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# Determination of the specific activity of CDK1 and CDK2 as a novel prognostic indicator for early breast cancer

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**Background:** We recently established a novel assay for specific activity (SA) of cyclin-dependent kinases (CDKs) using small tumor samples (≥8 $mm^3$). The aim of this study was to investigate the prognostic significance of CDK1SA and CDK2SA in human breast cancer.

**Methods:** CDK1SA and CDK2SA were determined in 284 breast cancer patients and their prognostic significance was investigated.

**Results:** Tumors with high CDK1SA and high CDK2SA showed significantly poorer 5-year relapse-free survival than those with low CDK1SA and low CDK2SA, respectively (66.9% vs 84.2% for CDK1SA; 43.6% vs 83.6% for CDK2SA). Moreover, combined analysis of CDK1SA and CDK2SA enabled the classification of breast tumors into high-risk and low-risk groups, where tumors in the high-risk group were strongly associated with unfavorable prognosis (5-year relapse-free survival 69.4% for the high-risk group and 91.5% for the low-risk group). Multivariate analysis showed that the risk determined by combined analysis of CDK1SA and CDK2SA is a significant (hazard ratio 3.09, $P < 0.001$) prognostic indicator for relapse, especially in node-negative patients (hazard ratio 6.73, $P < 0.001$).

**Conclusion:** Determination of CDK1SA and CDK2SA may be useful in the prediction of outcomes in breast cancer patients and has potential for use as a routine laboratory test.

**Key words:** breast cancer, cycline dependent kinase, prognosis

article

## introduction

It is well established that systemic adjuvant therapy for early breast cancer significantly reduces the risk of recurrence and death regardless of nodal status [1, 2]. However, the fact that approximately two-thirds of node-negative patients can survive without recurrence even without adjuvant therapy indicates that adjuvant therapy is administered to many patients who actually do not need it. To avoid unnecessary treatments, we need new and more powerful prognostic indicators [3, 4].

Recently, molecules involved in cell cycle regulation such as cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors have been attracting considerable attention as potential prognostic indicators [4–6]. Cyclin E appears to be the most promising of these molecules. High cyclin E expression detected by western blotting has been shown to be strongly associated with unfavorable prognosis, independent of nodal status [5]. However, it is not easy to reproducibly assay total cyclin E or low molecular weight cyclin E expression by western blotting, which does not seem to be suitable for routine laboratory tests.

We have been focusing on CDKs (CDK1 and 2) and investigating their prognostic significance in breast cancers because CDKs play a pivotal role in cell cycle regulation [7, 8]. The CDK expression levels are almost constant but their activities change markedly according to the cell cycle phase. Thus, it is necessary to measure CDK activity itself to accurately evaluate the role of CDKs in cell proliferation. Recently, we succeeded in developing a system that can assay the specific activity (SA) of CDKs using small tissue samples [9]. The aim of this study was to clarify the prognostic implications of CDKSA in breast cancers.

## patients and methods

### patients

For this study, 284 patients with primary invasive breast cancer who had undergone mastectomy or breast-conserving surgery between November 1996 and December 2002 were recruited. Of these 284 patients, 162 patients were given hormonal therapy (tamoxifen alone, 124; tamoxifen plus luteinizing hormone-releasing hormone analog, 31; other modalities, 7), 37 patients underwent chemotherapy (cyclophosphamide, methotrexate and 5-fluorouracil [CMF], 16; cyclophosphamide plus epirubicin [CE], 19; other modalities, 2) and 61

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patients received chemohormonal therapy (CMF plus tamoxifen, 17; CE plus tamoxifen, 25; other modalities, 19).

The median follow-up period was 56.6 (8–89) months, and the relapse-free survival rate at 5 years after surgery (5yRFS) was 80.9%. Forty-nine patients developed recurrence (liver, 6; lung, 9; bone, 11; soft tissue, 23). Ipsilateral breast recurrences after breast-conserving surgery were not counted as recurrences.

### assay for CDKSA

The assay of CDKSA consists of analyses of protein expression and kinase activity, as previously described [9]. In brief, lysates of frozen tissues were prepared with a homogenizer and stored at −80 °C until use. For expression analysis, the lysate was applied to an ImmobiChip (Sysmex, Kobe, Japan). The target protein was detected by sequential reactions with primary antibodies (anti-CDK1, anti-CDK2 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH); Sysmex, Kobe, Japan), biotinylated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and fluorescein-labeled streptavidin (Vector, Burlingame, CA). For kinase activity analysis, the CDK1 or CDK2 molecules in the lysate were first captured in a mini-column coupled with anti-CDK1 or anti-CDK2 antibody. Then an in-column kinase reaction and a fluorescein labeling reaction were performed sequentially, and the final reaction mixture was applied to the ImmobiChip. For quantification of both CDK expression and activity, catalytically active recombinant CDK1 or CDK2 (Upstate Biotechnology, Lake Placid, NY) was used as a standard. The CDKSAs were then calculated as kinase activity (U/μL lysate, where 1 U is equivalent to the activity of 1 ng of standard) divided by its corresponding expression (ng/μL lysate). The cut-off values for CDK1SA, CDK2SA and CDK2SA/CDK1SA ratio were defined as the points that gave the best discrimination in RFS. The optimal cut-off points were 100 U/ng for CDK1SA, 800 U/ng for CDK2SA and 5.6 for CDK2SA/CDK1SA. The distribution of breast tumors according to CDK1SA and CDK2SA is shown in Figure 1.

### assay for human epidermal growth factor receptor type 2 expression

HER2 expression was examined by HercepTest (DakoCytomation, Carpinteria, CA) in 195 patients and by western blotting in 87 patients whose primary tissues were not available for HercepTest. The insoluble membrane fraction of the lysate for CDKSA assay was solubilized by RIPA buffer-supplemented protease inhibitor cocktail (SIGMA-Aldrich, St Louis, MO). The resultant supernatant was electrophoresed followed by transfer to PVDF membrane. After blocking, the membrane was treated with polyclonal anti-HER2 antibody (Upstate Biotechnology, Lake Placid, NY), biotinylated anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and Alexa-Fluor488-streptavidin (Molecular Probes, Eugene, OR). Fluorescent signal intensities of HER2 were measured and normalized to GAPDH expression. HER2 expression was classified as negative, 1+ or 2+. A high concordance (82%) between score 3+ of HercepTest and 2+ of the western blotting was confirmed (data not shown), and both were defined as HER2-positive.

### statistical methods

RFS was calculated with the Kaplan–Meier method, and the differences were assessed with the log-rank test. The Cox proportional hazards model was used for both univariate and multivariate analyses. Test results were considered significant for $P \leq 0.05$.

## results

### relationship of various clinicopathologic parameters or CDK1/2SA with prognosis

The relationship of various clinicopathologic parameters with 5yRFS is shown in Table 1. Lymph node metastases, high histologic grade, estrogen receptor (ER) negativity, progesterone receptor (PR) negativity and HER2 positivity were significantly associated with poor 5yRFS. With respect to

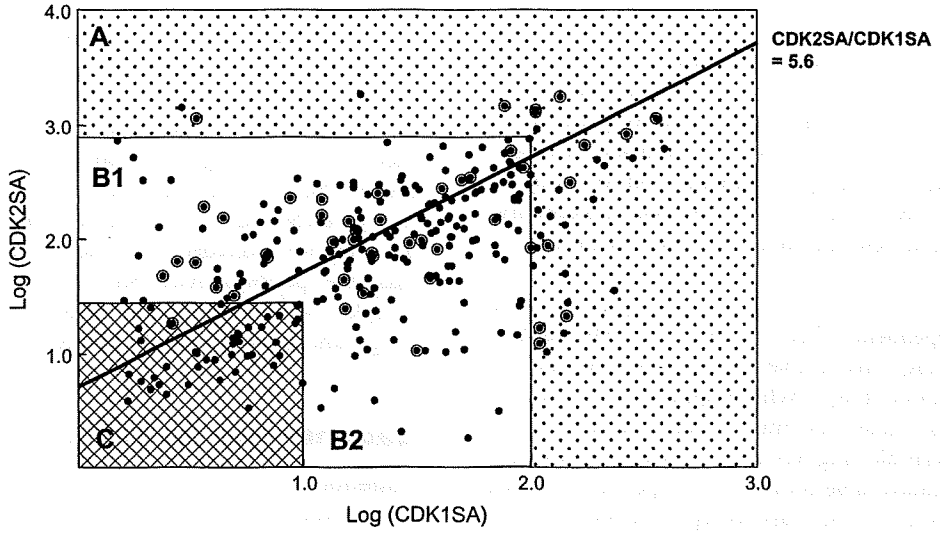


**Figure 1.** Distribution of breast tumors according to CDK1SA and CDK2SA. Tumors ($n$ = 284) are plotted in two dimensions (logarithmic scales) according to CDK1SA and CDK2SA. Area A includes tumors with high CDK1SA (>100 U/ng) and/or high CDK2SA (>800 U/ng) ($n$ = 37). Area C includes tumors where both CDK1SA and CDK2SA are less than lower measurement limits ($n$ = 33). The remaining tumors are divided into two groups (B1 [$n$ = 85] and B2 [$n$ = 129]) according to the CDK2SA to CDK1SA ratio, with a cut-off at 5.6. Tumors in areas A and B1 are considered to be high-risk for relapse (CDK-based high-risk group) and those in areas B2 and C to be low-risk (CDK-based low-risk group). Tumor without relapse; ●, tumor with relapse; ◉.

**Table 1.** Association between tumor parameters and 5-year RFS in all patients ($n = 284$)

| Parameters | Category | No. of patients ($n = 284$) | 5yRFS (%) | $P$ value* |
|---|---|---|---|---|
| Age | <50 years | 113 | 84.1 | 0.546 |
| | ≥50 years | 171 | 80.2 | |
| Tumor size | ≤2.0 cm | 118 | 85.5 | 0.082 |
| | >2.0 cm | 166 | 78.7 | |
| Lymph node status | Negative | 178 | 87.7 | 0.0006 |
| | Positive | 105 | 70.7 | |
| Histologic grade | 1 | 76 | 89.3 | 0.018 |
| | 2 + 3 | 206 | 78.6 | |
| ER[a] | Positive | 167 | 85.7 | 0.009 |
| | Negative | 111 | 76.2 | |
| PR[a] | Positive | 165 | 86.0 | 0.007 |
| | Negative | 113 | 75.7 | |
| HER2 | Negative | 247 | 82.4 | 0.028 |
| | Positive | 32 | 62.7 | |
| CDK1SA | Low | 251 | 84.2 | 0.004 |
| | High | 33 | 66.9 | |
| CDK2SA | Low | 273 | 83.6 | <0.0001 |
| | High | 11 | 43.6 | |
| CDK2SA/CDK1SA ratio | Low | 187 | 88.8 | 0.0001 |
| | High | 97 | 68.7 | |
| CDK-based risk[b] | Low | 162 | 91.5 | <0.0001 |
| | High | 122 | 69.4 | |

*$P$ value was evaluated by the log-rank test and was considered significant for $P \leq 0.05$.

[a]Estrogen receptor (ER) and progesterone receptor (PR) levels in tumors were measured with an enzyme immunoassay. The respective cut-off values for ER and PR were 13 and 10 fmol/mg protein.

[b]CDK-based risk was determined by the combination of CDK1SA and CDK2SA. CDK-based low-risk group was composed of patients with tumors showing both CDK1SA and CDK2SA less than lower measurement limits (area C in Figure 1) and those with a low ratio of CKD2SA/CDK1SA (area B2 in Figure 1). The CDK-based high-risk group was composed of patients with tumors showing high CDK1SA and/or high CDK2SA (area A in Figure 1) and those with a high ratio of CKD2SA/CDK1SA (area B1 in Figure 1).

HER2, Human Epidermal Growth Factor Receptor Type 2.

CDKSAs, patients with high CDK1SA and high-CDK2SA tumors showed a significantly lower 5yRFS than those with low CDK1SA and low-CDK2SA tumors, respectively. Moreover, patients with tumors with a high CDK2SA/CDK1SA ratio showed a significantly lower 5yRFS than those with tumors with a low CDK2SA/CDK1SA ratio.

Next, we studied the relationship of the combination of CDK1SA and CDK2SA with prognosis. Patients with high CDK1SA and/or high-CDK2SA tumors (area A in Figure 1) showed a poor prognosis (5yRFS rate 60%), whereas patients with tumors where both CDK1SA and CDK2SA were less than lower measurement limits (area C in Figure 1) were unlikely to develop recurrent diseases (5yRFS rate 96%). The remaining patients were able to be divided into the high- and low-risk groups according to the CKD2SA/CDK1SA ratio; that is, patients with tumors with a high CKD2SA/CDK1SA ratio (area B1 in Figure 1) were at high risk of relapse (5yRFS rate 73%) and those with a low CKD2SA/CDK1SA ratio (area B2 in Figure 1) were at low risk of relapse (5yRFS rate 91%). Accordingly, using the combination of CDK1SA and CDK2SA, all patients could be classified into a CDK-based low-risk group (area B2 and C in Figure 1) and a CDK-based high-risk group (area A and B1 in Figure 1). Patients in the CDK-based high-risk group showed a significantly lower 5yRFS than those in the CDK-based low-risk group (Table 1 and Figure 2A).

The prognostic impacts of various markers were evaluated by univariate and multivariate analyses (Table 2). In the univariate analysis, lymph node status, histologic grade, ER, PR, HER2 and CDK-based risk were significantly associated with relapse. In the multivariate analysis, however, only lymph node status and CDK-based risk had a significant correlation with relapse (hazard ratio 2.22 and 3.09, respectively).

*CDK1/2SA and clinicopathologic parameters.* The relationship of CDK-based risk with clinicopathologic parameters was evaluated with the chi-square test. CDK-based high risk showed a significant association with large tumor size ($P = 0.035$), lymph node involvement ($P = 0.046$), high histologic grade ($P = 0.0008$) and PR negativity ($P = 0.004$), but no significant association with ER ($P = 0.362$) and HER2 status ($P = 0.118$).

*CDK1/2SA and prognosis according to nodal status.* In both node-negative and node-positive subsets, patients in the CDK-based high-risk group showed a significantly lower 5yRFS than those in the CDK-based low-risk group (node-negative, 72.6% vs 97.8%; node-positive, 61.0% vs 79.0%) (Figure 2B and 2C).

In the node-positive group, univariate analysis showed that the number of metastatic lymph nodes, ER status and CDK-based risk were significantly associated with relapse, whereas multivariate analysis showed only that the number of metastatic lymph nodes and ER status were significant prognostic indicators for relapse (data not shown). In the node-negative group, univariate analysis showed that the CDK-based risk had a significant association with relapse, and that the histologic grade and PR status had a tendency to be associated with relapse. The multivariate analysis demonstrated that only CDK-based risk is a significant independent prognostic indicator (hazard ratio 6.73).

## prognostic factors for node-negative patients receiving hormonal therapy alone

Of 178 node-negative patients, 139 (78%) patients received hormone therapy alone as adjuvant therapy, and 14 of these 139 patients developed recurrences. Neither histologic grade nor the St Gallen's criteria [10], widely used as the risk classification especially for node-negative patients, showed a significant association with relapse in these 139 patients (Figure 2D and 2E). However, patients in the CDK-based high-risk group showed a significantly lower

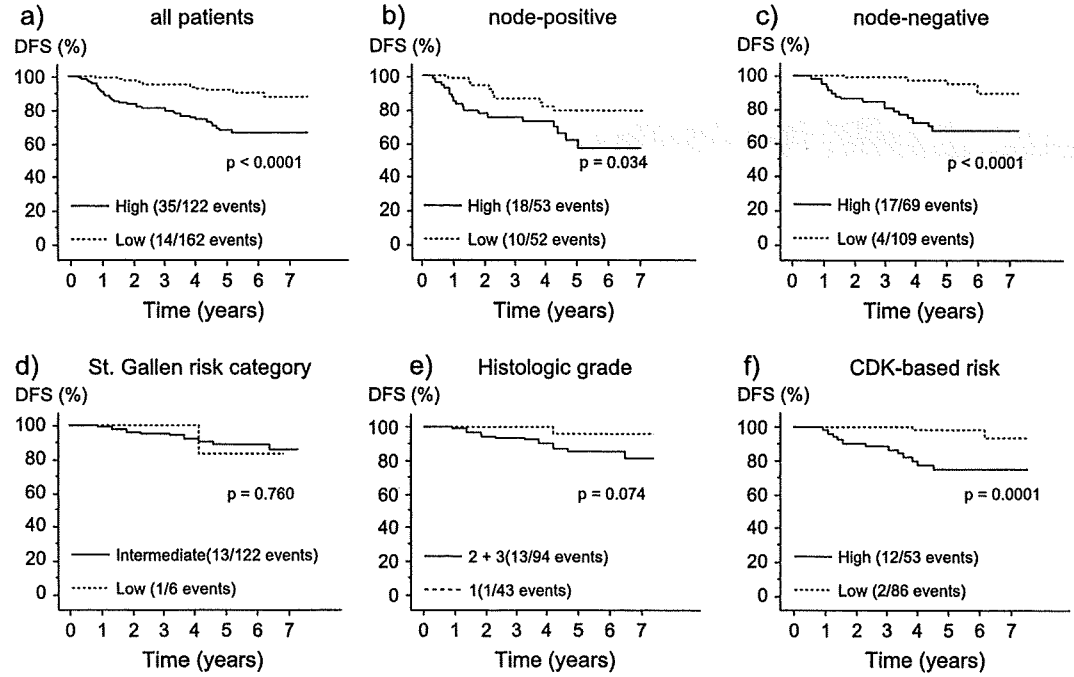


**Figure 2.** Relapse-free survival (RFS) rates according to the CDK-based risk and St Gallen's risk categorization. In (A) all, (B) node-positive and (C) node-negative patients, CDK-based high risk was strongly associated with poor prognosis compared to CDK-based low risk. In node-negative patients receiving hormone therapy alone as systemic adjuvant therapy, risk classification according to (D) St. Gallen risk category (2005 version) and (E) histologic grade failed to show a significant difference in RFS. (F) CDK-based risk was able to classify these patients into the high- and the low-risk groups, and their 5-year RFS rates were 74.9% vs 98.4%, respectively ($P = 0.0001$).

**Table 2.** Univariate and multivariate analyses for relapse in all patients ($n = 284$)

| Parameter | | Univariate | | | Multivariate | | |
|---|---|---|---|---|---|---|---|
| | | Hazard ratio | 95% CI* | *P* value | Hazard ratio | 95% CI* | *P* value |
| Age | < 50 vs ≥ 50 years | 0.84 | 0.47–1.50 | 0.546 | | | |
| Tumor size | >2.0 vs ≤2.0 cm | 1.70 | 0.93–3.13 | 0.086 | | | |
| Lymph node status | Positive vs negative | 2.59 | 1.47–4.56 | 0.001 | 2.22 | 1.24–3.95 | 0.007 |
| Histologic grade | 2 + 3 vs 1 | 2.69 | 1.15–6.32 | 0.023 | 1.88 | 0.79–4.48 | 0.155 |
| ER | Negative vs positive | 2.10 | 1.19–3.71 | 0.011 | | | |
| PR | Negative vs positive | 2.15 | 1.22–3.81 | 0.009 | 1.50 | 0.83–2.72 | 0.181 |
| HER2 | Positive vs negative | 2.21 | 1.07–4.59 | 0.033 | 1.87 | 0.87–3.99 | 0.108 |
| CDK-based risk | High vs low | 3.93 | 2.11–7.32 | <0.0001 | 3.09 | 1.64–5.82 | 0.0005 |

*CI, confidence interval; HER2, Human Epidermal Growth Factor Receptor Type 2.

5yRFS than those in the CDK-based low-risk group (74.9% vs 98.4%, $P = 0.0001$) (Figure 2F).

## discussion

In this study, we applied our novel assay system to breast cancers to find out whether determination of CDK1SA and CDK2SA could be useful for the prediction of patient outcomes. Although a high CDK1SA, a high CDK2SA and a high CDK2SA/CDK1SA ratio were significantly associated with a poor prognosis, the combination of these parameters (the CDK-based risk) has been found to predict patients' outcomes more accurately than each parameter alone. Multivariate analysis demonstrated that CDK-based risk was a significant prognostic indicator. More importantly, CDK-based risk was a highly significant and independent prognostic indicator for node-negative breast cancers.

The strength of this new indicator, CDK-based risk, is that it classified as many as 61% (109/178) of node-negative patients into the low-risk group where the RFS is extremely good, and the remaining 39% (69/178) into the high-risk group where the RFS is so low as to be equivalent to that seen in patients with one lymph node involvement [11]. This excellent capability for differentiation of the CDK-based risk sharply contrasts with that of St Gallen's risk classification of node-negative breast cancers. The latter categorized only 5% (8/166) of our subjects into the low-risk group, where recurrence was observed in 13% (1/8), and the remaining

95% (158/166) into the intermediate risk group, where recurrence was also observed in 13% (20/158).

We have focused on node-negative patients treated with hormonal therapy alone as systemic adjuvant therapy because this group represents the majority of node-negative cancers and includes some patients with unfavorable prognosis. For these patients, only the CDK-based risk was of significant use for the prediction of their prognosis (5yRFS 74.9% vs 98.4%). These findings seem to indicate that adjuvant hormonal therapy alone is under-treatment for node-negative and hormone receptor-positive patients with tumors belonging to the CDK-based high-risk group, who need chemotherapy in addition to hormonal therapy. By contrast, adjuvant hormonal therapy alone is an appropriate treatment for those in the CDK-based low-risk group. These preliminary findings obtained with a limited number of patients need to be confirmed in a future study including a larger number of patients.

Both CDK1 and CDK2 are considered to play an important role in cell proliferation and are expected to be associated with tumor aggressiveness and a poor prognosis [7, 8, 12, 13]. However, the prognostic impact of CDK1 in breast cancers still remains controversial [13–15]. Interestingly, some recent studies have shown that CDK1 may be required for apoptosis that is independent of the regulation of the cell cycle [16, 17]. Uncontrolled CDK1 activation might work as a brake for cancer cell growth in some tumors. Our present study has shown that a high ratio of CDK2SA to CDK1SA is associated with a poor prognosis and a low ratio is associated with a favorable prognosis. Although the real biological meaning of this ratio is still unclear, implication of CDK1 in apoptosis might partially explain why a low ratio of CDK2SA to CDK1SA is associated with a favorable prognosis. Several in vitro studies to clarify the biological meaning of this ratio are in progress in our laboratory.

Our results have demonstrated that tumors in the CDK-based high-risk group showed a significant association with unfavorable clinicopathologic features, such as high histologic grade, large tumor size, lymph node metastases and negative PR. CDK-based risk has a particularly strong association with histologic grade, suggesting that CDK-based risk may reflect the cell proliferation. It is well established that rapidly proliferating tumors are associated with a malignant potential to metastasize [4]. In fact, various parameters associated with cell growth have been identified as having the capability to serve as prognostic indicators in breast cancers. These parameters include mitotic index, DNA flow cytometry, $^{3}$H-thymidine/5-bromo-2′-deoxyuridine uptake and Ki-67 antigen immunohistochemistry [18, 19]. The main problem inherent in these methods is that they are of a subjective nature with significant inter-observer or inter-assay variations, and are thus too difficult to standardize for use in routine laboratory tests. By contrast, determination of CDK1SA and CDK2SA can be accomplished with a well-standardized method ready for use in laboratory tests [9]. Another strength of CDK1SA and CDK2SA assay is that it needs only a very small sample (minimum 8 mm$^{3}$).

In conclusion, we have shown that CDK-based risk determined by evaluating CDK1SA and CDK2SA is strongly associated with clinical outcome especially for node-negative breast cancer patients. We consider that the CDK-based risk has potential as a new prognostic factor independent of the conventional risk factors, and as a routine laboratory test. However, our results need to be validated in a study with a larger number of patients on a multicenter basis.

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# Association of GSTP1 expression with resistance to docetaxel and paclitaxel in human breast cancers

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**Abstract**

*Aims*: It has been reported that glutathione S-transferase P1 (GSTP1) expression is implicated in resistance to taxanes (docetaxel and paclitaxel) in human breast cancer cells in vitro. In the study presented here, we examine whether GSTP1 expression is associated with resistance to docetaxel or paclitaxel in human breast cancers. We also investigated the relationship between GSTP1 methylation status and response to these taxanes.

*Material and methods*: Sixty two primary breast cancer patients were treated with docetaxel or paclitaxel as primary systemic treatment (PST). GSTP1 expression was detected immunohistochemically and the hypermethylation status GSTP1 gene was identified with a methylation specific primer assay.

*Results*: The mean tumor reduction rate for all patients ($n = 62$) was significantly ($p < 0.001$) higher in GSTP1 negative ($0.73 \pm 0.04$; mean $\pm$ standard error) than GSTP1 positive ($0.31 \pm 0.09$) tumors. The subset analysis showed that the mean reduction rate was significantly ($p = 0.005$) higher in GSTP1 negative ($0.59 \pm 0.06$) than GSTP1 positive ($0.11 \pm 0.13$) tumors in the docetaxel group as well as in the paclitaxel group ($p = 0.006$; GSTP1 negative tumors: $0.84 \pm 0.05$; GSTP1 positive tumors: $0.56 \pm 0.08$). On the other hand, GSTP1 methylation showed no significant association with the reduction rate.

*Conclusion*: Our present study has suggested that GSTP1 protein expression, but not GSTP1 methylation status, might be associated with response to docetaxel and paclitaxel. This suggests that GSTP1 immunohistochemical expression might be a potentially clinically useful predictive factor for response to docetaxel and paclitaxel.


*Keywords:* GSTP1; Docetaxel; Paclitaxel; Primary systemic treatment; Breast cancer

## Introduction

Taxanes including docetaxel and paclitaxel are some of the most effective anticancer drugs for breast cancer. A growing number of breast cancer patients have recently been treated with taxanes not only in the metastatic setting but also in the adjuvant and neoadjuvant settings. Since the response rate to taxanes ranges from 22.9% to 43%[1–3] and not all patients benefit from taxane therapy, it is very important to select those patients who are likely to respond to taxanes in order to avoid unnecessary treatment. For this purpose, a reliable predictive factor for response to taxanes in human breast cancers needs to be developed.

Glutathione S-transferase P1 (GSTP1) belongs to a family of phase II metabolic enzymes that can detoxify several anticancer drugs by conjugating them with glutathione.[4] GSTP1 is therefore thought to confer resistance to chemotherapy. In fact, several studies have reported on the impact of GSTP1 expression on response to chemotherapy in breast tumor tissues.[5–8] Su et al. found that breast tumors with GSTP1 expression are resistant to anthracycline-containing chemotherapy in the neoadjuvant setting.[8] Huang et al. reported that patients with GSTP1 positive breast tumors showed a poorer prognosis than those with GSTP1 negative breast tumors when all the patients were

*Abbreviations:* ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor 2.

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treated with adjuvant chemotherapy, consisting of 49% anthracycline-containing regimen,[7] which suggests that GSTP1 can serve as a predictive factor for resistance to anthracycline-containing chemotherapy. Although contradictory results have also been reported,[9] a vast majority of the studies appear to indicate a significant relationship between GSTP1 expression and resistance to anthracycline-containing chemotherapy.[10]

On the other hand, the relationship between GSTP1 expression and response to taxanes has rarely been the subject of clinical studies of breast tumors. Recently, we were able to show that transfection of the GSTP1 expression vector into a human breast cancer cell line (MCF-7 cells) results in the acquisition of resistance to docetaxel.[11] Moreover, Mathieu et al. used an orthotopic model of a human non-small cell lung carcinoma cell line (A549 cells) to show that GSTP1 overexpression is associated with resistance to paclitaxel.[12] These results seem to suggest that GSTP1 plays a definite role in the acquisition of resistance to taxanes and that the identification of GSTP1 expression may thus be clinically useful. We therefore attempted to clarify the relationship between immunohistochemically identified GSTP1 expression and response to taxanes (docetaxel and paclitaxel) in the neoadjuvant setting. We also studied the GSTP1 methylation status and its association with response to taxanes since GSTP1 expression is often silenced by DNA promoter hypermethylation.

## Materials and methods

### *Patients*

Sixty two primary breast cancer patients (stage II, $n = 20$; stage III, $n = 35$; stage IV, $n = 7$) who were treated with docetaxel or paclitaxel monotherapy as primary systemic therapy (PST) at Osaka University Hospital between December 1999 and November 2005 were recruited for this study. Tumor tissue samples were obtained from primary breast tumors by means of core needle biopsy or vacuum-assisted core biopsy before PST. All patients were histologically diagnosed as invasive breast cancer, and no patients had been treated with chemotherapy and/or hormonal therapy before biopsy.

After informed consent had been obtained from all patients, 31 were treated with docetaxel (60 mg/m$^2$ every 3 weeks for 4 cycles), and the other 31 were treated with paclitaxel (80 mg/m$^2$ every week for 12 weeks) as PST. Of these 62 patients, 35 patients received anthracycline-containing chemotherapy after docetaxel ($n = 5$) or paclitaxel ($n = 30$), and all patients underwent breast conservative surgery or mastectomy after PST.

### *Assessment of clinical response*

Bi-dimensional (cm$^2$) breast tumor measurements were made before and after taxane (docetaxel or paclitaxel) treatment and mostly with MRI since we have shown that MRI is the most accurate modality for measuring breast tumor size.[13] Reduction rate was calculated as follows: (area before taxane − area after taxane)/area before taxane.

### *Immunohistochemical assay*

A total of 3 μm sections were cut and placed on silanized slides (DakoCytomation Inc., Carpinteria, CA). After dewaxing of the sections, endogenous peroxidase activity was inhibited with freshly prepared 0.5% hydrogen peroxide in distilled water for 10 min. The sections were then immediately incubated at 95 °C in citrate buffer (pH 6) for 15 min. Immunostaining was performed by using an immunoperoxidase method according to the manufacturer's instructions (EnVision+ Dual Link System Peroxidase; DakoCytomation Inc.). The incubation of the primary rabbit anti-GST-Pi polyclonal antibody (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) was performed overnight at 4 °C at a dilution of 1:1000 in 1% BSA in PBS. After incubation, the secondary antibody was added for 45 min to amplify the specific binding of the primary antibody. The sections were developed with a peroxidase substrate solution (0.05% 3,3-diaminobenzidine tetrahydrochloride, 0.01% $H_2O_2$ in PBS), counterstained with hematoxylin, dehydrated, and mounted. Appropriate control slides, positive and negative cases, were included in each series. Expression was considered to be positive when >10% of the tumor cells exhibited cytoplasmic or nuclear staining.[7]

### *Sodium bisulfite treatment*

Genomic DNA was extracted from frozen tumor specimens with the phenol/chloroform method. Sodium bisulfite conversion of 2 μg of genomic DNA was performed with a modified version of a method as described previously.[14] Briefly, DNA was denatured with 0.2 M NaOH and incubated for 30 min at 37 °C. A volume of 520 μl of freshly made bisulfite solution [3 M sodium metabisulfite and 10 mM hydroquinone (pH = 5.0)] was added to each sample, and the mixture was then incubated at 55 °C for 16 h in the dark. Modified DNA was purified using Wizard DNA purification resin according to the manufacturer's instructions (Promega Corp., Madison, WI) and eluted into 50 μl of water. Modification was completed by NaOH (final concentration: 0.3 M) treatment for 20 min at 37 °C, followed by ethanol precipitation. DNA was resuspended in water and used immediately or stored at −20 °C.

### *Methylation specific PCR (MSP) assay*

For PCR amplification, 1 μl of bisulfite-modified DNA was added to a final volume of 20 μl PCR mix containing 1 × PCR buffer [18 mM ammonium sulfate, 60 mM Tris (pH 8.9)], deoxynucleotide triphosphates (0.2 mM each), 1 unit Platinum TaqDNA polymerase (Invitrogen, Carlsbad,

CA), and primers. The primer sequences for GSTP1 for the unmethylated reaction were 5′-GAT GTT TGG GGT GTA GTG GTT GTT-3′ (upper primer) and 5′-CCA CCC CAA TAC TAA ATC ACA ACA-3′ (lower primer) and for the methylated reaction 5′-TTC GGG GTG TAG CGG TCG TC-3′ (upper primer) and 5′-GCC CCA ATA CTA AAT CAC GAC G-3′ (lower primer). PCR amplifications were carried out under the following conditions: 1 cycle at 95 °C for 5 min, and 38 cycles at 95 °C for 1 min, 64 °C for 1 min, and 72 °C for 1 min. The final extension was performed for 5 min at 70 °C.[15] DNA from MCF-7 breast cancer cells was used as a positive control for methylated alleles, and DNA from normal lymphocytes as a negative control for methylated genes.[16] PCR reactions were analyzed with 3% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV illumination.

*Statistical analysis*

The relationship between GSTP1 expression and clinicopathological parameters were examined by $\chi^2$ test or Fisher's exact test. Correlations between quantitative reduction rate and GSTP1 expression or methylation status were evaluated with the Mann–Whitney *U*-test. Statistical significance was defined as $p < 0.05$.

## Results

*GSTP1 expression and clinicopathological parameters of breast cancers*

Of the 62 breast tumors, 35 were found to be GSTP1 positive. There was a significant ($p < 0.05$) association between GSTP1 positive expression and large tumor size or clinical cancer stage, as well as between GSTP1 methylation and GSTP1 negative expression. No other clinicopathological parameters, such as age, menopausal status, histological grade, ER, PR, HER-2, were significantly associated with GSTP1 expression.

*Relationship between GSTP1 expression and response to docetaxel or paclitaxel*

For all the patients ($n = 62$) treated with docetaxel or paclitaxel, the mean reduction rate ($0.73 \pm 0.04$) in the GSTP1 negative tumors was significantly higher than that ($0.31 \pm 0.09$) in the GSTP1 positive tumors, as it was for the patients ($n = 31$) treated with docetaxel alone (corresponding mean reduction rates $0.59 \pm 0.06$ and $0.11 \pm 0.13$). And for those treated with paclitaxel alone ($n = 31$) (corresponding values: $0.84 \pm 0.05$, $0.56 \pm 0.08$). Tumor size and clinical stage were not significantly associated with reduction rate in all patients, in those treated with docetaxel, or in those treated with paclitaxel, while there was a significant association between GSTP1 expression and tumor size or clinical stage.

*Relationship between GSTP1 methylation status and response to docetaxel or paclitaxel*

Genomic DNA could be obtained from 48 breast tumors and was subjected to MSP assay. There ware 10 tumors (21%) with GSTP1 methylation. Of all the patients ($n = 48$) treated with docetaxel or paclitaxel, the reduction rate ($0.68 \pm 0.08$) for the GSTP1 methylated tumors was not significantly different from that ($0.47 \pm 0.07$) for the GSTP1 unmethylated tumors, nor was any significant difference found when the patients treated with docetaxel or paclitaxel were considered separately.

## Discussion

*Association of GSTP1 expression with resistance to docetaxel and paclitaxel*

We were able to show herein that GSTP1 negative breast tumors are significantly more closely associated with a higher reduction rate to docetaxel or paclitaxel than are GSTP1 positive breast tumors. Although several in vitro studies have suggested that GSTP1 expression is implicated in the acquisition of resistance to taxanes,[11,17,18] the significance of GSTP1 expression for resistance to taxanes has rarely been studied clinically in human breast cancers. Our study is thus the first to investigate the clinical significance of GSTP1 expression for taxane resistance in the PST setting. Until now, only one such study has been reported by Schmidt et al., who investigated the relationship between GSTP1 expression determined by immunohistochemistry and response to paclitaxel in metastatic breast cancers, but they could not show a significant association.[19] This discrepancy between their study and ours may be explained, at least in part, by the prior use of hormonal therapy and/or chemotherapy in Schmidt et al.'s study (73% of their patients had undergone therapy before paclitaxel administration) and the absence of such therapies in our study. Such prior therapies may have affected the GSTP1 expression. Our study, based on primary breast cancer patients treated with PST, seems to have an advantage over than based on metastatic breast cancer patients, who often have a history of prior therapy. When tumors are naïve to chemotherapy, the significance of a candidate predictive factor for response to chemotherapy can be evaluated under more relevant conditions not affected by prior therapy.

*GSTP1 and metabolism of docetaxel and paclitaxel*

CYP3A4 is involved in the inactivation of docetaxel and paclitaxel, and we were recently able to show that CYP3A4 expression in breast tumor tissues is associated with resistance to docetaxel.[20] GSTP1 is involved in the second phase of metabolism of docetaxel and paclitaxel, i.e., conjugation of docetaxel metabolites and paclitaxel metabolites with glutathione, suggesting a possibility that the

enhanced metabolism of these agents induced by GSTP1 up-regulation may lead to decreased anti-tumor activity.[17] The relationship of intra-tumoral concentrations of taxanes and their metabolites with GSTP1 expression thus need to be investigated in order to clarify the role of enhanced metabolism by GSTP1 in resistance to taxanes.

*GSTP1 and c-Jun N-terminal kinase (JNK)-mediated apoptosis*

Another reason which may explain the association of GSTP1 expression with resistance to taxanes is the anti-apoptotic effect of GSTP1. It has recently been shown[21] that monomeric GSTP1 can form a complex with JNK, which has a very important function in the control of cell survival and death pathways, while GSTP1 overexpression inhibits apoptosis induced by JNK.[22] Since both docetaxel and paclitaxel reportedly activate JNK in a dose-dependent manner and induce apoptosis,[23] we speculate that GSTP1 up-regulation may inhibit this JNK-mediated apoptosis, resulting in resistance to docetaxel and paclitaxel.

*GSTP1 methylation and response to docetaxel and paclitaxel*

It has been well established that GSTP1 gene expression is silenced by methylation of its promoter region in about 13–30% of breast cancers.[24–26] In our study, 10 tumors were found to have GSTP1 methylation. Although the reduction rate in tumors with GSTP1 methylation ($0.68 \pm 0.08$) was higher than in those without GSTP1 methylation ($0.47 \pm 0.07$), there was no statistically significant difference. In eight of the 10 tumors with GSTP1 methylation, immunohistochemistry detected no GSTP1 protein expression. On the other hand, in the 38 tumors without GSTP1 methylation, GSTP1 protein expression was present in 24 tumors and absent in 14 tumors, indicating that GSTP1 methylation is strictly associated with negative GSTP1 expression but that unmethylated GSTP1 does not necessarily mean positive GSTP1 expression. Consequently, the weak relationship ($p = 0.162$) between GSTP1 methylation status and its protein expression seems to explain why GSTP1 methylation status is not significantly associated with a response to taxanes.

*Limitations of the present study*

Since pathological complete response (pCR) achieved by PST is associated with a favorable patient prognosis,[27] pCR has recently been used more and more often as a marker of response in the PST setting. However, pCR rates achieved by docetaxel or paclitaxel monotherapy are too low[28] to be used as meaningful marker, so that we used clinical response instead. It has been reported that clinical response is also associated with a favorable patient prognosis.[29] We therefore believe that clinical response, especially if assessed accurately by MRI, can be a reliable marker of response to chemotherapy. Another limitation of the present study is a small number of patients analyzed. So, definitive conclusions are unlikely to be drawn from this study. Our findings need to be confirmed by a future study including a larger number of patients.

## Conclusion

In the present study, we have suggested, though preliminary due to a small number of patients, that GSTP1 protein expression, but not GSTP1 methylation status, can serve as a marker for resistance to both docetaxel and paclitaxel in primary breast cancers. Since GSTP1 protein expression can be determined by immunohistochemistry, it seems to have a potential to be applied to a routine clinical test after relevant clinical studies to confirm the clinical significance of GSTP1.

## Conflict of interest

This manuscript does not have any potential conflicts.

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ORIGINAL PAPER

# Low *LATS2* mRNA level can predict favorable response to epirubicin plus cyclophosphamide, but not to docetaxel, in breast cancers

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**Abstract**
*Purpose* Putative tumor suppressor genes LATS1 and LATS2 are implicated in the regulation of the cell cycle at the G2/M and G1/S phase, respectively. This study investigated possible correlations of intra-tumoral *LATS1* and *LATS2* mRNA levels with response to epirubicin plus cyclophosphamide (EC) or docetaxel (DOC) treatment.
*Methods* mRNA expression levels of *LATS1* and *LATS2* were determined by means of real-time PCR assay in 56 locally advanced breast cancers and 15 recurrent breast cancers treated with EC ($n = 32$) or DOC ($n = 39$).
*Results* Among the patients treated with EC, *LATS2* mRNA levels of responders ($0.72 \pm 0.11$, mean $\pm$ SE) were significantly ($P < 0.05$) lower than those of non-responders ($1.62 \pm 0.44$), and responders showed a tendency ($P = 0.05$) towards reduced *LATS1* mRNA levels. Patients with low *LATS2* mRNA levels ($n = 16$) showed a significantly ($P < 0.05$) higher response rate (75%) to EC treatment than those with high *LATS2* mRNA levels ($n = 16$; response rate = 31%). Positive predictive value, negative predictive value, and diagnostic accuracy of *LATS2* mRNA levels for prediction of response to EC were 75, 69, and 72%, respectively. On the other hand, neither *LATS1* nor *LATS2* mRNA levels were associated with response to DOC treatment.
*Conclusion* These results suggest the possibility that intra-tumoral *LATS2* mRNA levels may be clinically useful for the prediction of response to EC treatment by breast cancer patients. We speculate that disruption of the checkpoint function at the G1/S phase induced by down-regulation of LATS2 plays some part in the favorable response to EC.

**Keywords** LATS1 · LATS2 · Chemosensitivity · Breast cancer

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## Introduction

Anthracycline and taxanes are the most active chemotherapy components for breast cancers, and are often used for relieving symptoms and prolonging survival under metastatic conditions as well as improving survival in the adjuvant setting. These chemotherapies, however, are not necessarily effective for all patients. In fact, the response rates of metastatic breast cancers are 50–60% to anthracycline-containing regimens (A-regimens) (French Epirubicin Study Group 1988; Italian Multicentre Breast Study with Epirubicin 1988) and 50–60% to taxanes (Seidman et al. 1993; Adachi et al. 1996). The annual risk reduction rates improved by 30–40 and 40–50%, respectively, as a result of administering adjuvant A-containing regimens alone and A-containing regimens plus taxanes (Henderson et al. 2003; Mamounas et al. 2005). On the other hand, various side effects are observed essentially in all patients treated with these chemotherapies though the type, frequency, and grade of these side effects differ among patients. Thus, it is of vital importance to identify the factors, which can predict response to each of

the various chemotherapies. Such predictive factors would be useful for the selection of the most effective chemotherapy as well as for the elimination of ineffective chemotherapies on an individual patient basis. Although various biological parameters have been postulated as candidates for predictive factors of response to anthracyclines (Faneyte et al. 2001; Geisler et al. 2001; Egawa et al. 2003) or taxanes (Egawa et al.2001; Miyoshi et al.2002; Hasegawa et al.2003), their clinical value remains controversial, so that, at present, clinically useful predictive factors for these chemotherapies have yet to be established.

The *LATS1* and *LATS2* genes are human homologues of the Drosophila *lats* gene, which encodes a putative serine/threonine kinase (Tao et al. 1999; Yabuta et al. 2000; Hori et al. 2000). *LATS1* is thought to be a tumor suppressor gene since *LATS1*-deficient mice develop soft tissue sarcomas or ovarian stromal cell tumors (St John et al. 1999) and since frequent loss of heterozygosity (LOH) of this gene has been reported in human ovarian, cervical, and breast cancers (Cooke et al. 1996; Lee et al. 1990; Mazurenko et al. 1999; Fujii et al. 1996; Theile et al. 1996; Noviello et al. 1996). In addition, in vitro over expression of *LATS1* was found to cause G2/M arrest through the inhibition of CDK1 activity in a breast cancer cell line (Yang et al. 2001). *LATS2* (also known as KPM) is also considered a tumor suppressor gene since frequent LOH of this gene has been reported in various human cancers including breast, ovary, and liver (Lee et al. 1988; Sato et al. 1991; Wang and Roger 1988) and in vitro over expression of *LATS2* was seen to cause G1/S arrest through the inhibition of CDK2 activity (Li et al. 2003). Furthermore, we recently found that a reduced expression of *LATS1* mRNA or *LATS2* mRNA is associated with a biologically aggressive phenotype of breast cancer (Takahashi et al. 2005), indicating that the reduced function of these tumor suppressor genes leads to accelerated cell proliferation, resulting in a high incidence of distant metastases.

Several clinical studies have reported that tumors with increased cell proliferation detected by Ki-67 (or Mib-1) immunostaining and flowcytometry show an enhanced response to chemotherapy (Chang et al. 2000; Pohl et al. 2003), although contradictory results have also been reported (Chang et al. 1999; Bottini et al. 2001). These findings seem to suggest that factors involved in the regulation of the cell cycle may be useful for predicting response to chemotherapy. In fact, several cell cycle regulators such as p53, p21, p27, cyclin E, and BRCA1 are reportedly associated with response to chemotherapies including those using anthracylines or taxanes (Egawa et al. 2003; Colleoni et al. 1999; Taguchi et al. 2004). As mentioned earlier, LATS1 and LATS2 are tumor suppressor genes, which are implicated in the regulation of the cell cycle. Thus, it is speculated that the expression levels of *LATS1* mRNA and *LATS2* mRNA may be associated with chemosensitivity. In the present study, we therefore investigated possible correlations between *LATS1* mRNA and *LATS2* mRNA levels in breast cancer tissues determined with a real-time PCR assay with the response to epirubicin plus cyclophosphamide (EC) or docetaxel (DOC) monotherapy.

## Materials and methods

### Tumor specimens and patient treatments

For this study, 71 females breast cancer patients (56 locally advanced primary breast cancer patients and 15 locally recurrent breast cancer patients) were recruited. These patients were treated with either EC ($n = 32$) or DOC ($n = 39$). Tumor samples were obtained from primary breast tumors or locally recurrent lesions by means of incisional biopsy or vacuum-assisted core needle biopsy prior to chemotherapy. Part of each tumor sample was subjected to pathological diagnosis, and the rest was snap frozen in liquid nitrogen and kept at −80°C until use for RNA extraction and for estrogen receptor (ER) and progesterone receptor (PR) assay. An additional 41 primary breast tumor samples from patients without prior treatment were obtained interoperatively and also used for immunohistochemical analyses. Informed consent for these studies was obtained from all patients.

### Chemotherapy and evaluation of response

Four cycles of EC (epirubicin 60 mg/m$^2$ i.v. day 1 + cyclophosphamide 600 mg/m$^2$ i.v. day 1, q3w) or DOC (60 mg/m$^2$ i.v. day 1, q3w) were administered to the patients with locally advanced breast tumors before surgery or to patients with recurrent tumors until disease progression was observed. Chemotherapeutic response was evaluated according to the WHO clinical criteria: complete response (CR), disappearance of all known disease; partial response (PR), 50% or more decrease in tumor size; no change (NC), less than 50% decrease or less than 25% increase in tumor size; and progressive disease (PD), 25% or more increase in tumor size or appearance of new lesions (Miller et al. 1981). Patients showing CR or PR were considered responders, and those showing NC or PD non-responders.

### RNA extraction, reverse transcription, and real-time PCR assay of *LATS1* and *LATS2* mRNA levels

Total RNA was extracted from the frozen tumor specimens using TRIZOL reagent according to the protocol provided by the manufacturer (Molecular Research Center, Cincinnati, OH, USA). About 3 μg of total RNA was reverse-transcribed for single strand cDNA, using the oligo-$(dT)_{15}$ primer and Superscript II (Life Technologies Inc., Gaithersburg, MD, USA) at 42°C for 90 min, followed by heating at 70°C for 10 min. The ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) was used for real-time PCR reactions of *LATS1* and *LATS2*. The sequence of the primers and probes for *LATS1* and *LATS2* as well as the reaction conditions were described previously (Takahashi et al. 2005). *β-glucoronidase* transcripts for quantitative control were used to normalize the transcript content of the sample. The primer and probe mixture for *β-glucoronidase* was purchased from Perkin-Elmer Applied Biosystems and used according to the manufacturer's protocol. The standard curves for *LATS1*, *LATS2* and *β-glucoronidase* mRNA were generated using serially diluted solutions of plasmid clones inserted with *LATS1*, *LATS2* or *β-glucoronidase* cDNA as templates. The amount of target gene expression was then calculated from these standard curves with $10^{-9}$ μg of the PCR product for *LATS1* and *LATS2*, and $10^{-8}$ μg of the PCR product for *β-glucoronidase*, which was defined as 1. Real-time PCR assays were conducted in duplicate for each sample, and the mean value was used for calculation of the relative expression levels. The final expression levels of *LATS1* and *LATS2* mRNA were expressed as ratios to those of *β-glucoronidase*.

### Immunohistochemical staining of Mib-1, LATS1, LATS2, and Geminin

From the 71 tumors, 53 samples were available for immunohistochemical analysis for Mib-1 detection with the avidin–biotin–peroxidase method using a rabbit anti-human Mib-1 polyclonal antibody (MIB-1; Immunotech, Cedex, France) following a previously described method (Takamura et al. 2002). Another set of 60 formalin fixed paraffin embedded breast tumor tissues obtained interoperatively from patients without prior treatment were used for immunohistochemical study of LATS1, LATS2, and Geminin. Their expression was detected by using the Histofine Simple Stain system, Nichirei, Tokyo, Japan, for LATS1 and Geminin, or the CSA system (DAKO, Kyoto, Japan) for LATS2. Polyclonal antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA, for LATS1 and Geminin, and from Abgent, San Diego, CA, USA, for LATS2. Antigen retrieval was performed by incubating the sections in Histo VT One (Nakarai, Kyoto, Japan) for LATS1, or in a target retrieval solution (DAKO, Kyoto, Japan) for LATS2 and Geminin, in a hot water bath at 98°C for 40 min. After quenching endogenous peroxidase with 3% $H_2O_2$ in methanol for 20 min, non-specific binding was blocked by incubating the slides with Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) for 30 min. The slides were then incubated with primary antibody (1 μg/ml for LATS1 and Geminin, 5 μg/ml for LATS2) at 4°C overnight followed by incubation for 60 min with peroxidase-conjugated secondary antibody (Histofine Simple Stain MAX PO, Nichirei, Tokyo, Japan) for LATS1 and Geminin, or with peroxidase-conjugated anti-rabbit immunoglobulin G antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for LATS2. Next, the antibody complex was visualized with the 3, 3′-diaminobenzidine tetrahydrochloride (Merck KGaA, Darmstadt, Germany). The sections were counter-stained with hematoxylin, the lesions showing the most active staining selected microscopically and 1,000 tumor cells examined for calculation of the percentage of stained cells. Nuclear positive staining was determined for Mib-1 and Geminin, and 10% or more nuclear and/or cytoplasmic staining was judged to indicate positivity for LATS1 and LATS2. All the slides were examined by skilled observers blinded to the clinical data.

### Estrogen receptor (ER) and progesterone receptor (PR) assay

An enzyme immunoassay was used to measure ER and PR protein levels in breast cancers with the kits provided by Abbott Research Laboratories, Chicago, IL, USA, according to the manufacturer's instructions. The cut-off value for ER and PR was set at 5 fmol/mg protein.

### Statistical methods

Student's *t*-test was used for comparison of *LATS1* and *LATS2* mRNA expression levels as well as Mib-1 expression levels among various groups. The relationship between *LATS1* or *LATS2* mRNA high or low expression with response to EC or DOC, and the relationship between LATS1 or LATS2 expression and Geminin expression were analyzed with the chi-square test. Statistical significance was assumed for $P < 0.05$.

## Results

### Patient characteristics and *LATS1* and *LATS2* mRNA levels in breast cancer tissues

Characteristics of patients treated with EC ($n = 32$) or DOC ($n = 39$) were similar as shown in Table 1. *LATS1* and *LATS2* mRNA levels in the EC group ($1.91 \pm 0.28$ and $1.14 \pm 0.23$, respectively, mean ± SE) were not significantly different from those in the DOC group ($2.54 \pm 0.51$ and $0.98 \pm 0.14$, respectively) (Table 2). There were no significant associations between *LATS1* and *LATS2* mRNA levels and any clinical parameters including menopausal status, disease site, stage, ER, and PR status (Table 2).

### Relationship between Mib-1, *LATS1 mRNA* or *LATS2* mRNA levels and response to EC or DOC

In the EC group, percentages of cancer cells positive for Mib-1 immunohistochemistry were similar ($P = 0.96$) for responders ($30.25 \pm 4.25\%$, mean ± SE) and non-responders ($30.63 \pm 6.67\%$) (Table 3; Fig. 1). On the other hand, *LATS2* mRNA levels of responders ($0.72 \pm 0.11$) were significantly ($P < 0.05$) lower than those of non-responders ($1.62 \pm 0.44$), while *LATS1* mRNA levels of responders ($1.42 \pm 0.20$) were marginally significantly ($P = 0.05$) lower than those of non-responders ($2.47 \pm 0.51$) (Table 3; Fig. 1). The DOC group showed no significant differences between responders and non-responders in Mib-1 positivity, *LATS1* mRNA levels, or *LATS2* mRNA levels (Fig. 1).

Patients were divided into high and low expression groups for Mib-1, *LATS1* mRNA, or *LATS2* mRNA levels by using the median value as the cut-off value. Response rates to EC or DOC showed no significant differences between the Mib-1 high and low groups and between the *LATS1* mRNA high and low groups (Table 3). Patients with low *LATS2* mRNA levels showed a significantly ($P < 0.05$) higher response rate

**Table 1** Clinicopathological characteristics of patients treated with EC or DOC

| | EC[a] ($n = 32$) | DOC[b] ($n = 39$) |
|---|---|---|
| Age (years) [average (range)] | 50.1 (30–74) | 51.7 (34–67) |
| Menopausal status | | |
| Premenopausal | 17 (53)[c] | 16 (41) |
| Postmenopausal | 15 (47) | 23 (59) |
| Disease site | | |
| Locally advanced breast tumors | | |
| Stage II | 13 (41) | 11 (28) |
| Stage III | 7 (22) | 16 (41) |
| Stage IV | 5 (15) | 4 (10) |
| Locally recurrent tumors | 7 (22) | 8(21) |

[a] Epirubicin plus cyclophosphamide

[b] Docetaxel

[c] Percent (%)

**Table 2** *LATS1* and *LATS2* mRNA levels in breast cancer tissues

| | *n* | *LATS1* mRNA levels | *LATS2* mRNA levels |
|---|---|---|---|
| Chemotherapy | | | |
| EC[a] | 32 | $1.91 \pm 0.28$[c] | $1.14 \pm 0.23$ |
| DOC[b] | 39 | $2.54 \pm 0.51$ | $0.98 \pm 0.14$ |
| Menopausal status | | | |
| Premenopausal | 33 | $2.33 \pm 0.59$ | $1.01 \pm 0.18$ |
| Postmenopausal | 38 | $2.19 \pm 0.28$ | $1.08 \pm 0.18$ |
| Disease site | | | |
| Locally advanced breast tumors | | | |
| Stage II | 24 | $2.42 \pm 0.80$ | $1.02 \pm 0.16$ |
| Stage III | 23 | $2.04 \pm 0.36$ | $1.14 \pm 0.31$ |
| Stage IV | 9 | $2.10 \pm 0.55$ | $0.70 \pm 0.14$ |
| Recurrent tumors | 15 | $2.42 \pm 0.41$ | $1.18 \pm 0.25$ |
| ER status | | | |
| Positive | 27 | $2.90 \pm 0.73$ | $0.94 \pm 0.16$ |
| Negative | 39 | $1.86 \pm 0.23$ | $1.03 \pm 0.17$ |
| Unknown | 5 | $1.89 \pm 0.54$ | $1.87 \pm 0.89$ |
| PR status | | | |
| Positive | 20 | $2.56 \pm 0.95$ | $0.93 \pm 0.18$ |
| Negative | 46 | $2.17 \pm 0.25$ | $1.02 \pm 0.15$ |
| Unknown | 5 | $1.89 \pm 0.54$ | $1.87 \pm 0.89$ |

[a] Eepirubicin plus cyclophosphamide

[b] Docetaxel

[c] Mean ± SE

**Table 3** Relationship between Mib-1, *LATS1*, or *LATS2* expression levels and response to EC or DOC

| mRNA level | Responders (*n*) | Non-responders (*n*) | Response rate (%) | *P*-value |
|---|---|---|---|---|
| Mib-1 expression | | | | |
| EC[a] | | | | |
| High | 3 | 5 | 38 | 0.31 |
| Low | 5 | 3 | 63 | |
| DOC[b] | | | | |
| High | 11 | 8 | 58 | 0.41 |
| Low | 8 | 10 | 44 | |
| *LATS1* expression | | | | |
| EC | | | | |
| High | 8 | 8 | 50 | 0.72 |
| Low | 9 | 7 | 56 | |
| DOC | | | | |
| High | 13 | 7 | 65 | 0.15 |
| Low | 8 | 11 | 42 | |
| *LATS2* expression | | | | |
| EC | | | | |
| High | 5 | 11 | 31 | <0.05 |
| Low | 12 | 4 | 75 | |
| DOC | | | | |
| High | 10 | 9 | 53 | 0.88 |
| Low | 11 | 9 | 55 | |

[a] Epirubicin plus cyclophosphamide

[b] Docetaxel

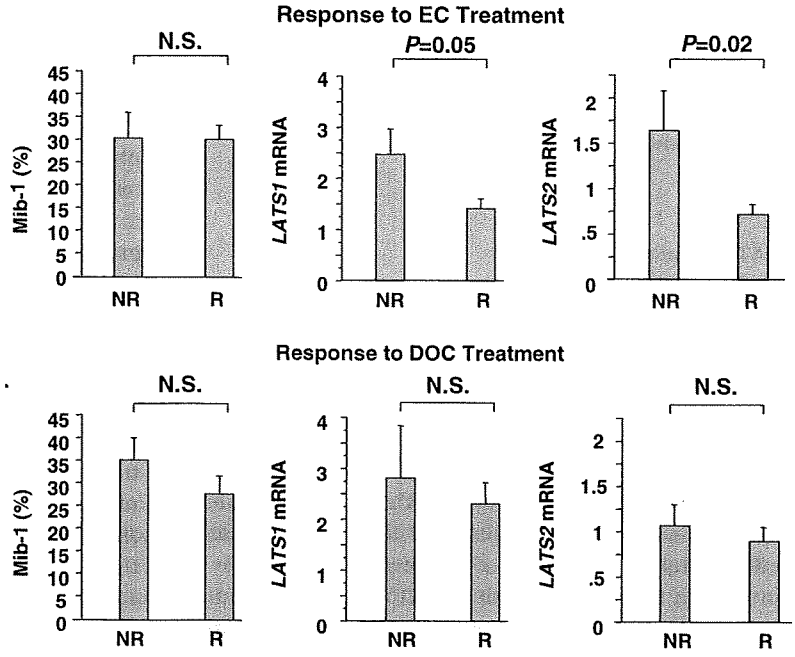


**Fig. 1** Mib-1 positivity (%) and *LATS1* or *LATS2* mRNA levels in non-responders (*NR*) and responders (*R*) to epirubicin plus cyclophosphamide (*EC*) treatment (*upper panel*) and docetaxel (*DOC*) treatment (*lower panel*)

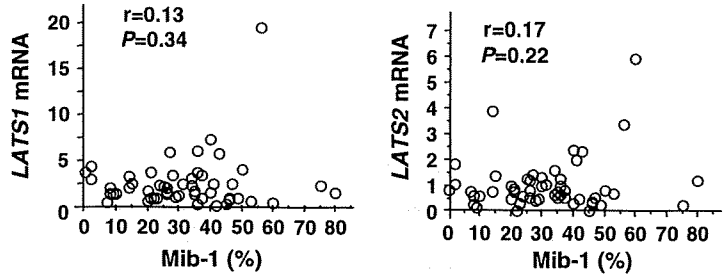


**Fig. 2** Correlation of Mib-1 positivity (%) with *LATS1* or *LATS2* mRNA levels in breast cancers

(75%) to EC than did those with high *LATS2* mRNA levels (31%) (Table 3), while, no significant association was found between *LATS2* mRNA levels and the response rate to DOC ($P = 0.88$). Positive predictive value, negative predictive value, and diagnostic accuracy of *LATS2* mRNA levels for the prediction of response to EC were 75, 69, and 72%, respectively.

### Relationship between Mib-1 expression levels and *LATS1* mRNA or *LATS2* mRNA levels in breast cancer tissues

There was no significant association between percentages of Mib-1 positive cells and *LATS1* mRNA ($r = 0.13$ and $P = 0.34$) or *LATS2* mRNA ($r = 0.17$ and $P = 0.22$) levels (Fig. 2).

### Immunohistochemical analyses of LATS1, LATS2, and Geminin in breast cancer tissues

In order to confirm the LATS1 and LATS2 expression in breast cancer cells, a different set of 41 tumor samples was subjected to immunohistochemical staining for further investigation. As shown in Fig. 3, nuclear and/or cytoplasmic staining of LATS1 and LATS2 were recognized in breast cancer cells in 29 (71%) and 24 (59%) tumors, respectively. Next, the expression of Geminin in cancer cells was examined and the relationship between Geminin expression and LATS1 or LATS2 expression is shown in Table 4. The proportion of Geminin-positive tumor cells tended to be higher ($P = 0.05$) in LATS1-negative than in LATS1-positiv tumors, while it was significantly ($P < 0.01$) higher in LATS2-negative than in LATS2-positive tumors.

## Discussion

In the study presented here, we were able to show that tumors with low *LATS2* mRNA expression are significantly associated with a high response rate to EC. The anti-neoplastic activity of EC is thought to be mostly attributable to epirubicin because the response rate of metastatic breast cancers to EC is similar to that to

**Table 4** Relationship between LATS1, or LATS2 expression and Geminin expression

| | Positive *n* (%) | Negative *n* (%) | *P*-value |
|---|---|---|---|
| LATS1 expression | | | |
| Geminin-positive cells (%) | | | |
| <10 | 24 (83) | 6 (50) | 0.05 |
| 10≤, <20 | 4 (14) | 3 (25) | |
| 20≤ | 1 (3) | 3 (25) | |
| LATS2 expression | | | |
| Geminin-positive cells (%) | | | |
| <10 | 22 (92) | 8 (47) | 0.006 |
| 10≤, <20 | 1 (4) | 6 (35) | |
| 20≤ | 1 (4) | 3 (18) | |

epirubicin monotherapy (Ormrod et al. 1999) and because the action of cyclophosphamide monotherapy is reported to be generally very low (Carter 1972). Anthracyclines including epirubicin exert their anti-neoplastic effect through formation of a complex with DNA by intercalating the DNA strands and inhibiting the function of topoisomerase II-alpha, which leads to the induction of DNA damage and, finally, the inhibition of DNA replication (Capranico et al. 1990). Since topoisomerase II-alpha is most strongly expressed in the S and G2/M phases (Boege et al. 1995), it is speculated that the cells in these phases are the most sensitive to anthracycline. This speculation is supported by Hill et al., who showed that cancer cells in the S-phase were most sensitive to anthracyclines in vitro (Hill and Whelan 1982). Since LATS2 negatively regulates CDK2 activity (Li et al. 2003), low *LATS2* mRNA expression is thought to lead to high levels of CDK2 activity, which then drives the transition of cancer cells from the G1 to the S phase, resulting in high sensitivity to anthracyclines.

Low expression of *LATS1* mRNA in our study tended ($P = 0.05$) to be associated with a high response rate to EC. Since LATS1 inhibits CDK1 activity (Yang et al. 2001), low *LATS1* mRNA expression is thought to lead to acceleration of the transition from the G2 to the M phase. Although the S-phase is the most sensitive to anthracylcines, the cancer cells in the early M-phase also reportedly show a relatively high sensitivity to anthracyclines (Hill and Whelan 1982). We therefore speculate that tumors with low *LATS1* mRNA expression are more sensitive to EC than those with high expression because the proportion of cancer cells in the M-phase is higher in tumors with low than in those with high *LATS1* mRNA levels. Another explanation is that the association between low *LATS1* mRNA expression and a high response rate to EC is not a causal relationship but simply an indirect association since *LATS1* mRNA expression significantly correlates with *LATS2* mRNA expression (data not shown). If this expression is low, it would then increase the population of cancer cells in the S phase when the cancer cells are most sensitive to anthracyclines.

The association of Mib-1 with sensitivity to anthracyclines has been studied by several investigators but not with consistent results. Some investigators reported a significant relationship between high Mib-1 expression and a favorable response to anthracyclines (Chang et al. 2000; Pohl et al. 2003) but others did not (Linn et al. 1997). We could not find a significant association either between Mib-1 expression and response to EC. Tumors with a high percentage of Mib-1 positive cells are generally considered to be characterized by high proliferation but the percentage of Mib-1 positive cells does not necessarily correlate with the percentage of cells in the S-phase since Mib-1 is expressed in all cells except those in the G0-phase (Gerdes et al. 1984). Thus, even if the percentage of Mib-1 positive cells is the same, the percentage of cells in the S phase

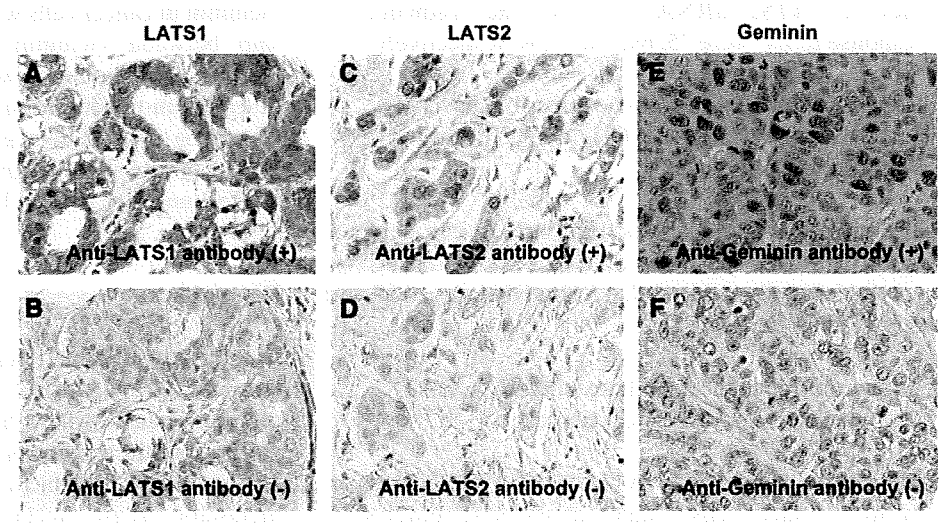


**Fig. 3** Representative results of immunohistochemical staining of LATS1, LATS2, and Geminin in cancer cells with (**a**, **c**, **e**) and without (**b**, **d**, **f**) antibodies (X400). Nuclear and/or cytoplasmic staining of LATS1 (**a**) and LATS2 (**c**), and nuclear staining of Geminin (**e**) were detected

can vary among tumors. This fact seems to explain, at least in part, the reason why the findings regarding the association between Mib-1 expression and response to anthracyclines are inconsistent. As mentioned earlier, we believe that a reduced expression of LATS2 correlates with the accelerated transition of cells from the G1 to the S-phase, thus rendering the cells more sensitive to anthracyclines. The absence of a significant association between *LATS2* mRNA expression and Mib-1 positivity appears to be consistent with our speculation that Mib-1 immunohistochemistry results do not necessarily reflect the proportion of cells in the S-phase. For these reasons, the reduction in the expression of *LATS2* mRNA might be a better marker than Mib-1 for the proportion of cells in the S phase. In order to verify this speculation, we made a direct comparison between LATS2 expression and Geminin expression, because Geminin has been demonstrated to be expressed mainly in S-phase (Kulartz and Knippers 2004). The inverse association observed in our study, namely that LATS2-negative tumors correlated with a high frequency of Geminin-positive tumor cells seems to provide support for the hypothesis that reduced expression of LATS2, leading to accelerated transition to the S phase, results in higher sensitivity to anthracyclines.

Since DOC promotes abnormal tubulin formation in the G1, S, and G2 phases (Hennequin et al. 1995), it has been speculated that DOC, in contrast with epirubicin, exerts its anti-neoplastic activity in a cell-cycle non-specific manner. This speculation seems to be compatible with our observation that the response rate to DOC, unlike that to EC, showed no significant association with *LATS1* or *LATS2* mRNA levels. On the other hand, LATS1 and LATS2 as negative regulators of tumor growth through induction of apoptosis (Xia et al. 2002, Ke et al. 2004) may affect response to chemotherapy. If this is the case, it can be speculated that tumors with reduced LATS1 or LATS2 might be associated with a poor response to EC. Contrary to this speculation, however, we detected an inverse correlation between a reduction in LATS2 expression and a favorable response to EC. These findings seem to suggest that apoptosis induced by LATS2 does not play a major role in response to EC.

In conclusion, we have been able to demonstrate that tumors with low *LATS2* mRNA levels show a significantly higher response rate to EC than those with high *LATS2* mRNA levels. Since LATS2 inhibits the transition from the G1 to the S-phase, the reduced expression of *LATS2* mRNA is thought to result in accelerated transition to the S phase when the cancer cells become most sensitive to anthracyclines. Our findings thus seem to suggest that *LATS2* mRNA levels may be useful predictors of response to EC. However, our preliminary results need to be confirmed by a future study of a larger number of patients as well as an in vitro study of the relationship between LATS2 levels and anthracycline sensitivity or the proportion of cells in the S-phase.

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