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ORIGINAL ARTICLE

Ki-67 expression in primary breast carcinomas and their axillary lymph node metastases: clinical implications

Daehoon Park · Rolf Kåresen · Tove Noren · Torill Sauer

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Abstract Proliferative activity of tumour cells assessed by immunohistochemical Ki-67 expression is one of several prognostic indicators in breast cancer. The major objective of this study was to investigate the prognostic impact of Ki-67 proliferative activity in the axillary lymph node metastases and in the matched primary breast carcinoma from 194 patients. There was a statistically significant up-regulation of Ki-67 protein in the metastatic deposit compared to where the primary tumour was found (p=0.001). A low Ki-67 index in both the primary and the metastatic tumours was a favorable prognostic factor. A high index in both primary and metastatic lesion and an up-regulation from a low index in the primary tumour to a high index in the metastatic deposit represented an unfavorable prognostic factor. Multivariate analysis showed that Ki-67 expression in the metastases was a superior independent prognostic factor of clinical outcomes compared to that in the primary tumours. Ki-67 expression in ≥10% of carcinoma cells in the primary tumours and ≥15% in the nodal metastases seems to be optimal cut-off levels. Ki-67 is of value as an independent prognostic factor in breast cancer.

Keywords Cell proliferation · Ki67 · Invasive breast cancer · Prognostic factors

D. Park (✉) · T. Noren · T. Sauer
Department of Pathology, Ullevaal University Hospital,
Kirkeveien 166,
N-0407 Oslo, Norway
e-mail: daehoon.park@ulleval.no

R. Kåresen
Department of Surgery, Ullevaal University Hospital,
Oslo, Norway

Introduction

The proliferative capacity of breast cancer is an important prognostic factor and can be evaluated by a variety of methods such as number of mitoses per 10 high power field (HPF), thymidine labeling index, bromodeoxyuridine labeling, S-phase fraction, and Ki-67 /MIB-1 antigen, Ki-S1 antigen, and proliferating cell nuclear antigen proliferative index [4, 11, 17, 24]. Determination of the proliferative index by means of immunohistochemistry (IHC) represents an easy and reliable method of assessing tumour cell proliferation in breast cancer [1, 23].

Ki-67 is a labile, nonhistone nuclear protein that is tightly linked to the cell cycle and is expressed in all continuously cycling cells of mid-G_1, S, and G_2 phase and in mitosis, but not in quiescent or resting cells in the G_0 and early G_1 phase [12]. As this protein is present in all proliferating cells (normal and tumour), it may serve as a marker to evaluate the growth fraction of a given cell population [7]. The functional significance of Ki-67 protein in cell cycle regulation still remains unknown [23]. Previous studies have shown controversial results concerning Ki-67 as an independent prognostic factor [18, 19, 25, 31]. To our knowledge, no study has investigated the relationship of Ki-67 expression in the metastatic lesions with clinical outcomes in breast cancer.

We have assessed the associations between Ki-67 immunoreactivity and other established clinicohistopathological parameters and the prognostic significance of Ki-67 both in primary breast carcinomas and in their corresponding axillary lymph node metastases (ALNM). We have also compared Ki-67 immunoreactivity in the primary tumours with the corresponding metastases and discuss the clinical implications of our findings.

Materials and methods

Patients

The study comprised 194 consecutive breast cancer cases that, from May 2000 to August 2004, underwent mastectomy or breast conserving surgery with complete axillary dissection at Ullevaal University Hospital for primary breast carcinomas with ALNM. Patients, 191, received postoperative adjuvant therapy according to national adjuvant treatment guidelines (http://www.nbcg.net/filer/79.ppt). Follow-up included clinical examination at 6–12 months intervals with annual mammography. Paraffin sections from the primary tumours and their corresponding metastases were retrieved from the archives. Data registered included age, tumour size, histological subtype, and grade, estrogen/progesterone receptor (ER/PgR) status, size of the largest metastatic focus and number of lymph nodes with metastasis. The characteristics of the study population and their specimens are summarized in Table 1. The median number of lymph nodes with metastasis was one (range : 1–27). The histological subtype and grade of the primary tumours had been evaluated according to the criteria of the World Health Organization [28] and the Elston and Ellis system [10].

Immunohistochemistry

Paraffin sections were immersed in Tris/ethylenediaminetetraacetic acid (EDTA) pH9 (HIER) in microwave oven (2.5 min at 750 W and 15 min at 160 W) for antigen retrieval. The IHC staining of Ki-67 was performed with the Ventana ES automated immunostaining system (Ventana Medical Systems, Inc., Tucson Arizona, USA), using mouse monoclonal antibodies with Ki-67 (Clone MIB-1; dilution 1:75; DAKO Norden A/S, Glostrup, Denmark).

The histological sections were reviewed to ensure representative tumour tissue for IHC from both primary tumour and its corresponding metastatic deposit. IHC analysis was carried out simultaneously on the primary mammary tumours and their corresponding metastases. The number of positively stained nuclei of all cases was assessed by one observer (DP). Another 35 randomly chosen cases were examined by TS to assess the inter-observer variation. Ki-67 positive nuclei were counted in several random areas including the periphery. At least 100 nuclei were counted in each area using a ×40 objective magnification. The results were expressed as percentages of positively stained nuclei over the total number of nuclei counted. The mean of the percentages of positively stained nuclei obtained in all areas was used for defining high/low Ki-67 index. A cell was considered positive if there was a clearly detectable brown colour in the nucleus. Cytoplasmic staining was considered nonspecific and was interpreted as negative. For high/low Ki-67 indices, four different cut-off values, 5, 10, 15 and 20%, were set and tested.

Statistical analysis

The results were analyzed using the statistical software SPSS 14.0 for Windows (SPSS Inc., Chicago, IL, USA). The comparison between the percentage of cells expressing Ki-67 in the primary tumour and ALNM was evaluated for statistical significance using Student's *t* test. The correlation between Ki-67 protein at the four different cut-off points and histopathological parameters was assessed using the χ^2 test and linear-by-linear test. For the interobserver agreement, the kappa (κ) statistic [15] was used. (Value of κ; poor to fair agreement: <0.4, moderate: 0.4–0.6, substantial: 0.6–0.8, almost perfect: >0.8). The clinical end points in the survival analysis were: disease/relapse free survival (DFS),

Table 1 Summary of the characteristics of the study population (N=194) and their specimens

Characteristics	Values
Age (years)	
Median (range)	54 (22–82)
Primary tumour type	
Invasive ductal carcinoma	172 (88.7%)
Invasive lobular carcinoma	22 (11.3%)
Primary tumour grade	
1	42 (21.6%)
2	112 (57.7%)
3	40 (20.6%)
Primary tumour size (cm)	
<1	29 (14.9%)
1–2	96 (49.5%)
>2	69 (35.6%)
Estrogen receptor status	
Positive	164 (84.5%)
Negative	30 (15.5%)
Progesterone receptor status	
Positive	143 (73.7%)
Negative	51 (26.3%)
Size of the largest axillary lymph node metastatic focus (mm)	
0.2–2.0	33 (17.0%)
2.0–5.0	47 (24.2%)
5.0–10.0	63 (32.5%)
>10.0	51 (26.3%)
Number of removed axillary lymph nodes	
Median (range)	13 (2–29)
Number of lymph nodes with metastasis	
1	99 (51.0%)
2	43 (22.2%)
≥3	52 (26.8%)
Total number of relapse (local/systemic)	28 (4/24)

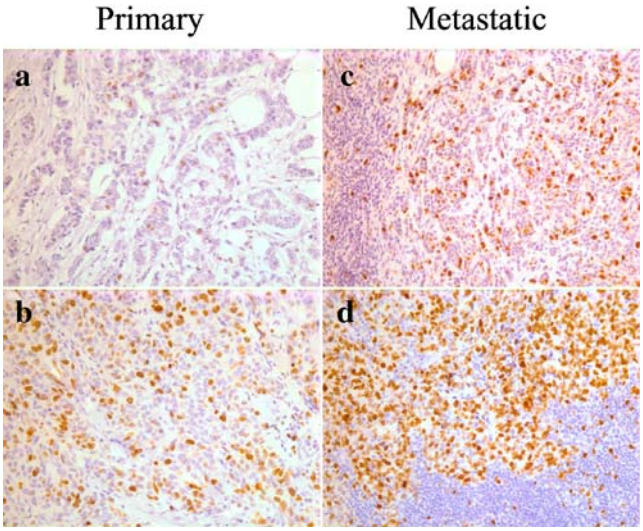

Fig. 1 Expression of Ki-67 protein in two primary tumours and their corresponding axillary lymph node metastases. (IHC, magnification ×200). Tumour 1 reveals a low number of positively stained nuclei (Ki-67 negativity) in the primary tumour (**a**), whereas the number of positively Ki-67 stained nuclei in the axillary lymph nodal metastases is significantly increased (**c**) (up-regulation). In tumour 2, both the primary (**b**) and metastatic carcinoma cells (**d**) show Ki-67 positivity, but the metastatic lesion reveals a higher number of Ki-67 positive cells than the primary tumour

distant disease free survival (DDFS), and breast cancer specific survival (BCSS). Kaplan–Meier survival curves were constructed, and the survival rates were compared using the log-rank test. Uni- and multivariate analyses of the independent prognostic factors were performed using the Cox proportional hazards regression model (stepwise backward elimination). A p-value of <0.05 was considered statistically significant.

Results

Method validation

The interobserver reproducibility was tested in 35 randomly chosen patients according to Ki-67 cut-off values and the tumour sites (i.e., in the primary tumours and in the ALNM). The agreements between the two observers were in the sufficient or good agreement range, irrespective of Ki-67 cut-off values and the tumour sites (k>0.6).

Expression of ki-67 in the primary tumours and their corresponding ALNM

There was a significant difference in the number of positively stained cells between the primary tumours and the corresponding ALNM (p=0.001) for Ki-67 immunoreactivity (Fig. 1). The mean percentage of positively cells was 11.4% in the primary breast tumours and 18.1% in the metastases (11.4±17.9 and 18.1±20.7%, respectively).

Correlation of ki-67 expression in primary and metastatic tumour with other histopathological parameters

Ki-67 expression in the primary tumours and the metastases correlated with a high histological grade (all Ki-67 cut-off values, p<0.001) and with a negative ER/PgR status (p=0.05~p<0.001). No correlation was found with age, cancer type, tumour size or number of lymph nodes involved. All cut-off values for Ki-67 expression in the ALNM correlated with the size of the metastases. The relationships between Ki-67 IHC staining and histopathological parameters are detailed in Table 2.

Table 2 Correlation between the expression of Ki67 and histopathological parameters

Parameters	5% Ki67 P + vs −	5% Ki67 M + vs −	10% Ki67 P + vs −	10% Ki67 M + vs −	15% Ki67 P + vs −	15% Ki67 M + vs −	20% Ki67 P + vs −	20% Ki67 M + vs −
Age (<55, ≥55)	NS	NS	NS	NS	NS	NS	NS	NS
Cancer type (IDC, ILC)	NS	NS	NS	NS	NS	NS	p=0.029[a]	NS
Grade (1, 2, 3)	p<0.001[b]	p<0.001[b]	p<0.001[b]	p<0.001[b]	p<0.001[b]	p<0.001[b]	p<0.001[b]	p<0.001[b]
Primary tumour size (<1, 1–2, >2)	NS	NS	p=0.013	NS	NS	NS	NS	NS
ER (+, −)	p=0.05[a]	p=0.019[a]	p=0.001[a]	p=0.003[a]	p<0.001[a]	p<0.001[a]	p<0.001[a]	p<0.001[a]
PgR (+, −)	p<0.001[a]	NS	p<0.001[a]	p=0.029[a]	p<0.001[a]	p=0.011[a]	p<0.001[a]	p<0.001[a]
Number of nodal involvement (1, 2, ≥3)	NS	p=0.016[b]	NS	NS	p=0.035[b]	NS	NS	NS
Size of largest metastases (0.2–2, 2–5, 5–10, >10)	p=0.027[b]	p<0.001[b]	NS	p=0.002[b]	p=0.045[b]	p=0.002[b]	NS	p=0.007[b]

P Primary tumour, *M* metastatic tumour, *%* cut-off value for defining low/high Ki-67 index, *NS* nonsignificant
[a] Chi-square test
[b] Linear-by-linear test

Prognostic value of ki-67 in primary and metastatic tumours

All 194 patients were included in the analyses for DFS, DDFS and BCSS. Twenty-eight patients had been diagnosed with recurrence within a median observation time of 40 months after primary surgery (range 8–73 months). A local relapse without distant metastasis was observed in 4 of 28 patients, while distant metastases occurred in 24. Nine had died of breast cancer.

Uni- and multivariate analyses

Age, cancer type, hormone status, histology grade, primary tumour size, size of largest metastatic focus and Ki-67 expression were all tested as prognostic factors in uni- and multivariate analyses. Hormone status, histology grade, primary tumour size and Ki-67 with 10 and 20% cut-off points both in the primary breast tumours and the nodal metastases were significantly correlated with both DFS and DDFS in univariate analyses. Ki-67 with 5 and 15% cut-off points in the primary tumours did not reveal association with DDFS. Multivariate Cox regression analyses based on four different Ki-67 cut-off values (Table 3) showed that primary tumour size and Ki-67 for all cut-off points in ALNM were significant prognostic factors for DFS. The same prognostic factors were found for DFS with exception of Ki-67 with 5% cut-off point where only histological grade and primary tumour size were independent prognostic factors. No independent prognostic factor was found for BCSS in uni- or multivariate analysis.

Survival analyses according to different cut-off values

Kaplan–Meier survival analyses based on four different Ki-67 cut-off points showed significantly reduced DFS and DDFS in patients with high Ki-67 index in the ALNM and in the primary tumours compared to those with low Ki-67 index ($p=0.036 \sim p<0.001$). In the primary tumours, the survival curves for Ki-67 with 5% and 15% cut-off points showed no significant difference in DDFS. The DDFS curves for Ki-67 with all four cut-off points are shown in Fig. 2. A high Ki-67 expression with cut-off points of 15 and 20% in the metastases was significantly associated with a shorter BCSS ($p=0.048$ and 0.037, respectively), whereas Ki-67 expression with the other cut-off values showed no difference in survival.

Survival analyses according to the combined results of both Ki-67 expressions in primary and in metastatic tumours

Most cases had a similar Ki-67 expression in the primary tumour (10% cut-off) and its corresponding metastasis

Table 3 The prognostic factors (multivariate analyses, stepwise backward elimination)

Variables	HR	DFS 95% CI	*p*-value	HR	DDFS 95% CI	*p*-value	HR	BCSS 95% CI	*p*-value
Ki67 with 5% cut-off value									
Age (<55 vs ≥55)		NS			NS			NS	
Cancer type (IDC vs ILC)		NS			NS			NS	
Hormone receptor status[a] (− vs +)	0.4	0.2–0.9	0.025		NS			NS	
Histology grade ($G_{1–2}$ vs G_3)		NS		2.5	1.1–5.7	0.034		NS	
Tumour size (T_1 vs $T_{2–3}$)	3.5	1.6–7.6	0.001	4.0	1.7–9.5	0.002		NS	
Size of largest metastatic focus (0.2–2 mm vs > 2 mm)		NS			NS			NS	
Ki67 in primary tumours (− vs +)		NS			NS			NS	
Ki67 in metastatic tumours (− vs +)	4.0	1.2–13.5	0.026		NS			NS	
Ki67 with 10% cut-off value									
Tumour size (T_1 vs $T_{2–3}$)	3.7	1.7–8.1	0.001	4.8	2.0–11.5	<0.001		NS	
Ki67 in metastatic tumours (− vs +)	5.9	2.0–17.4	0.001	5.8	2.0–17.2	0.001		NS	
Ki67 with 15% cut-off value									
Tumour size (T_1 vs $T_{2–3}$)	3.6	1.7–7.8	0.001	4.7	2.0–11.0	<0.001		NS	
Ki67 in metastatic tumours (− vs +)	4.1	1.6–10.3	0.003	5.1	1.9–13.6	0.001		NS	
Ki67 with 20% cut-off value									
Tumour size (T_1 vs $T_{2–3}$)	3.2	1.5–6.9	0.003	4.2	1.8–10.0	0.001		NS	
Ki67 in metastatic tumours (− vs +)	4.4	2.0–9.8	<0.001	5.1	2.1–12.5	<0.001		NS	

From Ki-67 with 10% cut-off value, the significant prognostic factor(s) were simply enrolled in the table.

DFS Disease free survival, *DDFS* distant disease free survival, *BCSS* breast cancer specific survival, *HR* hazard ratio, *CI* confidence interval, *IDC* invasive ductal carcinoma, *ILC* invasive lobular carcinoma, *NS* nonsignificant

[a] Hormone receptor status: positive—ER and/or PgR positive; negative—ER and PgR negative

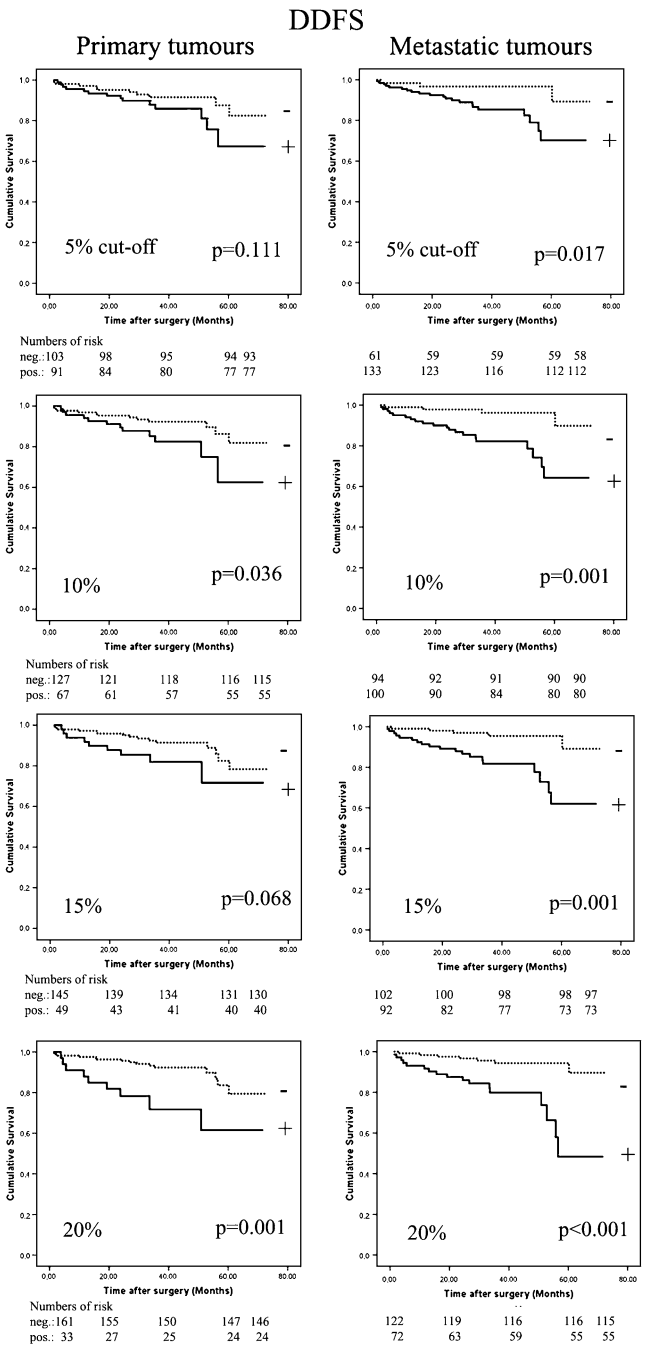

Fig. 2 Kaplan–Meier survival analyses based on four different Ki67 cut-off values show significantly shorter distant disease-free survival (DDFS) in patients with high Ki67 index in the nodal metastases and in the primary tumours compared to those with a low Ki67 index. No difference was found in 5 and 15% cut-off points in the primary tumours

(15% cut-off), either low (P−/M−: n=86, 44.3%) or high (P+/M+: n=53, 27.3%). A different Ki-67 expression between primary lesion and corresponding metastasis was detected in 55 cases (P+/M−: n=14, 7.2% and P−/M+: n=41, 21.1%, respectively). The correlation between the four groups and clinical outcome is detailed in Table 4. There was a highly significant difference in DFS and DDFS between P−/M- and the other groups; DFS: P−/M− vs P+/M− (p=0.002), P−/M− vs P−/M+ (p<0.001) and P−/M− vs P+/M+ (p<0,001), DDFS: P−/M− vs P+/M− (p=0.02), P−/M− vs P−/M+ (p=0.001) and P−/M− vs P+/M+(p=0,001). There was no difference among the three groups that had a high Ki-67 index either in the primary tumour or in the metastasis (i.e., P+/M−, P−/M+, and P+/M+), whereas a significant difference in BCSS was found between P−/M− and P−/M+ (p=0.006). The survival curves are shown in Fig. 3.

Survival analyses according to the presence of high Ki-67 expressions either in the primary or in the metastatic lesion compared to histological grade

The distribution of the four groups (i.e., P−/M−, P+/M−, P−/M+, and P+/M+) according to histological grade is shown in Table 4. Two groups were created according to the presence or absence of high Ki-67 index irrespective of the tumour sites (i.e., P−/M− vs the other group with P+ or/and M+: P+/M−, P−/M+ and P+/M+ were regarded as one group) and analyzed for the survival rates. Grade 2 P−/M− cases had a significantly better DFS than the other group. No significant differences in DDFS and BCSS were shown, but the difference in DDFS showed a borderline significance (p=0.057). No statistics were computed for grade 1 and grade 3 cases because they were all censored.

Discussion

Cell proliferation rate is a major determinant of the biologic behaviour of invasive breast carcinoma [25]. Ki-67 protein expression is strictly correlated to cell proliferation and to the active phases of the cell cycle [23]. Assessment of Ki-67 index represents an easy and reliable method for evaluating cell proliferative activity in breast cancer [1]. In the present study, we have assessed Ki-67 expression and compared the proliferative activity of cells in the primary breast carcinoma and their corresponding ALNM. We have detected significantly higher Ki-67 activity in the ALNM compared to the primary tumours (p=0.001). The finding corresponds with the result of Buxant et al. [5] who reported that Ki-67 expression was significantly increased in the ALNM. There was a significant difference in clinical outcome (DFS, DDFS and BCSS) between the groups with similar Ki-67 expression levels in primary and metastatic tumours and the groups with different expression levels. The group with similar expression was composed mainly of cases with P−/M− (73%, 72/98). As expected, this turned out to be a group with favorable prognosis (Fig. 3). Cases showing different Ki-67 expression in the primary tumour and the corresponding metastasis and cases with a high

Table 4 The high (+) or low (−) Ki-67 expression in primary (P) and metastatic tumours (M) and correlation to histological grade and clinical outcome

Groups	Number of patients (%)	Number of patients within histological grade			Number of any relapse (%)	Number of systemic relapse (%)	Number of breast cancer death (%)
		1	2	3			
P−/M−	86 (44.3)	35	48	3	3 (10.7)	3 (12.5)	1 (11.1)
P+/M−	14 (7.2)	2	10	2	2 (7.1)	2 (8.3)	1 (11.1)
P−/M+	41 (21.1)	2	28	11	10 (35.7)	9 (37.5)	5 (55.6)
P+/M+	53 (27.3)	3	26	24	13 (46.5)	10 (41.7)	2 (22.2)

expression in both lesions had an unfavorable prognosis. This finding supports Buxant et al.'s supposition [5] that a primary tumour is composed of multiple cell populations and that only those with the most aggressive potential (like a high proliferative index) are the most likely to escape from the primary tumour and establish itself as a lymph node metastasis.

Previous studies have reported significant associations between high Ki-67 index and clinicohistopathological parameters, such as age [18, 22], tumour size [18, 30, 32], histologic grade [2, 3, 18, 27, 32], ER/PgR status [6, 8, 18, 27, 30, 32] and lymph node status [32]. We found a statistically significant correlation of high Ki-67 expression in metastatic breast cancer with increasing histologic grade, negative ER/PgR status, size of largest metastatic focus, but not with age, cancer type, tumour size and the number of nodes involved. The high correlation between Ki-67 and grade is not surprising, as Ki-67 expression and mitotic rate are strongly interrelated [9, 18, 27]. In agreement with our findings, Ding et al. [9] reported that most ERα positive tumours were negative for the proliferation marker, Ki67. They suggested that such an inverse association between ERα expression and Ki-67 expression might be due to their different tumourigenic features; i.e., ERα positive tumours are more likely to be well differentiated, while Ki-67 positive breast tumours are frequently poorly differentiated [9].

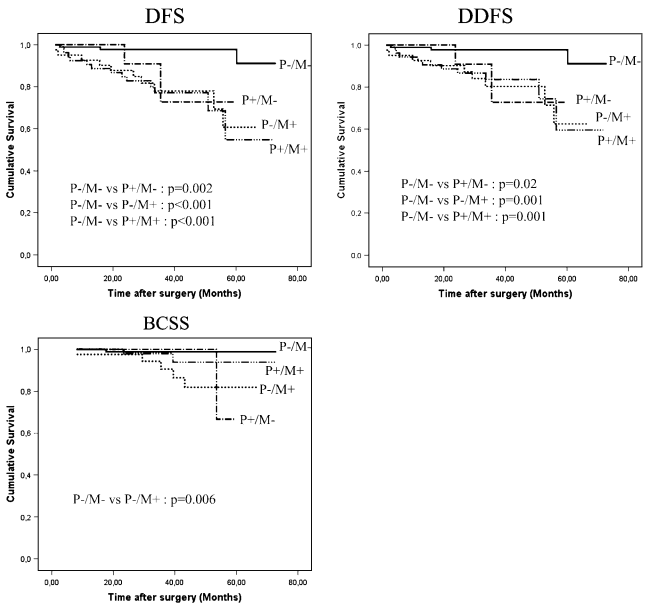

Fig. 3 The survival analyses (DFS, DDFS and BCSS) of all patients (n=194) according to the combined Ki67 status [high index (+) or low index (−)] in the primary tumours (P) and their nodal metastases (M). A significant difference in DFS and DDFS between P−/M− and the other groups was shown. No significant difference in DFS and DDFS among the combined groups (P+/M−, P−/M+ and P+/M+) was detected. In BCSS, the only survival difference between P−/M− and P−/M+ groups was significant

As there is no universally established standard Ki-67 cut-off value, we decided to treat Ki-67 expression as categorical variables by using four different Ki-67 cut-off points between 5 and 20% in 5% steps to be able to define a cut-off point for high/low proliferation rate in relation to clinical outcome.

Previous studies [3, 13, 14, 16, 21, 29] have shown statistically significant associations between Ki-67 immunoreactivity and clinical outcomes/survival. In our study, the associations between Ki-67 cut-off values and the predictabilities of DFS and DDFS were highly significant in all Ki-67 cut-off values both in the primary tumours and the metastases, except for Ki-67 expression of 5 and 15% cut-off in the primary tumours in predicting DDFS. The lack of significance in the two latter is probably due to too few cases in the group. BCSS correlated with Ki-67 expression at cut-off points of 15 and 20% in the ALNM. Ki-67 expression in more than 15% of tumour cells in the metastatic tumours pinpoint patients with a worse survival. Thus, it seems biologically "correct" to set the optimal "lower limit" of Ki-67 cut-off point to distinguish between a clinically relevant high and low proliferative activity at 15% in ALNM. In multivariate analysis, the Ki-67 expression in the metastatic tumours was a potential independent prognostic factor in predicting shorter DFS and DDFS. No association was found in BCSS, but the number of events with breast specific death (9/194) in the median follow-up of 40 months was low. A longer follow-up is probably required to see an eventual relation to BCSS.

Based on the statistical analysis, a cut-off of 10% seems to be optimal for Ki-67 expression in primary tumours. Ki-67 cut-off values have varied from one study to another. Some have used median values [14, 26], while others have chosen an arbitrary value (e.g., 10, 20% and so on) [9, 20, 25, 33]. Intriguingly, all the studies [14, 20, 26, 33] have shown a statistical correlation with clinical outcome irrespective of cut-off points. The reason might be explained by the fact that they all had chosen cut-off values that were found to be within the optimal range for evaluation of Ki-67 in the primary tumours.

On the basis of our results, we suggest that Ki-67 expression in ≥10% of carcinoma cells in the primary tumours and ≥15% in the ALNM should be used as cut-off levels for a clinically relevant prognostic difference. Evaluation of the Ki-67 index in the ALNM would pick up P−/M+ in addition to P+ cases. This would be in agreement with the finding that the level of Ki-67 expression in ALNM is a superior independent prognostic factor of clinical outcomes in metastatic breast cancer compared to that of Ki-67 expression in primary tumour.

The group with low Ki-67 index in both primary and metastatic tumour cells (P−/M−) is a favourable prognostic group albeit node-positive. Histological grade 2, P−/M− cases revealed significantly better survival than the other groups. The number of histological grade 1 P−/M− was probably too small to reach statistical significance. Histological grade 2 (and probably grade 1) tumours with a low Ki-67 expression in both the primary lesion and the corresponding metastasis (P−/M−) could be regarded as a low-risk group despite their ALNM and possibly with less need of adjuvant chemotherapy. However, further studies are necessary to confirm the association between low Ki-67 index (P−/M−) within grade 1 and 2 tumours and a good clinical outcome before such a conclusion might be drawn.

In conclusion, Ki-67 index has a potential to be used as an additional marker in metastatic breast cancer to select a high-risk (P+/M+, P−/M+ and P+/M−) and low-risk group (P−/M−) for distant metastasis that again might have consequences for adjuvant therapy strategies. Our results indicate that evaluation of Ki-67 expression in the axillary lymph node metastases is superior to evaluation of Ki-67 expression in primary breast carcinomas for predicting survival.

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