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Report

Long term prognostic value of growth fraction determination by Ki-67 immunostaining in primary operable breast cancer

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Summary

An immunohistochemical determination of the growth fraction (GF) with the Ki-67 monoclonal antibody has been performed in a prospective series of 140 patients with primary operable breast carcinoma. GF ranged from 0% to 43% Ki-67 stained cells with a median value of 8%. High GF (> 8%) was significantly associated with axillary node involvement (p = 0.006), aneuploidy (p = 0.008), histologic grade (p = 0.03), and S-phase fraction > 5% determined by flow cytometry (p = 0.01). After a median follow-up of 6 years, the univariate analysis did not show significant correlation between high GF and worse relapse-free survival (p = 0.10) or shorter overall survival. However, a multivariate analysis on relapse-free survival, performed in 127 comparable patients, showed that GF was an independent predictive factor (p = 0.03) together with nodal status (p = 0.00001), age under 45 years (p = 0.0008), and chemotherapy (0.006). In node negative patients, GF was still an independent prognostic indicator (p = 0.002) together with age under 45 years (p = 0.0003). Tumor proliferative activity evaluated by the monoclonal antibody Ki-67 appears to be an effective indicator of prognosis in breast cancer and could be of assistance in the decision making of adjuvant therapy in node negative patients.

Introduction

A number of studies have shown a correlation between the proliferative activity of human primary breast carcinomas and prognosis [1–3]. The method most commonly employed to estimate cell kinetics in human tumors is the evaluation of the S-phase (SPF) of the tumor population, either by the *in vitro* ^{3}H–thymidine labeling index (TLI) or by DNA flow cytometry [4–6].

The recent development of monoclonal antibodies that recognize antigens associated with cell proliferation has permitted the assessment of cell kinetics by easy and rapid *in situ* immunohistochemical assays [5].

The Ki-67 monoclonal antibody identifies a nuclear antigen that is synthesized during the late G1, S, M, and G2 phases of the cell cycle. Ki-67 antigen appears to be cell-cycle dependent and localized at the periphery of the chromosome scaffold and nuclear cortex. Two nuclear proteins (345 and 395 kDa) have been discovered reacting with Ki-67 antibody [7]. Full length cDNA has been cloned and sequenced in a recent study, suggesting that Ki-67 antigen defines a new category of cell cycle-associated nuclear nonhistone proteins [8]. Some authors reported a close correlation between Ki-67 proliferation index and the number of cells measured by ^{3}H-thymidine or bromodeoxyuridine incorporation [9, 10]. This immunostaining can be performed in

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Table 1. Clinico-pathologic characteristics of the patients

	No. of cases	%
Total evaluable Ki-67	140	100
Median age years (range)	58 (28–88)	
Menopausal status		
Pre meno	51	36.4
Post meno	89	63.6
TNM		
T1	50	35.7
T2	79	56.4
T3	11	7.9
N0	116	82.9
N1a	12	8.6
N1b	12	8.6
Node status[a]		
negative (pN −)	78	57.3
positive (pN +)	58	42.7
≤ 3 N +	37	64
> 3 N +	21	36
Tumor size median (range)	20 mm (7–70)	
Histological type		
ductal	118	84.3
lobular	8	5.7
others	14	10
SBR Grading[c]		
I	30	21.4
II	89	63.6
III	11	7.9
NA	10	7.1
Estrogen receptor (ER)		
negative	22	15.7
positive	110	78.6
NA	8	5.7
Progesterone receptor (PR)		
negative	51	36.4
positive	84	60
NA	5	3.6
Ploidy[b]		
diploid	59	48.8
aneuploid	61	51.2

[a] performed in 136 patients.
[b] performed in 120 patients.
[c] Scarff Bloom Richardson grading.
NA: Not Available.

frozen sections of tumors, thus allowing direct visual interpretation of labeling indices [11].

In breast cancer the histological status of axillary lymph nodes is widely accepted as the most reliable prognostic marker for the risk of relapse after surgery. It is used to select patients for adjuvant treatment. In node negative patients, there is a need to distinguish patients with good prognosis who need no further treatment after surgery from those patients with aggressive disease who are more likely to develop recurrence and who might benefit from adjuvant therapy [12]. There is increasing evidence that the assessment of proliferative activity is of considerable prognostic value and may be of particular interest in node negative patients [13].

The aims of this prospective study in breast adenocarcinomas treated by primary surgery were: 1) to correlate the Ki-67 immunohistologic staining (Ki-67 index) with other pathologic and biochemical prognostic factors, and 2) to evaluate the prognostic value of Ki-67 on overall survival (OS) and disease free survival (DFS) of these patients. Correlations between Ki-67 index and other prognostic indicators have been previously reported [5]. The prognostic value of Ki-67 on OS and DFS is reported here with a median follow-up of more than 5 years.

Patients and methods

Patients and tumor sampling

A prospective study of 148 consecutive patients with breast cancer undergoing primary surgery was conducted, within a short period of inclusion (September 1987 to February 1988) at the Institut Curie [5].

Six patients had cured contra-lateral breast carcinoma more than 6 years earlier. Three patients have been excluded for analysis (2 for metachronous breast carcinomas less than 5 years earlier, 1 for adenocarcinoma of the lung 2 years earlier). Measurable Ki-67 was obtained in 142 patients. Finally Ki-67 was evaluable in 140 patients whose characteristics are reported in Table 1.

Surgically removed breast tumor samples were obtained immediately after tumorectomy in 71 cases (50.7%) or mastectomy in 69 cases (49.3%), with lateral axillary node dissection in 136 patients (97%). Seventy-eight patients (55.7%) had adjuvant radiotherapy. Adjuvant chemotherapy was administered to node-positive patients: four cycles of cyclophosphamide, adriamycin, and 5-fluorouracil

(CAF) were delivered to 16 patients (11.4%) and 39 postmenopausal patients had endocrine therapy by tamoxifen 30 mg daily.

Estrogen and progesterone receptor assays, histologic examination, immunohistologic Ki-67 staining, and DNA flow cytometry were performed on the same tumor sample.

Histology and steroid receptor analysis

Tumor size was recorded as the largest diameter of the tumor at the time of trimming the fresh specimen.

Histologic examination was performed on hematein and eosin-stained paraffin-embedded sections. Tumors were classified by histologic type according to the criteria of the World Health Organization classification.

Nuclear and histologic grading was according to Scarff, Bloom, and Richardson classification [14].

Estrogen and progesterone receptor assays were performed by radioligand assays using the classical dextran-coated charcoal method as previously described [15].

Immunohistochemical determination of GF with Ki-67 monoclonal antibody

Cryostat sections (5 μm) were immediately prepared in embedding medium (Tissue-Tek, Miles Scientific) and fixed in cold acetone (– 20° C, 10 minutes), air-dried, and stored until use at – 80° C. For immunostaining, sections were washed briefly (1–2 minutes) and incubated 30 minutes with Ki-67 (Dakopatts, Copenhagen, Denmark) murine monoclonal antibody (dilution 1/10). Slides were then incubated successively with the biotinylated horse antimouse antibody and the avidin-biotinylated peroxidase complex (Vector Laboratories, Burlingame, CA). Incubations were at room temperature. Washings and dilutions were performed with phosphate-buffered saline (PBS), pH 7.6. Staining with the diaminobenzidine substrate was developed until labeling was clearly detectable. Control slides were incubated with an unrelated monoclonal antibody. Quantification of Ki-67 staining (Ki-67 index) was performed by ocular micrometry on a Leitz microscope (Orthoplan) at ocular magnification of × 40, with an eyepiece grid (Leitz). Counterstaining was hematoxylin. Positively stained cells were defined by a clear nuclear staining, be it homogeneous or dotted (see Fig. 1 in reference 5). Ki-67 index was based on a count of 300 consecutive tumor cells as identified by histopathologic criteria in the hematein and eosin-stained frozen sections and was expressed as the mean percentage of labeled cells per 100 cells between two independent pathologists (BD and PV in reference 5).

GF was assessed as a dichotomous variable using *a priori* the median value of 8% as a cut-off point to discriminate slowly versus rapidly proliferating tumors.

DNA flow cytometry

Fresh tumor specimens (30 mg) were minced with scissors in 1 ml Young's buffer. The suspension was then transferred and homogenized in 2 ml of Young's buffer with a Dounce-Potter (pestle A), centrifuged (600 xg, 15 minutes), and digested with preboiled RNase A (Sigma; 1 mg/ml in PBS, 37° C, 20 minutes). Samples were then repelleted, stained with propidium iodide (Sigma) (50 μg/ml in Isoton II; Coulter Electronics, Hialeah, FL), and filtered on nylon mesh (Graphosilk, Montreuil, France). Flow cytometric (FCM) study was performed on an FACS Analyzer I (Becton Dickinson), and DNA histograms were derived from an analysis of 30,000 cells on a Hewlett-Packard computer with the Consort 30 software (Becton Dickinson). The diploid peak of lymphoblastoid or fibroadenoma cells was set approximately on channel 55. The coefficients of variation (ratio of mid width to height) for G0/G1 peaks were always < 3.5%. Variations of the modal value of the peaks defined as diploid in each series never exceeded +/– 1 channel from that defined by the external standard. In case of ambiguity, a different sample of the same tumor was analyzed. Tumors displaying cells with DNA indices between 0.94 and 1.08 were considered to be DNA diploid, and the

presence of two aneuploid peaks was required to classify a tumor as multiploid. S-phase (SPF) was derived from flow cytometry histograms according to the rectangular model of Baisch *et al.* [16], with subtraction of an exponentially decreasing baseline by a home made program (H.M.). This procedure corresponds nowadays to the 'Cell fit' software. SPFs were considered uninterpretable in paucicellular samples, in histograms displaying overlapping stemlines, and in cases of large amounts of debris.

Statistical analysis

Ki-67, SPF, clinical, and statistical data were derived in blind by independent investigators.

The association between Ki-67 and other clinicopathologic variables was evaluated by Chi-square tests and analysis of variance.

The overall survival (OS) and the disease-free survival (DFS) were determined using a Kaplan Meier product-limit method [17]. Statistical significance between curves was assessed using the logrank test [18]. DFS was calculated from date of surgery to date of first event (local, metastatic, or node recurrence). Patients who did not undergo any event were censored at date of last follow up.

Multivariate analysis was carried out to assess the relative influence of prognostic factors on DFS, using the Cox proportional hazard model in a forward stepwise procedure [19].

Results

Immunocytochemical staining with the Ki-67 monoclonal antibody

Tumor cell nuclear and nucleolar staining of variable intensity and extent was demonstrated in 142 patients out of 148 (97%). Ki-67 labeling was obtained in 140 out of 145 evaluable patients. The percentage of labeled cells, calculated by ocular micrometry, ranged from 0% to 43% of labeled cells, with a median value of 8%. This median value of 8% was used as a cut-off between high GF and low GF.

Table 2. Comparison of Ki-67 index with prognostic factors

Parameter	p value
pN +	0.006
DNA ploidy	0.008
SBR	0.03
SPF	0.01
pT	0.12
ER	0.53
Age	0.55
Menopause	0.58
T	0.66
N	0.88
PR	0.95

Correlation of GF with SPF and with conventional prognostic factors

It was possible to evaluate the SPF in 95 of the 140 patients (68%). Median SPF was 6% (range: 0–30%). A significant but low correlation between Ki-67 index and SPF ($p = 0.01$) was observed. Comparison and correlations with prognostic factors have been previously reported for this series [5]. They are summarized in Table 2. Briefly, Ki-67 is correlated with the presence of a node involvement in the axillary dissection, aneuploidy, and histologic grading.

Table 3. Univariate analysis of prognostic factors: overall survival (OS) and disease free survival (DFS) (logrank test).

Parameter	OS p value	DFS p value
0N +/1–3 N +/> 3 N +	< 0.001	< 0.001
Age < 45 y	0.20	0.01
ER –	0.01	0.03
PR –	0.04	0.42
SPF > 5%	0.13	0.02
Ki-67 > 8%	0.41	0.11
T	0.80	0.32
pT	0.01	0.70
Histo	0.30	0.76
SBR	0.15	0.71
DNA ploidy	0.48	0.19
Tumorectomy	0.08	0.64
Radiotherapy	0.82	0.56
Chemotherapy	0.94	0.52
Hormonetherapy	0.35	0.36

Clinical results

Median follow-up was 70 months, ranging from 11 to 78 months. Two patients have been lost from follow-up at 11 and 18 months. The 5-year probability of overall survival (OS) and disease-free survival (DFS) for this population was 83% and 65% respectively. Fifty-one patients had recurrence of disease, 38 had distant metastases, and 25 patients died, 2 from causes unrelated to breast carcinoma (included in survival analysis).

Univariate analysis

Ki-67 was not a significant prognosticator of overall survival or disease-free survival in this series in univariate analysis. Prognostic relevance of the different variables is given in Table 3. Factors that had significant influence both on DFS and OS were axillary node status and estrogen receptor status. SPF had a prognostic value only for DFS with a 5% value used as cut-off (p = 0.02).

Multivariate analysis

This analysis was performed for DFS alone. GF score was adjusted for the variables which had prognostic relevance and that were correlated with it (variables in Table 3), using Cox proportional hazard model in a forward stepwise procedure. Missing values were coded as a separate variable for ploidy and SPF in the aim of conserving more information. This procedure was not performed when missing data were less than 10 for a given parameter. The final statistical model contained data on 127 comparable patients. Results of this analysis are given in Table 4. Patients with tumors with high Ki-67 GF had a relative risk of relapse of 1.90 (p = 0.03) compared to those with low GF. Axillary node status (pN + > 3) (p = 0.00001), age under 45 years (p =

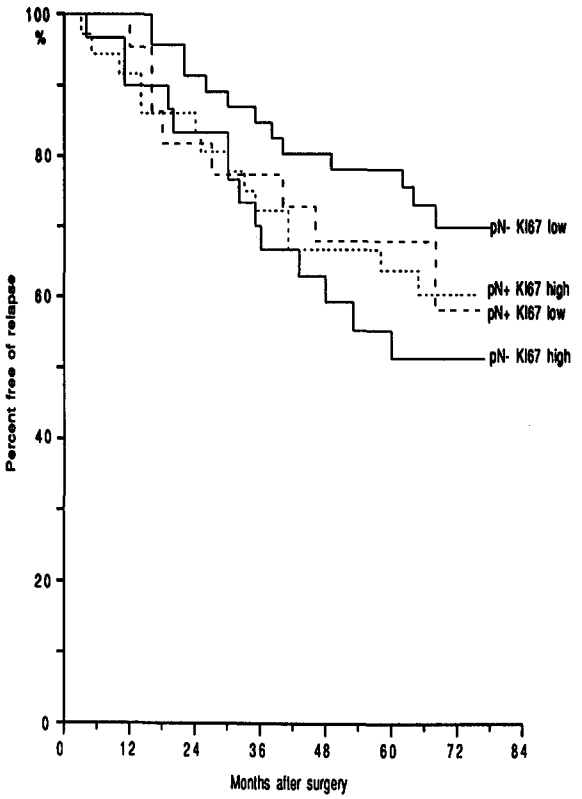

Fig. 1. The global difference between groups is not significant by the Mantel-Cox test (p = 0.41).
The log-rank test of paired groups was:
pN – Ki-67 low vs. pN– Ki-67 high: p = 0,06
pN + Ki-67 low vs. pN + Ki-67 high: p = 0,89
pN – Ki-67 low vs. pN + Ki-67 high: p = 0,37
pN – Ki-67 high vs. pN + Ki-67 high: p = 0,52
Number of patients in differents subgroups:
pN – Ki-67 low: 48
pN – Ki-67 high: 30
pN + Ki-67 low: 22
pN + Ki-67 high: 36

Table 4. Multivariate analysis: disease free survival in 127 comparable patients (Cox model)

Parameter	Relative risk	CI (95%)	p value
N + > 3	5.9	[2.8–12.7]	< 0.00001
Age < 45 years	3.1	[1.7–5.9]	0.0008
Chemotherapy	3.9	[1.4–11.1]	0.006
Ki-67 high	1.9	[1.1–3.5]	0.03

Table 5. Multivariate analysis: disease free survival in node negative patients in 71 comparable patients (Cox model)

Parameter	Relative Risk	CI (95%)	p value
Age < 45 years	5.7	[1.9–17.2]	0.0003
Ki-67 high	3.7	[1.6–8.7]	0.002

0.0008), and absence of chemotherapy (p = 0.006) also retained their pejorative prognostic value.

We compared the DFS curves of patients distributed by nodal status, with Ki-67 cell proliferation index below or above the median value. The overall difference between groups by the Mantel-Cox test was not significant (p = 0.41). For node negative patients (pN –), when patients with high GF were compared to patients with low GF, the difference was nearly significant (p = 0.06) (Fig. 1).

A multivariate analysis was performed in the subgroup of node negative patients (pN –). None of these patients had received adjuvant chemotherapy. The analysis was performed in 71 comparable patients for the different prognostic factors. Ki-67 (p = 0.002) and age under 45 years (p = 0.0003) emerged as independent prognostic factors (Table 5).

Discussion

Different studies on breast cancer [20–22], including ours [5], have found a direct correlation between Ki-67 staining and other prognostic variables, such as mitotic index, tumor size, histologic grading, receptor status, and lymph node involvement. However, the data referring to the prognostic value of Ki-67 proliferation index are scanty. Earlier works including clinical follow-up are summarized in Table 6. The present results of a prospective study with a long follow-up, confirm in multivariate analysis the clinical prognostic value of Ki-67 on DFS reported by others [10, 23–25], except for a recent study [26].

In our series, we have observed a significant influence of adjuvant treatment on recurrences although a minority of patients (16/140) received chemotherapy. We may tentatively propose that a larger proportion of these patients should have benefited from cytotoxic therapy. But the aim of

Table 6. Collected studies on Ki-67 proliferation index in prognosis

Author	Year	Pts	Tumor	Age (years)	Mean follow-up (months)	Median Ki-67	Cut-off	Univariate (p)		Multivariate (p)	
								OS	DFS	OS	DFS
Sahin [28]	1991	42	primary operable, node negative	59 (36–83)	> 60	6%	< 4% vs 4–12% (vs > 12%	NA	< 0.01	NA	NA
Weikel [22]	1991	193	primary operable and recurring tumor	58 (29–91)	24	NA	< 10% vs 10–20% vs > 20%	0.0019	0.0084	NA	NA
Wintzer [23]	1991	63	operable	62 (40–83)	37	12%	16%	< 0.01	NA	< 0.01	0.01
Gasparini [24]	1992	165	operable, stage I and II	56 (31–71)	36	7.5%	7.5%	NS	0.0027	NA	0.04
Veronese [10]	1993	129	primary operable	NA	42	12%	20%	NA	NA	0.00005	NA
Railo [25]	1993	327	primary operable	57 (25–86)	32	NA	0% vs > 10%	< 0.005	NA	0.02	NA
Gaglia [9]	1993	385	primary operable	59 (30–88)	31	9%	9%	NA	NA	NA	0.038
Rudas [26]	1994	184	primary operable	NA	73	5.25	< 3.5% vs 3.5–10.5% vs > 10.5%	NS 0.18	NS 0.1	NS	NS
Present study	1994	140	primary operable	58 (28–88)	70	8%	8%	NA	NS 0.10	NA	0.03

NA: Not Available.
NS: Not Significant.

this work was not to evaluate prognostic value of a given chemotherapy regimen, and patient characteristics were not stratified according to the treatment. In the population of this study, patients who received chemotherapy were younger (< 45 years, $p = 0.05$), had histologic node involvement (pN +, $p < 0.0001$), and had high Ki-67 value ($p < 0.001$). After adjusting the analysis for the treatment received by the patients, using the Cox model, we observe that Ki-67 becomes a significant, independent prognostic value. Age appears to be an important independent prognostic factor in this series. This point has been previously observed in our institution [27].

Both lymph node involvement and cell kinetics should be taken into consideration when adjuvant therapies have to be planned. Our results suggest, like others [9], that Ki-67 labelling should permit within the N- group, separation of patients with a good prognosis from those (Ki-67 > 8%) with a risk of recurrence comparable to N+ patients. Women of the latter group would be candidates for systemic adjuvant therapy. Among N+ patients, Ki-67 could not discriminate groups with different prognosis. Significant prognostic value of Ki-67 in node negative patients has been reported in previous studies: in a small series of 42 patients reported by Sahin [28] and in a more recent and larger series of 385 women reported by Gaglia [9].

Ki-67 antibody labeling technique has technical advantages over other methods of assessing cell kinetics in that it does not require a large amount of material, does not require the use of live cells such as for bromodeoxyuridine, iododeoxyuridine, or thymidine labeling, and can also be determined in samples from fine-needle aspirates. This could be extremely useful in neoadjuvant treatments, as it provides a significant prognostic parameter before the beginning of therapy. Also, using Ki-67, GF can be assessed in nearly all tumors (97% in the present series), whereas flow cytometry is non informative in approximately one fourth to one third of tumors [3, 5]. We obtained 68% informative S phase on fresh tumor samples at the time of accrual in the present series. Although the rate of informative S phase can be slightly increased with experience and new devices, it will hardly exceed 80% in prospective studies due to such causes as multiploidy, necrosis, etc... The main limitation of Ki-67 labeling is the subjective nature of its evaluation [24]. We did not get any additional prognostic information by taking the labeling intensity into account, at least in this series of a limited number of patients and events.

Prognostic information was obtained considering the percentage of Ki-67 labeled cells as a dichotomous variable, with a cut off set a priori at the median value. Although the search for an optimized cut off by maximization of the log-rank test gave a value of 7.6%, such internal derivation is liable to statistical bias. The median value offers the possibility of interlaboratory comparisons. The initial Ki-67 antibody could only be used on frozen section. The presently available antibody equivalent to Ki-67 in its immunoreactivity, MIB1, works on paraffin sections. Although our results are not transferable to MIB1, they are encouraging for confirmative studies with this latter monoclonal antibody.

In conclusion, multivariate analysis demonstrated that the percentage of Ki-67 positive cells and nodal status were both significant and independent prognostic factors. Cells kinetics measured by immunohistochemical techniques could be used to define groups of patients at different risks of recurrence and in the design of chemotherapy protocols.

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