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 mm3). 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

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], 15; cyclophosphamide plus mitomycin C [CM], 15; cyclophosphamide plus etoposide [CE], 19; other modalities, 2) and 61...
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/L lysate, where 1 U is equivalent to the activity of 1 ng of standard) divided by its corresponding expression (ng/L 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
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). 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).

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

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

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

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Category</th>
<th>No. of patients (n = 284)</th>
<th>5yRFS (%)</th>
<th>P value*</th>
</tr>
</thead>
</table>
| 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
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

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, 3H-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 mm³).

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.

references