Immunologic Proliferation Marker Ki-S2 as Prognostic Indicator for Lymph Node-Negative Breast Cancer

Pierre Rudolph, Per Alm, Hans-Jürgen Heidebrecht, Hendrik Bolte, Virgo Ratjen, Bo Baldetorp, Mårten Fernö, Håkan Olsson, Reza Parwaresch

Background: Proper treatment of lymph node-negative breast cancer depends on an accurate prognosis. To improve prognostic models for this disease, we evaluated whether an immunohistochemical marker for proliferating cells, Ki-S2 (a monoclonal antibody that binds to a 100-kd nuclear protein expressed in S, G2, and M phases of the cell cycle), is an accurate indicator of prognosis. Methods: We studied 371 Swedish women with lymph node-negative breast cancer; the median follow-up time was 95 months. The fraction of tumor cells in S phase was assessed by flow cytometry, and tumor cell proliferation was measured immunohistochemically with the monoclonal antibodies Ki-S2 and Ki-S5 (directed against the nuclear antigen Ki-67). A combined prognostic index was calculated on the basis of the S-phase fraction, progesterone receptor content, and tumor size. Results: In multivariate analyses that did or did not (263 and 332 observations, respectively) include the S-phase fraction and the combined prognostic index, the Ki-S2 labeling index (percentage of antibody-stained tumor cell nuclei) emerged as the most statistically significant predictor of overall survival, disease-specific survival, and disease-free survival (all two-sided P<0.0001). In the risk group defined by a Ki-S2 labeling index of 10% or less, life expectancy was not statistically significantly different from that of age-matched women without breast cancer, whereas the group with a high Ki-S2 labeling index had an increased risk of mortality of up to 20-fold. Conclusions: Cellular proliferation is a major determinant of the biologic behavior of breast cancer. Prognosis is apparently best indicated by the percentage of cells in S through M phases of the cell cycle. Measurement of the Ki-S2 labeling index of a tumor sample may improve a clinician’s ability to make an accurate prognosis and to identify patients with a low risk of recurrence who may not need adjuvant therapy. [J Natl Cancer Inst 1999;91:271–8]

Because of improved diagnostic methods and screening programs, lymph node-negative mammary carcinomas presently account for more than two thirds of all breast cancer cases in Western countries, and the percentage is increasing (1–4). Women with lymph node-negative breast cancer constitute a low-risk group, approximately 70% of whom are cured by surgical resection (1). However, in a substantial number of patients, the disease may be fatal, and thus adjuvant systemic therapy is sometimes mandatory. Such therapy is costly and has the risk of overtreating patients who are not destined to relapse and would consequently suffer from its toxic effects without receiving any benefit. Therefore, an accurate prognosis is crucial for the clinical management of lymph node-negative breast cancer (1,5).

The prognostic indicators currently in common use are tumor size and histopathologic grade, hormone receptor status, and the fraction of cells in the S phase of the cell cycle, termed the S-phase fraction (1,6). Immunohistochemical proliferation indices, although reported to be of relevance (7–15), have not yet become established prognostic indicators. The inevitable inclusion of G1-phase cells with uncertain destinies (16) might account for the limited utility of the determination of the global growth fraction, defined as the cell population in G1, S, G2, and M phases of the cell cycle, and for the relatively stronger prognostic impact of the S-phase fraction (17).

In this study, we test a unique immunohistochemical proliferation marker, Ki-S2—a monoclonal antibody against a 100-kd nuclear protein that is expressed in the S, G2, and M phases of the cell cycle (18,19)—as a prognostic indicator for lymph node-negative breast cancer in a long-term follow-up of a cohort of 371 lymph node-negative breast cancer patients. We evaluate this marker in relation to the standard prognostic criteria considered as individual parameters and as a combined score (20) and to the Ki-67 index determined by immunostaining with the monoclonal antibody Ki-S5 (21,22). By means of a multivariate analysis, we show that a single factor, the Ki-S2 index, may identify a subset of patients with excellent prognosis, for whom adjuvant therapy may be unnecessary.

Patients, Materials, and Methods

Patients and Tumors

We examined 371 consecutive patients from the health care region of southern Sweden who were diagnosed with lymph node-negative breast cancer during the period from September 1975 through January 1986. In this district, hormone receptor analyses are routinely performed on fresh-frozen tumor specimens from patients with primary mammary carcinomas, and frozen tumor samples are stored in a bank at the Department of Oncology, University Hospital of Lund. We excluded any patients with purely intraductal carcinomas or with recurrent cancers and patients in whom distant metastasis occurred within 2 months after primary surgery. Ages of patients at diagnosis ranged from 23 years to 91 years (median age, 61 years). The menstrual status was premenopausal in 110 patients (29.6%) and postmenopausal in 261 patients (70.4%).

Lymph node status and tumor size were obtained from the original pathology reports, the maximum diameter of the tumor having been measured on the unfixed resection specimen in most instances. A median number of nine axillary lymph nodes (range, five to 57 nodes) was examined. The median tumor size was 20 mm (range, 5–90 mm). In addition, the histologic tumor type was determined on hematoxylin–eosin-stained sections, and a histopathologic grade was assigned to each tumor according to the Bloom and Richardson grading system (23,24). The routine baseline evaluation of the patients included laboratory analyses, chest radiography, mammography, and often a bone scan. Clinical follow-up evaluation included radiography, mammography was performed for each patient at least once a year, and bone scanning or other assays were performed only if clinically indicated.

The last update of follow-up was January 15, 1993; the update includes all details of treatment and clinical events, the site and date of any recurrence, and any date of death. Recurrent disease was con...
sidered to be the cause of death only if the disease was clinically confirmed before death. The presence of other neoplastic disease was recorded from the cancer registry, and the date of death was from the population registry. The median duration of follow-up was 95 months (24–208 months).

**Treatment**

Local treatment was given in accordance with the guidelines drawn up by the South Swedish Breast Cancer Group and consisted either of modified radical mastectomy with axillary dissection (n = 332) or, in some patients with tumors that were less than 20 mm in diameter (n = 37), of conservative breast surgery with axillary dissection and postoperative irradiation of the breast (54 Gy, usually applied by a tangential technique over a 7-week period). Some patients (n = 72), most of whom had tumors that were greater than 20 mm in diameter, underwent irradiation with the four-field technique (45–48 Gy to the axillary, supraclavicular, infraclavicular, and parasternal lymph nodes and the thoracic wall, given as a split-course treatment over a 7-week period) after a modified radical mastectomy. Eighty-six patients received adjuvant endocrine therapy (usually 30 mg of tamoxifen per day) for 1 year; 44 of these patients received this treatment in combination with the extended-field radiotherapy and the remaining 42 received only single adjuvant treatment. Written informed consent concerning the treatment was obtained from all patients.

**Antibodies**

The tumor cell growth fraction as defined by Ki-67 expression was assessed by means of a monoclonal antibody (Ki-S5) that was produced in our laboratory (Fig. 1B); Ki-S5 binds to a formalin-resistant epitope of the proliferation-associated Ki-67 protein (21,22,25). This antibody yields reproducible results on archival material (21,26), and in our experience, its reactivity spectrum does not differ substantially from the reactivity spectrum of the widely used antibody MIB-1 (Dianova, Hamburg, Germany).

Monoclonal antibody Ki-S2 (19) detects p100, a nuclear protein of unknown function that is initially expressed in proliferating cells at the transition between G1 and S phases, persists through G2 and M phases, is rapidly degraded after mitosis (18), and thus becomes undetectable in G1 cells (Fig. 1, A and C–F). In mitosis, Ki-S2 binds to sites associated with the poles and fibers of the mitotic spindle. In S and G2 phases, the antigen is diffusely distributed throughout the cell nucleus (18). Specificity for S, G2, and M phases was verified by flow cytometry analysis of growth-stimulated normal lymphocytes (19) and by dual-parameter flow cytometry with a human cancer cell line (19). After adequate antigen retrieval (19), antibody Ki-S2 produces identical results on fresh and formalin-fixed tissues. The cells that react with Ki-S2 represent variable subsets of the population of cells that react with Ki-S5 (Ki-67-positive population) (19), which suggests that the fraction of cells in G1 phase may differ widely between individual tumors.

**Laboratory Assays**

**Determination of hormone receptor content.**

We determined the concentrations of estrogen receptors (ERs) and progesterone receptors (PgRs) in fresh or fresh-frozen specimens by use of isoelectric focusing, the dextran-coated charcoal assay, and Scatchard analysis (27). The values obtained were accepted only if the tumor specimen had been kept at the proper temperature before processing and correctly diluted during processing.

**DNA analysis.**

Fresh or fresh-frozen tumor tissue was disrupted mechanically in phosphate-buffered saline (PBS), and chicken red blood cells were added as an internal standard. After staining with propidium iodide, the DNA content of individual cell nuclei was quantitated with the use of an Ortho 50H flow cytometer (Ortho, Neckargmuend, Germany), and the percentage of nuclei in S phase was calculated by planimetry from the cytometry data in

![Fig. 1. Ki-S2 immunohistochemical staining of breast tissue. A) The infiltrating margin of a ductal carcinoma of the breast is shown; a substantial portion of tumor cells (well above the cutoff level of 10%) exhibits nuclear staining with Ki-S2 (right). The normal ductal epithelium (left) shows no reactivity, whereas the surrounding lymphoid infiltrate contains single positive cells. B) Ki-S5 staining of the same area is shown. Many more tumor cells (>25%) are stained, more lymphocytes are stained, and a few ductal epithelia show nuclear staining. C) In this example of a well-differentiated carcinoma, Ki-S2 staining is confined to a few scattered cells (<10%). D) A much higher percentage of cells—yet still less than 25%—react with Ki-S5 in the same area of the well-differentiated carcinoma. E) Moderately well differentiated carcinoma with remarkably low Ki-S2 reactivity (<10%) is shown. F) High-grade ductal carcinoma with a high Ki-S2 index (far greater than 10%) is shown; a cluster of mitotic figures is apparent in the center. All sections were stained with the peroxidase technique and counterstained with hematoxylin. Original magnifications were ×140 for A and B and ×350 for C–F.](https://example.com/fig1.jpg)
the region between peaks for G1-phase and G2/M-phase nuclei, as described by Baish et al. (28). Tumors were defined as DNA diploid (one cell population) or aneuploid (two or more cell populations) according to the procedure of Hiddemann et al. (29). In nondiploid tumors, the S-phase fraction was determined for the aneuploid cell populations exclusively. The S-phase fraction was not calculated when cell debris was higher than 15% or when the coefficient of variation exceeded 5%.

Immunostaining procedures. Sections (4 μm thick) were cut from paraffin-embedded tumor specimens, mounted on silane-coated slides, routinely deparaffinized in xylene, and rehydrated in graded ethanol. Endogenous peroxidase activity was blocked by treating the sections with 3% hydrogen peroxide in a solution of methanol and PBS for 10 minutes. Antigen retrieval was achieved by boiling the slides in 0.01 M citric acid (pH 6.0) for 2 minutes in a pressure cooker (30). Slides were incubated with the primary antibodies (Ki-S5 and Ki-S2, both as lyophilized hybridoma cell culture supernatants reconstituted with 5 mL of H2O) for 60 minutes at room temperature, and the immunoreaction was enhanced by means of the peroxidase technique (31) with an avidin–biotin complex and rabbit antikeratin antiserum. Tissue structures were visualized by counterstaining with Mayer’s hematoxylin.

Evaluation of immunolabeling indices. Only unequivocal nuclear staining was considered a positive reaction. The slides were scanned at low magnification for the most evenly and intensely labeled areas. A minimum of 1000 cells in these fields was then counted at high power (×350) by use of a blood cell counter (Statistest L10; Ferrari, Berlin, Germany), and the labeling index was calculated as the percentage of labeled nuclei. Counting was done by two independent pathologists (P. Rudolph and R. Parwaresch) who did not have knowledge of the patient data; the overall interobserver agreement was 98%.

Statistical Analysis

The SPSS software package was used for statistical analysis. Correlations between continuous variables were analyzed by means of the Spearman rank-correlation coefficient and the Kendall τ coefficient of concordance. The Kruskal–Wallis nonparametric analysis of variance was used to characterize relationships between categorical and continuous variables. Considered as covariates were patient’s age and menstrual status, tumor size and histopathologic grade, Ki-S2 and Ki-S5 immunolabeling scores, ER content (status), PgR content (status), the S-phase fraction, and a combined score based on tumor size, S-phase fraction, and PgR status (20). Complete sets of data could be obtained in most cases. However, for technical reasons (20), the S-phase fraction could be assessed in only 279 cases.

Survival was expressed as the number of months from the date of primary surgery to the occurrence of an event and was analyzed in the following three ways: 1) as overall survival based on all deaths regardless of their cause, 2) as disease-specific survival based on deaths due to or with advanced breast cancer, and 3) as disease-free survival based on clinically confirmed local, regional, or distant recurrence.

Covariates were examined as discrete and as continuous variables whenever possible, and their potential prognostic significance was estimated by means of a univariate Cox regression analysis. Subsequently, cutoff points were used to define different risk groups. Thus, S-phase fraction values were divided into three categories corresponding to 1) less than 7%, 2) 7%–11.9%, and 3) 12% or more, respectively, according to previous observations (20), and three age groups corresponding to 1) 49 years or younger, 2) 50–74 years, and 3) 75 years or older were formed on the basis of an evaluation of breast cancer mortality in Scandinavia (32). Cutoff points for Ki-S2 and Ki-S5 immunoreactive scores were set at 10% and 25%, respectively, according to their respective median values in this study, which were rounded off to the nearest 5%. In addition, a three-score model (combined prognostic index) (20) was included in the analyses; it was based on the S-phase fraction values (<7% = 0, 7%–11.9% = 0.5, and >12% = 1), PgR content (>10 fmol/mg = 0 and <10 fmol/mg = 1), and tumor size (<20 mm = 0 and ≥20 mm = 1) designed to distinguish low (0 risk factors), intermediate (0.5–1.5 risk factors), and high (2–3 risk factors) risk groups.

The prognostic value of the different covariates was evaluated by means of the product-limit estimate of the survival function (Kaplan–Meier method), and differences between the survival functions were assessed with the Mantel–Haenszel log-rank test and confirmed by the generalized Wilcoxon test. Multivariate analysis was performed by use of the Cox proportional hazards model. The proportionality of baseline hazard functions was assessed for each covariate by means of the log-minus-log survival plot, and all variables were considered time independent. Covariates were selected in a stepwise (backward conditional) fashion, with the use of the maximum likelihood ratio. Only variables achieving statistical significance (P < 0.05) in the univariate analysis were allowed to enter the model. Cases with missing values (except for S-phase fraction) were excluded from the multivariate analysis. All P values were two-sided.

The independent prognostic significance of the covariates was assessed in two ways: First, the established prognostic factors (tumor size, tumor grade, and hormone receptor content) were used to build a model in which the proliferation characteristics (S-phase fraction and Ki-S5 and Ki-S2 indices) were integrated alternatively. Second, all covariates were analyzed in one model containing either the combined prognostic index or its individual constituents tumor size, S-phase fraction, and PgR status. Finally, the assumptions of the Cox were validated with a jackknife procedure.

Relative survival was computed with the SURV2 Relative Survival Analysis Program Version 2.0β (Finnish Cancer Registry, Institute for Statistical and Epidemiological Research, Helsinki) and reference data from the South Swedish Population Registry according to the providers’ guidelines (33–35).

RESULTS

By the last update, 68 patients with advanced breast cancer had died of the clinically verified advanced breast cancer, 44 patients (of whom two had recurrent but not generalized breast cancer) had died of other causes, 17 patients were alive with evidence of disease, and 242 patients were alive and free of disease. The median survival of the patients who died of disease-unrelated causes was 72.5 months.

Tumor Characteristics

The majority of the tumors were typical invasive ductal carcinomas without special differentiation characteristics. Four carcinomas were purely tubular, and 19 carcinomas were of the mucinous type. Pure papillary or medullary carcinomas were not present. Thirty-two tumors (8.6%) were grade 1, 251 tumors (67.7%) were grade 2, and 88 tumors (23.7%) were grade 3. The median Ki-S2 labeling index was 9.0% (range, 0.5%–44%), and the median Ki-S5 score was 23.5% (range, 3%–93%). The median S-phase fraction was 5% (range, 0%–29%). The pure tubular and mucinous carcinomas, none of which became fatal, consistently displayed a low proliferative activity, with Ki-S5 and Ki-S2 labeling indices of less than 12% and less than 5%, respectively. Fig. 1 shows typical examples of Ki-S2 and Ki-S5 immunostaining.

Correlation of Covariates

Ki-S2 and Ki-S5 labeling indices were closely covariant (τ = 0.617; P < 0.0001), and both correlated with the S-phase fraction (τ = 0.254 and 0.295, respectively; P < 0.0001). An inverse correlation was found with the ER status (for Ki-S2, τ = −0.209 and P < 0.0001; for Ki-S5, τ = −0.248 and P < 0.0001) and PgR status (for Ki-S2, τ = −0.136 and P = 0.0015; for Ki-S5, τ = −0.148 and P < 0.0005). ER status and PgR status were also closely interrelated (τ = 0.439; P < 0.0001).

Age correlated positively with ER status (τ = 0.215; P < 0.0001) and negatively with Ki-S5 (τ = −0.247; P < 0.0001) and Ki-S2 (τ = −0.265; P < 0.0001) labeling indices but not with any of the other variables.

Tumor size showed an inverse correlation with ER status (τ = −0.103; P = 0.005) and PgR status (τ = −0.101; P = 0.006) and correlated positively with the S-phase fraction (τ = 0.114; P = 0.002) and with the labeling indices for Ki-S5 (τ = −0.103; P = 0.04) and Ki-S2 (τ = 0.175; P < 0.0001). The Bloom and Richardson grade correlated strongly with the S-phase fraction and the Ki-S2 and Ki-S5 labeling indices (P < 0.0001), correlated weakly with tumor size (P = 0.017), and correlated inversely with ER status (P < 0.0001), PgR status (P = 0.002), and patient’s age (P = 0.0009).
Univariate and Multivariate Analyses of Survival

In the univariate analysis, all parameters except patient’s age significantly predicted survival in the three models. The highest statistical relevance was achieved by Ki-S2 and Ki-S5 immunolabeling scores (Fig. 2, A–C), a high score being associated with an approximately 20-fold and fivefold increased risk for cancer mortality, respectively. Patient’s age was strongly predictive with respect to overall survival but lacked statistical significance in the other models. Men-
strual status was not statistically significant. The results are detailed in Table 1.

Three hundred thirty-two cases were available for multivariate analysis without the S-phase fraction and combined prognostic index. If the S-phase fraction and combined prognostic index were included, the 263 cases were available. In this way, two major cohorts were formed. The results are listed in Table 2; the statistical significance and the hierarchy of the covariates remained virtually unchanged when these were examined as either categorical or continuous variables.

When compared individually with the standard prognostic criteria, all proliferation parameters were selected as independent prognostic indicators (Table 2, A); the highest relative risk was associated with a Ki-S2 score of greater than 10%. Accordingly, in the model including patient’s age, tumor size, Bloom and Richardson grade, PgR content, and Ki-S5 and Ki-S2 scores (332 observations), the Ki-S2 labeling index emerged as the most statistically significant prognostic indicator in all three survival models (Table 2, B). When Ki-S2 was removed from the model, the Ki-S5 index and tumor size were selected as independent prognostic factors in all three survival models, patient’s age remained statistically significant for overall survival, and a PgR-negative status additionally predicted reduced disease-specific survival. Inclusion of the ER status had no influence.

In the second series, in which a combined prognostic index and its constituent elements were alternatively considered as covariates (263 observations), the results remained largely similar when Ki-S2 was included in the analysis, except that the combined prognostic index and the S-phase fraction were added in turn as independent predictors of overall survival and disease-specific survival but not of disease-free survival (Table 2, B). After exclusion of the Ki-S2 index, the Ki-S5 index, the S-phase fraction, and the combined prognostic index remained statistically significant in all three survival models, and again old age was associated with a shortened overall survival.

Because the inhomogeneity of treatment protocols could influence the impact of the prognostic factors, we sought to exclude treatment bias in two ways. First, treatment characteristics were included as covariates in the multivariate models. They, however, were not selected as an independent prognostic factor and their inclusion in no way modified the results of the multivariate analysis.

Second, we excluded all patients who had received any kind of adjuvant therapy. This reduced the cohort to 218 patients who had been treated with radical mastectomy alone and for whom complete datasets were available. The median follow-up time was 94.5 months (range, 24–133 months). In this group, the hormonal receptor status lost statistical significance in the univariate analysis of all three survival models (all $P > .1$), which resulted in a concomitant decline in the prognostic strength of combined prognostic index (for overall survival, $P = .012$; for disease-specific survival, $P = .001$; for disease-free survival, $P = .011$). The S-phase fraction and the Ki-S5 index remained statistically significant prognostic indicators of overall survival, disease-specific survival, and disease-free survival (all $P < .01$; Fig. 1, D and E). The highest prognostic impact, however, was achieved by the Ki-S2 score in all three models ($P < .0001$; Fig. 1, F) with an approximately 20-fold increased risk for breast cancer-related mortality. Conversely, the PgR status was highly signifi-

### Table 1. Univariate analysis of prognostic covariates of survival in 371 patients with lymph node-negative breast cancer

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Group</th>
<th>No. of patients*</th>
<th>Overall survival</th>
<th>Disease-specific survival</th>
<th>Disease-free survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>OR†</td>
<td>$P$‡</td>
<td>OR†</td>
</tr>
<tr>
<td>Patient’s age</td>
<td>≤49 y</td>
<td>86</td>
<td>1.0§</td>
<td>.001</td>
<td>1.0§</td>
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<tr>
<td></td>
<td>50–74 y</td>
<td>192</td>
<td>0.91</td>
<td>1.39</td>
<td>0.94</td>
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<tr>
<td></td>
<td>≥75 y</td>
<td>93</td>
<td>1.62</td>
<td>0.01</td>
<td>1.0§</td>
</tr>
<tr>
<td>Tumor size</td>
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<td>1.0§</td>
<td>.01</td>
<td>1.0§</td>
</tr>
<tr>
<td></td>
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<td>179</td>
<td>1.89</td>
<td>1.95</td>
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<td>Estrogen receptor content</td>
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<td>.034</td>
<td>1.0§</td>
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<td></td>
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<td>1.46</td>
<td>2.26</td>
<td>1.30</td>
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<td>Progesterone receptor content</td>
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<td>1.0§</td>
<td>.017</td>
<td>1.0§</td>
</tr>
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<td>1.60</td>
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<td>3</td>
<td>88</td>
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<td>S-phase fraction</td>
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<td>90</td>
<td>1.0§</td>
<td>.0004</td>
<td>1.0§</td>
</tr>
<tr>
<td></td>
<td>7.0%–11.9%</td>
<td>116</td>
<td>1.27</td>
<td>1.05</td>
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<tr>
<td></td>
<td>≥12%</td>
<td>73</td>
<td>1.90</td>
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<tr>
<td>Combined prognostic index</td>
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<tr>
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<td>1.12</td>
<td>1.0</td>
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<td></td>
<td>2.0–3.0</td>
<td>93</td>
<td>2.66</td>
<td>5.08</td>
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<td>Ki-S5 index</td>
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<td>.0001</td>
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<td></td>
<td>&gt;25%</td>
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<td>2.26</td>
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<td>Ki-S2 index</td>
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<td>261</td>
<td>1.0§</td>
<td>.0001</td>
<td>1.0§</td>
</tr>
<tr>
<td></td>
<td>&gt;10%</td>
<td>110</td>
<td>4.85</td>
<td>20.2</td>
<td>6.66</td>
</tr>
</tbody>
</table>

*Values for each covariate or category were not available for all patients.
†OR = odds ratio.
‡Comparison of defined risk groups with patient survival characteristics shown (logrank test; all $P$ values are two-sided).
§Reference category.
\[See Fig. 1.\]
cant in the group of patients who received adjuvant therapy (for overall survival, \( P = .0047 \); for disease-specific survival, \( P = .0001 \); for disease-free survival, \( P = .00125 \)), whereas the significance levels of the other parameters were virtually unchanged.

The results of the multivariate analysis were comparable to those obtained for the entire cohort. In the subset of patients treated with radical mastectomy alone, the Ki-S2 index was selected as the single most relevant independent predictor of disease-specific survival and disease-free survival, with relative risks of 12.6 and 4.34, respectively (\( P = .0001 \)). For overall survival, both the Ki-S2 index and patient’s age were independent prognostic factors, with respective odds ratios of 5.6 (\( P < .0001 \)) and 2.9 (\( P = .0002 \)). Similar results were found in the group of patients who received adjuvant treatment, except that PgR status was included as an independent predictor of disease-specific survival (relative risk = 2.98; \( P = .027 \)). The odds ratios of a Ki-S2 index greater than 10% for overall survival, disease-specific survival, and disease-free survival were 8.15, 9.75, and 9.29, respectively (\( P < .0001 \)). The S-phase fraction or combined prognostic index did not enter the final selection in either group.

We further performed an internal validation with a jackknife technique for model building. Cox regression analysis selected identical independent factors (Ki-S2 index and patient’s age for overall survival; Ki-S2 index and tumor size for disease-specific survival and disease-free survival) in the subsets as formed, the maximum divergence of their respective odds ratios in corresponding survival models (overall survival, disease-specific survival, and disease-free survival) being 15%. The relative risk associated with a Ki-S2 index of greater than 10% was entirely comparable to that determined in the overall analysis.

Finally, we compared the cumulative survival of the patients with a low Ki-S2 index (\( \leq 10% \)) with the life expectancy of an age-matched population of Swedish women without breast cancer. The two survival curves were almost superimposable, and the difference was statistically insignificant (\( P = .67 \)).

**DISCUSSION**

Our results show that cellular proliferation is important for the progression of breast cancer. The S-phase fraction is widely accepted as a prognostic indicator (1). However, this method is not universally applicable, which inevitably results in the exclusion of a subset of cases (15,36,37).

The use of immunohistochemical proliferation markers is rarely handicapped by technical or tumor-inherent factors. It is therefore surprising that breast cancer studies that use this approach reflect little unanimity (7,8,10–13,15,17,38–45). Small case series, insufficient follow-up times, inhomogeneous study designs, and the use of different antibodies might account for these discrepancies. On the other hand, standardization of antigen retrieval and immunostaining techniques is an absolute prerequisite for the reproducibility of results.
Considering our previous experience with proliferation markers (7,8,21,25,26, 37,46–50), the high prognostic relevance of the Ki-S5 (Ki-67) immunolabeling index in this study was not unexpected. Remarkably, it even superseded the prognostic impact of the S-phase fraction. Although difficult to interpret, this result is in line with an earlier investigation (37) and another report showing that the fraction of cells in S and G2 phases is more closely associated with the clinical outcome than the S-phase fraction alone (40). However, 16% of the patients with a low-risk Ki-S5 index (≤25%) suffered a relapse, with fatal consequences in 9% of all patients (which is 56.3% of the patients who had a relapse). An inadequate estimation of the fraction of proliferating cells in a tumor, due to uncertainties in the destiny of G1-phase cells (16), may account for such a failure.

This drawback can be avoided by use of monoclonal antibody Ki-S2, which is directed against a 100-kd protein expressed exclusively in S, G2, and M phases (18,19). In all models tested, the Ki-S2 index emerged as the most statistically significant independent indicator of prognosis. In the low-risk group (Ki-S2≤10%), we found no statistically significant reduction in the survival probability (P = .98 after 5 years and P = .95 after 10 years), whereas a high Ki-S2 index was associated with an increase of up to 20-fold in the mortality risk. The prognostic impact of Ki-S2 remained virtually unchanged when patients who had received any kind of adjuvant treatment (irradiation or endocrine therapy) and patients treated solely by mastectomy were examined as separate groups.

This implies that the Ki-S2 index, possibly in combination with the hormone receptor status, may be used to select treatment protocols for patients with lymph node-negative breast cancer. All other parameters failed to add essential information, except that patient’s age played a role in overall survival, with a substantial proportion of disease-unrelated deaths occurring in elderly patients.

Although the impact of the Ki-S2 index was slightly weakened as a result of the close association with the S-phase fraction and the Ki-S5 index, the Ki-S2 index remained the most statistically significant independent prognostic indicator when these factors were included in the analysis. This suggests that the proliferation characteristics measured by Ki-S2 are fundamentally different from those assessed by other techniques. It also appears that the fraction of cells in S through M phases carries the strongest prognostic information, compared with the fraction of cells in S and M phases or the fraction of proliferating cells in the tumor. This may be explained by the exclusion of cells programmed for quiescence or apoptosis and by a more reliable appraisal of the distribution of cells in the phases of the cell cycle. Furthermore, an increased expression of the Ki-S2 antigen p100 might reflect an advanced loss of cell cycle-inhibitory mechanisms resulting in a more aggressive cancer (19).

When compared with the combined prognostic index, which was reported to identify a group of patients with a mortality ratio comparable to that of the age-matched control population in Sweden (33), the Ki-S2 index proved to be a better indicator of prognosis. Although a cure is difficult to ascertain, 5- and 10-year survival rates in the group with a low Ki-S2 index did not differ essentially from the life expectancy reported for the general Swedish population of comparable age. This may be interpreted as showing that breast cancer evolves in two distinct ways—one way that is fatal and another way in which patients have a longevity similar to that of women without breast cancer (51,52). Among all covariates, the Ki-S2 index was the unique parameter that allowed this degree of discrimination. If our findings are corroborated by further studies, then the use of the immunologic proliferation marker Ki-S2 might be able to considerably improve the accuracy of breast cancer prognosis. Because of the technical simplicity of the detection method, Ki-S2 immunohistochemistry appears to be a particularly useful prognostic indicator, which might have similar relevance in other types of neoplastic diseases.

**References**


(17) Dettmar P, Harbeck N, Thomsen C, Pache L,


NOTE

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