RESEARCH ARTICLE

Ki67 Index in Breast Cancer: Correlation with Other Prognostic Markers and Potential in Pakistani Patients

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Abstract

Introduction: Breast cancer aggressiveness can be correlated with proliferation status of tumor cells, which can be ascertained with tumor grade and Ki67 indexing. However due to lack of reproducibility, the ASCO do not recommend routine use of Ki67 in determining prognosis in newly diagnosed breast cancers. We therefore aimed to determine associations of the Ki67 index with other prognostic markers like tumor size, grade, lymph node metastasis, ER, PR and HER2neu status. Methods: A total of 194 cases of newly diagnosed breast cancer were included in the study. Immunohistochemical staining for ER, PR, HER2neu and Ki67 was performed by the DAKO envision method. Associations of the Ki67 index with other prognostic factors were evaluated both as continuous and categorical variables. Results: Mean age of the patients was 51.7 years (24-90). Mean Ki67 index was 26.9% (1-90). ER, PR, HER2neu positivity was noted in 90/194 cases (46.4%), 74/194 cases (38.1%) and 110/194 cases (56.70%) respectively. Significant association was found between Ki67 and tumor grade, PR, HER2neu positivity and lymph node status, but no link was apparent with ER positivity and tumor size. There wasan inverse relation between Ki67 index and PR positivity, whereas a direct correlation was seen with HER2neu positivity. However, high Ki67 (>30%) was associated with decreased HER2neu positivity as compared to intermediate Ki67 (16-30%). The same trend was established with lymph node metastasis. Conclusion: Our study indicates that with high grade tumors, clinical utility of ki67 is greater in combination with other prognostic markers because we found that tumors with Ki67 higher than 30% have better prognostic profile compared to tumors with intermediate Ki67 level, as reflected by slightly lower frequency of lymph node metastasis and HER2neu expression. Therefore we suggest that Ki67 index should be categorized into high, intermediate and low groups when considering adjuvant chemotherapy and prognostic stratification.

Keywords: Ki67 index - breast cancer - ER - PR - HER2neu

Asian Pac J Cancer Prev, **14 (7)**, 4353-4358

Introduction

Biomarkers expression in breast cancer is used as a prognostic indicator and predictor of response to hormonal and chemotherapy. To date, the leading parameters that guide adjuvant therapy in breast cancer are estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor (HER2neu). In recent years, gene expression analysis studies have demonstrated the vitality of proliferation signatures not only in the prognosis of breast cancer but also as a predictive response to subsequent therapy (Dai et al., 2005; Whitfield et al., 2006; Bonnefoi et al., 2009).

In terms of tumor biology, proliferation has been recognized as a distinct hallmark of cancer and act as an important determinant of cancer outcome (Hanahan et al., 2000; Desmedt et al., 2004; Van Diest et al., 2006). Increased tumor cell proliferation is accompanied by cell matrix remodeling and neo-angiogenesis, which together form the basis for an aggressive tumor phenotype (Ellis et al., 1996; Eppenberger et al., 1998). Since tumors that exhibit increased proliferation tend to be more aggressive clinically, measures of proliferation are often incorporated into histological grading systems. The simplest and most widely used method is the mitotic count. In recent years immunohistochemistry for Ki-67 has also been used to determine tumor proliferation. Ki-67 is a nuclear non-histone protein which was first identified after immunization of mice with Hodgkin's lymphoma (Gerdes et al., 1983; 1991). The murine monoclonal antibody Ki-67 reacts with a human nuclear antigen that is expressed in G1, S, G2, and mitosis, but not in G0 (Gerdes et al., 1984). In breast cancer, a strong correlation has been found between the percentage of cells positive for Ki-67 and nuclear grade and mitotic rate (Sahin et al., 1991; Keshgegian 1995).

Several studies have investigated the prognostic significance of Ki67 in breast cancer. Studies have shown that over expression of Ki67 correlates with poor disease free survival (Colozza et al., 2005). Conversely patients

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with tumors that have a very high level of proliferation have a better response to chemotherapy (Bottini et al, 2005). Furthermore this marker could help select patients who are unable to benefit from chemotherapy, such as those with HER2neu-negative and hormone receptor-positive tumors with low proliferation (Fasching et al., 2011).

Moreover there is a lack of consensus regarding cut off values of Ki67 for the administration of chemotherapy and there appears to be a grey zone (intermediate level Ki67) regarding initiating adjuvant therapy based on proliferation index (Goldhirsch et al., 2009; 2011). Therefore correlation of Ki67 expression with other prognostic markers including hormone receptor status and HER2neu expression will be helpful in making clinical decisions regarding institution of adjuvant therapy especially with intermediate level Ki67 status. The aim of the present study is to correlate Ki67 expression with clinic-pathologic and prognostic markers of breast cancer like tumor grade, lymph node metastasis, ER, PR and HER2neu receptor status. This will help stratify patients into prognostic subgroups with a better predictive response to adjuvant and neoadjuvant hormonal and chemotherapy.

Materials and Methods

Patients and tumors

It is a comparative cross sectional retrospective study performed at Liaquat National Hospital, Histopathology Department was carried out from June 2010 till May 2011. This includes 194 cases of primary breast cancer which includes mastectomies, lumpectomies, trucut, incisional and wedge biopsies. All non-epithelial tumors and post-chemotherapy patients were excluded. Histologic type of tumors was determined by WHO classification of breast tumors and graded by Modified Bloom-Richardson grading system. One representative section from each tumor is selected for immunohistochemical staining.

Immunohistochemistry

Four millimeter thick sections were deparaffinized in xylene and dehydrated. Antigen retrieval was done by boiling target DAKO Envision retrival solution (high PH 50×) for 40mins at 96-99°C. Endogenous peroxidase activity was blocked by treatment with DAKO Envision flex peroxidase blocking reagent. The slides were incubated for 20-30mins at room temperature in humidity chamber with appropriate dilutions of primary antibodies along with their positive and negative controls. The slides were then incubated with secondary antibody (Envision horse reddish peroxidase) for coupling reaction for 20-30mins at room temperature. The substrate (Diamino benzidine+Chromogen) was used to produce crisp brown color at the site of target antigen. The hematoxylin (1-2 dips) was used as a counter stain.

The results for ER and PR were scored in a semi quantitative fashion incorporating both the intensity and the distribution of specific staining (Collins, 2005). The evaluations were recorded as percentages of positively stained tumor cells in each of the five intensity categories denoted as zero (no staining), 1+(weak but detectable), 2+(mildly distinct), 3+(moderately distinct) and 4+(strong). For each tissue a value designated as HSCORE was derived by summing up the percentages of cells staining intensity multiplied by the weighted intensity of staining. An HSCORE of less than 50 was established as negative, between 51 to 100 as mild (weak positive), 101 to 200 as moderate (intermediate positive), while 200 and more as strong positive.

HER2neu were scored based on the intensity and percentage of positive cells on a scale of 0 to 3+. Cases were reported 0 (negative) if no staining or membrane staining in less than 10% of invasive tumor cells was seen, 1+(negative) if faint/barely perceptive membrane staining was detected in more than 10% of invasive tumor cells, 2+(positive) if weak to moderate complete membrane staining in more than 10% tumor cells or <30% with strong complete membrane staining, or 3+(positive) if strong complete membrane staining in more than 30% invasive tumor cells was seen (Wolff, 2007).

Ki-67 immunoreactivity was recorded as continuous variables, based on the proportion of positive tumor cells (0-100%) regardless of staining intensity. Besides evaluating Ki-67 as continuous variable, levels of Ki-67 were quantified as high (immunostaining ≥30%), low (immunostaining <15%) and intermediate (between 16 to 30%) approach adopted by St Gallen International Expert Consensus (Goldhirsch et al., 2009; 2011).

Statistical analysis

One way Anova was employed to examine the correlation of Ki67 as a continuous variable with other prognostic markers (tumor size, histologic type, tumor grade, ER, PR, HER2neu expression and lymph node status) and correlation of Ki67 as a categorical variable was determined by chi square test. Data was expressed as mean and standard deviation. p value<0.05 was considered as to be significant.

Results

Among 194 patients included in the study, 100 patients underwent modified radical mastectomy or lumpectomy with axillary dissection, 6 cases were those of simple mastectomy/lumpectomy while 94 cases were of incisional/trucut biopsies. Mean age of the patients was 51.76 years (24-90). Mean Ki67 index was 26.91% (1-90). Detailed tumor characteristics with mean Ki67 index are presented in Table 1. Infiltrating ductal carcinoma was the most common histological subtype, comprising 174/194 cases (89.7%), followed by infiltrating lobular carcinoma accounting for 10/194 cases (5.1%). Majority of the tumors were in the range of 2-5cm (pT2). Among 94 cases in which axillary dissection was done, 35/94 cases (37.2%) were lymph node negative, while 89/94 cases (62.8%) were lymph node positive (N1-N3). Grade II tumors were most common accounting for 105/194 cases (54.1%). ER, PR, HER2neu positivity was noted in 90/194 cases (46.4%), 74/194 cases (38.1%) and 110/194 cases (56.70%) respectively. As a continuous variable, significant association was found between mean Ki67 index and histologic tumor type, lymph node metastasis,

Table 1. Tumor Characteristics with Mean Ki67 Index

		N	Ki67_index	95% Confidence Interval for Mean		p value
			Mean±S.D	Lower level	Upper level	
Histologic tumor type	Infiltrating Ductal Carcinoma	174	27.76±23.70	24.22	31.31	0.031*
	Infiltrating Lobular Carcinoma	10	23.00±22.49	6.92	39.08	
	Others	10	8.10±5.97	3.83	12.37	
Tumor size (cm)	pT1 <2	4	24.50±17.64	-3.56	52.56	0.641
	pT2 2-5	65	15.91±18.14	11.41	20.4	
	pT3 >5	31	18.13±22.45	9.89	26.36	
Lymph node Involvement	No positive nodes	35	11.57±16.13	6.03	17.11	0.014*
(positive nodes)	N1 1-3	22	16.09±17.26	8.44	23.74	
	N2 4-9	21	28.67±23.62	17.91	39.42	
	N3 >9	16	15.81±19.03	5.67	25.96	
Tumor grade	I	48	17.29±17.81	12.12	22.46	0.001*
	II	105	26.97±22.23	22.67	31.27	
	III	41	36.10±28.13	27.22	44.98	
Estrogen receptor status	Negative	104	30.57±26.48	25.42	35.72	0.038*
	Week Positive	31	25.61±19.09	18.61	32.62	
	Intermediate Positive	21	17.29±18.47	8.88	25.69	
	Strong Positive	38	21.21±17.43	15.48	26.94	
Progesterone receptor status	Negative	120	30.83±25.81	26.17	35.5	0.008*
	Week Positive	23	15.74±16.09	8.78	22.7	
	Intermediate Positive	20	22.65±18.53	13.98	31.32	
	Strong Positive	31	20.23±16.23	14.27	26.18	
Her2_neu receptor status	Negative	35	13.06±21.84	5.55	20.56	0.001*
	1	49	31.78±27.62	23.84	39.71	
	2	54	26.74±20.18	21.23	32.25	
	3	56	30.07±20.47	24.6	35.55	

*p value is statistically significant. One Way ANOVA

Table 2. Coorelation Of Ki67 Index with Tumor Grade

	Tumor grade				p value
	I	II	III	Total	
Low (0-15%)	28	38	12	78	
Intermediate (16-30%)	13	31	10	54	0.010*
High (>30%)	7	36	19	62	
Total	48	105	41	194	

*p value significant at <0.05 level

Table 3. Coorelation of Ki67 Index with Tumor Size

	Tumor size				p value
	pT1 <2 cm	pT2 2-5 cm	pT3 ≥5 cm	Total	
Low (0-15%)	1	40	18	18	0.665*
Intermediate (16-30%)	2	15	7	7	
High (>30%)	1	10	6	6	
Total	4	65	31	31	

*P-value is not significant at <0.05 level

tumor grade, ER, PR and HER2neu positivity (Table 1). Ki67 was categorized into high (>30%), intermediate (16-30%) and low (<15%) levels. Significant association was found between Ki67 and tumor grade, PR, Her2neu positivity and lymph node status (Table 2 and 4). However no significant association was found between Ki67 index with ER positivity and tumor size (Tables 3 and 4). There was inverse relation between Ki67 index and PR positivity (Table 4), whereas direct relation was seen with HER2neu positivity, however high Ki67 (>30%) was associated with decreased HER2neu positivity as compared to intermediate Ki67 (16-30%) (Table 4). The same trend was found with lymph node metastasis (Table 4).

Table 4. Coorelation of Ki67 Index

		Negative	Positive	Total	p value
Estrogen receptor status*					
Low	(0-15%)	37	41	78	
Intermediate	(16-30%)	27	27	54	0.108*
High	(>30%)	40	22	62	
Total		104	90	194	
Progesterone receptor status*					
Low	(0-15%)	42	36	78	
Intermediate	(16-30%)	31	23	54	0.021*
High	(>30%)	47	15	62	
Total		120	74	194	
Her2neu receptor status					
Low	(0-15%)	46**	32	78	
Intermediate	(16-30%)	15**	39	54	0.001*
High	(>30%)	23**	39	62	
Total		84**	110	194	
Lymph node status					
Low	(0-15%)	27	28	55	
Intermediate	(16-30%)	4	19	23	0.017*
High	(>30%)	4	12	16	
Total		35	59	94	

*p value is not significant at <0.05 level. **1+ Her2neu is considered negative

Discussion

Routine assessment of cell proliferation is recommended in the pathological evaluation for all breast cancers. This has traditionally taken the form of mitotic activity scoring, which is an integral component of histologic grading and considered as an established prognostic marker in breast cancer. Role of Ki67 immunohistochemistry as a prognostic and predictive marker in breast cancer is being investigated; we found

statistically significant association of Ki67 expression with tumor grade, lymph node metastasis, PR and HER2neu status. This is evident by significant p values as observed in our study.

Breast cancer aggressiveness appears to be directly related to the percentage of Ki67 positive cancer cells. The same fact is depicted in our results. Because we observed that immunohistochemical expression of Ki67 appears to be associated with the grade of differentiation, lymph node metastasis, and absence of PR expression and Her2neu positivity. These findings underlined the relationship between Ki67, a relatively new biological marker and other valuable already tested predictive factors.

After introduction of Ki67 in clinical practice, several studies investigated the prognostic significance of Ki67 specifically as a predictor of chemotherapeutic response. A study conducted in Italy demonstrated that baseline elevated Ki67 is associated with complete pathological and clinical response (Bottini et al., 2005). Dowsett et al. concluded that Ki67 level at 2 weeks of treatment is a better predictor of recurrence free survival than pre-treatment levels (Smith et al., 2005; Dowsett et al., 2006; 2009). On the other hand a trial involving 211 patients, did not find any statistically significant association of Ki67 index with clinical response rate (Learn et al., 2005). These differences may be due to heterogeneous group of population, different methods for assaying Ki67, or different cutoffs to designate high or low Ki67. As a result, the American Society of Clinical Oncology (ASCO) Tumor Marker Guidelines Committee proposed that the evidence supporting the clinical utility of Ki67 was insufficient to recommend routine use of this marker for prognosis in patients with newly diagnosed breast cancer (Harris et al., 2007). Therefore Ki67 losses its significance in isolation and it should be assessed in correlation with other prognostic factors in more narrowly defined tumor subgroups. In a similar context a group of investigators have generated an IHC-based assay of four markers, designated IHC4, which consists of ER, PR , HER2, and Ki67 and validated its prognostic value compared to 21-gene Genomic Health recurrence score (GHI-RS) (Cuzick et al., 2009).

In a metaanalysis of 71 studies from 1990 to 2010, Ki67 was found to be an independent prognostic factor for disease free survival and the greatest benefits from Ki-67 assessment could be observed in patients with ER+ breast cancers. It is not predictive for chemotherapy, but high Ki-67 was found to be associated with immediate complete response in the neoadjuvant setting (Luporsi et al., 2012).

Histological grade can unequivocally subdivide tumors into low and high risks groups (grade 1 vs. grade 3) in terms of outcomes. However, about 40-50% of breast cancers are classified as grade 2 with a less well-defined risk. The use of Ki-67 index in a grade 2 population could be particularly useful to sub-classify them.

Another important issue is the choice of the cut-off value for Mib-1 (Ki67) positivity, as it determines which patients are classified as 'high Ki-67', and therefore which have a poorer prognosis. These patients will generally receive more aggressive therapy. Different cut-off points were chosen in different studies on the basis of the median value, which maximizes the difference between the survival curves or on arbitrary percentages, usually 10% or 20% (Trihia et al., 2003; Railo, 2007).

The use of data-derived 'optimal' cut-points can result in serious bias due to different patient populations in each series. It should be stressed that transforming continuous variables, such as the Ki-67 index, into two categories can lead to a loss of power of the biomarker (Royston, 2006; Viale, 2008). In addition, this is unrealistic at the individual level, since it suggests that patients, who have tumors with Ki-67 levels close to the cut-point but on either side of the cut-point, are very different, and in turn receives different therapy, whereas in reality they are probably very similar. Few investigators specifically directed their analysis to Ki-67 cut-off values but failed to individuate a single optimal value, while demonstrating a linear association between increasing staining counts and poorer outcome (Molino, 1997). We adopted the similar approach as proposed by the St. Gallen International Expert Consensus using 2 cuff points at 15% and 30%, subcategorizing Ki67 level into low, high and an intermediate risk category. This approach is particularly useful as it defines a central grey zone in between low and high values, where other factors may be considered to make therapeutic decisions. We after adopting the same approach found that with two cut off values of Ki67 index, a subset of patients with high Ki67 (30%) may be better prognostically than intermediate Ki67 as they are associated with negative Her2neu and lymph node status. The same fact was demonstrated by other co-workers who found better response to chemotherapy with high Ki67 index (Bottini et al., 2005).

Although tumor grade, a parameter easily assessed on core biopsies is not sufficient to define prognosis and it cannot be assessed optimally in post neoadjuvant settings (Matsubara et al., 2013). Furthermore, as more conservative surgeries and staging techniques increasingly are introduced into the management of breast carcinoma e.g increasing use of fine needle aspiration over tissue biopsies, much useful prognostic information, including tumor size, tumor grading, vascular invasion and lymph node involvement, will not be available. In this setting new markers such as Ki67, p53 etc that can be applied on small samples and they may be of prognostic significance which will be invaluable (Bilgren et al., 2002).

This study also confirms the value of Ki67 evaluation as an objective means for prediction of prognosis as other recently published studies (Ferguson et al., 2013; Reyel et al., 2013; Strand et al., 2013).

Our study elucidate that measurement of Ki67 alone cannot provide data of significant value to other important prognostic indicators such as grading and pathologic staging. What is demonstrated is that Ki67 is a very reliable replacement for mitotic counts and would be easier to apply in FNAC and core biopsies, in which there is limited number of cells present. In addition there are also many other possible parameters to asses such as p53, but there is a need for a large, controlled study to assess markers in small biopsies and FNACs that can substitute for parameters in classic grading.

We have attempted to elucidate the relationship between Ki67, ER and PR content. Other workers

have shown that the Ki67 is positively correlated with histological grade and negatively correlated with ER and PR content determined immunohistochemically and data obtained from our study are in agreement with these findings (Haerslev et al., 1996).

Although to the best of our knowledge, no such study was conducted in our population. A study carried out in Iranian population showed significant correlation between PR and Ki67 but correlation with other hormone receptors i.e., ER was not found (Sharifi et al., 2006). We also found significant inverse correlation with HER2neu positivity and lymph node metastasis. Among rare types as in our cases, the higher expression of Ki67 was observed in invasive lobular carcinoma and the least in papillary carcinoma. Again representing the same fact that Ki67 is a bad prognostic marker therefore its expression is strong in tumor types with bad prognosis.

A similar study conducted in African population in Sudan, revealed significant association of Ki67 index with tumor grade, however they failed to reveal any significant association of Ki67 with hormone receptors, tumor size and stage of the disease (Awadelkarim et al., 2012).

In conclusion, although prognostic and predictive value of Ki67 index is well established, but the clinical utility of Ki67 is more useful in the combination of other prognostic markers especially hormone receptor status and HER2neu expression as a subset of high grade tumors (Ki67>30%) may have a better prognosis inspite of high Ki67 status as demonstrated by slightly lower frequency of HER2neu expression and lymph node metastasis. Therefore we suggest that Ki67 should be categorized into high, intermediate and low risk groups when considering adjuvant chemotherapy and prognostic stratification. Future studies will enable us to better define Ki67 index as a high or low risk group and prognostic stratification of the patients. Moreover Ki67 is particularly useful in limited tissue samples like trucut biopsies and FNAC samples where traditional grading may not be very accurate.

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