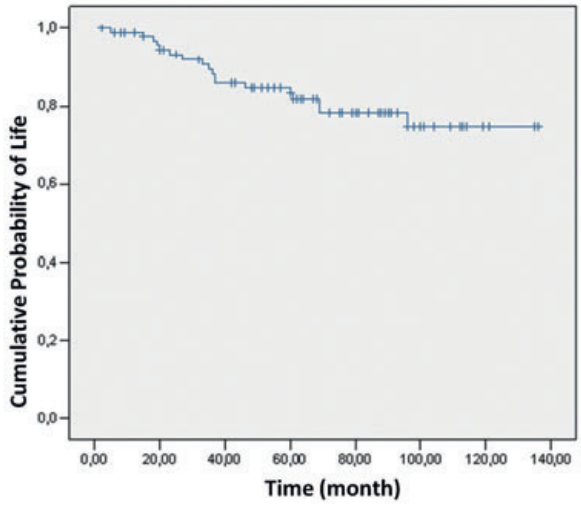

Fig. 3. Distribution of patients' duration of survival according to months.

The approval of the ethics committee of Faculty of Medicine, Uludag University was obtained for the present study (Date: February 13th 2007, No: 2007 – 2/15). Sections were prepared from the paraffin blocks found in the archives of Department of Pathology. Hematoxylin–eosin stained sections were re-examined. Proper samples were selected for immunohistochemical stains4-micron sections prepared from paraffin blocks of subjects were deparaffinized with xylene and Ki67 immunohistochemical staining was performed using Streptavidin-biotin technique.

Olympus light microscope with plan objective was used for the assessment of used stain. Brown nuclear staining was observed in the tumor cells found in the mitotic cycle with Ki67 immunohistochemical staining (Figs 1 and 2). In each preparation, 10x40 magnification, staining of the busiest areas, starting next to the field in this area and these areas, and starting from the most intensely stained and neighboring regions 1000 tumor cells were counted and the number of Ki67-positive stained nuclei was found. Regardless of staining intensity, all stained nuclei were included to the number. Ki67 was reported as the proliferation index, and the number of stained nuclei were stated to be / 1000.

The statistical analysis of the data was performed with SPSS13.0 statistical package program. Shapiro-Wilk test was

used to examine if the data followed a normal distribution. Mann-Whitney U test was performed to compare two groups to examine the data following an abnormal distribution. The correlations between variables were evaluated using Pearson's and Spearman's correlation coefficient. The Pearson's Chi-square and Fisher's Exact tests were used to examine the categorical data. To examine the effects of prognostic factors on length of survival, threshold values were derived by using ROC analysis and considering duration of survival. On condition of finding a significant threshold value, minimum effect of duration of survival was compared with the Log-rank test using Kaplan-Meier analysis. The significance level was chosen to be α=0.05.

Results

Features of the patients

A total of 95 patients were recruited, treated and followed-up in Uludag University Faculty of Medicine Department of General Surgery with stage II–III breast cancer between May 1997 and December 2002. Patients were between 26 and 80 years of age (mean age ± SD: 51.2±12.6). According to type of operations, out of 95 patients 48 (50.5 %) underwent a modified radical mastectomy, 47 (49.5 %) had breast-saving surgery. Postoperatively, our patients were classified by the AJCC TNM staging system. Distribution of patients according to stages has been exhibited in Table 1.

Tab. 1. Distribution of patients according to stages.

	Number of Patients (%)			Number of Patients	(%)
Stage II	54 (56.8%)	Stage IIA	T1N1M0	32	33.7
		Stage IIB	T2N1M0	22	23.2
Stage III	41 (43.2%)	Stage IIIA	T1N2M0	1	1.1
			T2N2M0	2	2.1
			T3N1M0	8	8.4
			T3N2M0	1	1.1
		Stage IIIB	T4N1M0	23	24.2
			T4N2M0	6	6.3

Tab. 2. Features of the metastatic lymph node.

			Minimum	Maximum	Mean	SD*
Number of Lymph Nodes	Level 1	Total	2	34	12.97	6.53
		Benign	0	25	8.86	6.20
		Metastatic	0	34	4.11	4.54
	Level 2	Total	0	14	3.65	3.23
		Benign	0	14	2.84	3.11
		Metastatic	0	9	0.81	1.75
	All lymph nodes	Total	3	34	16.63	7.09
		Benign	0	26	11.72	7.13
		Metastatic	1	34	4.90	5.304
LN Ki67 staining pattern			39	920	265.43	136.62
Ki67 staining pattern of the tumor			15	920	202.41	150.75

* SD: standard deviation **LN: lymph node

22 of our patients (23.2 %) received neoadjuvant chemotherapy. All patients received adjuvant chemoradiotherapy. Distribution of therapy according to diagrams used during chemotherapy is respectively as follows: 90 (94.7 %) patients were treated with FEC (Fluorouracil + Epirubicin + cyclophosphamide) therapy, CMF (cyclophosphamide + methotrexate + Fluorouracil) was administered to 3 (3.1 %) patients and 2 (2.2 %) were given AC (doxorubicin + cyclophosphamide). Postoperatively, 35 (36.8 %) patients received endocrine therapy. Anti-estrogenic agents - tamoxifen or nolvadex- were used in 28 patients, 6 patients were treated with aromatase inhibitors in combination with anti-estrogenic agents and a1 patient received only aromatase inhibitor. 92 (96.8 %) patients received postoperative radiotherapy. The detected carcinomas in our study were: 85.3 % invasive ductal carcinoma, then respectively (8.4 %) invasive lobular, (3.2 %) medullary, (2.1 %) mucinous and (1.1 %) invasive papillary carcinoma.

Features of the metastatic lymph node have been exhibited in Table 2.

Our patients were followed-up for average 66.2 (2–136) months. During the follow-up period 9 (9.5 %) patients had local recurrence, 4 patients (4.2 %) developed a second primary tumor, 30 (31.6 %) patients developed distant metastases, and 18 (18.9 %) patients died. Bone metastases (10 patients 33.3 %) was the most frequently detected distant organ metastases. When the last state of survival obtained from clinics, where they were followed-up, were examined, out of 95 patients 42 (44.3 %) were healthy and 15 (15.8 %) developed metastases. Additionally, a total of 20 patients, 12 (12.6 %) healthy and 8 (8.4 %) with metastases, were followed-up during a period shorter than 60 months because they did not attend follow-up appointments. These patients were evaluated according to their follow-up periods. Distribution of the duration of survival of these patients has been exhibitedin (Fig. 3).

The cut-off values were examined for distant metastasis, local recurrence, second primary tumor, time of death and the staining pattern of Ki67 proliferative index in the tumor and lymph node. The cut-off values for Ki67 in the tumor and in the lymphatic node were established 141 and 227 respectively.

The effects of risk factors of breast cancer on mortality and duration of survival, we investigated in our study, have been exhibited in Table 3.

In our patients with local recurrence, distant metastasis and second primary the proliferative index Ki67 in the tumor and lymph node was not determined significantly higher than those of other patients (p>0.05). Ki67 values of the patients with local

Tab. 3. Risk factors for breast cancer.

		Number (n)	Mortality (%)	5 Year survival (%)	Mean length of life (month)	SD	p
Tumor Ki67 proliferation index	Staining below 141	38	2 (%5.3)	94.7	129.62	4.38	0.006*
	Staining over 141	57	16 (%28.1)	80.7	101.66	6.85	
Ki67 proliferation index in lymph node	Staining below 227	43	4 (%9.3)	93	125.62	4.96	0.013*
	Staining over 227	52	14 (%26.9)	80.8	101.36	7.39	

* p<0.05

Tab. 4. Ki67 values of the patients with local recurrence. distant metastasis and second primary.

	Local recurrence	
	Present (n=9) Mean value ± SD	Absent (n=86) Mean value ± SD
Tumor Ki67	258.44 ± 251.79	196.54 ± 137.10
Lymph node Ki67	288.11 ± 150.26	263.05 ± 135.86
	Distant metastasis	
	Present (n=30) Mean value ± SD	Absent (n=65) Mean value ± SD
Tumor Ki67	185.90 ± 88.40	210.03 ± 172.19
Lymph node Ki67	245.16 ± 86.41	274.78 ± 154.11
	Second Primary	
	Present (n=4) Mean value ± SD	Absent (n=91) Mean value ± SD
Tumor Ki67	202.00 ± 206.11	202.42 ± 149.39
Lymph node Ki67	287.00 ± 168.58	264.48 ± 136.11

SD: Standard Deviation

recurrence, distant metastasis and second primary have been exhibited in Table 4.

No significant correlation was determined between the proliferative index of proliferative index Ki67 in the tumors and metastatic lymph nodes and number of metastatic lymph nodes, tumor diameter, lymphatic invasion and extracapsular spread ($p>0.05$). There was a significant correlation between the histological grade and Ki67 proliferative index of the tumor and Ki67 proliferative index of the lymph node ($p=0.001$, $r=0.334$). There was a significant correlation between the nuclear grade and Ki67 proliferative index of the tumor ($p=0.047$, $r=0.207$) and Ki67 proliferative index of the lymph node ($p=0.011$, $r=0.264$).

The correlation between Estrogen Receptor, Progesterone Receptor, lymphatic invasion and extracapsular spread and Ki67 values of tumor and lymph node has been exhibited in Table 5.

Discussion and conclusion

Today it is not possible to arrange the treatment of breast cancer only according to tumor size, type and axillary lymph node involvement. 2005 St. Gallen Consensus focuses on the targeted breast cancer treatment, tumor endocrine response and risks of progressive disease. Clinician should determine the existing prognostic and predictive parameters. Histopathological features (size, type, grade, number of involved lymph nodes), biological parameters, hormone receptor status and HER2/neu gene overexpression or amplification concerned with tumor must be determined (8).

In a study performed on the changes in breast cancer incidence and mortality in the literature, incidence rates for breast cancer were established to decrease between 35 and 49 years of age and increase between 50 and 69 years of age in women (9). Ages of our patients were between 26 and 80 (Mean age ± SD: 51.2±12.6).

Standard surgery is composed of axillary dissection in breast conserving surgery and radiotherapy. But in recent years, due to the increasing morbidity of patients following axillary dissection, biology of the primary tumor has been studied to find predictive factor of lymph node metastasis (10). Thus, the status of the lymph nodes can guide the physician to prediction without lymph node dissection and choice of postoperative treatment. Fehm et al. (10) found that high level HER-2 expression, staining with Ki67 >18 % and Progesterone Receptor negativity was a predictive factor of lymph node metastasis in 655 patients.

The presence of Lymphovascular Invasion, staining with Ki67 ≥18 % (high proliferative activity of tumor nucleus) and histological grade III was reported to be a predictive factor for lymph node metastases in another study (11) examining predictive factors for lymph node metastases in 358 patients who had tumor T1 and underwent level I–II axillary dissection.

Nuclear pleomorphism, mitosis and differentiation measurements of the tumor cell is determined grading, Ki67 proliferative index determines the proliferation of tumor cell more accurately than grading system. Ki67 proliferative index was observed to increase by value of cell proliferation before tumor cells exhibited breast cancer symptoms and after they became symptomatic (5).

Ki67 proliferative index of tumor cells of our patients was 202.41± 50.75. The obtained cut-off value was 141. Patients with proliferative Ki67 index ≤141 had the mortality rate of 5.26 % and mean duration of survival of 129.62±4.38 months which is better than patients with proliferative index >141. Ahlin et al. indicated cut-off values between 150 and 220 for Ki67 in 570 patients with T1-4 N0-1 M0., but an optimal value could not be given in numerous studies because there are many prognostic factors affecting the studied groups. Prognostic factors are estimated in relation to duration of survival,

Tab. 5. The correlation between estrogen receptor. progesterone receptor. lymphatic invasion. extracapsular spread and stage and Ki67 in the tumor and lymph node.

		Ki67 value in the Tumor			Ki67 value in the Lymph node		
		n	Mean ± SD	P	n	Mean ± SD	p
Estrogen Receptor	negative	33	249.69 ± 164.11	0.014*	33	249.69 ± 164.11	0.001*
	positive	62	177.24 ± 137.97		62	236.19 ± 131.44	
Progesterone Receptor	negative	46	205.54 ± 143.26	>0.05	46	289.13 ± 127.29	0.034*
	positive	49	199.46 ± 158.88		49	202.42 ± 149.39	
Lymphovascular Invasion	negative	59	189.64 ± 153.65	>0.05	59	253.81 ± 149.14	>0.05
	positive	36	223.33 ± 145.54		36	284.47 ± 112.58	
Extracapsular Spread	negative	48	196.66 ± 165.45	>0.05	48	268.75 ± 158.55	>0.05
	positive	47	208.27 ± 135.64		47	262.04 ± 111.51	
Stage	II	54	177.90 ± 153.09	0.004*	54	241.79 ± 135.62	0.029*
	III	41	234.68 ± 143.13		41	296.56 ± 133.22	

n: number of patients. * $p<0.05$

the usage of prognostic factors is calculated by measuring its effects on the proliferation of tumor (4). In our patients the estimated Ki67 cut-off values were 141 in tumor tissue and 227 in lymph node.

The Ki67 proliferative index increased with proliferating tumor. Buxant et al (6) found the Ki67 proliferative index in the metastatic lymph node higher than the Ki67 proliferative index in the primary tumor. We compared the Ki67 proliferative index, a prognostic value, in the primary tumor and the axillary metastatic lymph nodes with length of survival in our patients with breast cancer. Ki67 proliferative index in the lymph node was 265.43±Std. 136.62. The obtained cut-off value was 227. The mortality rate of patients with Ki67 proliferative index ≤227 was 9.3 % and mean duration of survival was 125.62±4.96 months and better than those of patients with Ki67 proliferative index >227. Ki67 proliferative index (265.43±136.62) in the metastatic lymph node was significantly higher than that of Ki67 proliferative index of the tumor cell (202.41±150.75). In patients with local recurrence, distant metastasis and second primary tumor, Ki67 proliferative index in the lymph node of our patients was not significantly higher than that of other patients.

The correlation between Ki67 and other biomarkers were studied in numerous studies. Naturally, if we consider that mitotic count is described as a component of three criterion of the Nottingham Combined Histological grading system, there is a good correlation with tumor grade (3).

Ki67 proliferative index was shown to be associated with mitotic index, tumor size, histological grade and lymph node status (6). In our study no significant correlation was determined between Ki67 proliferative index in the primary tumors and metastatic lymph nodes and number of metastatic lymph nodes, tumor diameter, lymphatic invasion, lymph nodes and extracapsular spread. A significant correlation was found to exist between histological grade and Ki67 proliferative index in the tumors and in the lymph nodes. An inverse relationship was defined to exist between low proliferative activity and Ki67 in estrogen-positive tumors (3). We determined high levels of Ki67 proliferative index in the tumors and lymph nodes in patients with estrogen receptor-negative. High level of Ki67 in the tumors was found insignificant in patients with progesterone receptor-negative; however, level of Ki67 in the lymph node was determined to be high.

Apoptosis and proliferation are commonly used markers in tumor cells of cyclooxygenase 2, Ki67 and p53. In specimens obtained from tumors before and after celecoxib treatment, followed by 5-flurourasil, epirubicin and cyclophosphamide these three markers were observed to decrease after treatment. The effect of the treatment can be investigated using these markers (12). The benefits of neoadjuvant chemotherapy using the Ki67 proliferative markers were examined in the literature. (13). All patients received adjuvant chemotherapy and 22 of them received neoadjuvant chemotherapy. Patients receiving the neoadjuvant chemotherapy experienced worse duration of survival. Ki67 values of patients receiving the neoadjuvant chemotherapy were higher but no statistical significance was determined. We believe that we obtained inadequate data. 92 patients received postoperative radiotherapy.

In conclusion, each patient and tumor behaves differently in breast cancer due to numerous effective factors. A lot of prognostic factors should be considered for the choice of treatment. We can have knowledge of biological behavior of the tumor by detecting the mitotic activity of the lymph node obtained by sentinel lymph node biopsy. If biological behavior of tumor can be predicted before diagnosis of the tumor, the better choice of treatment can be determined, or if the effect of the applied treatment can be assessed, patients benefit from the treatment more effectively. We cannot determine a significant relationship between Ki67 value in the lymph node and duration of survival in the multivariate analysis since the patients included to our study did not comprise a homogenous and crowded group. There were also many variations. We determined that Ki67 proliferative index in the lymph node evaluated in the univariate analysis had shorter duration of survival above cut-off value of 227. We evaluated that, consistent with the literature, our patients had better duration of survival when tumor Ki67 proliferative index was below 141 and the patient was free from distant metastasis. Ki67 proliferative index in the lymph node was determined to increase more with increasing histological and nuclear grade, receptor negativity of estrogen and progesterone and at stage III.

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Received October 6, 2011.
Accepted August 18, 2013.