

# Ki-67, p53, Er-Receptors, Ploidy and S-Phase as Prognostic Factors in T1 Node Negative Breast Cancer

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The prognostic value of Ki-67, p53 and ER immunohistochemical labelling and flow-cytometric S-phase fraction and ploidy was evaluated in 212 pT1N0M0 breast carcinomas. The mean follow-up time was 8.3 years. Patients with breast carcinomas with high Ki-67 expression ( $\geq 10\%$ ) had a less favourable disease-free survival than those with low Ki-67 expression ( $< 10\%$ ) ( $p = 0.008$ ). A positive p53 staining and high SPF were associated with a less favourable disease-free survival although it did not reach statistical significance. The subset of patients with ER negative, Ki-67  $\geq 10\%$  and p53  $\geq 20\%$  tumours, had a shorter disease-free survival compared with that of all the other patients ( $p = 0.03$ ). We conclude that the potential value of Ki-67 labelling for prognostic evaluation of T1N0M0 breast carcinoma is good.

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The monoclonal antibody, Ki-67, is reactive with a human nonhistone nuclear protein associated with cell proliferation (1). A positive correlation exists between Ki-67 immunolabelling, S-phase fraction (SPF) and node status in breast cancer (2, 3). A positive correlation with mitotic index and tumour necrosis (4), tumour differentiation (3, 5) and disease-free survival (DFS) (6) has also been described. The Ki-67 staining has been reported to be an independent prognosticator when compared with tumour size, nodal status and steroid receptor (ER, PR) expressions (5).

The tumour suppressor gene p53 is the most frequent site of genetic changes in breast cancer (7, 8). High p53 expression is found in up to 50% of node positive and invasive cancers, and in 15% of node negative and in-situ carcinomas (9). The alteration of the p53 gene leads to the loss of its negative growth regulatory function (10). The p53 expression correlates with a shorter DFS (9, 11), also in small node negative breast cancers (12–15). p53 accumulation is associated with negative ER and PR status (9, 12, 16) high histological grade (9, 12) and a higher proliferative fraction (12, 14). Nuclear p53 is an independent prognostic factor in breast cancer (9, 12).

The flow-cytometric S-phase fraction (SPF) correlates to poor histological differentiation, particularly in medullary and ductal carcinomas (17). The SPF fraction can be used in combination with tumor size and ER receptor status to evaluate the risk of relapse also in node negative tumours (18).

Our aim is to find a prognostic factor or a combination of prognostic factors that would differentiate node negative patients into those with good and those with less favourable prognosis. We have determined the prognostic value of the markers of proliferation, Ki-67 and SPF, ploidy, p53 and the ER receptor status either singly or in different combinations.

## MATERIAL AND METHODS

### Patients

A total of 212 patients with pT1N0M0 breast carcinoma were included in the study. The patients were operated on at the Fourth Department of Surgery, Helsinki University Central Hospital, between 1975 and 1989. The mean age was 60 years (range 29–86). Twenty-two percent of the patients were under the age of 50, and 26% were between

Table 1

Association between high Ki-67 immunolabelling ( $\geq 10\%$ ) and SPF, p53, ploidy, and ER in patients with T1 node negative breast cancer

Covariate	Number of patients	Number with Ki-67 $\geq 10\%$	$\chi^2$	p-value
SPF				
<3%	106	22 (21%)	39.2	<0.0001
$\geq 3\%$	106	67 (63%)		
p53				
<20%	169	56 (33%)	26.8	<0.0001
$\geq 20\%$	43	33 (77%)		
Ploidy				
Diploid	138	45 (33%)	14.3	0.0002
Aneuploid	74	44 (59%)		
ER				
<30%	43	28 (65%)	11.8	0.0006
$\geq 30\%$	169	61 (36%)		

50 and 60 years. The mean follow-up time was 8.3 years (range 0.2–18.7). Disease-free survival data were available on all patients. Until 1984, all patients were treated with modified radical mastectomy without radiation therapy but after 1984 the patients were able to choose the alternative of breast conserving surgery followed by radiation therapy. Neither preoperative nor postoperative systemic adjuvant therapy was given. The patients were followed up for recurrence and survival.

#### Histological specimens

Haematoxylin-eosin stainings of all carcinoma specimens were reviewed by one of the authors (S.N.). The most representative specimens were chosen for immunohistochemistry and flow cytometry. All specimens had been fixed in formalin and embedded in paraffin according to the routine method of the laboratory.

#### Immunohistochemical methods

Four  $\mu\text{m}$  thick paraffin sections were mounted on 3-aminopropyltriethoxy-silane (APES) (Sigma, St. Louis, MO) coated slides and dried for 12–24 h at 37°C. The sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol to distilled water. To enhance immunostaining the slides were thereafter incubated in a microwave oven (Moulinex), as described earlier (19).

Immunohistochemical stainings were performed with a commercial ABC kit (Vectastain Elite, Vector Laboratories, Burlingame, CA) using the monoclonal anti-human ER antibody (clone 1D5, Dako, Glostrup, Denmark) diluted 1:800, the monoclonal antibody DO-7, which recognizes both mutant and wild-type p53, diluted 1:300, and the polyclonal Ki-67 antibody diluted 1:500 (Dako, Glostrup, Denmark). The Ki-67 and p53 immunostaining was visualized with 3-amino-9-ethyl-carbazol (Sigma, No

A-5754) solution (0.2 mg/ml in 0.05M acetate buffer containing 0.03% perhydrol, pH 5.0). ER immunostaining was visualized with DAB chromogen tablets (Abbot Laboratories, Chicago, IL.) Positive control specimens were included in every staining batch. The staining results were interpreted independently by a pathologist (S.N.), unaware of the clinical outcome of the patients. The level of immunoreactivity was expressed as the percentage of Ki-67, p53 or ER-positive cancer cell nuclei estimated from at least five medium power microscopic fields. The intensity of the staining was not recorded. When samples from more than one site of the tumour were available, the mean percentage of positive nuclei was used. The cut-off value for Ki-67 was 10%, for p53 20% and for ER 30%. The cut-off value for Ki-67 was on the bases of our previous experience (20) and that of p53 was taken from the literature (21). In addition very few tumours have a p53 staining between 1 and 20%. The cut-off value for ER was chosen because there are few breast carcinomas with values between 1 and 30%.

#### DNA flow cytometry

Two 50  $\mu\text{m}$  thick sections were treated with 10 mg/ml proteinase K (Sigma, St Louis, MO, USA) for 30 min at room temperature. After filtration, the nuclei were treated with 10 mg/ml RNAse and stained with 25 mg/ml ethidium bromide (Sigma) for at least 1 h. The DNA content was determined by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA, USA) using 15 mW excitation at 488 nm, and the total emission above 560 nm was recorded. As the staining intensity of fixed nuclei varies from one sample to another, no internal standard was added. The lowest peak was assigned a DNA index (DI) value of 1.00 and the DI values of other peaks were calculated with this as a reference. Therefore, possible hypodiploid peaks were identified as diploid and the nor-

**Table 2**  
Association ( $\chi^2$ ) between p53, SPF, ER and ploidy

Covariate	Ki-67	SPF	p53	Ploidy	ER
SPF	39.2 p < 0.0001				
p53	26.8 p < 0.0001	31.8 p = 0.0001			
Ploidy	14.3 p = 0.0002	36.6 p < 0.0001	10.4 p = 0.0013		
ER	11.8 p = 0.0006	24.5 p < 0.0001	60.3 p < 0.0001	6.3 p = 0.012	
Age	4.4 p = 0.036	0.03 p = 0.87	1.03 p = 0.31	1.56 p = 0.21	0.36 p = 0.55

mal diploid peak as hyperdiploid. For analyses, tumours with one peak were considered as diploid and those with more than one peak as aneuploid. The histograms were interpreted by one of us (S.N.) without knowledge of the clinical outcome. The SPF was calculated either using the Cellfit program of the FACScan flow cytometer or manually by a modified rectilinear method. The SPF of the stem line with the highest DI was calculated. If the automatic and the manual methods gave different results, the lower SPF was chosen. At least 10 000 nuclei from each specimen were analyzed. The cut-off value for the SPF fraction was the median value (3%).

#### Statistical methods

The  $\chi^2$ -test was used to test the strength of the association between Ki-67 expression and the different prognostic factors.

Life tables were calculated according to Kaplan-Meier. Deaths were those due to breast carcinoma, deaths due to other causes were censored. The statistical significance of the differences in the univariate survival analysis was calculated using the log-rank test.

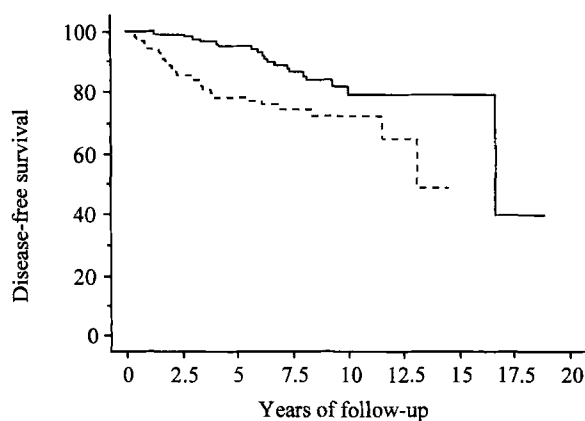


Fig. 1. Disease-free survival related to Ki-67 immunolabelling. High Ki-67 labelling tumours ( $\geq 10\%$ ) are associated with a significantly shorter disease-free survival than low Ki-67 ( $< 10\%$ ) patients ( $p = 0.008$ ). Numbers below x-axis are patients at risk.

Multivariate survival analyses were performed with the Cox proportional hazards model entering the following covariates: p53 immunoreactivity level ( $< 20\%$  positive nuclei = 0,  $\geq 20\%$  = 1), Ki-67 immunoreactivity level ( $< 10\%$  positive nuclei = 0,  $\geq 10\%$  positive nuclei = 1), ploidy (diploid tumour = 0, aneuploid = 1), SPF fraction ( $< 3\%$  = 0,  $\geq 3\%$  = 1), ER status ( $\geq 30\%$  = 0,  $< 30\%$  = 1) and age as a continuous variable. Covariates were selected in a stepwise fashion with use of the maximum likelihood ratio. A p-value of 0.05 was adopted as limit for inclusion of a covariate.

#### RESULTS

There were 42% high Ki-67 ( $\geq 10\%$ ), 20% p53 positive ( $\geq 20\%$ ) and 80% ER positive ( $\geq 30\%$ ) tumours. The mean value of the SPF was 5.8% (range 0.40–32.6) and that of Ki-67 12% (range 0–70%). There were 65% diploid tumours.

#### Association between Ki-67, p53, SPF, ploidy and ER

There was an association between a high Ki-67 immunoreactivity, high SPF ( $\chi^2 = 39.2$ ;  $p < 0.0001$ ), p53 ( $\chi^2 = 26.8$ ;

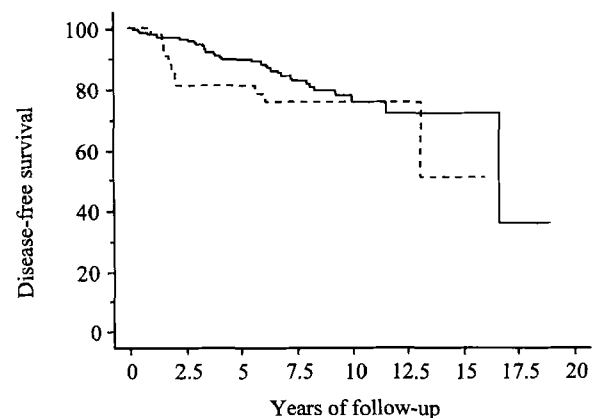
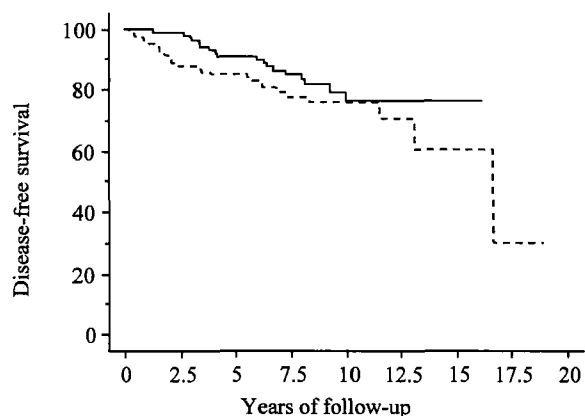


Fig. 2. Disease-free survival related to p53 immunolabelling. ( $p = 0.28$ ) Numbers below x-axis are patients at risk.



SPF < 3%	106	102	90	55	28	7	1	0
SPF ≥ 3%	106	92	86	53	28	10	3	1

Fig. 3. Disease-free survival related to the S-phase fraction. ( $p = 0.22$ ) Numbers below x-axis are patients at risk.

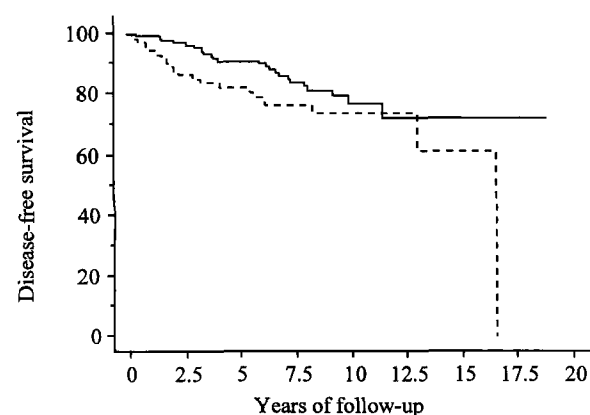
$p < 0.0001$ ), aneuploidy ( $\chi^2 = 14.3$ ;  $p = 0.0002$ ) and a negative ER status ( $\chi^2 = 11.8$ ;  $p = 0.0006$ ) (Table 1). There was also an association between p53, ER, SPF and ploidy as well (Table 2). Age was not associated with the different factors.

#### Disease-free survival analysis

In the univariate DFS analysis patients with high Ki-67 ( $\geq 10\%$ ) breast carcinomas had a less favourable prognosis compared with that of patients with low Ki-67 ( $< 10\%$ ) breast carcinomas ( $p = 0.008$ ) (Fig. 1).

The prognostic significance in the DFS analysis of p53 (Fig. 2), SPF (Fig. 3) and ploidy (Fig. 4) were not statistically significant (Table 3).

The subsets of patients were further analyzed by grouping ER negative, high Ki-67 ( $\geq 10\%$ ) and p53 positive



DI < 1	138	131	118	72	33	10	2	1
DI ≥ 1	74	63	58	36	23	7	2	0

Fig. 4. Disease free survival related to ploidy (DI) ( $p = 0.12$ ) Numbers below x-axis are patients at risk.

tumours together (Group 2). The DFS of patients with such tumours was lower than the DFS of those with all other types of tumours (Fig. 5).

In the Cox's multivariate analysis the only independent significant prognostic factor was Ki-67 ( $p = 0.008$ , RR = 2.23, Confidence interval 1.21–4.08). SPF, p53, ER status, ploidy and age were not statistically significant.

## DISCUSSION

This study shows a prognostic significance for Ki-67 in T1 node negative breast carcinoma. The statistical significance of the Ki-67 expression alone was similar to a subgroup analysis, comparing high Ki-67, p53 positive and ER negative tumours with all others. This study focused on the prognostic significance of Ki-67 expression in small, T1 ( $\leq 20$  mm) N0 tumours. Ki-67 is found to be a good prognosticator in other series of ANN breast cancer as well, but the tumour size in these studies can be up to 5 cm (5, 22). The T1 tumour size in this series enhances the prognostic significance of Ki-67 expression as compared with p53 and SPF and ER.

The expression of p53 has been reported to correlate with a less favourable DFS (9, 11) also in patients with small, node negative breast cancers (12–14). In the present study, the prognostic value of p53 was not significant, but there was a trend indicating an adverse prognosis in p53 positive tumours. The lack of prognostic value of p53, even in non-selected breast carcinoma, is reported as well (23). The diversion of the arms in the life-table analysis indicates that p53 has a trend as a prognosticator for early recurrence. In this study, the p53 positive patients who recurred, all except for three patients, developed their recurrence within two years. The maximum size of the tumours in the present study was 20 mm. A high prognostic significance of p53 in node negative patients has mainly been found in series with larger tumours, up to 50 mm in diameter (12, 14, 24). A recent study on T1N0M0 tumours also showed a prognostic significance of p53 (25). In a large series of more than one thousand node negative tumours, the 5-year survival rate declined from 96.3% to 82.2% when the tumour diameter increased from less than 20 mm to 50 mm (26). In this context, even though p53 did not reach absolute statistical significance, the rigorous choice of small size carcinomas enhances the trend of its significance.

In large series of ANN breast tumours, the stratification of the tumours by ploidy alone and the combination of ploidy and SPF revealed a significant prognostic difference between aneuploid and diploid tumours (18, 27) The SPF and ploidy were not significant prognosticators for DFS in the present study. The trend of the arms in the univariate analysis of both SPF and ploidy is diverging, unfortunately without statistical significance. On the other hand,

**Table 3**  
Univariate survival analysis of Ki-67, p53, SPF, ploidy, ER and age

Covariate	Number of patients	$\chi^2$	p-value
Ki-67			
< 10%	123	7.05	0.008
≥ 10%	89		
p53			
< 20%	169	1.15	0.28
≥ 20%	43		
SPF			
< 3%	106	1.51	0.22
≥ 3%	106		
Ploidy			
Diploid	138	2.38	0.12
Aneuploid	74		
ER			
< 30%	43	0.07	0.80
≥ 30%	169		
Age			
< 50 years	47	0.008	0.93
≥ 50 years	165		

the association between SPF and both Ki-67 and p53 was statistically highly significant.

The arms in the univariate life-table analysis of DFS in the p53, SPF and DI tend to parallel at 10–12 years of follow-up. This indicates that they are not prognosticators in a longer follow-up in T1N0 tumours. On the other hand, the number of patients at risk decreases after 8 years of follow-up, which might influence the prognostic potential of these markers.

We conclude that Ki-67 is a prognosticator in T1 ( $\leq 20$  mm) ANN breast carcinomas. The prognostic value of Ki-67 alone seems to be similar to that of a combination of the different factors.

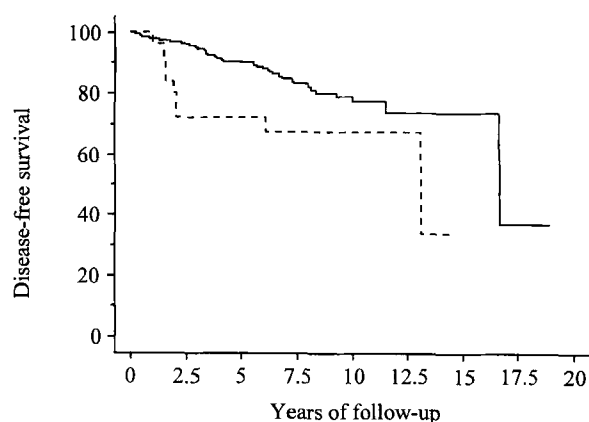


Fig. 5. Grouping ER negative, Ki-67 and p53 positive patients together, revealed that the disease-free survival of Ki-67/p53 positive and ER negative patients (Group 2) was clearly shorter than that of all other patients ( $p = 0.03$ ). Numbers below x-axis are patients at risk.

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