Elevated thymidine kinase 1 in serum following neoadjuvant chemotherapy predicts poor outcome for patients with locally advanced breast cancer

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Abstract. Patients with locally advanced breast cancer (LABC) commonly have an unfavorable prognosis. A molecular predictor for the identification of at-risk patients is urgently required. Thymidine kinase 1 in serum (S-TK1) is an enzyme involved in the synthesis of DNA precursors. In studies using immunohistochemistry, it was reported to be a more useful proliferation marker than Ki-67 in breast, lung and colorectal carcinoma. In the present study, we extended the research of prior breast carcinoma studies by postulating that in patients with LABC, overexpression of S-TK1 following neoadjuvant chemotherapy predicts cancer outcome. An experimental design consisting of 48 patients with LABC was prospectively constructed and analyzed. All patients received neoadjuvant chemotherapy and definitive surgical therapy. Study homogeneity was maintained by standardized treatment, surveillance and compliance protocols. The S-TK1 concentration was detected using the anti-TK1 chicken IgY antibody, using a dot-blot immuno-assay. After a median follow-up of 30 months, the results indicated a statistically significant trend (unadjusted). Patients with high S-TK1 overexpression had a significantly higher incidence of recurrence (P=0.006) and cancer death (P=0.0128) than those with low S-TK1 overexpression. A multivariate analysis provided identical results. The hazards ratio for developing recurrence in patients with higher S-TK1 expression was 6-7 times higher than the hazards ratio in patients with lower expression. In conclusion, our results indicate that a high S-TK1 concentration in sera from LABC patients receiving neoadjuvant chemotherapy is predictive of cancer outcome.

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Introduction

Neoadjuvant chemotherapy is now the preferred approach for treating patients with inflammatory breast cancer and/or locally advanced breast carcinoma (LABC) (1,2). There are multiple advantages to this approach, including the down-staging of an inoperable cancer to an operable one, an increase in the availability of breast conservation for those patients otherwise required to undergo a mastectomy, and provision of *in vivo* testing of the efficacy of reduction in the primary tumor volume during treatment, a surrogate marker for a reduction in micrometastatic disease (3,4). Another advantage with the neoadjuvant approach is the short observation time for response to therapy when compared to adjuvant chemotherapy (5). Finally, neoadjuvant chemotherapy allows for the genetic profiling of tumors prior to treatment coupled with the subsequent assessment of the responsiveness of a particular chemotherapy regimen, thereby providing the potential for individualized therapy for patients with LABC (6). Despite these advantages when compared with adjuvant chemotherapy, neoadjuvant chemotherapy has not demonstrated significant survival advantages (4,7-11); regardless of treatment, the majority of patients with LABC usually succumb to these diseases.

Thymidine kinase (TK) is an enzyme in the pyrimidine salvage pathway and catalyzes the phosphorylation of thymidine monophosphate (12). There are two forms of TK: A cytosolic (TK1) and a mitochondrial form (TK2) (13). The level of TK1 is very low in non-proliferating cells but increases dramatically at late G1 to late S-phase/early G2 phase during the cell cycle in proliferating cells and tumor cells. This makes TK1 a noteworthy marker for cell proliferation and tumor growth. In patients with malignancies, >95% of the TK1 activity in serum (S-TK1) is derived from malignant cells (14). Thus, S-TK1 should be a good marker for tumor cell proliferation. S-TK1 activity has been used to monitor the extent of tumor metastasis and prognosis in patients with acute leukemia, chronic leukemia, Hodgkin's and non-Hodgkin's disease, bladder carcinoma, and cervical carcinoma (15-21). Yet, no study to date has examined the significance of S-TK1 as a prognostic indicator for LABC patients who have undergone

neoadjuvant chemotherapy. Therefore, we sought to determine whether a high S-TK1 level in cancer specimens retrieved from LABC patients receiving neoadjuvant chemotherapy is an independent predictor of poor outcome.

Materials and methods

Patients. A total of 48 patients with LABC were recruited. None of the patients had inflammatory breast cancer. Treatment and surveillance protocols were standardized to ensure study homogeneity. Compliance with treatment and surveillance protocol was 95 and 99%, respectively. All patients underwent standard treatment protocol for neoadjuvant chemotherapy. The majority of patients received four cycles of cyclophosphamide and epirubicin, followed by four cycles of docetaxel. Surgical treatment consisted of either a modified radical mastectomy or breast conservation therapy (BCT) (lumpectomy with tumor-free margin, axillary node dissection, and breast irradiation). Adjuvant axillary irradiation, systemic chemotherapy, and anti-estrogen therapy were offered and administered as indicated according to the current standard of care. Surveillance protocol consisted of a history and physical examination every 3 months during the first year, every 6 months during the second year and annually thereafter. Annual chest X-ray, mammogram, complete blood count, and liver function test were obtained. Any additional radiological and/or histological evaluation was performed based on the patient's examination and history. Clinical data were accrued and recorded prospectively and included age at diagnosis, comorbid conditions, stage of disease, treatment protocol, surveillance protocol compliance, and study endpoints. Study primary endpoints were cancer recurrence and cancer-related death. Serum samples were obtained from the patients at the following time points: prior to neoadjuvant chemotherapy, after each cycle of neoadjuvant chemotherapy, after surgery, after each cycle of adjuvant chemotherapy, and 3, 6, 12 and 24 months post-adjuvant chemotherapy. For logistical reasons, 48 patients were analyzed at the time point before neoadjuvant chemotherapy, before surgery, before adjuvant chemotherapy and 3 months after adjuvant chemotherapy.

The characteristics of the patients are shown in Table I. During the follow-up, a total of 11 patients developed distant recurrence, while 7 patients developed loco-regional recurrence within 3 years of surgery. One patient developed distant recurrence after 2 years of surgery. The tumors were scored for patient age, ER and nodal status. The blood sera were stored at -80˚C. Sera from 15 healthy individuals were used as negative controls. At the time of analysis, the sera were thawed and immediately assayed for S-TK1. Informed consent was obtained from all patients, and the study was approved by the Committee on Research Ethics at Shenzhen University Hospital, China.

Assay for S-TK1. The concentration of S-TK1 was measured by enhanced chemiluminescence (ECL) dot blot assay provided by Sino-Swed Molecular Bio-Medicine Research Institute, Shenzhen, China. Briefly, 3 μl of serum sample was applied to a nitrocellulose membrane, in duplicate. The sera were probed with and without anti-TK1 chicken immunoglobin Y (IgY) antibody, the latter were used as negative controls. Sera from 13 healthy individuals were also used as negative controls. We also used anti-TK1 mouse immunoglobin G (IgG) monoclonal antibodies, with identical results. 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 area of the spots were equally defined by integration computer program of the GS-700 Imaging Densitometer. From the three different concentrations of TK1, a standard curve was created, permitting calculation of S-TK1, as pmol/l (pM). The accuracy of the assay was 4-6%. 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. Fig. 1 shows an example of the dot-blot and western blot analyses.

Table I. Patient characteristics.

	Number	Percent (%)
Mean age	49±8.5 years	
Median follow-up	28 months	
Surgical procedure		
BCT	2	4
MRM	46	96
Post-neoadjuvant T stage		
T0 (pCR)	3	6
T1	5	10
T2	24	50
T3	14	29
T4	2	5
Nodal status		
N0	13	27
N1	14	29
N2	12	25
N3	9	19
Receptor status		
ER^+	28	58
PR^+	24	50
$HER\text{-}2^+$	16	33

pCR, pathologic complete response; BCT, breast conservation therapy; MRM, modified radical mastectomy.

Assay for HER-2 expression. A positive HER-2 status was defined as a value ≥2, using FISH method.

Estrogen and progesterone receptor status. Estrogen receptor (ER) and progesterone receptor (PR) status was determined using immunohistochemical methods. Activity >10% was considered positive.

Statistical analysis. Statistical analyses were performed using SPSS software. Level of S-TK1, tumor size, tumor grade, nodal status, HER-2, ER and PR statuses were correlated using the samples t-test, Chi-square test and Spearman rank correlation.

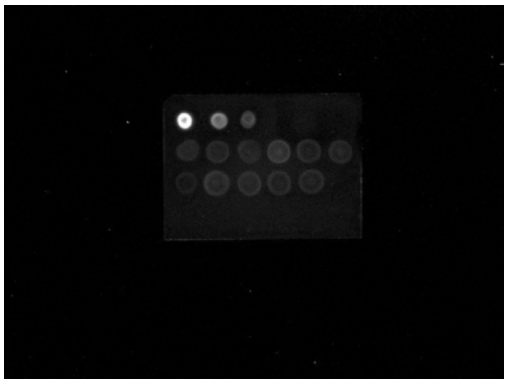

Figure 1. TK1 overexpression in LABC specimens. Note that TK1 overexpression was observed in varying degrees in breast cancer specimens.

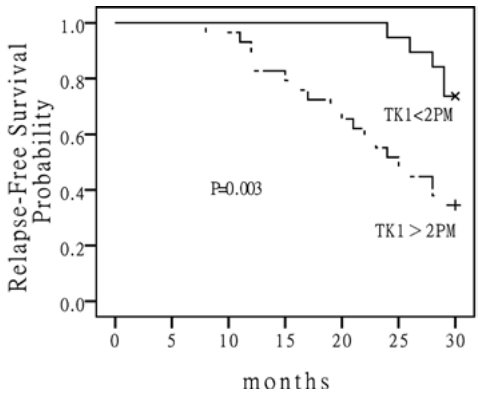

Figure 2. Influence of TK1 overexpression on disease-free survival for LABC patients who were treated with neoadjuvant chemotherapy. This Kaplan-Meier survival curve compares the disease-free survival, based on the degree of TK1 overexpression, in LABC patients who underwent neoadjuvant chemotherapy. Note that patients whose tumors had a high TK1 concentration in their serum had a worse disease-free survival when compared to those whose tumors had low serum TK1 concentration (P=0.003).

Survival analysis was performed using the Kaplan-Meier method, and the log-rank test was used to compare the curves, and Cox proportional hazard regression models were applied for multivariate analysis. Risk ratios and 95% confidence intervals (CI) were calculated from the model. A P-value ≤0.05 was considered statistically significant.

Results

Forty-eight patients were investigated for this study. The mean age at diagnosis was 49 years, and the mean follow-up was 28 months. Due to the advanced nature of the disease, the majority of patients (46 patients) underwent a modified radical mastectomy. There were 22 patients who developed recurrent disease, of which 18 patients (81.8%) had distant disease. The median disease-free survival (DFS) and overall survival (OS) were 38 months for each. Note that the concentration of S-TK1 was observed in varying degrees in breast cancer specimens. Based on our previous study (20), we used 2.0 as our cut-off value. Patients were distributed into two groups: a low S-TK1 group (<2.0 pM, n=19 patients) and a high S-TK1 group (≥2.0 pM, n=29 patients).

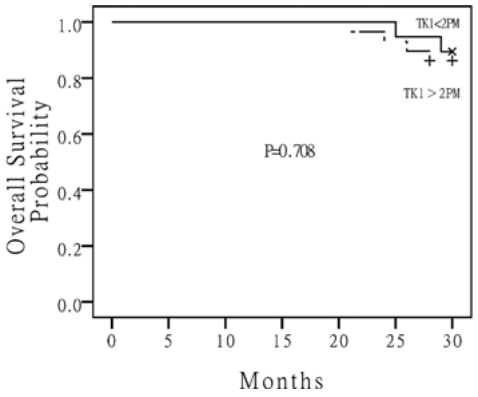

Figure 3. Influence of TK1 overexpression on overall survival for LABC patients who were treated with neoadjuvant chemotherapy. This Kaplan-Meier survival curve compares the overall survival, based on the degree of TK1 overexpression, in LABC patients who underwent neoadjuvant chemotherapy. Note that patients whose tumors had high TK1 concentration in serum had a relatively worse overall survival when compared to those whose tumors had low TK1 concentration although there were no significant differences.

The breakdown of patients as grouped by tumor size (T stage) and nodal status (N) following neoadjuvant chemotherapy is shown in Table I. The T stage distribution was as follows: T0 lesions (n=3), T1 lesions (n=5), T2 lesions (n=24), T3 lesions (n=14), and T4 lesions (n=2). There were 35 node-positive patients and 13 node-negative patients. The N stage distribution was as follows: N0=13 patients, N1=14 patients, N2=12 patients and N3=9 patients.

To assess the robustness of our sample set, we evaluated outcome with known traditional prognostic markers. Among traditional clinicopathologic factors, nodal status was the strongest predictor of outcome; patients who had a high number of positive pathological nodes after neoadjuvant chemotherapy had a worse DFS (P=0.006) and OS (P=0.036) than those who had none or minimal nodal disease. These results indicate that our sample size was adequate.

Note that patients who had elevated S-TK1 levels in their serum had a higher rate of recurrence and cancer-related death when compared to those who had low S-TK1 levels. The 5-year DFS for the low S-TK1 group versus the high S-TK1 group were 56 and 21%, respectively (P=0.003) (Fig. 2). The median DFS had not been reached for the low S-TK1 group and was 23 months for the high S-TK1 group. The median OS for the low S-TK1 group and high S-TK1 group had not been reached (Fig. 3). There were no statistically significant differences between the two groups. Although a high S-TK1 concentration appeared to be predictive of outcome, we assessed whether S-TK1 was a covariant of nodal status. In other words, was a high S-TK1 level a surrogate marker of advanced nodal disease? There was no statistical correlation between the two variables (P=0.11), thus confirming that a high S-TK1 concentration was not a covariant of nodal status. Next, we evaluated whether a high S-TK1 concentration was correlated with any known clinicopathological factors such as tumor size, nodal status, estrogen and progesterone receptor status, and HER-2 receptor status. There were no correlations between the degree of S-TK1 concentration with tumor size (P=0.15), nodal status (P=0.11), ER status (P=0.63), PR status

Table II. TK-1 and cancer recurrence (Cox regression analysis).

	Score	Significance
High S-TK1	8.633	P=0.003
Tumor size	2.839	P=0.158
Estrogen receptor	1.6	P=0.206
Progesterone receptor	3.126	P=0.077
Nodal status	9.282	P=0.002
Her-2	1.030	P=0.310

High S-TK1 was an independent predictor of cancer recurrence in patients with locally advanced breast cancer.

Table III. TK-1 and cancer death (Cox regression analysis).

	Score	Significance
High S-TK1	0.140	P=0.708
Tumor size	2.011	P=0.156
Estrogen receptor	0.230	P=0.632
Progesterone receptor	0.556	P=0.456
Nodal status	4.099	P=0.043
Her-2	0.019	P=0.891

High S-TK1 was not an independent predictor of cancer death in patients with locally advanced breast cancer.

(P=0.45), or HER-2 status (P=0.89), thus suggesting that the S-TK1 concentration is an independent predictor of outcome.

Finally, to further strengthen our hypothesis that a high S-TK1 concentration in serum following neoadjuvant chemotherapy is a noval independent prognostic indicator of poor cancer outcome in patients who have LABC, we performed a Cox regression analysis to compare the relative risks of cancer recurrence (Table II) and cancer-related death (Table III) between S-TK1 and known clinicopathological factors. Note that for both DFS and cancer-related death, S-TK1 overexpression out-performed nodal status as a predictor of outcome. Patients whose serum had a high S-TK1 concentration following neoadjuvant chemotherapy had a higher risk of cancer recurrence compared with patients whose S-TK1 concentration was low (P=0.003). In comparison, patients who had evidence of nodal disease had a higher risk of cancer recurrence compared with patients who had no evidence of nodal disease (P=0.002).

Discussion

Patients with locally advanced breast cancer (LABC) are at risk of cancer recurrence and death. Neoadjuvant chemotherapy has become the mainstay treatment. Even with this approach, the 5-year survival rates remain disappointingly low, ranging between 20 and 55% (22-24). Irrespective of postoperative chemotherapy, outcome remains dismal due to the spread of metastases (24). Apart from nodal status and a complete pathological response (pCR), there are virtually no additional prognostic factors available, either clinicopathological or molecular biological, that can assist in identifying subgroups of patients at a heightened risk of cancer recurrence and death. Furthermore, if one were to consider that only 8 to 20% of all LABC patients achieve pCR following neoadjuvant chemotherapy, then the prognostic indication of the majority of LABC patients depends solely on nodal status. An additional discriminating factor independent of nodal status would greatly assist clinicians in identifying subgroups of high risk patients to be targeted for either more intensive and/or novel targeted therapy.

In general, prognostic factors are those that predict patient outcome regardless of the treatment administered, while predictive factors indicate responsiveness to a specific treatment (25). Past studies have yielded highly variable results on the utility of predictive factors to predict response to neoadjuvant chemotherapy (26-29). A previous study of 89 patients with LABC found that the recurrence score (RS) developed by Paik *et al* (30) was positively associated with the likelihood of a pCR (P=0.05) following neoadjuvant paclitaxel and doxorubicin. These findings suggest that the greatest benefits of chemotherapy are reserved for those LABC patients at the greatest risk of developing recurrence (27). Similarly, Hess *et al* (27) reported the utility of using a 30-probe genomic profile to identify patients who achieve pCR in response to paclitaxel, fluorouracil, doxorubicin, and cyclophosphamide neoadjuvant regimens. However, Sorlie *et al* (28), using a large-scale gene expression profile on 81 tumors of patients with LABC, were unable to demonstrate a convincing evidence that could reliably predict response to neoadjuvant regimens. Similarly, Tiezzi *et al* (22) evaluated 60 patients who received neoadjuvant docetaxel and epirubicin and found that there were no reliable molecular markers that could predict response to therapy. Finally, Piega *et al* (29) utilized a microarray-based comparative genomic hybridization technique using 44 cancer specimens and were unable to establish a correlation between DNA copy number changes and clinical response to doxorubicin and cyclophosphamide neoadjuvant chemotherapy.

While studies concerning predictive molecular markers for LABC are few, publications on prognostic molecular markers are even fewer. Most have concentrated on more traditional clinicopathologic prognostic features such as extent of nodal diseases, presence of inflammatory breast cancer, or poor pathologic response to neoadjuvant chemotherapy (31,32). Our study is unique in that we identified a molecular marker that is able to prognosticate outcome for patients with LABC.

The concentration of S-TK1 has been used as a serological tumor marker, particularly in leukemia and lymphoma and in breast cancer patients. We recently demonstrated its clinical utility in patients with HER-2-negative tumors, mainly in primary tumors indicating a poor outcome, independent of HER-2 status, ER/PR status, and nodal status (33). As an extension of this study, we evaluated the prognostic significance of S-TK1 in patients with LABC who received neoadjuvant chemotherapy. We found that, among patients with LABC, those who had a high S-TK1 concentration following neoadjuvant chemotherapy exhibited a poorer survival outcome than those who had a low S-TK1 concentration. Apart from predicting a significantly higher relative risk for cancer

recurrence (P=0.003), the concentration of S-TK1 also appeared to be a predictor of cancer-related death. Although a significant difference was not achieved between the high and low S-TK1 concentration in predicting cancer-related death (P=0.708), we proposed that if the period of follow-up had been prolonged, perhaps a significant outcome may have been achieved. These findings have tremendous importance as, to our knowledge, this report is the first to demonstrate that a single molecular marker is a predictor of outcome independent of nodal status, a factor that long has been held to be the strongest prognostic indicator of cancer outcome.

The clinical significance of this finding is that the activity of S-TK1 can be used as a molecular prognostic marker in addition to nodal status and pCR since it appears to be independent of HER-2 status, ER/PR status, tumor size, and nodal status. The lack of a correlation between S-TK1 concentration and HER-2 status remains an important observation. These findings appear to contradict a recent preclinical report that linked HER-2 expression to S-TK1 activity. It is plausible that although S-TK1 activity may be influenced by HER-2, there may be other stronger factors that control S-TK1 activity.

Although our dataset had only 48 patients, we believe that results from this dataset are reliable since we were able to verify that outcome was dependent on nodal status, a well-established prognosticator. We found that nodal status significantly influenced both disease-free survival and overall survival in these patients. Furthermore, comparable to other, larger series, our overall 5-year survival rates and the percentage of patients who had pCR were similar to theirs. Finally, our results, although retrospective, were based on a prospective database.

Although we are highly encouraged by these results, we are nevertheless cautious not to overstate their importance. The prognostic significance of S-TK1 for patients with LABC who have undergone neoadjuvant chemotherapy should be validated either by a future prospective clinical trial or by an independent database.

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