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Original Paper

Urokinase Plasminogen Activator, a Strong Independent Prognostic Factor in Breast Cancer, Analysed in Steroid Receptor Cytosols with a Luminometric Immunoassay

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Urokinase plasminogen activator (uPA) is involved in the activation of different proteases which participate in the degradation of extracellular matrix, thereby enhancing the invasive capacity of tumour cells. uPA has been shown to be of prognostic importance in breast cancer. We have analysed uPA with a new luminometric immunoassay (LIA), applicable in cytosol samples routinely used for oestrogen-receptor (ER) and progesterone-receptor (PgR) analyses. At a cut-off value of 0.62 ng uPA/mg protein, 33% (230/688) samples were classified as representing high uPA tumours. High uPA content was found to be associated with shorter recurrence-free survival (median observation time: 42 months), ER and PgR negativity, increased p53 expression, DNA non-diploidy and a high S-phase fraction (SPF), but not with lymph node involvement or tumour size (≤20 mm versus >20 mm). In the subgroup of patients not treated with systemic adjuvant therapy, multivariate analysis showed uPA to be an independent prognostic factor together with lymph node status and SPF. If these results can be reproduced, uPA may be a factor suitable for inclusion in a prognostic index. Copyright © 1996 Elsevier Science Ltd

Key words: urokinase plasminogen activator, luminometric immunoassay, prognosis, breast cancer
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INTRODUCTION

TUMOUR METASTASIS remains the major cause of death among cancer patients. Metastasis has occurred in many patients by the time of cancer diagnosis and surgery. The metastatic potential of a tumour is dependent on the ability of the tumour cell to penetrate basement membrane. The invasiveness of tumour cells involves changes in their adhesion to other cells and to the extracellular matrix (ECM), extracellular proteolytic activity and motility, where degradation products of ECM components such as fibronectin, laminin, collagen and elastin have been reported to induce tumour cell motility [1, 2].

In attempts to elucidate the invasion of tumour cells, the factors most widely studied have been proteases participating in the degradation of ECM and basement membrane, such as plasminogen activators, plasmin, collagenases, gelatinases and cathepsins. Some act directly on substrate molecules in the ECM and some by activating other proteases (e.g. the plasminogen activators that activate plasmin, and cathepsins that cleave the pro-enzyme forms of plasminogen activators to active enzyme [3]). A characteristic feature of cancer invasion is the overproduction of proteases in response to multiple hormonal, cytokine or growth factor signals [4].

Of the two kinds of plasminogen activators that have been identified, the tissue-type plasminogen activator (tPA) is mainly active in blood vessels in the initial solubilisation of fibrin clots, whereas the urokinase-type plasminogen activator (uPA) participates in the mechanisms for tissue remodelling such as wound healing, and in cell migration. Since the first evidence that uPA is a key determinant of tumour invasiveness was published by Åstedt and coworkers in 1976 [5], evidence has accumulated suggesting that tumour uPA content might serve as an independent prognostic indicator of outcome in breast cancer patients. Duffy and colleagues, who in 1988

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reported that the disease-free interval in breast cancer patients was shorter in those with high levels of tumour uPA activity than in those with low levels [6], confirmed their findings in 1990 in a study where immunoreactive uPA was measured, concluding that uPA is an independent risk factor for recurrence, independent of tumour size, node status and oestrogen receptor level [7]. The level of uPA as a prognostic factor has also been reported by other groups. In a study of 104 breast cancer patients, based on cytosols extracted from tumour tissue using the detergent-containing buffer, Triton X-100, Jänicke and coworkers found tumour uPA content to correlate with recurrence-free survival, and to be of greater prognostic value than receptor content or the extent of vascular invasion [8]. In another study of breast cancer patients ($n = 671$) by Foekens and colleagues, based on cytosols extracted with the (non-detergent) method normally used for evaluating hormone receptor content, the prognostic value of tumour uPA content was demonstrated both in node-positive and node-negative subgroups, as well as in postmenopausal patients and in patients with steroid receptor-positive tumours [9]. When this group in another study included plasminogen activator inhibitor (PAI-1) in the multivariate analysis, PAI-1 but not uPA was found to be an independent prognostic factor [10]. In yet another study of breast cancer patients, also based on cytosols extracted with the (non-detergent) method for receptor analysis, Gröndahl and colleagues found tumour uPA content to correlate with overall survival, but not to recurrence-free survival [11]. Spyratos and coworkers found the level of tumour uPA content to predict metastasis-free survival [12]. Finally, Bouchet and colleagues found uPA to be of prognostic value, which was further strengthened in subgroups by the addition of PAI-1 and PAI-2 [13].

The aim of our study was to investigate further the prognostic importance of uPA in breast cancer, especially with regard to adjuvant endocrine treatment with tamoxifen and the prognostic value of the S-phase fraction (SPF), and to investigate whether uPA might be a factor suitable for inclusion in a prognostic index. We analysed tumour uPA content with a new luminometric immunoassay in cytosol samples prepared for routine oestrogen receptor (ER) and progesterone receptor (PgR) analyses.

PATIENTS AND METHODS

Patients and treatment

The series comprised 688 patients diagnosed between 1984 and 1988 with clinical follow-up data available. During that time period, samples from approximately 80% of all diagnosed breast cancers were sent to our laboratory for steroid receptor analyses. Residual cytosols were stored at −70°C and used for uPA analysis in the present study. Most of the patients had stage II tumours and were participating in prospective randomised clinical trials launched by the South Sweden Breast Cancer Group (SSBCG), where the effect of adjuvant tamoxifen (TAM) at 20 mg daily for 2 or 5 years was investigated. Local treatment was given in accordance with guidelines adopted by the SSBCG. Surgery consisted either of modified radical mastectomy with axillary dissection, or of conservative segmental resection with axillary dissection in some patients with tumours less than 20 mm in diameter, combined with postoperative radiation of the remaining breast tissue. In general, patients with metastasis to the axillary lymph nodes received postoperative radiation of regional lymph nodes and of the chest wall. Of the series as a whole ($n = 688$), information on adjuvant endocrine treatment was available for 621 patients, of whom 77% (479/621) received adjuvant treatment with TAM [33% without lymph node metastasis (N0) and 67% with lymph node metastasis (N+)], and 23% ($n = 142$) did not (49% N0 and 51% N+). No patient received adjuvant cytostatic treatment.

Patient and tumour characteristics are summarised in Table 1.

The median follow-up was 42 months. Twenty-five per cent (173/688) of the patients had developed recurrence (155 distant and 18 locoregional). Recurrence-free survival (RFS) was chosen as the endpoint owing to the relatively short duration of follow-up. Moreover, we have previously shown similar prognostic information to be obtained with different endpoints (RFS, distant RFS, overall survival and breast cancer survival [14]).

Analytical methods

uPA. At our department, residual cytosol and tumour tissue from steroid receptor analysis are routinely stored at −70°C. In the present study, uPA analysis was performed on such frozen cytosol samples, using a luminometric immunoassay (uPA LIA, AB Sangtec Medical, Bromma, Sweden). The assay is based on tubes precoated with monoclonal antibody to uPA, and a detection reagent containing monoclonal antibodies conjugated to a isoluminol derivate. It detects uPA in the pro-enzyme form, the active two-chain uPA, uPA bound to its receptor (uPAR) and uPA in complex with the inhibitor, PAI-1. In this one-step assay, 100 μl of the sample and 200 μl of detection reagent are added to the tubes, incubated for 2 h at room temperature, washed three times with 0.9% NaCl, and the uPA value is read in a luminometer. Catalyst reagents that induce light emission from the bound isoluminol derivate are added automatically in the luminometer, and the light signal is read immediately thereafter for 5 s. Total assay time is normally under 3 h. The signal is measured in relative light units (RLU). The amount of uPA detected in the cytosols is expressed as ng uPA/mg protein, determined according to Lowry and coworkers [15]. With the LIA, the range of uPA detection is 0.05–40.0 ng/ml, the lowest detection limit (3 S.D. above background RLU) normally being 5 pg/ml; the intra-assay coefficient of variation (CV) is 2.8%, and the interassay CV 7.7% (mean values for six samples). The cytosol samples that were used for evaluation of precision were extracted with the buffer used for routine analysis of ER and PgR (see below).

ER and PgR. ER content was measured with isoelectric focusing (IF) in polyacrylamide gels and enzyme immunoassay (EIA), and PgR content with EIA and a dextran-coated charcoal method (DCC) with Scatchard analysis ([16]; EIA was performed according to kit instructions; Abbot Laboratories, Diagnostic Division, Chicago, Illinois, U.S.A.). The buffer used for cytosol extraction after IF and DCC analysis contained Tris–HCl 10 mM, EDTA 1.5 mM, mercapto-ethanol 10 mM; pH 7.4), whereas the buffer for cytosol extraction after EIA analysis contained Tris–HCl 10 mM, EDTA 1.5 mM, Na_2MoO_4 5.0 mM, monothioglycerol 1.0 mM; pH 7.4 (final concentrations). Samples with receptor content values of ≥10 (IF and DCC) or ≥25 (EIA) fmol/mg protein were classified as ER or PgR positive, and samples with values below these levels as ER or PgR negative [14].

Table 1. Characteristics of the 688 breast cancer patients

					P-values	
		n	High uPA	%	No assoc.	Eq. med.
All		688	230	33	—	—
Age	<50 years	224	86	38		
	≥50 years	464	141	30	0.038	0.67
Lymph node status	N0	265	92	35		
	N1–3	256	79	31		
	N ≥ 4	167	59	35	0.54	0.36
Tumour size	≤20 mm	256	82	32		
	>20 mm	432	148	34	0.56	0.01
ER status	Negative	229	98	43		
	Positive	459	132	29	<0.001	0.008
PgR status	Negative	304	121	40		
	Positive	384	109	28	0.002	0.19
DNA ploidy status	Diploid	182	43	24		
	Non-diploid	309	121	39	<0.001	<0.001
S-phase fraction	Low	262	73	28		
	High	195	83	43	0.001	<0.001
ERBB2	No amplification	177	54	31		
	Amplification	42	24	57	0.002	0.004
INT2	No amplification	187	71	38		
	Amplification	31	7	23	0.11	0.12
c-myc	No amplification	33	13	39		
	Amplification	4	3	75	0.30	0.41
Cathepsin D	<45 pmol/mg protein	102	23	23		
	≥45 pmol/mg protein	202	69	34	0.047	<0.001
p53	<0.27 ng/mg protein	182	39	21		
	≥0.27 ng/mg protein	25	16	64	<0.001	<0.001

For each pair of uPA and another prognostic factor, two hypotheses were tested: no association in the 2 by 2 or 3 by 2 tables (No assoc.), and no difference in median uPA content (Eq. med.). Fisher's exact test was used in the former case and the Kruskal–Wallis test in the latter. ER, oestrogen receptor; PgR, progesterone receptor; uPA, urokinase plasminogen activator.

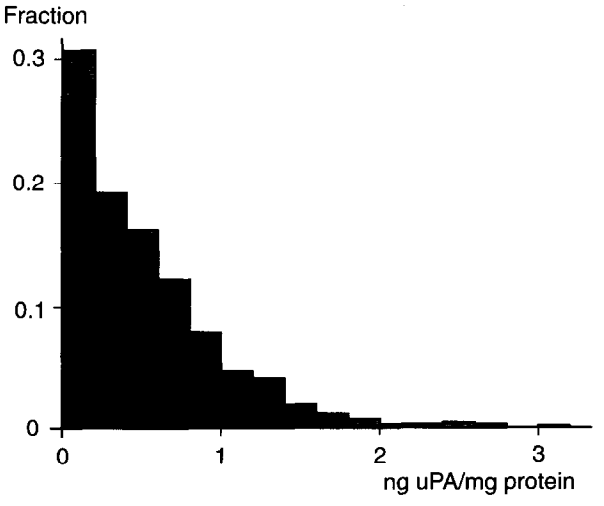

Figure 1. The frequency distribution of urokinase plasminogen activator (uPA) values (ng/mg protein).

Flow cytometry (FCM) DNA analysis. Residual tumour tissue after the routine ER and PgR analysis was available in 71% (491/688) of cases in the present series. These tumour samples were used for FCM DNA analysis in an Ortho 50H instrument after staining with propidium iodide [17]. In accordance with the Convention of Nomenclature for DNA Cytometry [18], ploidy status was defined as follows: one DNA cell population = diploid, two or more cell populations = non-diploid. The percentage of nuclei corresponding to the SPF was estimated planimetrically in 457 (93%) of the 491 DNA FCM analysed samples [19]. Non-diploid samples with an SPF ≥12% and diploid samples with an SPF ≥7.0% were classified as high SPF, and the respective samples with values below these levels as low SPF [14, 20].

Gene analysis. Gene amplification was analysed with the Southern blot and slot blot techniques as described previously [21]. Filters were sequentially hybridised with probes for the *ERBB2*, *INT2* and *C-MYC* genes, and with probes for control markers located on the same chromosomal arm as the respective proto-oncogenes (i.e. for *ERBB2* at 17q-myeloperoxidase,

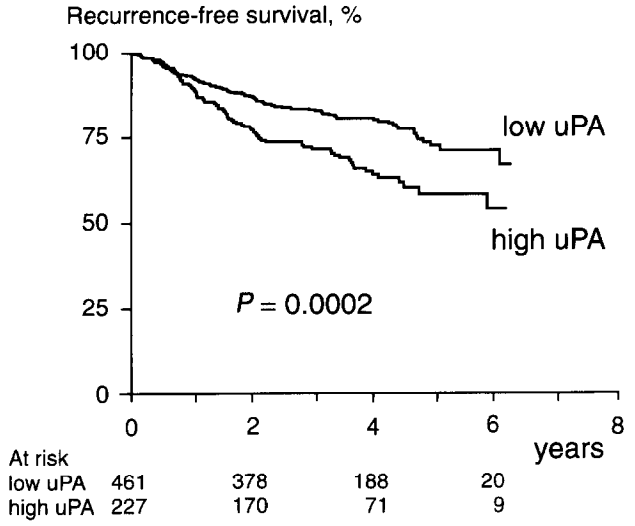

Figure 2. The prognostic importance for recurrence-free survival (RFS) of tumour urokinase plasminogen activator (uPA) content when the present series is subgrouped according to a cut-off value of 0.62 ng/mg protein. ($\chi^2(1) = 14.05$; $P = 0.0002$).

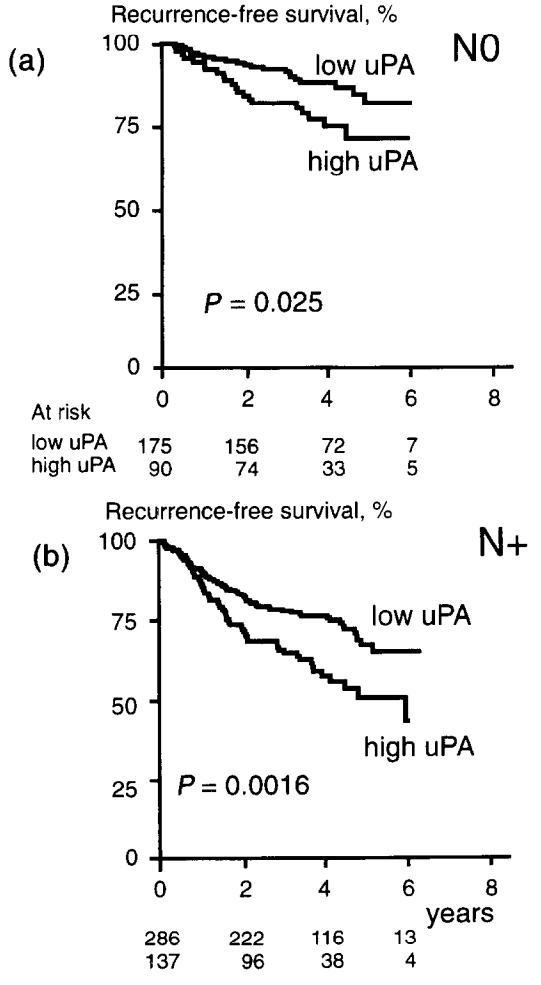

Figure 3. The prognostic importance for recurrence-free survival (RFS) of tumour urokinase plasminogen activator (uPA) content for (a) N0 patients ($\chi^2(1) = 5.03$; $P = 0.025$; $n = 265$), and (b) N+ patients ($\chi^2(1) = 9.91$; $P = 0.0016$; $n = 423$).

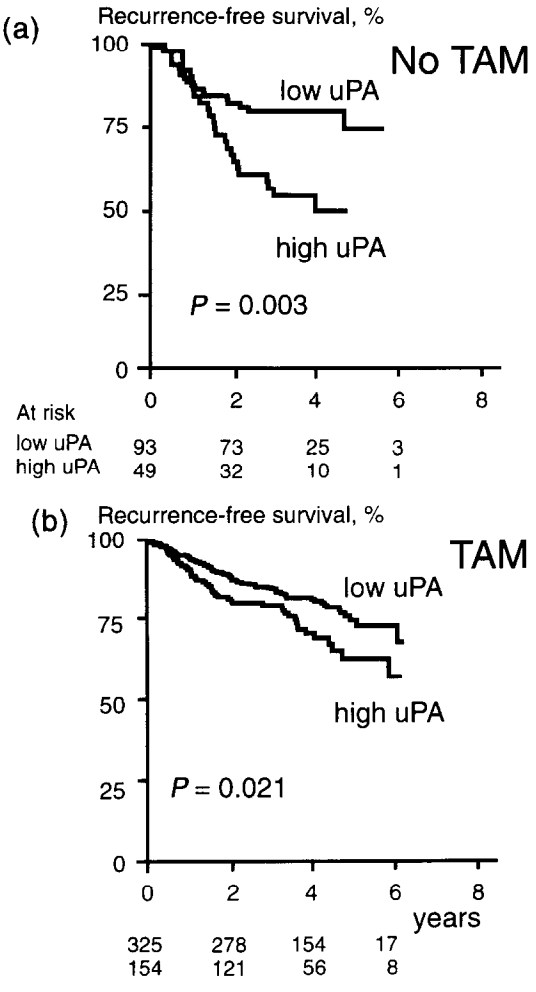

Figure 4. The prognostic importance for recurrence-free survival (RFS) of tumour urokinase plasminogen activator (uPA) content for (a) patients not treated with adjuvant tamoxifen (TAM) ($\chi^2(1) = 8.83$; $P = 0.0030$; $n = 142$), and (b) patients treated with adjuvant TAM $\chi^2(1) = 5.30$; $P = 0.021$; $n = 479$).

for *INT2* at 11q-progesterone receptor, and for *C-MYC* at 8q-mos). The degree of amplification was evaluated with densitometric analysis of slot blot autoradiograms, by comparing the signals from each proto-oncogene and control gene. Tumours were classified as having a single or an amplified (≥ 2) gene copy number of the haploid genome.

p53 and cathepsin D. A sandwich type LIA procedure was used for measuring both mutant and wild type p53 protein (p53 LIA, AB Sangtec Medical, Bromma, Sweden [22]), and an immunoradiometric assay for measuring total cathepsin D content (ELSA-cath-D-kits; CIS-BIO International, Gif-sur-Yvette, France [23]) were used in cytosols from routine ER and PgR analysis.

Statistics

Fisher's exact test (two-sided), the Mann–Whitney *U* test, the Kruskal–Wallis test and Spearman's rank correlation were used to assess associations between tumour uPA content and other prognostic factors. The non-parametric Kaplan–Meier method [24] was used to estimate RFS, and the log-rank test to compare survival in different subgroups [25]. The size of the population at risk is given along the time axis. As

Table 2a. Recurrence-free survival, according to univariate and multivariate analysis (Cox's proportional hazards model) of prognostic covariates for 416 patients

Covariate	Univariate	Multivariate		95% confidence interval
	P-value	*P*-value	RR	
Lymph node status	<0.001*			
1–3 versus 0		0.040	1.7	1.0–2.9
≥4 versus 0		<0.001	3.3	2.0–5.7
Endocrine therapy				
TAM versus no TAM	0.0017	<0.001	0.5	0.3–0.7
uPA				
High versus low	0.0003	0.004	1.8	1.2–2.8
S-phase				
High versus low	0.0002	0.013	1.7	1.1–2.6
PgR status				
Positive versus negative	0.0001	0.009	0.6	0.4–0.9
Tumour size				
Large versus small	0.042	0.059†		
ER status				
Positive versus negative	<0.0001	0.44†		
Ploidy status				
Non-diploid versus diploid	0.82	0.52†		
Patient age				
≥50 versus <50 years	0.0006	0.13†		

RR, relative risk; uPA, urokinase plasminogen activator; PgR, progesterone receptor; ER, oestrogen receptor; TAM, tamoxifen. * Log rank test comparison of recurrence-free survival in the three groups simultaneously (0,1–3, 4+). † Likelihood ratio test for inclusion of the covariate in a model including lymph node status, endocrine therapy, uPA, S-phase and PgR status.

recommended by Altman [26] survival estimates were continued as long as at least 5 patients remain at risk.

Cox's proportional hazards model was used for multivariate analyses [27]. The proportionality assumptions have been checked graphically, by plotting the log cumulative hazard for each level of one factor at a time versus time, and none of the assumptions seemed to be grossly violated, a conclusion verified by applying Schoenfeld's test to the final models presented in Table 3 below [28]. To compare the prognostic importance of uPA in two separate subgroups (in this case TAM- and non-TAM-treated), two Cox models were fitted. Both models included uPA, treatment and a set of other important covariates, but one model also included an interaction term between uPA and adjuvant treatment (uPA × treatment). The hypothesis: "no difference in prognostic importance of uPA" between the two subgroups can in this setting be tested with a likelihood ratio test comparing the models with those without the interaction term. All tests were two-sided, and unless otherwise stated, *P*-values <0.05 were considered significant.

RESULTS

Choice of the cut-off value for uPA

Cytosol uPA content varied from 0 to 3.19 ng/mg protein (median 0.40 ng/mg protein; Figure 1). Using univariate techniques for analysis of RFS, different methods of using uPA as a categorical variable were evaluated.

The quartile with the highest uPA content manifested worse prognosis than the remaining patients ($P = 0.0014$). When that quartile was excluded from the analysis, no significant difference in RFS was obtained between the remaining three uPA quartiles ($P = 0.30$). A similar pattern was obtained by subgrouping into thirds instead, the third with the highest uPA values (≥0.62 ng/mg protein) manifesting a worse prognosis than the other two thirds ($P = 0.0003$; 3-year recurrence rate 28% versus 17%), whereas no significant difference was found between the latter two subgroups ($P = 0.14$). The choice of 0.62 ng/mg protein as a cut-off value seems to be appropriate also for two other reasons. First, the proportion of samples with a high uPA content was similar to that obtained in two other studies; 32% [9] and 36% [8]. Gröndahl and coworkers have used the median value as the cut-off level [11]. Secondly, when testing cut-off values at different uPA concentration levels, the best prognostic separation was obtained with a cut-off level of 0.62 ng/mg protein. Estimates of RFS for high and low uPA are presented in Figure 2.

Association between uPA and other prognostic factors

Tests of homogeneity in cross tables revealed tumours with a high uPA content to be more often ER and PgR negative, p53 overexpressed, non-diploid, highly proliferating, *ERBB2* amplified and from patients under the age of 50. Similar results for all factors but tumour size, age and PgR were obtained when the non-grouped uPA measurements were used to compare the distribution of uPA across groups of other prognostic factors (Table 1). A positive dependence

Table 2b. Recurrence-free survival, according to univariate and multivariate analysis (Cox's proportional hazards model) of prognostic covariates for patients not *treated with adjuvant TAM* (n = 74)

	Univariate	Multivariate		
Covariate	*P*-value	*P*-value	RR	95% confidence interval
Lymph node status*	0.063†			
≥4 versus 0–3		0.001	9.0	2.4–33
uPA				
High versus low	0.009	<0.001	20	3.7–111
S-phase				
High versus low	0.010	<0.001	12	2.8–51
uPA × S-phase	—	0.004	0.08	0.01–0.45
uPA × lymph node status	—	0.024	0.12	0.02–0.76
PgR status				
Positive versus negative	0.24	0.99‡		
Tumour size				
Large versus small	0.077	0.23‡		
ER status				
Positive versus negative	0.25	0.73‡		
Ploidy status				
Non-diploid versus diploid	0.63	0.18‡		
Patient age				
≥50 versus <50 years	0.72	0.51‡		

For abbreviations, see legend to Table 2a. * No significant difference in prognosis between N0 and N1–3 in the multivariate analysis. † Log rank test comparison of recurrence-free survival in the three groups simultaneously (0,1–3, 4+). ‡ Likelihood ratio test for inclusion of the covariate in a model including lymph node status, uPA, S-phase and interactions.

between uPA and tumour size was found in the latter case, but not in the former. The observed proportions of large tumours were approximately the same in the two uPA subgroups (62% versus 64%). Nevertheless, the median uPA content was significantly higher in large tumours than in small ($P = 0.01$). High uPA tumours seem to be more frequent in younger patients (<50 years) than in older (≥50 years), but the hypothesis of equal median uPA content in the two age groups could not be rejected. The contradictory results concerning the association between uPA and PgR reveal that the dependence between these factors is not as simple as the highly significant result of the test of homogeneity indicated. The Spearman rank correlation coefficient between the continuous factors uPA and PgR is positive in the low-uPA subgroup ($r_s = 0.16$), whereas it is negative in the high uPA subgroup ($r_s = -0.10$).

The prognostic importance of uPA in relation to lymph node status

In both the N0 ($n = 265$, 43 recurrences) and N+ ($n = 423$, 130 recurrences) subgroups, patients with low uPA tumours manifested a better prognosis than those with high uPA tumours (N0: $P = 0.025$, 3-year recurrence rate 7.8% versus 18%, Figure 3a; N+: $P = 0.0016$, 3-years recurrence rate 22% versus 35%, Figure 3b).

The prognostic importance of uPA in relation to adjuvant endocrine treatment

When investigating prognostic factors in breast cancer, the importance of adjuvant systemic therapy needs to be considered, since if adjuvant therapy is given it is also the predictive value of the factor in relation to the effect of treatment which is studied. In the present study, it was shown that adjuvant endocrine therapy may affect the outcome, as there was a weak tendency ($P = 0.20$) for the difference in prognosis between patients with low versus high uPA tumours to be more pronounced in the subgroup not treated with TAM ($n = 142$, 44 recurrences) than in the TAM-treated subgroup ($n = 479$, 112 recurrences). In both subgroups there was, however, a significant difference in prognosis between the two uPA subgroups (no TAM: $P = 0.003$, 3-year recurrence rate 21% versus 47%, Figure 4a; TAM: $P = 0.021$, 3-year recurrence rate 15% versus 21%, Figure 4b).

Multivariate analysis

Multivariate analysis, performed on the results for 416 patients (95 recurrences), where information on all variables, except *ERBB2*, *C-MYC*, *INT2*, p53 and cathepsin D, was available, showed lymph node status, TAM treatment, uPA, SPF and PgR to be independent prognostic factors for RFS, whereas tumour size (border-line significance; $P = 0.059$), ER, ploidy status and patient age were not (Table 2a).

In the small subgroup of patients not treated with adjuvant TAM ($n = 74$, 25 recurrences) multivariate analysis was performed in two steps. First, all factors except uPA were evaluated in a stepwise Cox regression, which showed lymph node status and SPF to be independent prognostic factors, whereas menopausal status, tumour size, ER, PgR and ploidy status

Table 2c. Recurrence-free survival, according to univariate and multivariate analysis (Cox's proportional hazards model) of prognostic covariates for patients treated with adjuvant TAM (n = 342)

	Univariate	Multivariate		
Covariate	*P*-value	*P*-value	RR	95% confidence interval
Lymph node status*	<0.0001†			
≥4 versus 0–3		<0.001	2.7	1.7–4.5
PgR status				
Positive versus negative	0.0001	<0.001	0.4	0.3–0.7
uPA				
High versus low	0.0079	0.028	1.7	1.1–2.8
Patient age				
≥50 versus < 50 years	0.016	0.020	0.5	0.3–0.9
Tumour size				
Large versus small	0.13	0.24‡		
S-phase				
High versus low	0.0054	0.21‡		
ER status				
Positive versus negative	0.0002	0.41‡		
Ploidy status				
Non-diploid versus diploid	0.72	0.98‡		

For abbreviations see legend to Table 2a. * No significant difference in prognosis between N0 and N1–3 in the multivariate analysis. † Log rank test comparison of recurrence-free survival in the three groups simultaneously (0,1–3, 4+). ‡ Likelihood ratio test for inclusion of the covariate in a model including lymph node status, PgR status, uPA and patient age.

Table 3. The relative risk (RR), 95% confidence interval (CI) and P-value for low versus high urokinase plasminogen activator (uPA) in different subgroups of lymph node status and S-phase fraction (SPF) for patients not treated with adjuvant tamoxifen (TAM) (n = 74)

Subgroup	*n*	RR	CI	*P*-value
High SPF, N ≥ 4	6	0.19	0.03–1.0	0.05
High SPF, N ≤ 3	27	1.5	0.5–4.6	0.46
Low SPF, N ≥ 4	9	2.5	0.4–14	0.31
Low SPF, N < 3	32	20	3.7–111	<0.001

RR, relative risk.

were not. Secondly, as interactions between uPA and lymph node status and SPF (the only independent prognostic factors according to above) were suspected, uPA was introduced into the multivariate model as a single factor (uPA), but also under the assumption that its prognostic importance may be dependent both on lymph node status and SPF (uPA × node status and uPA × SPF). This multivariate analysis showed uPA, both alone and adjusted for lymph status and SPF, to be an independent prognostic factor, but also that its prognostic importance was dependent on these two factors, being more pronounced in the subgroup with less than four positive lymph nodes and in the subgroup with low SPF (Table 2b). This interaction is further illustrated in Table 3, which shows the prognostic value of uPA to be greater in the subgroup with low SPF and N ≤ 3 (no additional prognostic information was yielded by separating N0 and N1–3 subgroups). uPA was also an independent prognostic factor in the subgroup treated with adjuvant TAM (Table 2c, $n = 342$, 70 recurrences), no interactions between uPA and other factors being of any importance. In this subgroup, the effect of PgR status was also found to be significant.

Prognostic index

We have previously developed a prognostic index (PI) based on SPF, PgR and tumour size, which is currently being tested in a prospective controlled clinical trial for premenopausal patients with lymph node negative breast cancer [14]. The results obtained so far concerning uPA indicate that it may be a factor suitable for inclusion in a PI. Based on a Cox model for the subgroup not treated with TAM ($n = 74$), the following PI was calculated:

$$\begin{aligned} PI = {} & 2.5 \times I_{\{\text{high SPF}\}} + 2.2 \times I_{\{N \geq 4\}} \\ & + 3.0 \times I_{\{uPA \geq 0.62\}} \\ & - 2.6 \times I_{\{\text{high SPF and } uPA \geq 0.62\}} \\ & - 2.1 \times I_{\{N \geq 4 \text{ and } uPA \geq 0.62\}} \end{aligned}$$

where $I_{\{\text{high SPF}\}}$ is 1 if high SPF and 0 if low SPF, $I_{\{N \geq 4\}}$ is 1 if $N \geq 4$ and 0 if N0–3, $I_{(uPA \geq 0.62\}}$ is 1 if $uPA \geq 0.62$ and 0 if $uPA < 0.62$, etc. The different combinations of N-status, SPF and uPA, and recurrence rate in each subgroup are shown in Table 4. By dividing this material at two cut-off levels (PI = 0 and PI = 2.6), three subgroups with similar numbers of patients were obtained, differing markedly from each other in

Table 4. Based on a Cox-model for the subgroup not treated with tamoxifen (TAM) a prognostic index was calculated (see Results). The index ('risk points') for the different combinations of the factors included in the prognostic index are listed in this table, together with number of patients and recurrences in each subgroup

SPF	N	uPA	Number	Recurrences	Riskpoints	Risk group
L	0–3	L	25	2	0	Low
L	4+	L	6	1	2.2	Intermediate
H	0–3	L	14	4	2.5	Intermediate
H	0–3	H	13	7	2.9	High
L	0–3	H	2	1	3.0	High
H	4+	H	7	4	3.0	High
L	4+	H	3	2	3.1	High
H	4+	L	4	4	4.6	High

SPF: L = low and H = high; uPA L = low and H = high; N ≤ 3 = 0–3 and N ≥ 4 = 4+.
SPF, S-phase fraction; uPA, urokinase plasminogen activator.

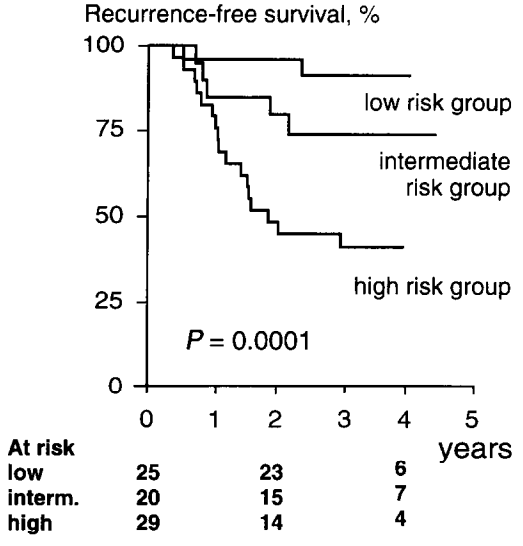

Figure 5. The prognostic importance for recurrence-free survival (RFS) of tumour urokinase plasminogen activator (uPA) content for three subgroups defined according to a prognostic index based on lymph node involvement, S-phase fraction (SPF) and uPA ($\chi^2(2) = 17.95$; $P = 0.0001$; $n = 74$).

recurrence-free survival (Figure 5). As this index has been evaluated in a rather small series, the results can only be considered preliminary and its prognostic value needs to be confirmed in a new, preferably larger series.

DISCUSSION

The study showed tumour uPA content to be a strong independent prognostic factor in breast cancer, when measured with a LIA in cytosol samples used for routine analysis of ER and PgR. The prognostic importance of uPA was demonstrated for patients with both lymph node-negative and lymph node-positive breast cancer. In the node-negative subgroup, prognostic factors may be clinically useful in selecting a high-risk group in need of adjuvant cytostatic treatment, and among N+ patients the effect of high dose chemotherapy combined with autologous stem cell support is currently being tested among patients with an expected 5-year RFS of less than 30%. uPA may prove to be a useful prognostic factor for the identification of this high-risk group.

The main results in our study are in agreement with those previously published by others [8–11]: (1) a similar proportion of high uPA tumours (33% versus 32–36%); (2) no correlation between uPA and N-status and tumour size but similar correlations with other prognostic factors; and (3) uPA is a strong independent prognostic factor.

In the present study, it was shown that adjuvant endocrine therapy may affect the outcome, as there was a weak tendency ($P = 0.20$) for the difference in prognosis between patients with low versus high uPA tumours to be more pronounced in the subgroup not treated with TAM than in the TAM-treated subgroup. The effect of adjuvant TAM on the prognostic importance of uPA should be further investigated in a new study, preferably consisting of patients participating in controlled randomised clinical trials.

Multivariate analysis showed uPA and SPF (together with lymph node status) to be independent prognostic factors, and consequently both may be included in a prognostic index to select high-risk patients in need of additional adjuvant treatment. Such an index yielded a good separation into prognostic subgroups. The prognostic instrument used today in our health care region for premenopausal lymph node-negative patients consists of SPF, PgR and tumour size. If two of these factors indicate poor prognosis, the patient is considered to belong to a 'high-risk' group [14]. Comparison of this index and that obtained in the present study should be performed in a new series of patients, preferably consisting of lymph node-negative breast cancer patients not treated with adjuvant systemic therapy. This question, of whether uPA and/or SPF are suitable for prognostic purposes, has also been discussed by Jännicke and coworkers [8], but owing to the very few SPF samples analysed in their series ($n = 35$), they could draw no conclusions. In contrast to our study, their SPF values did not correlate with uPA content. This may be explained by the fact that they excluded a large proportion of samples because of the poor quality of the DNA histograms, especially among aneuploid cases, the inferior quality perhaps having been due to the use of paraffin-embedded tissue sections. The additional prognostic information yielded by measuring PAI-1, besides uPA, has been demonstrated in some studies [8–11,13]. In a recent study, Yamashita and coworkers found that uPA was not an independent prognostic factor when compared to three other products of human

breast cancer cells, including membrane-associated phospholipase A2, polymorphonuclear leucocyte elastase and tPA [29].

Another issue is the optimal way to analyse uPA, which is currently being investigated by several groups, both with regard to the choice of buffer (i.e. pH, containing detergent or not, etc.) and the importance of different methodological principles, LIA (present series) and ELISA [8, 9, 11]. The effect on the uPA value of using detergent containing buffers is demonstrated by comparing the median uPA values from different studies: 2.3 ng/mg protein (with Triton X-100 [8]) versus 0.7 or 0.4 (without triton X-100 [9, present series]). With their procedure, Rosenquist and coworkers found only approximately 12% of the potentially extractable uPA antigen to be obtained without using detergent [30]. Although there is a strong correlation between the amount of uPA found in Triton X-100 extracts and that in cytosolic extracts of breast cancer [30], Jänicke and colleagues have demonstrated that the former yielded slightly more prognostic information [31].

In conclusion, a new easily performed, sensitive and quantitative LIA suitable for routine measurements of uPA in steroid receptor cytosol preparations was evaluated in 688 tumour samples. uPA was found to be an independent prognostic factor, especially in the subgroup of non-TAM-treated patients with ≤ 3 positive lymph nodes and low SPF. The latter finding needs to be confirmed in a new study.

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