Thymidine Kinase 1 in Serum Predicts Increased Risk of Distant or Loco-regional Recurrence Following Surgery in Patients with Early Breast Cancer

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Abstract. *Background: The prognostic value of the concentration of serum thymidine kinase 1 (S-TK1) with regard to recurrence in low risk breast cancer patients, 3 months after surgery was evaluated. Patients and Methods: The concentration of S-TK1 in serum was determined in 120 breast cancer patients at the time of surgery and in 67 patients 3 months after surgery, by anti-TK1 chicken IgY antibody, using a dot-blot immuno-assay. The S-TK1 concentration was compared with the serological activity of thymidine kinase (STK) and of carbohydrate antigen (CA 15-3). Results: A statistically significant trend (unadjusted) was found for recurrence (distant or loco-regional) in patients with a higher S-TK1 concentration, as compared with patients with a lower S-TK1 concentration. A multivariate analysis gave the same results. The hazard rate ratio for developing distant and/or loco-regional recurrence in patients with a higher S-TK1 concentration was about six to seven times higher than in patients with a lower S-TK1 concentration. Conclusion: Our results indicate that the S-TK1 concentration is higher in patients developing distant and/or loco-regional recurrence 3 months post-surgery.*

Although a number of potential tumour markers for breast malignancy have been tested, only a few of these markers have reached the point where they are of clinical use for screening, diagnosis, prognosis, monitoring and surveillance. They include oestrogen receptor (ER) (1-3) and human epidermal growth factor receptor 2 (HER-2/neu) (4, 5), carbohydrate antigen 15-3 (CA 15-3) (6, 7) and 27.29 (CA 27.29) (8), and carcinoembryonic antigen (CEA) (9). In 2002, the American Society of Clinical Oncology (ASCO) discussed relevant prognostic markers for breast cancer (10). Their recommendation included only ER and Her-2/neu, but not CEA, CA 15-3 and p53, because of controversial results. Furthermore, since only 25-30% of breast cancer patients are HER-2/neu - positive (11, 12), the usefulness of this marker may be limited. Detection of metastatic tumour cells in bone marrow is another method used, but has not yet been proved to be efficient. These data highlight the need for more sensitive and specific prognostic indicators.

For more than two decades, the activity of thymidine kinase in serum (STK) has been used as a serological tumour marker, particularly in leukaemia and lymphoma (13-15), and in pre-operative breast cancer patients (16), but not in solid human tumours during therapy. Thymidine kinase 1 (TK1) is an enzyme involved in deoxyribonucleic acid (DNA) synthesis, and therefore, it is proliferation-related. In our previous studies on serum from patients with breast cancer (17) and gastric cancer (18), we found that the STK activity was elevated in patients pre-operatively, but was irregular following surgery and adjuvant treatment. The concentration of S-TK1 (17), as determined by poly/monoclonal anti-TK1 antibodies, showed a closer correlation to the response of the therapy post-operatively, than did the STK activity.

Abbreviations: ASCO, American Society of Clinical Oncology; CEA, carcino-embryonic antigen; CI, confidence interval; DNA, deoxyribonucleic acid; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; ER, oestrogen receptor; HER2/neu, human epidermal growth factor receptor 2; IgG, immunoglobulin G; IgY, immunoglobulin Y; N+, nodal-positive; pN0, nodal-negative; NSCLC, non-small cell lung cancer; HTK1, recombinant human thymidine kinase 1; RIA, radio-immunoassay; STK, activity of thymidine kinase in serum; S-TK1, concentration of thymidine kinase 1 in serum; STO5, Stockholm 5 Clinical Study of Breast Cancer; TK1, thymidine kinase 1; TK2, thymidine kinase 2; TNM, extent of tumour (T), presence of lymph node (N), or distant metastasis (M).

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In this paper we therefore extended our studies on breast cancer patients, regarding the use of the concentration of S-TK1 post-operatively, as a marker for increased risk of recurrence. In a follow-up study of low-risk breast cancer patients, *i.e.* patients with a relatively small tumour burden pre-operatively, serum was used 3 months after surgery to investigate the time to recurrence in patients with different S-TK1 concentrations.

Patients and Methods

Patients. In 1990-1994 a randomised study of three different endocrine approaches in the adjuvant setting for pre-menopausal women with node-negative or node-positive breast cancer was performed in the Stockholm area in Sweden. The inclusion criteria were invasive breast cancer, pre-menopausal menstrual status, primary surgery consisting of a mastectomy or lumpectomy plus axillary dissection, histopathological tumour size >10 mm and no clinical evidence of distant metastases. The exclusion criteria were inoperable breast cancer, prior radiotherapy or neo-adjuvant chemotherapy and prior or concurrent endocrine therapy. Stratification was done according to nodal status, so that patients with one to three positive lymph nodes also received adjuvant chemotherapy and patients with four or more positive nodes received both chemotherapy and regional radiotherapy. Patients in all three strata were randomly assigned to treatment with tamoxifen alone (20 mg/day for 2 years), treatment with goserelin (3.6 mg subcutaneously over 28 days for 2 years), a combination of tamoxifen/goserelin or no endocrine treatment. Within this study 120 consecutive patients were asked to participate in a study of bonemineral density effects and serum markers. These 120 patients were included in a local study of changes in bone mineral density and other side effects of endocrine treatment, in pre-menopausal women. Besides regular measurements of bone mineral density, sera were investigated post-operatively around the time of randomisation, and at 3, 6, 12, 24 and 36 months. For logistical reasons, 67 patients were analysed at 21 days and at 3 months, respectively, for S-TK1 concentrations, while 40 patients were tested for S-TK activity and CA 15-3.

The characteristics of the patients are shown in Table I. During the follow-up, a total of eleven patients developed distant recurrence, while seven patients developed loco-regional recurrence within 5 years of surgery. One patient developed distant recurrence after 10 years. The tumours were scored for the patient's age, ER, nodal status and tumour size. The blood sera were stored in a bio bank at –80˚C. Sera from 13 healthy individuals were used as negative controls. At the time of determination the sera were thawed and immediately assayed for S-TK1. The study was conducted in accordance with the Helsinki Declaration of 1983. Informed consent was obtained from all patients and the study was approved by the Committee on Research Ethics at Huddinge University Hospital, Sweden (No. 388/01).

Enhanced chemiluminescence dot-blot analysis of thymidine kinase 1 concentration in serum. The concentration of S-TK1 was measured by enhanced chemiluminescence (ECL) dot blot assay, as previously described (17). Briefly, 3 μl of serum sample were applied to a nitrocellulose membrane (Amersham Pharmacia, Uppsala, Sweden), in duplicate. The sera were probed with and without anti-TK1 chicken immunoglobulin Y (IgY) antibody (19), the latter were used as negative controls. Sera from 13 healthy individuals were also used as negative controls, provided by Dr Siamak Haghdoost, Stockholm University, Sweden, while recombinant human TK1 (HTK1), provided by Prof. Staffan Eriksson, Biomedical Centre, Uppsala, Sweden, was used as positive control. We also used anti-TK1 mouse immunoglobulin G (IgG) monoclonal antibodies (1D11/1E3) (20), with identical results (data not discussed). The ECL-treated membranes were exposed to X-ray films, taking into account the variation in S-TK1 concentration of the samples. The intensities of the spots on the films were determined using a GS-700 Imaging Densitometer (Bio-Rad, USA). The areas of the spots were equally defined by the integration computer program of the GS-700 Imaging Densitometer. From the three different concentrations of HTK1, a standard curve was created, permitting calculation of S-TK1, as pmol/l (pM). The accuracy of the assay was 4-6% (17). The sensitivity varied from 0.75 to 1.0, depending on the type of malignancy, and the specificity was found to be 1.0 at a cut-off value of 2 pM (21). Figure 1 shows an example of the dot-blot and Western blot analyses.

Western blot of thymidine kinase 1. Western blot of native gel was performed, as previously described (17).

Table I. *Patient characteristics.*

Characteristics	Number of patients (%)
Age	
<45 yrs	29 (43)
≥45 yrs	38 (57)
Mean age, yrs (range)	45 (28-54)
Nodal status[a]	
pN0	56 (84)
N+	11 (16)
Tumour size	
<20 mm	39 (58)
≥20 mm	28 (42)
ER status	
ER–	20 (30)
ER+	40 (60)
Unknown	7 (10)
Endocrine treatment[b]	
No	25 (37)
Yes	42 (63)
S-TK1[c]	
<0.78	22 (33)
0.78-1.08	22 (33)
>1.08	23 (34)
Total	67 (100)

[a]Stratification factor in STO5. Node-negative patients from list A and node-positive patients from lists B and C.
[b]Allocated treatment. "No" responds to C and "yes" to T, Z or TZ in the randomised ATO5 study.
[c]Ratio between S-TK1 measured at 3 months and S-TK1 measured 3 weeks after surgery. Cut-off points were obtained by dividing the distribution into three equally sized groups.

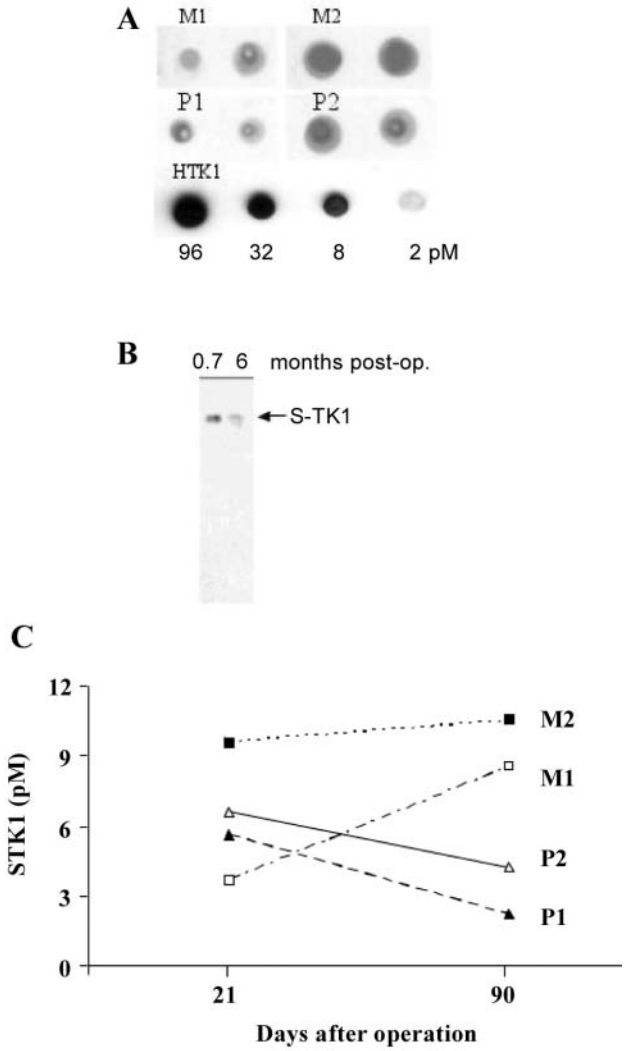

Figure 1. *Examples of results of the dot-blot assay of patients with (M1, M2) and without known metastatic disease (P1, P2), at 21 days and 3 months after surgery. A) Original dot-blot results, with purified recombinant human TK1 (HTK1) used as a standard, B) Western blot of native gel of thymidine kinase 1 in serum (S-TK1) from patients at 0.7 and 6 months after surgery, C) results of A) scanned and re-calculated as pmole/l (pM).*

Thymidine kinase 1 activity. The activity of TK1 in serum was determined by a radio-immunoassay (RIA) (Sangtec Molecular Diagnostics AB, Bromma, Sweden, now DiaSorin, Saluggia, Italy).

Carbohydrate antigen 15-3. The concentration of CA 15-3 in serum was determined by a radio-immunoassay (RIA) (Sangtec Molecular Diagnostics AB, Bromma, Sweden).

Statistical methods. The relative change in S-TK1 for each patient during 3 months post-surgery was calculated by taking the ratio of the S-TK1 concentration determined at 3 months and to that determined at 3 weeks after surgery. Cut-off points were obtained by dividing the ratio distribution into three equally sized groups.

In the analysis, time to recurrence was calculated from the date of the second S-TK1 measurement (at 3 months post-operatively) to the date of loco-regional recurrence, distant metastases or intercurrent death (whichever came first). In the absence of an event, the time was calculated from 3 months post-operatively to the end date of follow-up, 4 September, 2003. In the analysis of distant recurrence, the event of loco-regional recurrence was ignored.

Crude cumulative incidence rates were estimated using Kaplan-Meier technique generalised to include competing risks (22). The influence of prognostic factors on the event of interest was estimated using Cox's proportional hazards model, treating other types of failure, as well as withdrawn alive and event-free, as censored observations. Test for trend was performed by coding the S-TK1 category into the values 0, 1 and 2, and treating the variable as continuous in the proportional hazard model. Results are presented as hazard rate ratios with 95% confidence intervals (95% Cis). Reported p-values correspond to likelihood ratio tests (22).

Results

The absolute concentration of S-TK1 in the healthy individuals (n=13) was 0.6±0.4 pM, ranging from undetectable to 2 pM. In the breast cancer patients the S-TK1 concentration was significantly elevated ($p<0.001$), when compared with the healthy individuals however, it varied extensively between patients (0.2–37 pM) (Figure 2). The mean S-TK1 value at 21 days post-surgery was 7.9±9.8 pM. No difference was found between the mean values of patients with and patients without recurrence. After surgery the mean S-TK1 value of the patients without recurrence decreased to about 6 pM at 3 months and remained at this level until 36 months. The S-TK1 in patients with a recurrence was about 8 pM during this time period, however, the difference between the two groups of patients was not statistically significant. To more accurately compare the different patients after surgery, the post-surgery value of each patient was related to the corresponding individual value at 21 days post-surgery, *i.e.* to the internal control. Significantly higher, mean relative S-TK1 values, were found up to 18 months after surgery in the group of patients with recurrence, as compared with the group of patients without recurrence (Table II). It is obvious from this finding that the results depend on how the S-TK1 is analysed. In the present study, we prefer to use the relative S-TK1 concentration values, since they are standardised to the internal control, *i.e.* S-TK1 values at 21 days after surgery. In 40 out of the 67 patients (16 patients with recurrence and 24 without recurrence) the S-TK1 concentration was also compared with the activity of STK and CA 15-3. While 63% (10/16) of the patients with recurrence showed a higher S-TK1 concentration compared with the patients without recurrence, no such difference was seen for STK activity or CA 15-3 (Figure 3).

To establish whether the S-TK1 value could be used for early identification of those patients developing recurrence, at 3 months post-operatively the patients were divided into three equally sized subgroups, according to their relative

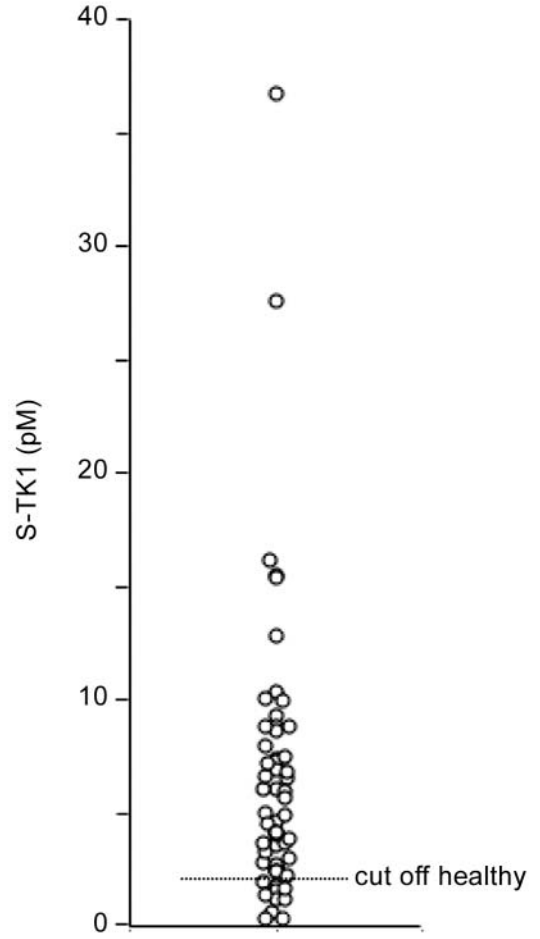

Figure 2. *Concentration of thymidine kinase 1 in serum (S-TK1) (pM) of breast cancer patients 21 days after surgery.*

concentrations of S-TK1, <0.78, 0.78-1.08 and >1.08. These S-TK1 values were used in all of the following analyses.

The estimated crude incidence of distant recurrence at 5 years was 0.09 for patients with a relative S-TK1 concentration <0.78 (Table III). The corresponding rate for patients with a relative S-TK1 concentration >1.08 was 0.26 (Table III).

In an unadjusted analysis of time to distant recurrence, the hazard rate ratio was 3.8 (95% CI: 0.8-18.1), when comparing patients with a relative S-TK1 concentration >1.08 with patients with a relative S-TK1 concentration <0.78 (Table III). In a multivariate analysis controlling for patient age, nodal status, tumour size, ER-status and endocrine treatment, the hazard ratio was 2.5 (95% CI: 0.5-12.1) (Table III). There was no significant difference between the groups with high (>1.08) and low (<0.78) relative S-TK1 concentrations, and in both analyses the test for trend was not significant (Table III).

With regard to time to recurrence (distant or loco-regional), the estimated crude cumulative incidence at 5

Table II. *Levels of thymidine kinase 1 in serum (S-TK1) at different time points after surgery. The mean concentration (pM)± standard deviation (SD) are expressed in relation to S-TK1 concentration (pM) at 21 days post-surgery. The statistical significance was determined by Student's t-test.*

	S-TK1 (%)				
Time after surgery (months)	No recurrence	n	Recurrence	n	*p*-value
0.7	100	93	100	18	
3	0.93±0.53	51	1.81±1.05	16	0.005
6	0.93±0.56	44	1.71 ±1.32	15	0.04
9	0.97±0.57	38	1.67±1.24	12	0.08
12	0.92±0.63	44	1.80±1.16	11	0.032
18	0.82±0.34	28	2.20±0.95	8	0.004
24	1.01±0.66	38	2.22±2.02	8	0.146
36	0.94 ±1.12	31	1.68±0.76	6	0.075

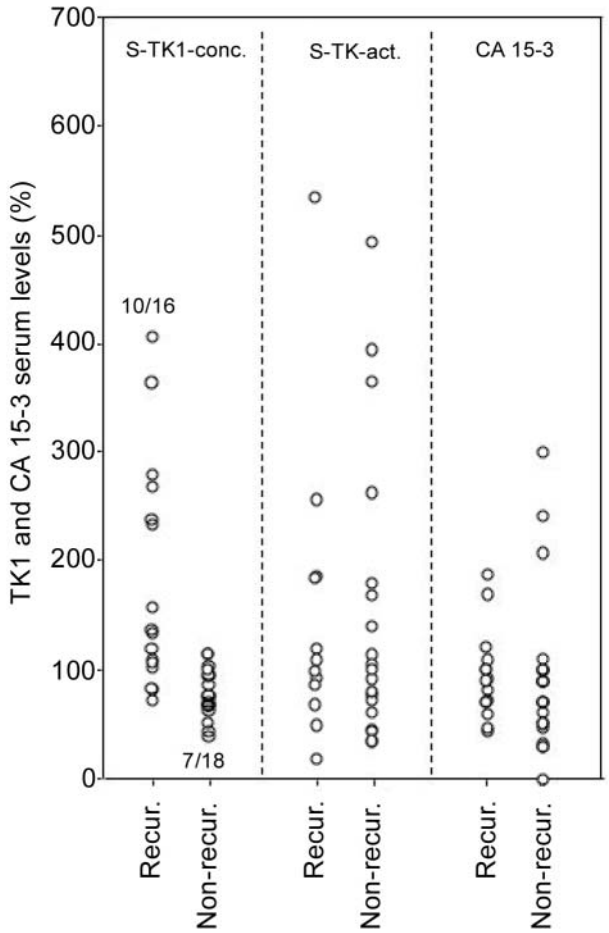

Figure 3. *Individual values of thymidine kinase 1 (S-TK1) concentration (pM), S-TK activity and carbohydrate antigen 15-3 (CA 15-3) in serum of breast cancer patients with and without recurrence, 3 months after surgery. The values are expressed in relation to the corresponding values at 21 days after surgery, and given as a percentage. Recur. = recurrence; non-recur. = non-recurrence.*

Table III. *Thymidine kinase 1 in serum (S-TK1) in relation with different types of events.*

		Unadjusted effect		Adjusted effect[a]	
Type of 1st event	Events/ patients	Hazard rate ratio (95% CI)	*p*-value	Hazard rate ratio (95% CI)	*p*-value
S-TK1 Distant recurrence					
S-TK1 concentration					
<0.78	2/22	1		1	
0.78 – 1.08	3/22	1.5 (0.3 – 9.0)		1.2 (0.2 –7.3)	
>1.08	7/23	3.8 (0.8 – 18.1)	0.056	2.5 (0.5 – 12.1)	0.19
Any recurrence (loco-regional or distant)					
S-TK1 concentration					
<0.78	2/22	1		1	
0.78 – 1.08	5/22	2.6 (0.5 – 13.6)		2.3 (0.4 – 12.3)	
>1.08	12/23	7.7 (1.7 – 34.5)	<0.001	6.1 (1.3 – 28.5)	0.004

[a]Adjusted for age (<45, ≥45 years), nodal status (pNO, pN+), tumour size (<20 mm, ≥20 mm), treatment (yes, no), ER status (ER-, ER+) and endocrine treatment. Seven patients were excluded from this analysis due to missing information on ER status.
[b]Ratio between S-TK1 measured at 3 months and at 3 weeks after surgery. Cut-off points were obtained by dividing the S-TK1 distribution into three equally sized groups.
[c]Test for trend.

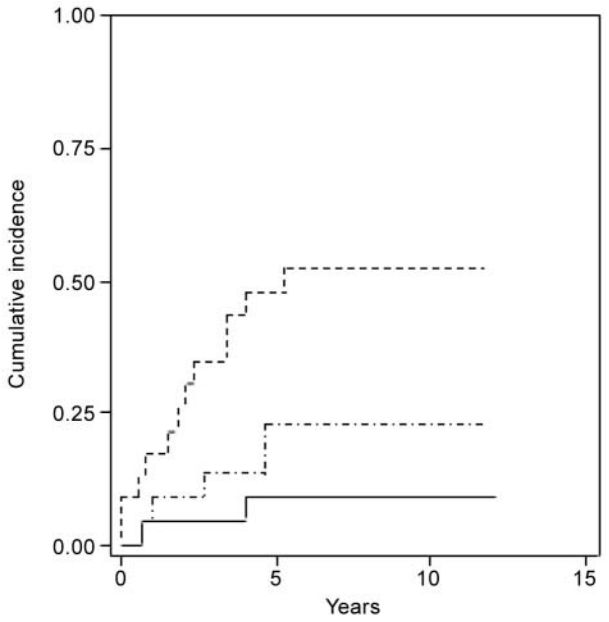

Figure 4. *Cumulative incidence of any recurrence (loco-regional or distant recurrence) in breast cancer patients with different relative concentratuion of thymidine kinase 1 (S-TK1) in a controlled clinical trial of adjuvant endocrine therapy. S-TK1 value <0.78 (———), 0.78 – 108 (– – – – –) and >1.08 (-------).*

years in the group of patients with a relative S-TK1 concentration >1.08 was 0.48. For patients with a relative S-TK1 concentration <0.78 it was 0.09 (Figure 4). In the unadjusted analysis of time to any recurrence, the hazard rate ratio was 7.7 (95% CI:1.7-34.5), when the group with the relative S-TK1 concentration >1.08 was compared with the group with the relative S-TK1 concentration <0.78 (Table III). In the multivariate analysis the hazard rate ratio was estimated to be 6.1 (95% CI: 1.3-28.5) (Table III). Although the width of the CI indicates low precision in both analyses, the recurrence rate was significantly higher in the group with a relative S-TK1 concentration >1.08, than in the group with concentration <0.78 (Table III). In both of these analyses (unadjusted and multivariate), the test for trend was significant ($p<0.001$ and $p=0.004$, respectively) (Table III). These data were also analysed by a non-parametric test, obtaining the same results as described above.

Discussion

In this study, the possibility to use the concentration of S-TK1, instead of STK activity, as a tool for early prediction of distant and/or loco-regional recurrence in low-risk breast cancer patients post-operatively, has been explored. To this purpose, we have developed anti-TK1 mouse mono/polyclonal IgG antibodies (17, 23) and an anti-TK1 chicken IgY antibody (19). Both types of antibody were used with similar results; consequently, only the results for the anti-TK1 chicken IgY antibody are shown. As an immune-detection system, the sensitive dot-blot ECL immunoassay, allowing detection of as low as 0.3 pM of S-TK1 was used, a sensitivity necessary for using TK as a tumour marker in solid malignancies. Single-way ELISA was performed, but it was found, unexpectedly, that the sensitivity was no less than 20 pM of S-TK1 (17, 19), as also reported by Kuroiwa and *et al.* in 2001 (24). These anti-TK1 antibodies are also useful for immuno-histochemical staining of tumour sections of different types of cancer, showing the close relation between these antibodies and proliferation (20, 25, 28).

The patients in this study were characterised as low-risk patients, *i.e.* they had relatively small tumour burden before surgery. The contribution of TK1 to serum from their tumours was therefore smaller than would be expected in patients with larger tumours. The concentration of S-TK1 in persons with no known history of malignancy, *i.e.* healthy persons, is about 1 pM (ranging from undetectable values to 2 pM). Sixty-seven percent of our breast cancer patients showed S-TK1 concentrations of 2-10 pM at 21 days post-surgery. Despite the elevated S-TK1 values, these patients should be regarded as having

fairly low S-TK1 concentrations. Concentrations of S-TK1 ≥100 pM are common in many cancer patients (17, 29). Only a few patients in our study showed S-TK1 values ≥10 pM (7/67), ranging from 10 pM to 36 pM. The rest 22% of the patients had an S-TK1 concentration in the same range as the healthy persons (0.3-2 pM). Therefore, the low concentration of S-TK1 seems to well reflect the low tumour burden of the breast cancer patients, in this study. However, low S-TK1 concentrations at the start of the therapy may reduce the ability to predict changes in the tumour burden. Patients with larger tumours may benefit more from such an assay.

The concentration of S-TK1 in patients with the lowest and patients with the highest S-TK1 concentration in this study differed by a factor of 30-40 (Figure 2). To compare the results of the surgery and the subsequent adjuvant therapy between the various patients, the S-TK1 value should optimally be normalised to the S-TK1 value pre-operatively. However, because the collection procedure had started more than one-decade previously, no blood samples had been drawn before surgery. Consequently we normalised the S-TK1 values to the S-TK1 concentration at 21 days after surgery, the first time point available. This may interfere with the evaluation of our results. In a previous study on the concentration of S-TK1 in breast cancer patients the half-life of S-TK1 in serum of recurrence-free patients after surgery was approximately 1 month (17). This means that in the recurrence-free patients the S-TK1 concentration should be expected to be reduced by about 25-30% at 21 days post-surgery. If the value to this reduced concentration is normalised, the relative decrease should be less pronounced than if it is normalised to the higher concentration, at the time before surgery. Therefore, the relative concentration of S-TK1 after surgery in our recurrence-free patients should be expected to be lower, by 25-30%, than what was calculated. Consequently, the difference between the group with recurrence and the recurrence-free group would be more pronounced, improving our results.

According to our hypothesis, S-TK1 should decrease with time after surgery in recurrence-free patients. However, in this study the S-TK1 concentration did not decrease up to 3 months post-operatively in 25% of the recurrence-free patients. This indicates a more complex regulation of degradation of S-TK1 in the serum of some patients. It may also be possible that these patients had a recurrence, not yet manifested.

In this study, S-TK1 concentration was not correlated to cancer stage or grade. This would have been of interest, since stage and grade are important parameters for diagnosis and prognosis of malignancy. However, when the samples were collected (1990-1994), stage and grade were not part of the diagnostic protocol. In our recent studies of adenocarcinoma, in a type of non-small cell lung cancer (NSCLC) (27), and of bladder cancer (29), S-TK1 concentrations were found to be correlated to cancer stage, but not to grade. In squamous cell carcinoma of the lungs, no correlation to stage or grade was found (28). We also know that the cytosolic concentration and activity of TK1 correlate to stage and grade in breast cancer (16, 20), colorectal cancer (25) and NSCLC (28). Therefore, it is reasonable to believe that the S-TK1 concentration of the breast cancer patients in this study would also correlate to stage at least.

Optimal cut-off values were not seeked. The statistical analysis on patients, performed divided into three equally sized subgroups representing low, medium and high relative S-TK1 concentrations. Although a significant difference was found between the patients with low and high relative S-TK1 concentrations, the CI was wide, which makes the conclusion less reliable. However, there was a significant trend towards an increased risk of distant and/or loco-regional recurrence in the subgroup with a high relative S-TK1, compared with the subgroup of low relative S-TK1 concentration. The results from this study indicate that it may be possible to identify about 40% of patients who will later develop distant and/or loco-regional recurrence, and also, those patients who will not. To further demonstrate the possibility of using S-TK1 concentration for early detection of recurrence, S-TK1 was compared with serological CA 15-3, a marker used frequently in breast cancer patients, as well as with STK activity. Higher CA 15-3 or STK activity values were not found in patients with recurrence, as we expected. This indicates that these markers are not as sensitive as the concentration of S-TK1 in detecting recurrence early after surgery. Therefore, monitoring of the post-operative S-TK1 concentration, would improve the treatment of patients both with and without recurrence, *i.e.* avoid, prolong or change adjuvant therapy, leading, not only to reduced therapy-related stress, but also, to enhanced survival.

Although the number of patients included in the study is limited, the results point to the possibility that there could be a significant trend toward higher relative S-TK1 concentrations, in patients developing distant and/or loco-regional recurrence. Since this allows improved risk assessment, such information can be used to decide on adjuvant therapy.

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