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Report

Thymidine labeling index and Ki-67 growth fraction in breast cancer: Comparison and correlation with prognosis

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Summary

In situ determination of proliferative activity was performed on 184 consecutive primary invasive breast cancers. Methods used were monoclonal antibody Ki-67 in immunohistochemistry and thymidine labeling index. Tumor proliferation correlated between both methods ($p = 0.0001$). For thymidine labeling index and Ki-67, respectively, significant correlations existed with histologic tumour grade and steroid hormone receptors (Tumor grade: TLI $p = 0.0001$; Ki-67 $p = 0.0001$. ER-ICA: TLI = 0.0001; Ki-67 $p = 0.014$. PgR-ICA: TLI $p = 0.0001$; Ki-67 $p = 0.0008$).

For thymidine labeling index a significant correlation was demonstrated for overall survival ($p = 0.001$) and recurrence free survival ($p = 0.01$). No statistical significance was observed for clinical outcome and Ki-67 (overall survival $p = 0.18$; recurrence free survival $p = 0.1$). None of the factors, TLI or Ki-67, was an independent prognostic factor as demonstrated by multivariate analysis.

Introduction

One of the unsolved and poorly understood problems of breast cancer is the variability of its behavior. Even within similar stages of the disease there exist remarkable differences of clinical outcome. In the literature there exist reports on different prognostic factors which are useful for subclassification. The most accepted are axillary lymph node status, tumor size [1], histologic tumor grade according to Bloom and Richardson [2, 3], and estrogen receptor status [4–6]. But none of these factors is able to provide sufficient prognostic information for a single patient.

Over the past years there have been many studies providing evidence that also proliferative activity of breast cancer is of importance. For determination of proliferative activity there are different methods available. A simple method is the light microscopic mitotic index [7, 3]. Problems with this method are appropriate tissue fixation for preservation of mitotic figures and histologic interpretation, because the mitotic phase during the cell cycle is very short compared to other phases of the cell cycle. Therefore large tumor areas need to be examined to get reliable results. A more modern method is the DNA flow cytometry [8, 9]. It is an elegant method where in a short time large numbers of cells can be analyzed. But it is limited with respect to widespread use because sophisticated and expensive technical equipment is used. Another limitation is that the tissue needs to be homogenized and it is not known exactly which tissue components are analyzed. A method where the growth fraction can be

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demonstrated on the basis of histologic sections is thymidine labeling index (TLI). It has been used a long time and is quite well documented. Moreover it has been shown that TLI is a reliable and reproducible method [10]. With the TLI, cells in S-phase of the cell cycle are determined [11]. Problems with this method arise because radioactivity is needed and viable tumor tissue is necessary. Another histologic method more recently developed is immunohistochemistry using the monoclonal antibody Ki-67 [12]. Ki-67 detects proliferating cells in the S, G_2, M, and G_1 phases of the cell cycle. It does not detect resting cells in the G_0 phase. For both histologic methods there exist reports on significant correlations with other prognostic factors of breast cancer [13–17]. For TLI significant correlations are reported for survival [18–22]. But unfortunately there is very little information known about Ki-67 and survival data [23, 24].

Therefore we wanted to examine how both methods work in our hands, to compare both methods with each other, and most importantly to determine their prognostic significance.

Material and methods

Tumor specimens derived from 184 consecutive primary invasive breast cancers. All tumor samples were obtained by quadrantectomy or mastectomy. One slice each was used for frozen section, paraffin histology, and thymidine labeling.

Histologic sections of tumor tissue were prepared according to routine histologic techniques and stained with hematoxylin-eosin. Tumor classification was performed according to Azzopardi [25], tumor grading according to Bloom and Richardson [2]. Steroid hormone receptors were determined by immunohistochemical method using ER-ICA and PgR-ICA-Kit from Abbott Laboratories (Chicago, Ill., USA).

The TLI was measured according to the method of Meyer [11]. Slices less than 1 mm thick were cut free-hand with a razor blade from the periphery of the carcinoma immediately following surgical incision. Slices were stored less than 3 hours at 4° C in Hank's balanced salt solution (HBSS) prior to incubation. For incubation hyperbaric oxygen (3 to 4 atmospheres) and the thymidylate synthetase inhibitor 5-fluoro-2′-deoxyuridine (10^{-6} mole per liter) was used. 5-fluoro-2′-deoxyuridine was used to facilitate uptake of ^{3}H-TdR by the S-phase cells. The concentration of tritiated thymidine, specific activity 6 to 60 Ci/mmole, was 5 µCi per ml of HBSS. The incubation was carried out for two hours at 37° C with shaking. After the incubation period the slices were washed with HBSS free of excess ^{3}H-TdR and fixed in phosphate-buffered 8% formaldehyde pH 7.0. Tissue was processed according to routine paraffin embedding method, and slices were cut at 5 µm and mounted on glass slides. Following removal of paraffin the slides were dipped in liquid photographic emulsion (NTB-2, Eastman Kodak Co., Rochester, New York) and incubated in the dark for 7 days. Then they were developed and stained with hematoxylin-eosin. The TLI was determined by counting at least 1000 carcinoma cell nuclei over a 1 cm^2 ocular grid under 400 × magnification. Areas highly and evenly labeled were assumed to be representative. Background counts were fewer than 1 grain per nucleus and cells with 5 or more grains per nucleus were designated as S-phase cells. In all cases the mean grain count of nuclei designated as labeled exceeded 20. TLI was calculated as percentage of labeled tumor cells.

For measurement of Ki-67, frozen sections adjacent to the sections used for TLI were cut at 4 µm. Slices were mounted on glass slides pretreated with poly-L-lysine, air-dried, and fixed in acetone for 10 minutes at room temperature. Immunohistochemistry was performed using the ABC-method. Briefly slides were incubated with the primary antibody Ki-67 diluted 1 : 20 (Dakopatts, Glostrup, Denmark) for 1 hour. After incubation for 30 minutes with a biotinylated anti-mouse antibody, the slides were incubated with the ABC-complex for one hour. Between the incubations slides were washed 3 times in phosphate–buffered saline. The reaction product was developed with diaminobenzidine–tetra-hydrochloride. Counterstaining was performed using Harris' hematoxylin. All steps of incubation were performed at room temperature. The Ki-67 growth fraction (KGF) was determined by counting at least 1000 tumor cells over a 1 cm^2

ocular grid under 400 × magnification. Nuclei in which brown color could be detected were scored as positive. The selection of areas for examination was performed in the same way as for TLI. KGF was calculated as percentage of labeled tumor cells. Slides for determination of TLI and KGF, respectively, were scored blindly to the results of the other.

Statistical analysis

Survival was expressed as time from the date of primary treatment of breast cancer to the occurrence of an event and was analyzed in two ways: as overall survival and as recurrence-free survival. Recurrence-free survival was defined as the interval between the date of operation and the first recurrence of breast cancer. Patients who died due to other reasons than breast cancer, without any signs of breast cancer recurrence, were considered censored for all analyses. Overall survival and disease-free survival were estimated by the Kaplan Meier method [26] and possible prognostic differences between groups were analyzed by the generalized Wilcoxon test [27] for censored data, using the programs of the BMDP statistical software package (Biomedical Computer Programs, BMDP Statistical Software, University of California, Berkeley, Calif.). The cutpoint values for grouping for TLI and KGF used in recurrence and survival analyses were based on formal statistical cutpoint analyses. All other statistical analyses were done using the statistical software SAS (SAS Institute Inc., Cary, NC, USA). The prognostic effects of TLI and Ki-67 on overall and on disease-free survival were also determined by the proportional hazards regression model of Cox [28], which allows the simultaneous adjustment for other factors of assumed prognostic importance. All p-values are results of two-sided tests. Continuous, monotone associations between two factors were examined by the test for Kendall's tau [29].

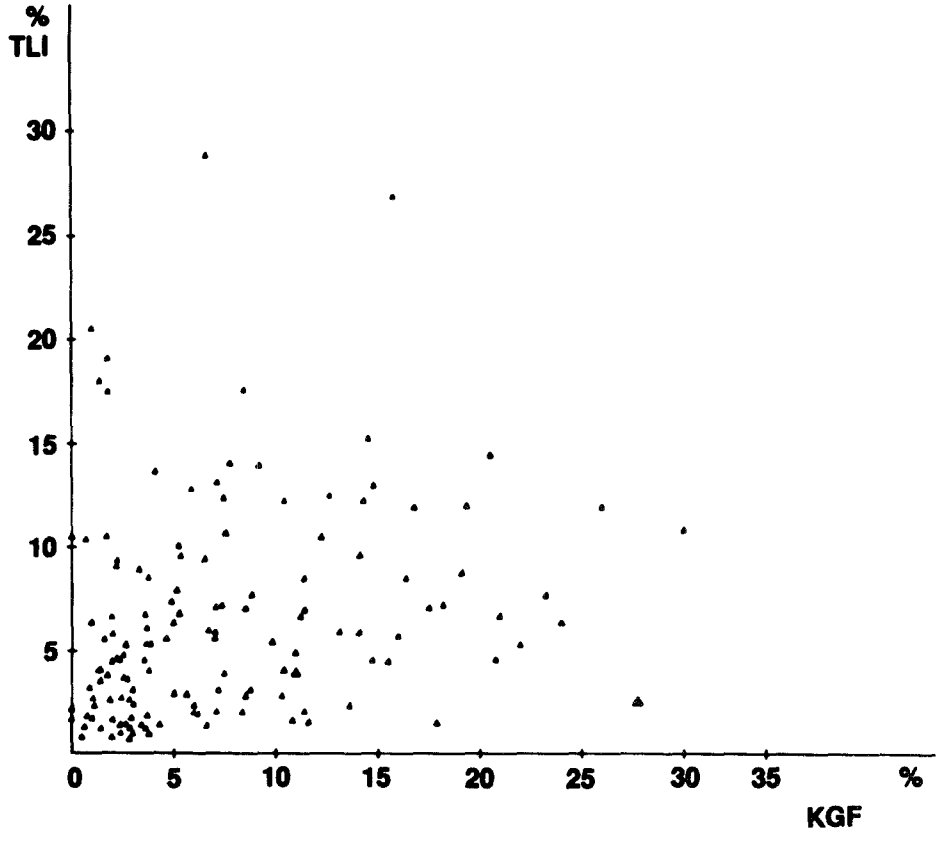


Fig. 1. Plot of KGF against TLI. n = 136, ▲ = 1 value, Δ = 2 values; p = 0.0001, r = 0.22.

Table 1. Histologic classification of carcinomas and TLI and KGF

Classification	TLI			KGF		
	n (%)	Median	Range	n (%)	Median	Range
NOS stellate	76 (51)	5.1	0.7–28.9	86 (52)	3.7	0.0–27.7
NOS circumscribed	30 (20)	6.5	0.9–13.9	33 (20)	9.4	2.0–30.0
Lobular	20 (13)	3.2	0.8–10.5	25 (15)	5.2	0.5–27.7
Mixed lobuloductal	10 (7)	3.9	1.4–10.5	6 (4)	8.7	2.6–17.5
Medullary	2	11.2	10.5–12.0	4	7.4	1.7–19.4
Mucoid	4	3.7	1.8– 6.7	3	6.0	0.8– 9.8
Tubular	3	0.9	0.6– 1.8	2	3.4	2.0– 4.8
Papillary	3	1.8	1.2– 2.1	2	7.2	2.9–11.4
Comedo	2	10.0	7.7–12.2	5	8.6	4.0–22.8

Patients and follow-up

The patient cohort consisted of one hundred eighty-four consecutive patients with primary operable breast cancer (T1–3, N0–1, M0, UICC stages I and II), who were treated by modified radical mastectomy or quadrantectomy and complete axillary dissection. Patients with breast conserving surgery routinely received adjuvant radiotherapy. Most of the patients were accrued to several clinical study protocols comparing different adjuvant therapy regimens in a prospective randomized fashion as described previously [30]. In general, premenopausal patients received cytotoxic chemotherapy with risk-adapted aggressiveness or hormonal therapy including hormonal ovarian ablation, while postmenopausal women received adjuvant hormonal treatment. Detailed descriptions of these protocols have been published extensively [31]. All patients were regularly followed at least every three months for the first three years and with six month interval thereafter. The routine evaluation of the patients included clinical examination and laboratory analyses, mammography every six months or more frequently if indicated [32], and chest x-rays, liver ultrasound, and bone scanning whenever clinically indicated. Since missing information from a few patients was retrieved through the Central Population Registry of Austria, for this analysis no patient was lost to follow-up [33].

Results

TLI was obtained in 166 breast cancers and KGF in 184 cases. The proportion of TLI positive cells varied from 0.5 to 28.9%, the proportion for KGF from 0 to 30.0%. The median for all carcinomas was 5.05% for TLI and 5.25% for KGF. In Fig. 1 TLI is plotted against KGF. There was a statistically significant correlation between TLI and KGF. However, there was also some scattering of values determined by each method.

The frequency distribution of various histologic types of breast carcinoma is given in Table 1. The range of TLIs and KGFs in each histologic tumor type was wide. In TLI low values were associated with lobular, mixed lobuloductal, tubular, and papillary carcinomas. Medullary and comedo carcinomas had distinctively higher values than NOS carcinomas. These differences were not found as clearly in KGF.

A statistically significant correlation existed for histologic tumor grading and both TLI and KGF (Fig. 2). Also each single factor of tumor grade showed a significant correlation to both growth fractions (Nuclear anaplasia: TLI $p = 0.0001$; KGF $p = 0.0005$. Tubular differentiation: TLI $p = 0.003$; KGF $p = 0.03$. Mitotic count: TLI $p = 0.0001$; KGF $p = 0.0003$).

Steroid hormone receptors showed a significant inverse correlation with both TLI and KGF (Figs 3 and 4). While the significance for progesterone receptor was comparable for TLI and KGF, for estro-

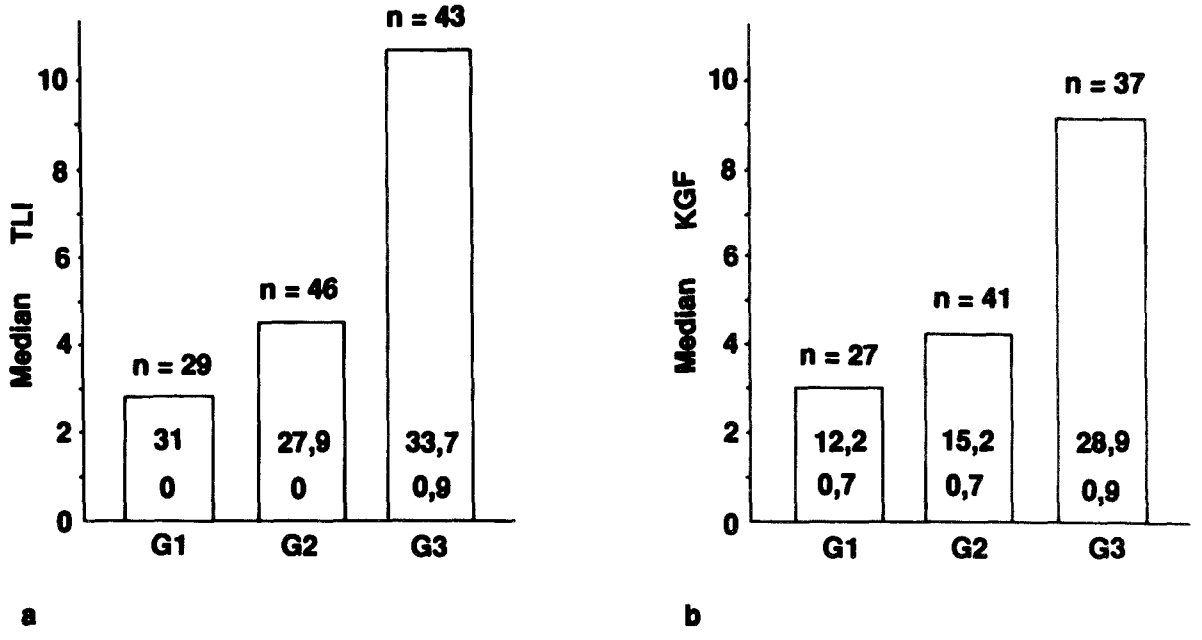


Fig. 2. Correlation between histologic tumor grade and growth fraction. Numbers in columns represent highest and lowest values in each group. a) TLI, p = 0.0001, r = 0.46; b) KGF, p = 0.0001, r = 0.26.

gen receptor it was two orders of magnitude higher in TLI than in KGF.

In Figs 5 and 6 the relationship between survival data and TLI and KGF is demonstrated. Median follow up was 73 months. For overall survival a statistically highly significant correlation was found for TLI. After 91 months 75% of patients with low TLIs were still alive, compared to 48% of patients

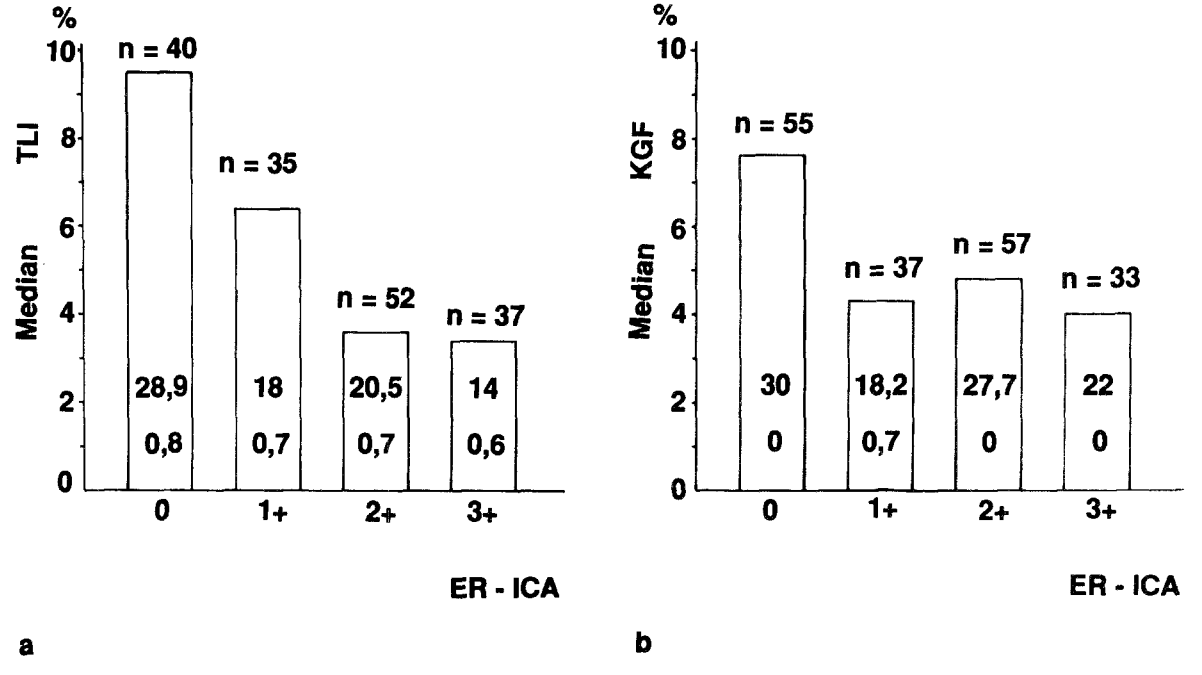


Fig. 3. Correlation between ER-ICA and growth fraction. Numbers in columns represent highest and lowest value in each group. a) TLI, p = 0.0001, r = – 0.34; b) KGF, p = 0.014, r = – 0.14.

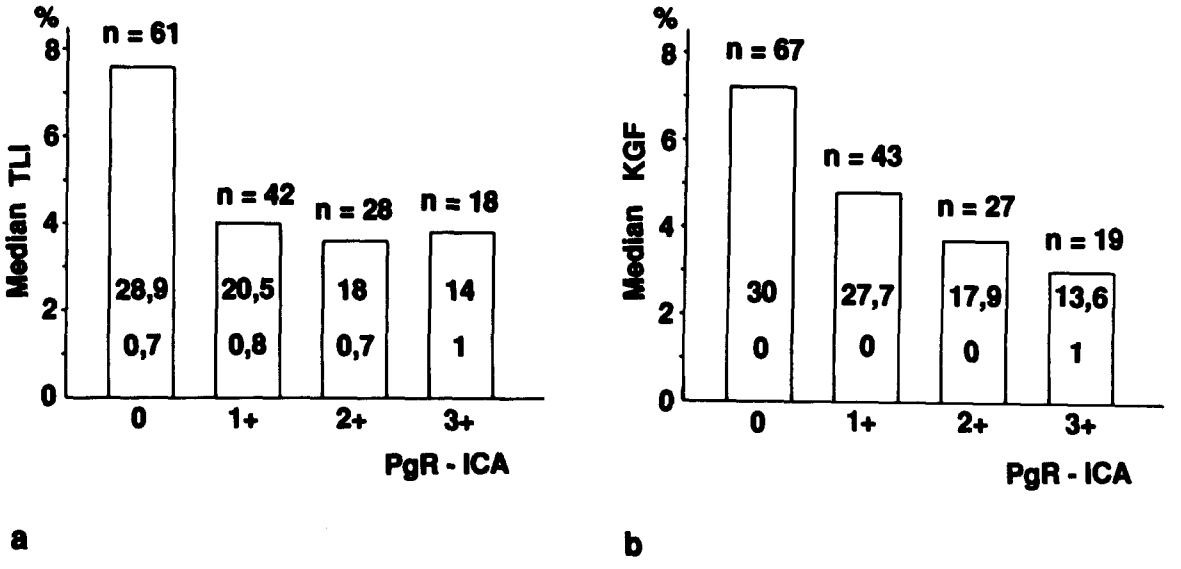


Fig. 4. Correlation between PgR-ICA and growth fraction. Numbers in columns represent highest and lowest value in each group. a) TLI, $p = 0.0001$, $r = -0.24$; b) KGF, $p = 0.003$, $r = -0.19$.

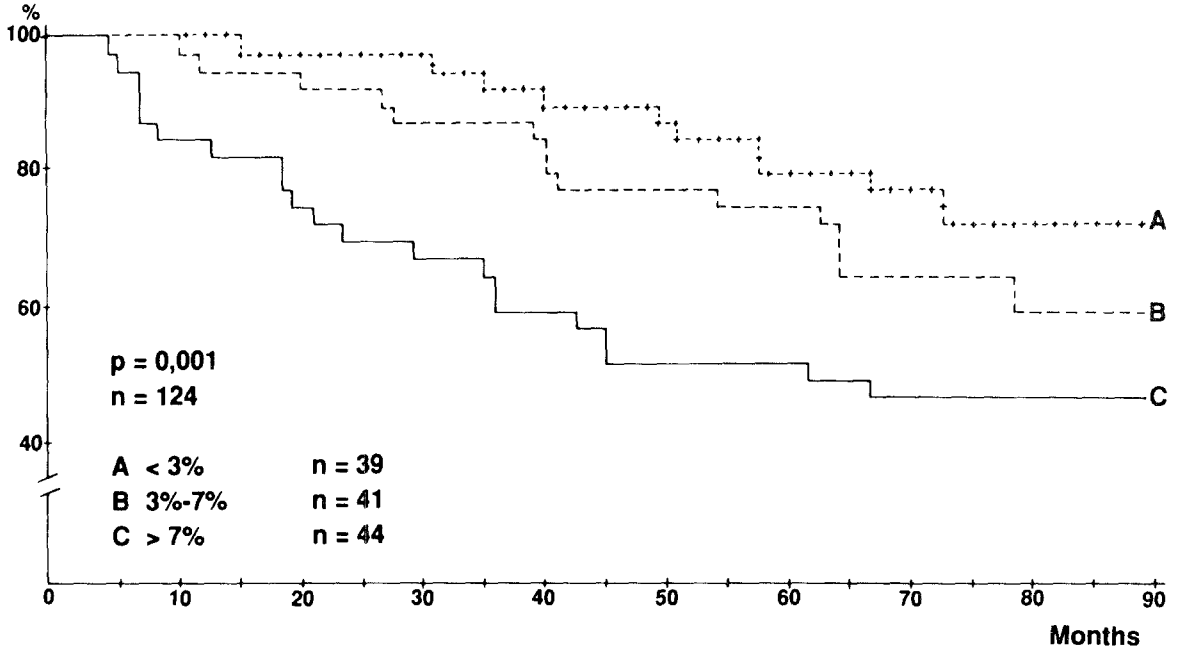
with high TLIs (Fig. 5a). No significant correlation was found for KGF and overall survival (Fig. 5b); the probability of survival at 91 months for patients with low KGFs was 65%, and for patients with high KGFs 52%.

For recurrence-free survival the probability of being free of relapse at 91 months for patients with low TLIs and KGFs was 77% and 75% and for patients with high TLIs and KGFs 55% and 59%, respectively. However, the data reached statistical significance for TLI, but not for KGF.

Results on the prognostic effect of various factors obtained from Cox analyses are summarized in Table 2. The results for TLI are based on 94 patients, and those for KGF on 114 patients. The results from the regression model are all adjusted for the following factors: axillary lymph node status, histological tumor grade, tumor size, steroid hormone receptors and adjuvant therapy being either none, chemoendocrine, or either chemo- or endocrine therapy. As demonstrated, neither TLI nor KGF were independent prognostic factors for overall survival, independent factors for overall survival were only axillary lymph node status and estrogen receptors. However, for recurrence-free survival in the regression model the single prognostic factor was TLI. Treatment was not significant in any analysis.

Discussion

Until recently the presence or absence of axillary lymph node metastases was the most important prognostic factor. Only a few other factors like estrogen receptor status were also important prognosticators. But these factors did not fully account for the variations in histologic behavior. Consequently, other factors were needed. One field which gained increasing interest was proliferative activity of tumors. Several methods either on cell suspensions or on histologic specimens are now available. We wanted to examine the clinical significance of proliferative activity which is directly localized on tumor cells in histologic specimens. This is possible using TLI, which has been used already for a long time and is well documented in the literature. However it cannot be used widely because of the need of radioactivity which results in relatively high costs and because viable tumor specimens are needed. One of the more recently developed methods for determination of growth fraction in histology is immunohistochemistry by monoclonal antibody Ki-67 [12]. The major advantage is that it is a simple immunohistochemical nonradioactive procedure, which can be performed by pathologic laboratories. But in the literature there is only little known about its prognostic significance [23, 24].



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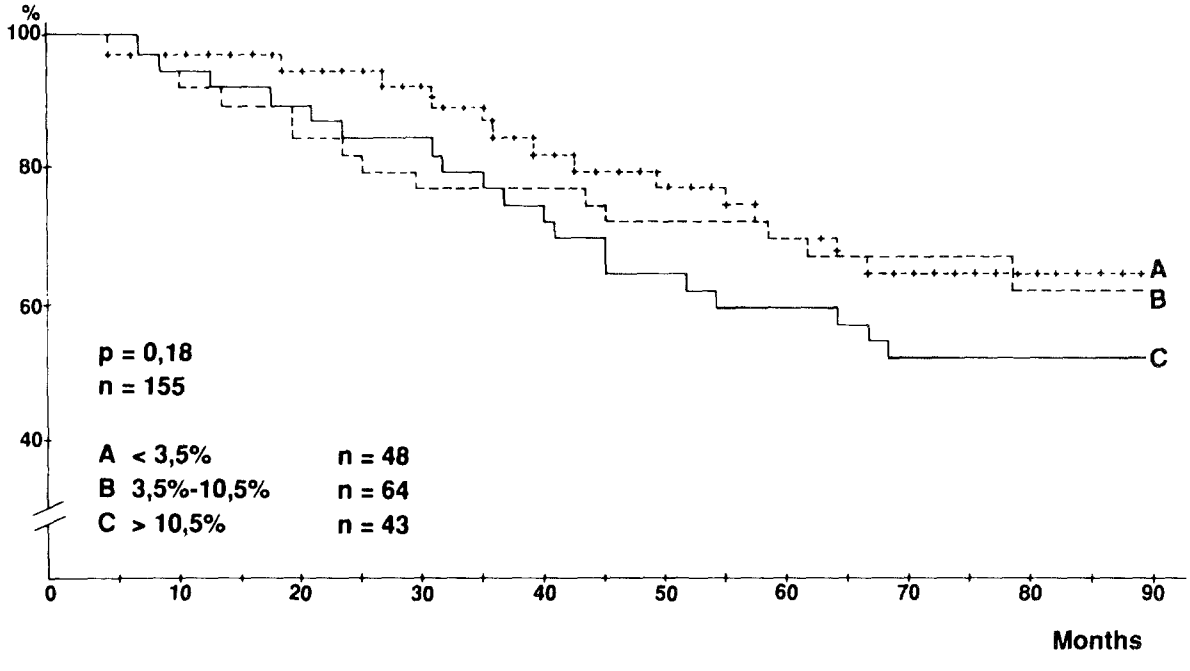

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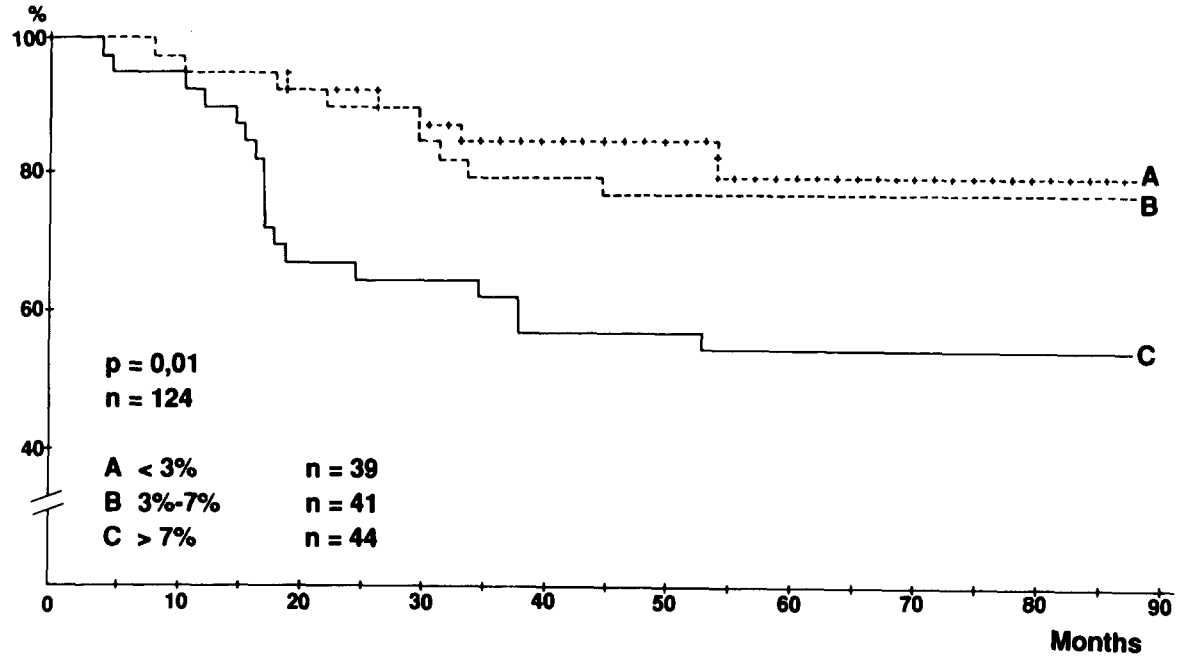
Fig. 5. Overall survival and growth fraction: a) TLI, b) KGF



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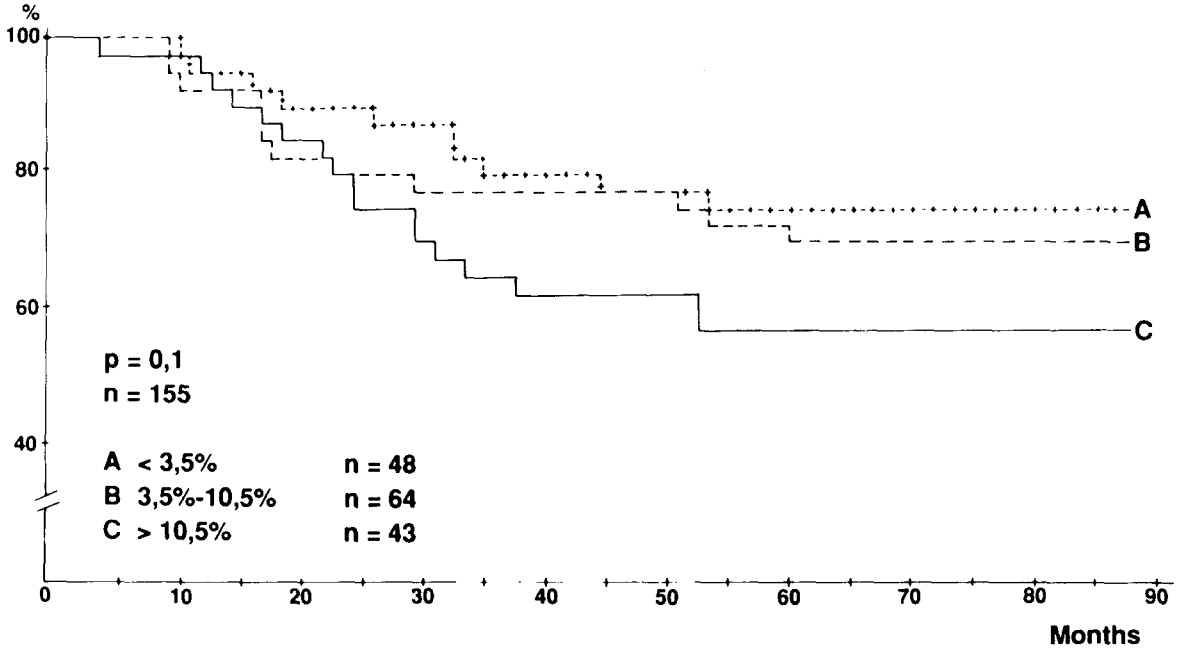

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Fig. 6. Recurrence free survival and growth fraction: a) TLI, b) KGF

Table 2. Comparison of prognostic effect of various factors obtained by Cox analysis

a) Overall survival

Prognostic factor	Univariate analysis		Regression model	
	Relative risk	P	Relative risk	P
TLI	1.09	0.02	n.s.	
KGF	1.02	0.35	n.s.	
Axillary nodes	2.03	0.001	2.19	0.0005
Tumor grading	1.84	0.01	n.s.	
ER-ICA	1.77	0.0009	1.85	0.0003
PgR-ICA	1.38	0.08	n.s.	
Tumor size	1.36	0.04	n.s.	

b) Recurrence free survival

Prognostic factor	Univariate analysis		Regression model	
	Relative risk	P	Relative risk	P
TLI	1.11	0.01	1.11	0.01
KGF	1.03	0.17	n.s.	
Axillary nodes	1.48	0.09	n.s.	
Tumor grading	1.62	0.08	n.s.	
ER-ICA	1.56	0.02	n.s.	
PgR-ICA	1.12	0.56	n.s.	
Tumor size	1.17	0.36	n.s.	

In our study a correlation could be found between TLI and KGF. This correlation is similar to the literature [34]. But in contrast to other reports where KGF is greater than TLI, in our study KGF was in the same range as TLI. This may be due to problems dealing with histologic interpretation. KGF is determined on frozen sections where the morphology for interpretation on a single cell level is not always as clear as on paraffin sections. Therefore we have interpreted cells only as Ki-67 positive in cases of distinct nuclear staining.

We found significant correlations of various histopathologic parameters with both TLI and KGF. The correlation between the nuclear tumor grade and proliferative activity is well documented for TLI [35] as well as for KGF [36, 37]. The synthesis of estrogen and progesterone receptors by breast cancer cells can be understood as a manifestation of biochemical differentiation. Thus it seems reasonable that cancer cells producing receptors are associated with low proliferative activity. This inverse relationship between steroid hormone receptors and proliferation is documented for TLI and KGF and is in accordance with reports in the literature [13, 15, 38–40].

For several types of breast cancer low values of proliferative activity are reported for TLI. This is true for tubular, lobular, and lobuloductal carcinomas. Also mucinous carcinomas show slightly lower values than ductal carcinomas. In contrast medullary and comedo carcinomas have distinctively higher labeling indices. These findings are in accordance with the literature [41, 42]. For KGF correlations with histologic tumor types could not be demonstrated as clearly (Table 1).

The clinical significance of TLI is well documented [18–22] and widely accepted. In our material TLI also could be demonstrated to correlate significantly with overall survival and at lower significance also with recurrence-free survival. This correlation was found after a relatively long observation period with a median follow-up of 73 months. The fact that TLI correlates to clinical outcome and also to various histoprognostic parameters led to speculation that KGF also may correlate to prognosis of breast cancer since it correlated to TLI. These speculations legitimately were further supported by the correlations which existed between KGF and other histoprognostic factors. But surprisingly in our study we could not demonstrate significant correlations for clinical outcome and KGF. This was true for both overall and recurrence-free survival. We found a trend that patients with low KGFs had a better clinical outcome and especially patients with high KGFs had a worse prognosis. But the differences did not reach significance. These results may partly be explained by the fact that our data are based on longer observation periods than the few reported in the literature [23, 24]. One also has to consider that by KGF cells in all phases of the cell cycle except G_0 are detected, while by TLI only cells in S-phase are labeled. Moreover Ki-67 is directed against a cell proliferation associated antigen. By immunohistochemistry only the presence of this antigen is detected; it does not give information about the cell function. In contrast TLI detects the S-phase-fraction in functioning cells by incorporation

of thymidine to the DNA. This difference in methology may also explain why KGF failed to be of prognostic significance. However, as a last point one has to mention that neither TLI nor KGF had independent prognostic significance as demonstrated by multivariate analyses.

In conclusion one can say that Ki-67 is easier to perform than TLI and therefore can be widely used in pathology laboratories. But its clinical significance remains yet unclear despite the fact that it shows significant correlations to other prognostic factors. It is necessary to consider its clinical importance cautiously until further widespread clinical studies are performed.

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