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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original article

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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

original 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

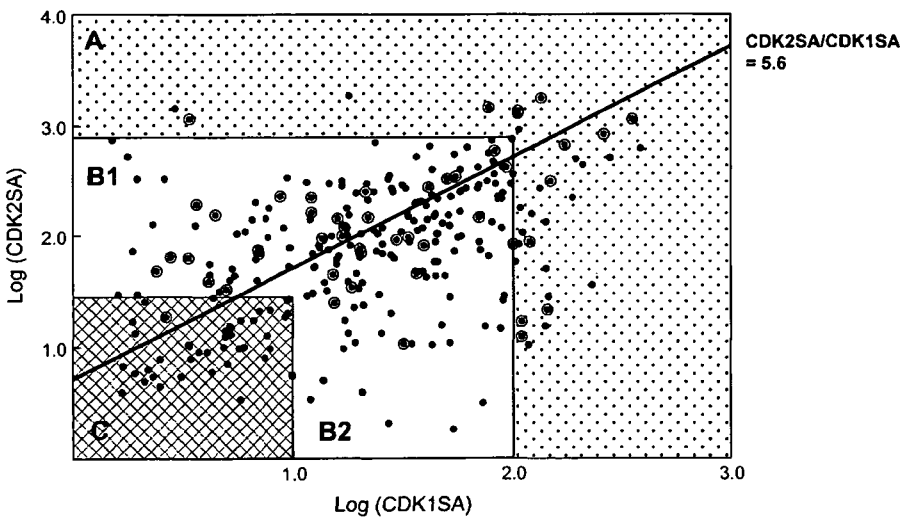

Figure 1. Distribution of breast tumors according to CDK1SA and CDK2SA. Tumors (n = 284) are plotted in two dimensions (logarithmic scales) according to CDK1SA and CDK2SA. Area A includes tumors with high CDK1SA (>100 U/ng) and/or high CDK2SA (>800 U/ng) (n = 37). Area C includes tumors where both CDK1SA 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; ◉.

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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

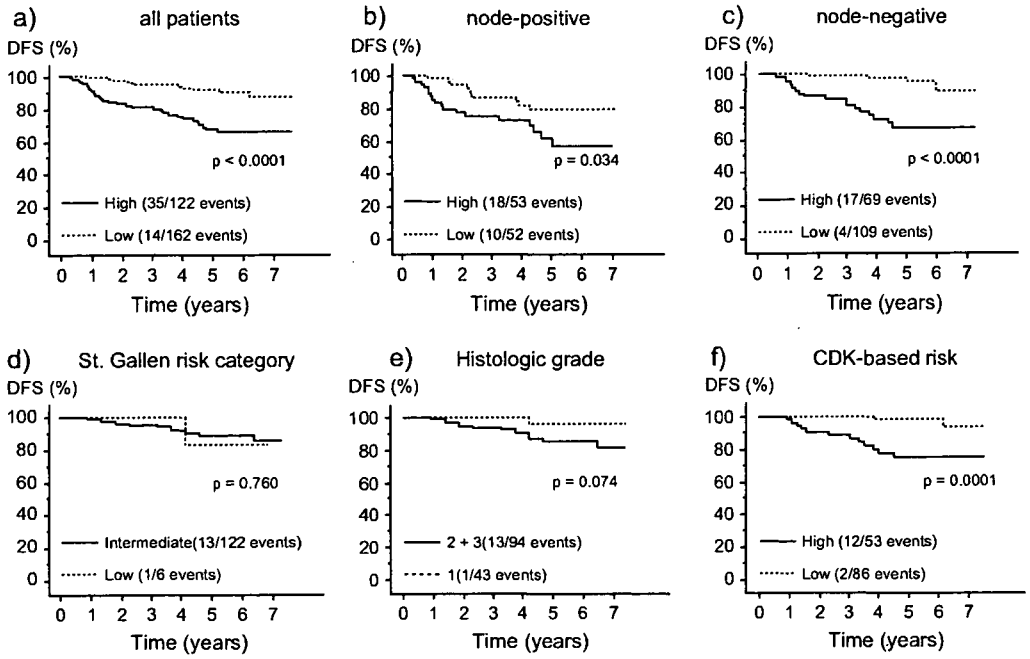

Figure 2. Relapse-free survival (RFS) rates according to the CDK-based risk and St Gallen's risk categorization. In (A) all; (B) node-positive and (C) node-negative patients, CDK-based high risk was strongly associated with poor prognosis compared to CDK-based low risk. In node-negative patients receiving 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

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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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Connexin26 expression is associated with aggressive phenotype in human papillary and follicular thyroid cancers

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Abstract

Connexin26 (Cx26), a component of GAP junctions and until recently believed to be a tumor suppressor gene, has been shown to play an important role in lymphatic invasion as well as lymph node and distant metastases in squamous lung cancer and breast cancer. In the study presented here, we investigated Cx26 expression in human papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC) and its relationship with various clinicopathological parameters. Of 69 PTCs, 33 were positive for Cx26 (47.8%), as were five of 11 FTCs (45.5%), all follicular thyroid adenomas ($n = 22$) and normal thyroid tissues ($n = 20$) were negative for Cx26. A statistically significant association was observed between Cx26 expression and large tumor size ($p = 0.028$ for PTC) and lymph node metastases ($p = 0.053$ (marginally significant) for PTC and $p = 0.035$ for FTC). Presence of intra-glandular dissemination of tumor cells was significantly ($p = 0.048$) more frequent in Cx26-positive (30.3%) than Cx26-negative PTCs (11.1%). Lymphatic vessel invasion was more frequent in Cx26-positive PTCs (6.1%) than in Cx26-negative PTCs (0%) though the difference was not statistically significant. These results suggest that Cx26 may be implicated in the pathogenesis of PTC and FTC and is associated with the biologically aggressive phenotypes of these tumors.

Keywords: Connexin26; Thyroid cancer; D2-40; CD34

1. Introduction

Gap junctions form channels between adjacent cells, which allow the exchange of ions, nucleotides, metabolites and other small molecules (<1 kDa) including second messengers such as Ca^{2+}, cAMP, and IP3 [1–3]. Gap junctional intercellular communication (GJIC) plays an important role in a variety of cellular processes including homeostasis, morphogenesis, cell differentiation, and growth control

Abbreviations: Cx, connexin; PTC, thyroid papillary carcinoma; FTC, thyroid follicular carcinoma; FTA, thyroid follicular adenoma; GJIC, gap junction intercellular communication.

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[4]. Gap junction channels are composed of two hemi-channels (connexons) and each connexon consists of six connexins. Gap junctions may be composed of different Cx isotypes (heterotypic) or of more than one Cx isotype (heteromeric) [1]. To date, 20 members of the connexin transmembrane protein family have been identified [5].

It has been shown that, loss or reduced function of GJIC is generally implicated in the progression of a variety of tumors, and is usually induced by down-regulation of connexins. Reduced expression of Cx32 in human gastric cancer [6], Cx43 in human prostatic adenocarcinoma cells [7] and human brain glioma cells [8], Cx32 and Cx43 in human lung cancer [9], and Cx43 in human breast cancer [10] have been reported. In addition, restoration of function in these connexins has been shown to result in the retardation of cell growth and induction of more normal phenotypes [11,12]. Thus, connexins are generally considered to be tumor suppressor genes [13].

Cx26 is a member of the connexin family and was initially isolated as a gene with down-regulated expression in breast cancer cell lines as compared with normal human breast epithelial cell lines [14]. Furthermore, transfection of Cx26 into various tumor cell lines including breast cancer cell lines has been shown to confer growth suppression [15–17]. Analogous to other connexins, Cx26 has thus also been considered to serve as a tumor suppressor gene. However, recent studies have disclosed a unique feature of Cx26 in tumor progression. Ito et al. [18] found that abnormally augmented expression of connexin26 is responsible for the enhanced spontaneous metastasis of mouse BL6 melanoma cells, and that the exogenous expression of a dominant negative form of Cx26 results in an increase in the spontaneous metastases of BL6. They also suggested that formation of heterologous gap junctions between Cx26 and Cx43, which are expressed in melanoma cells and vascular endothelial cells, respectively, may facilitate the invasion of tumor cells into the blood vessels. They based their suggestion on the observation that melanoma cells could transfer dye into vascular endothelial cells.

Very recently, Ito et al. also reported the up-regulation of Cx26 in human lung squamous cell cancer as well as its association with poor prognosis [19] and suggested that Cx26 may play an important role in the acquisition of malignant phenotypes. Furthermore, we were able to show that Cx26 expression is associated with lymphatic vessel invasion, large tumor size, high histological grade, and poor prognosis of human breast cancers, indicating that Cx26 seems to enhance metastasis, probably through the promotion of lymphatic vessel invasion [20].

Putting all these observations together leads to the speculation that Cx26, unlike other connexins, may be implicated in the acquisition of malignant phenotypes such as tumor invasion and metastases and that the association between Cx26 expression and malignant phenotypes may also apply to other human cancers. Since no studies of the expression of Cx26 expression in human thyroid cancers have been reported yet, we investigated, in the study presented here, the relationship between Cx26 expression and various clinicopathological parameters including lymph node metastases, lymphatic invasion, and blood vessel invasion in papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC).

2. Materials and methods

2.1. Patients and tumor tissues

Surgical specimens obtained from 102 patients, who underwent hemithyroidectomy or total thyroidectomy due to PTC ($n = 69$), FTC ($n = 11$) or follicular thyroid adenoma (FTA) ($n = 22$) at Osaka University Hospital, were fixed in 10% buffered formalin and embedded in paraffin. Fresh tumor tissues and normal thyroid tissues, obtained from three PTCs, three FTAs and three normal thyroid tissues adjacent to PTC, were snap frozen in liquid nitrogen and kept at −80 °C until used for Western blotting. This study was approved by the institutional review board of Osaka University Graduate School of Medicine.

2.2. Immunohistochemistry

Paraffin sections (4 μm) of tumor tissues were subjected to immunohistochemical staining of Cx26 protein with the avidin–biotin-peroxidase method. In brief, endogenous peroxidases were quenched by incubating the sections for 20 min in 3% H_2O_2. Antigen retrieval was performed by heating the samples in 10 mmol/L citrate buffer (pH 6.0) at 95 °C for 40 min. After treatment with Block Ace (Dainippon Sumitomo Pharmaceutical, Osaka, Japan) for 30 min at room temperature, the sections were incubated at 4 °C overnight with a mouse monoclonal anti-Cx26 antibody (Catalog No.13-8100, working dilution 1:500; purchased from Zymed Laboratories Inc., South San Francisco, CA, USA). The avidin–biotin-peroxidase complex system (Vectastain® Elite ABC kit; Vector Laboratories Inc., Burlingame, CA, USA) was used for color development. The entire tumor lesion was observed with special attention to the periphery of tumors since tumor cells in the periphery of other tumors are reportedly more likely to be Cx26-positive

than those in the center [19]. For each tumor, 1000 tumor cells were counted and, when more than 10% were clearly positive for Cx26 staining, the tumor was considered to be Cx26-positive.

2.3. Lymph and blood vessel density

After immunohistochemical staining of lymphatic vessels by anti-D2-40 and of blood vessels by anti-CD34 antibodies [20], vessel density in each case was determined by counting all immunostained vessels at a magnification of 200× from five areas, and the mean was used as the blood or lymphatic vessel density of the case. Since Yasuoka et al. reported that lymphatic vessel density differs in the intratumoral and peritumoral areas in human thyroid tumors [21], blood vessel density or lymph vessel density in the intratumoral and the peritumoral areas was analyzed separately.

2.4. Immunoabsorption test

Antibody blocking experiments were performed to confirm antibody specificity. Cx26 antibody blocking peptide was purchased from Alpha Diagnostic International (San Antonio, TX, USA). Ten micrograms control peptide per 1 μg of this antibody was used and incubated at 37 °C for 1 h and centrifugal separation was conducted at 7000 rpm for 10 min. This was followed by immunohistochemistry performed according to the protocol described above.

2.5. Western blotting

Western blotting was also performed to confirm antibody specificity. After surgery, thyroid tissues were rapidly frozen and stored at −80 °C until use. In preparation for SDS–polyacrylamide gel electrophoresis (SDS–PAGE), tissue was homogenized in Tris buffer. Homogenates were sonicated, and total protein was determined. Nine lanes were prepared on the gel. Lanes 1–3 were for thyroid papillary cancer tumors which were immunohistochemically positive for Cx26, lanes 4–6 were for thyroid adenomas and lanes 7–9 were for normal thyroid tissues adjacent to thyroid cancers. Proteins were resolved by SDS–PAGE on 5–20% gel (SuperSep TM HG; Wako, Osaka, Japan), 20 μg per lane, and samples not boiled prior to loading were transblotted to 0.2 mm nitrocellulose in transfer buffer, pH 8.3. Immunoblots were blocked for 2–3 h in 4% block ace (Yukijirushi, Osaka, Japan) and incubated with anti-Cx26 antibodies (1:500 dilution) overnight at 4 °C in Can Get Signal Solution 1 (Taiho Pharmaceutical, Osaka, Japan). Membranes were washed in TBS-T and then incubated for 1 h at room temperature with a secondary antibody (anti-mouse horseradish peroxidase-conjugated IgG at 1:3000 dilution) in Can Get Signal Solution 2 (Taiho Pharmaceutical). Membranes were again washed and incubated for 5 min with the ECL Plus Western Blotting Detection System (GE Healthcare UK Ltd., Buckinghamshire, UK) for detection of immunoreactive protein.

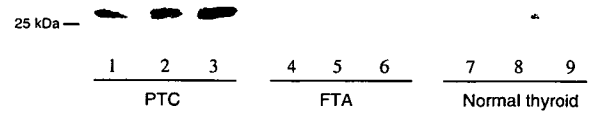

Fig. 2. Western blot analysis of Cx26. Lanes 1–3, papillary thyroid cancers immunohistochemically positive for Cx26. Lanes 4–6, follicular thyroid adenomas. Lanes 7–9, normal thyroid tissues.

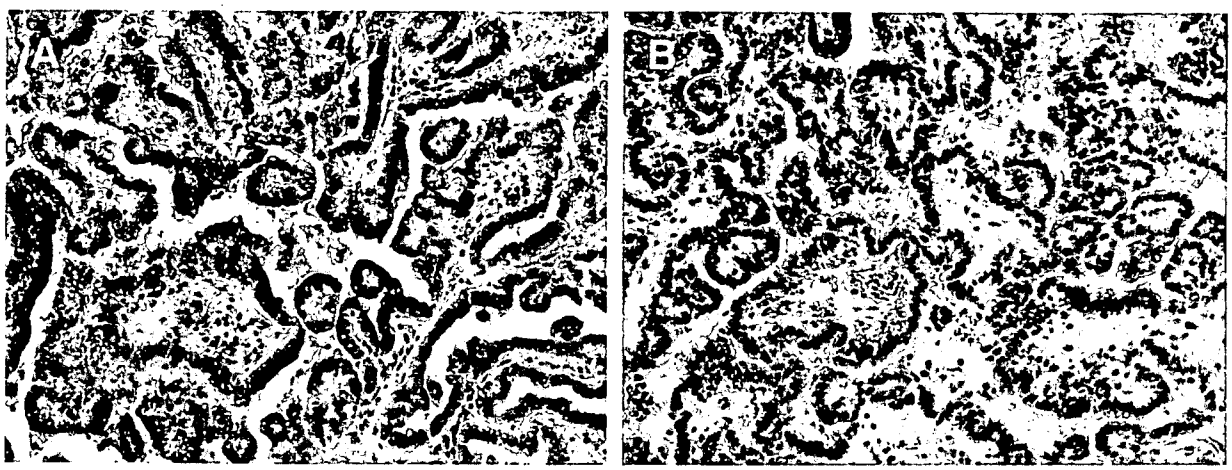

Fig. 1. Immunoabsorption study. Paraffin sections from papillary thyroid cancer were incubated with anti-Cx26 antibody in the absence (A) or presence (B) of pretreatment with excess amount of the same peptide used to generate this antibody (200×).

2.6. Statistics

Chi-square test or Mann–Whitney's *U*-test was used for analysis of the relationship between Cx26 expression and clinicopathological parameters of thyroid tumors. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Specificity of immunohistochemical examination of Cx26

In order to prove the specificity of immunostaining of Cx26, we carried out an immunoabsorption study, i.e., paraffin sections from Cx26-positive PTCs were incubated with anti-Cx26 antibody in the absence (Fig. 1A) or presence (Fig. 1B) of pretreatment with an excess amount of the same peptide which had been used to generate this antibody. The positive signal in tumor cells seen in Fig. 1A (without pretreatment) almost completely disappeared as seen in Fig. 1B (with pretreatment), indicating that immunohistochemical examination using this antibody is specific to Cx26. Western blotting of Cx26 using fresh tissue samples from PTCs, FTAs, and normal thyroid tissues was also performed. Cx26 expression was observed only in PTCs but not in FTAs or normal thyroid tissues (Fig. 2), so that these results were consistent with those obtained by immunohistochemistry.

3.2. Immunohistochemical examination of Cx26 expression in PTC, FTC, and FTA

Of the 69 PTCs, 33 were positive for Cx26 (47.8%), and of the 11 FTCs, five were positive for Cx26 (45.5%). All FTAs ($n = 22$) and normal thyroid tissues ($n = 20$) were negative for Cx26. Representative results of immunohisto-

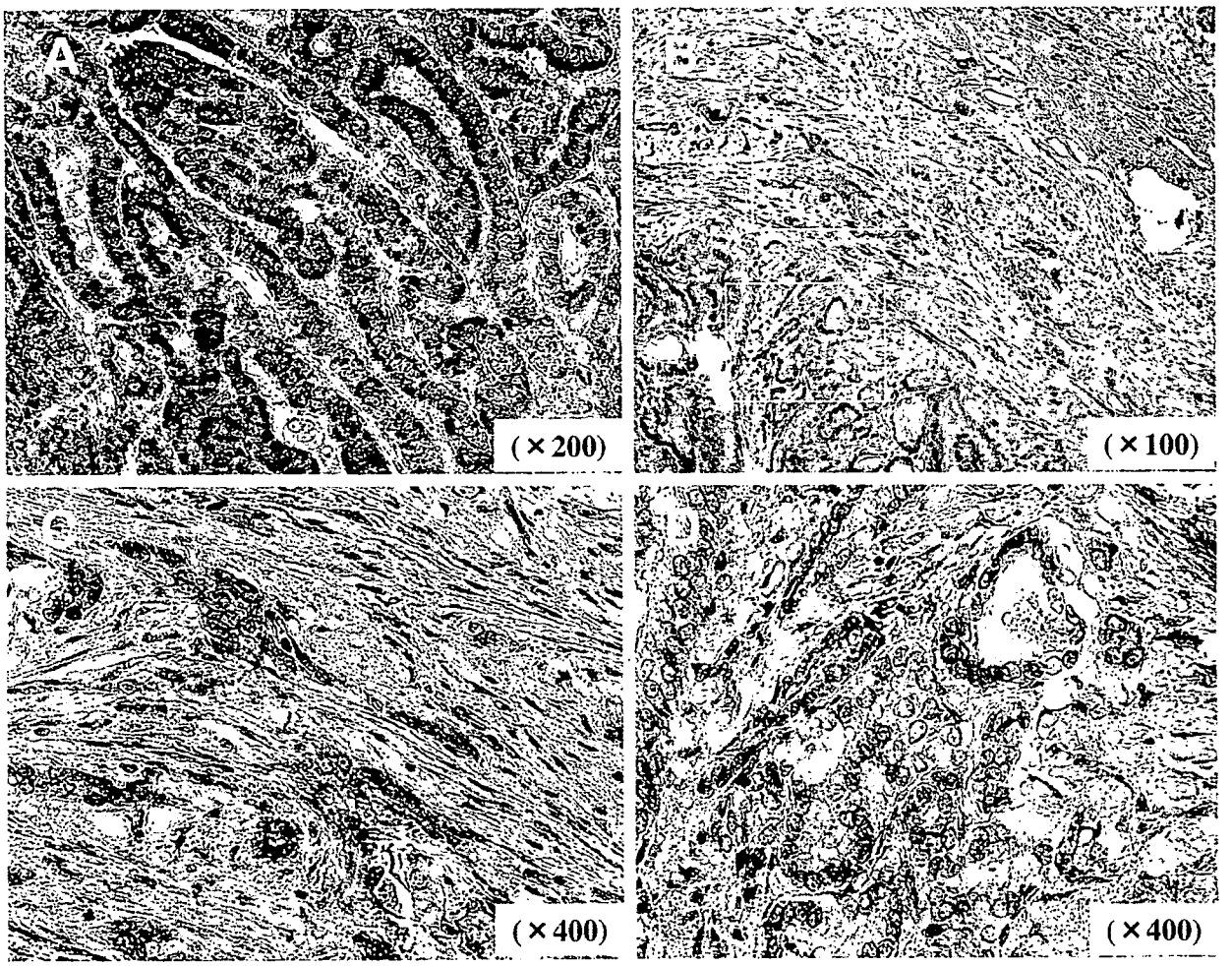

Fig. 3. Representative results of immunohistochemical staining of Cx26. (A) Papillary thyroid cancer strongly positive for Cx26 immunostaining (200×). (B) When a small percentage of tumor cells (about 10% in this tumor) was positive for Cx26 immunostaining, positive tumor cells were mostly observed in the periphery (100×). (C) Cx26-positive tumor cells in the periphery and (D) Cx26-negative tumor cells in the center of a tumor (400×).

chemical examination of Cx26 expression in thyroid cancer tissues are shown in Fig. 3. Fig. 3A shows a PTC positive for Cx26 immunostaining with cytoplasmic staining observed in almost all tumor cells. Fig. 3B shows a PTC with about 10% of tumor cells positive for Cx26 staining. In such a weakly positive tumor, Cx26-positive tumor cells were mostly observed in the periphery (Fig. 3C), but not in the center (Fig. 3D).

3.3. Relationship between Cx26 expression and clinicopathological features of thyroid cancers

A statistically significant association was observed between Cx26 expression and large tumor size ($p = 0.028$ for PTC) and lymph node metastases ($p = 0.053$ (marginally significant) for PTC and $p = 0.035$ for FTC) (Tables 1 and 2). Presence of intra-glandular dissemination of tumor cells was significantly ($p = 0.048$) more frequent in Cx26-positive (30.3%) than in Cx26-negative PTCs (11.1%). There was no significant association between Cx26 expression and histological type of PTCs (Table 1) or invasion type of FTCs (Table 2).

Tumor cell invasion into the lymphatic vessels and blood vessels was immunohistochemically evaluated by using anti-D2-40 and anti-CD34 antibodies to visualize the lymphatic vessels and blood vessels, respectively. Representative results of lymphatic vessel invasion and blood vessel invasion are shown in Fig. 4, where Cx26-positive tumor cells are seen in both lymphatic and blood vessels. Lymphatic vessel invasion was more frequent in Cx26-positive PTCs (6.1%) than in Cx26-negative PTCs (0%) and blood vessel invasion was more frequent in Cx26-positive PTCs (12.1%) than in Cx26-negative PTCs (5.6%) though neither was statistically significant (Table 1).

Table 1
Relationship between Cx26 expression and clinicopathological parameters in thyroid papillary cancers

	Cx26 expression		*p*
	Negative	Positive	
Tumor size			
T1 (≦2 cm)	23	13	0.028
T2 (>2, ≦ 4cm)	9	11	
T3 (>4 cm)	4	8	
T4 (Invasion)	0	1	
Lymph node metastasis			
Positive	18	24	0.053
Negative	18	9	
Lymphatic vessel invasion			
Positive	0	2	0.145
Negative	36	31	
Blood vessel invasion			
Positive	2	4	0.334
Negative	34	29	
Dissemination			
Positive	4	10	
Negative	32	23	0.048
Histological type			
Classical type	28	31	0.203
Follicular variant	6	1	
Encapsulated variant	1	1	
Oxyphilic cell variant	1	0	

Table 2
Relationship between Cx26 expression and clinicopathological parameters in thyroid follicular cancers

	Cx26 expression		*p*
	Negative	Positive	
Tumor size			
T1 (≦2 cm)	2	2	0.632
T2 (>2, ≦4 cm)	1	0	
T3 (>4 cm)	3	3	
T4 (Invasion)	0	0	
Lymph node metastasis			
Positive	1	4	0.035
Negative	5	1	
Lymphatic vessel invasion			
Positive	0	0	...
Negative	6	5	
Blood vessel invasion			
Positive	1	0	0.338
Negative	5	5	
Invasion type			
Minimally invasive	4	5	0.154
Widely invasive	2	0	

3.4. Relationship between Cx26 expression and lymphatic or blood vessel density

A statistically significant ($p = 0.023$) association was observed between peritumoral, but not intratumoral, lymphatic vessel density and lymph node metastasis in PTCs (Table 3). There was a non-significant ($p = 0.099$) tendency for peritumoral lymph vessel density to be associated with Cx26 expression in PTCs (Table 3).

3.5. Comparison of Cx26 expression in primary tumor and lymph node metastases

In 22 PTCs with lymph node metastases, Cx26 expression was compared in primary tumors and lymph node metastases, but no significant difference was observed (Fig. 5).

4. Discussion

We were able to demonstrate immunohistochemically that Cx26 was positive in 47.8% of PTCs and

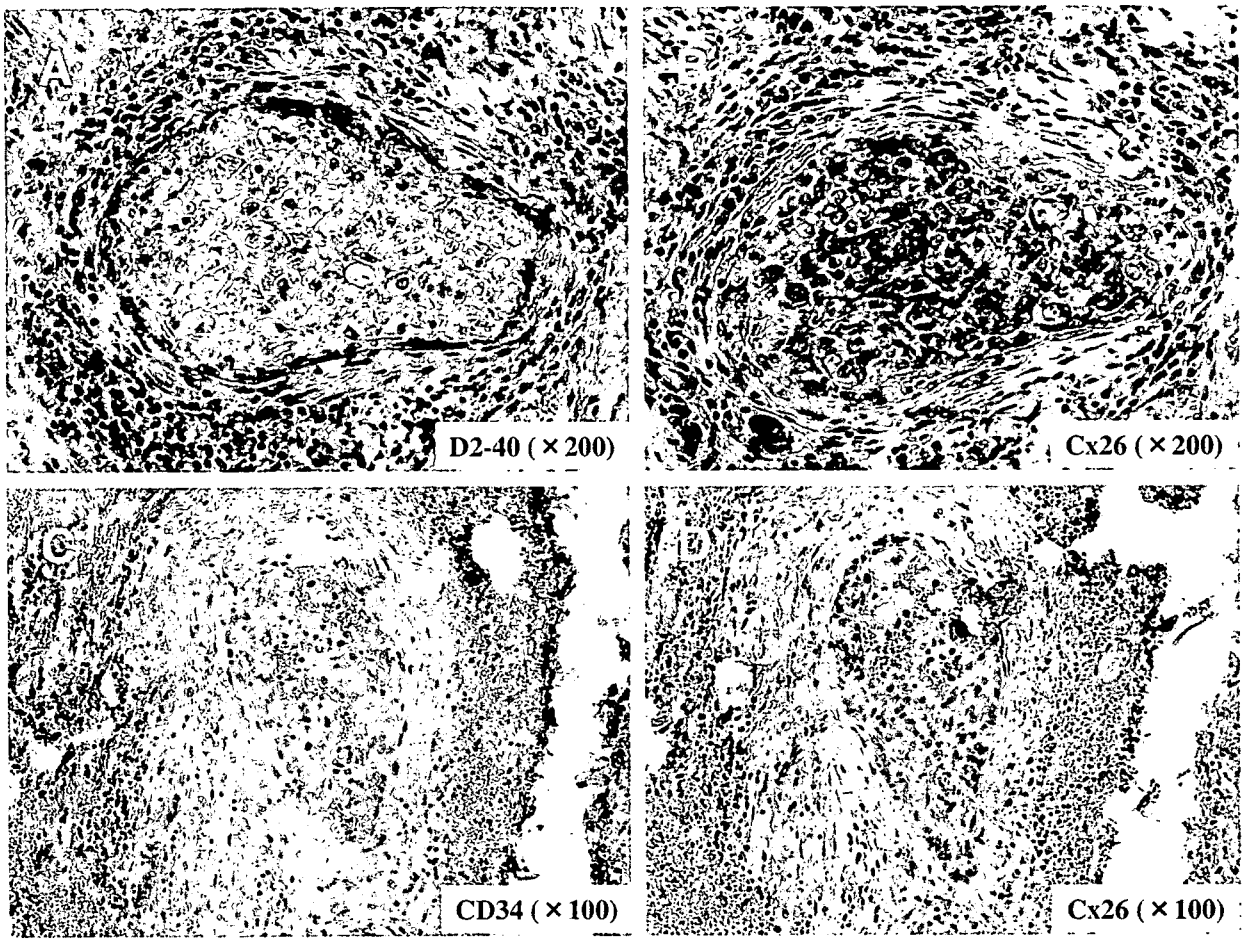

Fig. 4. Representative findings of lymphatic vessel invasion and blood vessel invasion. (A) Lymphatic vessel invasion detected by immunostaining with an anti-D2-40 antibody (200×). (B) In the section next to (A), Cx26-positive tumor cells were observed in the lymphatic vessel (200×). (C) Blood vessel invasion detected by immunostaining with an anti-CD34 antibody (100×). (D) In the section next to (C), Cx26-positive tumor cells were observed in the blood vessel (100×).

Table 3
Relationship between lymph node metastasis and vessel density

	Lymph node metastasis			Cx26 expression		
	Negative	Positive	*p*-Value	Negative	Positive	*p*-Value
Blood vessel density						
Intratumoral	223 ± 15.6[a]	17.6 ± 11.9	0.257	21.2 ± 15.8	17.6 ± 10.7	0.371
Peritumoral	26 ± 2.2	3.7 ± 4.6	0.981	3.9 ± 4.6	2.6 ± 2.9	0.232
Lymphatic vessel density						
Intratumoral	09 ± 2.1	1.9 ± 4.3	0.329	1.7 ± 3.7	1.2 ± 3.6	0.509
Peritumoral	4.8 ± 5.6	8.2 ± 7.7	0.023	5.6 ± 6.6	8.1 ± 7.4	0.099

Mann–Whitney *U*-test was used to examine the relationship between lymph node metastasis and the density of each vessel.
[a] Mean ± SD/field.

45.5% of FTCs but not in FTAs or normal thyroid tissues. These findings seem to be incompatible with the generally accepted thesis that Cx26 is a tumor suppressor gene, but rather to indicate a possible involvement of Cx26 in the pathogenesis of thyroid cancers. Furthermore, we could show in this study a significant association between Cx26 expression and large tumor size, lymph node metastasis and intra-

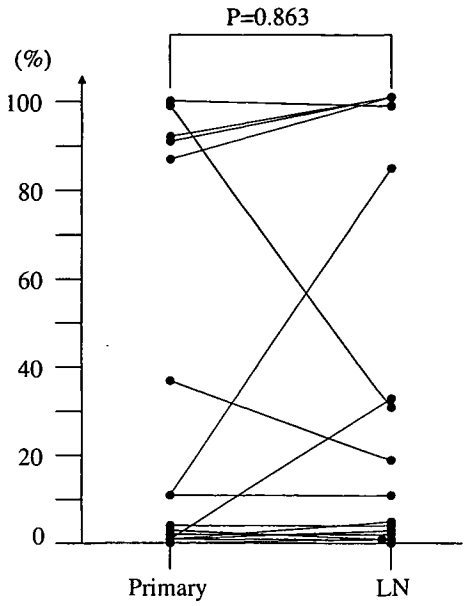

Fig. 5. Comparison of Cx26 expression in primary papillary thyroid cancers and matched lymph node metastases. Paired *t*-test was used to examine the association.

glandular dissemination in PTC, suggesting that Cx26 expression is associated with a biologically aggressive phenotype. These observations are consistent with recent reports that Cx26-positive squamous cell lung cancers are associated with a high recurrence rate [19], that Cx26-positive BL6 mouse melanoma cells increase metastatic properties [18], and that Cx26-positive breast cancers are associated with lymphatic vessel invasion, large tumor size, high histological grade, and poor prognosis [20]. We therefore speculate that the association of Cx26 expression with biologically aggressive phenotype is not a rare phenomenon seen in a few types of tumors but rather a ubiquitous occurrence in a variety of tumors. Although connexins have been characterized as a tumor suppressor gene in several types of cancers [7–9,11,12,22–26], our observations reported here seem to indicate the need for a re-evaluation of the role of Cx26 in the pathogenesis of these tumors.

Staining of Cx26 was mostly observed in the cytoplasm of tumor cells (Fig. 1A), as previously reported for colorectal cancer [3], human breast cancer [20] and squamous lung cancer [19]. Cytoplasmic expression of Cx26 may constitute indirect evidence of a lack of functional gap junction channels between cancer cells which may thus facilitate the local invasion of tumor cells. Interestingly, we were able to show in a previous study on breast cancer that lymphatic vessel invasion was significantly ($p = 0.001$) more frequent in Cx26-positive tumors (39.7%) than in Cx26-negative tumors (14.9%). In this study on thyroid cancer, we could show a non-significant tendency for Cx26 expression to be associated with lymphatic vessel invasion as well as a significant association between Cx26 expression and intra-glandular dissemination or lymph node metastases. Since intra-glandular dissemination of tumor cells is considered to be a sign of lymphatic vessel invasion, our findings seem to suggest that, like Cx26-positive breast cancer cells, Cx26-positive thyroid cancer cells also have a propensity to invade the lymphatic vessels, resulting in the development of lymph node metastases. It is speculated that such a propensity stems from the formation of a heterologous gap junction between Cx26-expressing tumor cells and Cx43-expressing vascular endothelial cells [27,28], leading to the invasion of tumor cells into the lymphatic vessels.

We previously reported [20] that Cx26-positive tumor cells in breast cancer are mostly localized in the periphery (invasion front) of a tumor when Cx26 is weakly positive. Such a peripheral localization of Cx26-positive tumor cells was also observed in PTC. In addition, a statistically significant association was observed between peritumoral lymph vessel density and lymph node metastases in PTC. Putting these finding together makes it tempting to speculate that Cx26-positive tumor cells localizing in the invasion front, even if their number is small, seem to have a good opportunity to invade the adjacent lymphatic vessels. Interestingly, there was also a tendency for Cx26 positivity to be associated with peritumoral lymphatic vessel density. Cx26-positive thyroid cancer cells appear to be very likely to invade the lymphatic vessels. Their intense invasiveness is due to lack of GJIC, formation of heterologous gap junctions with lymphatic vessels, and easy accessibility of lymphatic vessels in the periphery of a tumor.

Koda et al. reported that Cx26 expression is higher in lymph node metastases than in primary tumors in breast cancers, suggesting that Cx26-positive tumor cells are more likely than Cx26-negative tumor cells to invade the lymphatic vessels and metastasize to the lymph nodes [29]. We therefore compared Cx26 expression in primary tumors and lymph node metastases in PTC, but were unable to detect an increased expression of Cx26 in lymph node metastases. Although the reason for this discrepancy is currently unknown, the role of Cx26 in the development of

lymph node metastases may be much more important in breast cancer than in thyroid cancer.

In conclusion, our results suggest that Cx26 may be involved in pathogenesis and is associated with biologically aggressive phenotypes such as large tumor size and lymph node metastases in PTC and FTC. Cx26 seems to function not as a tumor suppressor gene but as an oncogene in these tumors. The limitation of the present study is such a small number of analyzed tumors, especially, FTCs, that a definitive conclusion can not be drawn. Thus, our preliminary observation needs to be validated in a future study including a larger number of tumors with various histological types.

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ORIGINAL ARTICLE

Akt-dependent nuclear localization of Y-box-binding protein 1 in acquisition of malignant characteristics by human ovarian cancer cells

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Y-box-binding protein 1 (YB-1), which is a member of the DNA-binding protein family containing a cold-shock domain, has pleiotropic functions in response to various environmental stimuli. As we previously showed that YB-1 is a global marker of multidrug resistance in ovarian cancer and other tumor types. To identify YB-1-regulated genes in ovarian cancers, we investigated the expression profile of YB-1 small-interfering RNA (siRNA)-transfected ovarian cancer cells using a high-density oligonucleotide array. YB-1 knockdown by siRNA upregulated 344 genes, including *MDR1*, *thymidylate synthetase*, *S100 calcium binding protein* and *cyclin B*, and downregulated 534 genes, including *CXCR4*, *N-myc downstream regulated gene 1*, *E-cadherin* and *phospholipase C*. Exogenous serum addition stimulated YB-1 translocation from the cytoplasm to the nucleus, and treatment with Akt inhibitors as well as Akt siRNA and integrin-linked kinase (ILK) siRNA specifically blocked YB-1 nuclear localization. Inhibition of Akt activation downregulated *CXCR4* and upregulated *MDR1* (*ABCB1*) gene expression. Administration of Akt inhibitor resulted in decrease in nuclear YB-1-positive cancer cells in a xenograft animal model. Akt activation thus regulates the nuclear translocation of YB-1, affecting the expression of drug-resistance genes and other genes associated with the malignant characteristics in ovarian cancer cells. Therefore, the Akt pathway could be a novel target of disrupting the nuclear translocation of YB-1 that has important implications for further development of therapeutic strategy against ovarian cancers.

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Introduction

The Y-box-binding protein 1 (YB-1), which is a DNA/RNA-binding protein also known as dbpB, regulates transcription, translation, DNA damage repair and other biological processes in both the nucleus and cytoplasm (Matsumoto and Wolffe, 1998; Kohno *et al.*, 2003). In the cytoplasm, YB-1 regulates mRNA stability and translational regulation (Evdokimova *et al.*, 2001; Ashizuka *et al.*, 2002; Fukuda *et al.*, 2004), while in the nucleus, it plays a pivotal role in transcriptional regulation through specific recognition of the Y-box promoter element (Ladomery and Sommerville, 1995; Kohno *et al.*, 2003). Interaction of YB-1 with its cognate Y-box-binding site (inverted CCAAT box) is promoted by cytotoxic stimuli, including actinomycin D, cisplatin, etoposide, ultraviolet (UV) and heat shock, leading to the activation of a representative ABC transporter *MDR1/ABCB1* and DNA topoisomerase IIα genes (Asakuno *et al.*, 1994; Furukawa *et al.*, 1998; Ohga *et al.*, 1998). YB-1 also selectively interacts with damaged DNA or RNA, and protects from cytotoxic effects following cellular exposure to cisplatin, mitomycin C, UV and oxygen radicals (Ohga *et al.*, 1996; Ise *et al.*, 1999).

Royer and co-workers were the first to report that nuclear localization of YB-1 is associated with intrinsic *MDR1* expression in human primary breast cancer (Bargou *et al.*, 1997). Immunostaining analysis of various human cancers also supported this result, and showed that nuclear expression of activated YB-1 was closely associated with the acquisition of P-glycoprotein-mediated multidrug resistance (Kuwano *et al.*, 2004). YB-1 has also been shown to induce basal and 5-fluorouracil-induced expression of the major vault protein (*MVP/LRP*) gene, the promoter of which contains a Y-box (Stein *et al.*, 2005). In human malignancies, vault proteins are involved in acquiring drug resistance (Mossink *et al.*, 2003). Taken together, these findings suggest that nuclear localization of YB-1 might play a key role in the acquisition of global drug resistance through transcriptional activation of relevant genes and the repair of damaged DNA (Kuwano *et al.*, 2004).

The nuclear localization of YB-1 is required for transcription and DNA repair in response to various environmental stimuli, such as adenovirus infection (Holm *et al.*, 2002), DNA-damaging agents, UV irradiation, hyperthermia (Stein *et al.*, 2001) and serum stimulation (En-Nia *et al.*, 2005). However, as a nucleocytoplasmic shuttling protein, it is important to understand which signalling molecules are involved in the translocation of YB-1 into the nucleus. Koike *et al.* (1997) first reported the possible role of protein kinase C in YB-1 nuclear translocation in cancer cells exposed to UV irradiation, and highlighted the importance of the YB-1 C-terminal region in cytoplasmic retention. Other studies have suggested the involvement of additional molecules: thrombin-mediated YB-1 nuclear translocation was shown to be inhibited by protein tyrosine phosphatase inhibitor in endothelial cells (Stenina *et al.*, 2000), while Dooley *et al.* (2006) demonstrated the involvement of Jak1 in YB-1 nuclear translocation. Sutherland *et al.* (2005) recently reported that phosphorylation of YB-1 by Akt at serine 102 in the cold-shock domain is required for YB-1 nuclear translocation in cancer cells. Another mechanism for nuclear translocation of YB-1 was shown to be promoted by various cytotoxic anticancer agents, which trigger the proteolytic cleavage by the 20S proteasome of the YB-1 C-terminal fragment containing the cytoplasmic retention signal (Sorokin *et al.*, 2005). In our present study, we have provided evidence that Akt activation is one of the mechanisms for nuclear translocalization of YB-1, and also that YB-1 regulates expression of various cell growth and malignant progression-related genes as well as global drug resistance-related genes including *MDR1*.

Results

Suppression of YB-1 leads to an enhancement of MDR-1 expression and decrease of CXCR-4 expression

We previously reported that YB-1 was expressed in the nucleus in almost 30% of serous ovarian cancers, and that YB-1 nuclear-positive patients had a poor prognosis (Kamura *et al.*, 1999). As nuclear translocation of YB-1 is highly susceptible to environmental stimuli, we first examined whether the stress-inducing exogenous addition of serum could stimulate nuclear translocation of YB-1 in seven serum-deprived human ovarian cancer cell lines. Among the seven cell lines, nuclear YB-1 translocation was stimulated more than twofold in two: RMG-III and SKOV-3 (Figure 1a). In these two lines, serum incubation markedly enhanced Akt phosphorylation and increased translocation of YB-1 into the

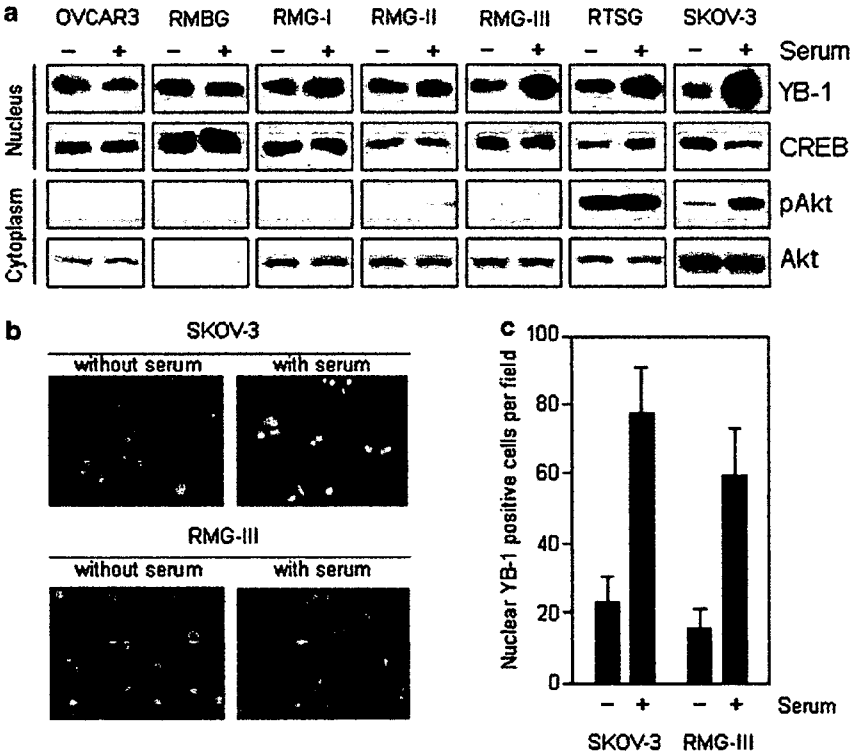

Figure 1 Levels of Akt phosphorylation and nuclear localization of YB-1 in ovarian cancer cell lines with or without serum stimulation. (**a**) Cytoplasmic and nuclear extracts were prepared 1 h after 10% serum stimulation. Anti-YB-1 and anti-CREB immunoblots were performed on nuclear extracts, and anti-pAkt and anti-Akt immunoblots were performed with cytoplasmic extracts. CREB and Akt are shown as a loading control. (**b**) Immunofluorescent staining of YB-1 in ovarian cancer cells. Cells stimulated with or without serum for 1 h were fixed and permeabilized, incubated at 4°C with the primary YB-1 antibody, then with the Alexa Flour 546-labelled secondary antibody. (**c**) Quantitative analysis of YB-1 nuclear localization as shown in Figure 1b. Data are mean of three independent experiments; bars ±s.d.

nucleus, as shown by immunofluorescense analysis (Figure 1b and c).

Although YB-1 is known to regulate the expression of several genes at the transcriptional level, the complete network of genes associated with YB-1 has not been elucidated. We therefore, explored the expression profile of YB-1 siRNA-treated SKOV-3 cells and mock-treated SKOV-3 cells using a high-density oligonucleotide microarray. We transfectd YB-1 siRNA into SKOV-3 cells at a concentration of 200 and 400 nM. Transfection of 200 nM YB-1 siRNA decreased expression of YB-1 mRNA by only 45%, whereas 400 nM YB-1 siRNA decreased by 70% (Figure 2). Of the 54 675 RNA transcripts and variants in the microarray, we identified 344 genes that were increased more than twofold and 534 genes that were decreased 0.5-fold or less in both 200 and 400 nM YB-1 siRNA-transfected cells (Supplementary Table S1). Upregulated genes were classified into 'cell cycle' ($P<0.0001$), 'cytoskeleton organization and biogenesis' ($P=0.0003$), 'cell growth and/or maintenance' ($P=0.0005$), and GO SLIMS Biological Process' ($P=0.0013$). Downregulated genes were classified into 'catalytic activity' ($P=0.0007$) and 'transferase' ($P=0.0010$). We selected 46 genes that we expected to be associated with drug resistance, cell growth, cancer malignant progression and cell signalling (Table 1), and chose three of these for further study: *MDR1*, *MVP/LRP* and chemokine (C-X-C motif) receptor 4 (*CXCR4*).

We used quantitative real-time PCR (QRT-PCR) to confirm whether expression of these three genes was modulated in YB-1 siRNA-transfected cells. Expression of *CXCR4* decreased by 67%, whereas expression of *MVP/LRP* was unaffected by the siRNA (Figure 2). *MDR1* expression was increased approximately 30-fold in 400 nM YB-1 siRNA-transfected cells compared with control siRNA-transfected cells. The results of QRT-PCR are broadly consistent with those of the microarray analysis.

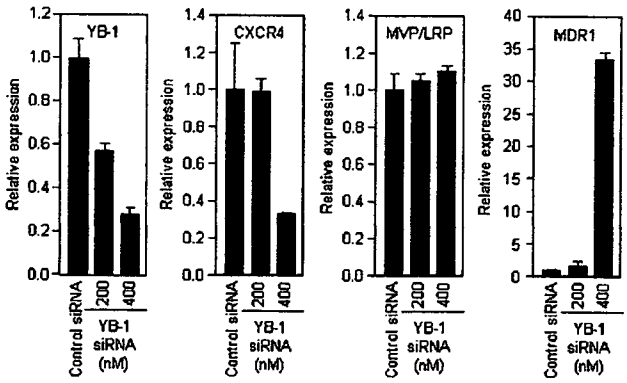

Figure 2 Effect of YB-1 knock down on expression of MDR1, MVP/LRP and CXCR4. SKOV-3 cells were treated with YB-1 siRNA for 48 h and then total RNA was prepared. QRT-PCR was performed for MDR1, MVP/LRP, CXCR4, YB-1 and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative gene expression for each sample was determined using the formula $2^{(-\Delta C_t)} = 2^{(C_t(\text{GAPDH})-C_t(\text{target}))}$ which reflected target gene expression normalized to GAPDH levels. Data were mean of three independent experiments; bars ± s.d.

Pearson correlation and hierarchical cluster analysis of selected NCI-60 genes

We next examined a database containing the expression profile of the National Cancer Institute (NCI)-60 panel from the Developmental Therapeutics Program (http://www.dtp. nci.nih.gov/), shown as a log of mRNA expression level in the NCI screen. When the Pearson correlation coefficients were calculated, YB-1 was negatively correlated with *MDR1* expression, positively correlated with *CXCR4* expression and showed little correlation with *MVP/LRP* (Figure 3). Moreover, the hierarchical dendrogram of gene expression revealed that *YB-1* and *CXCR4* belong to the same cluster, whereas *MDR1* and *MVP/LRP* are clustered in a separate group (Figure 4). Together, these NCI-60 panels suggest that cellular levels of YB-1 negatively modulate expression of *MDR1* and positively regulate expression of *CXCR4*. In this cluster analysis, six ovarian cancer cell lines including SKOV-3 showed various correlation coefficiencies with YB-1 expression. Our oligonucleotide array analysis was performed only with SKOV-3, and correlation coefficiencies among ovarian cancer cell lines would depend upon which cell line was analysed.

Akt activity is prerequisite for nuclear translocation of YB-1and transcriptional regulation by YB-1

Phosphorylation of YB-1 by Akt is a necessary requirement for its translocation from the cytoplasm into the nucleus (Sutherland *et al.*, 2005). We therefore investigated the effect of two inhibitors of Akt activation (LY294002 and 1L-6-hydroxymethyl-*chiro*-inositol 2(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate) on serum-stimulated SKOV-3 cells. Both Akt inhibitors markedly blocked the nuclear accumulation of YB-1, whereas treatment with inhibitors of MEK (U0126), p38MAPK (SB203580) and JNK (SP600125) had no effect on nuclear translocation (Figure 5a). In addition, phosphorylation of Akt was inhibited by LY294002 and octadecylcarbonate, but not by U0126, SB203580 and SP600125. Immunofluorescence analysis with a YB-1 antibody also demonstrated the predominant accumulation of YB-1 in the cytoplasm when treated with LY294002 and octadecylcarbonate (Figure 5b and c). As Akt inhibitors blocked the nuclear translocation of YB-1, we examined whether they could also affect expression of YB-1-regulated genes. *CXCR4* expression was found to be downregulated in a dose-dependent manner following treatment with the Akt inhibitors when determined by QRT-PCR analysis (Figure 5d). Treatment with Akt inhibitors upregulated the expression of *MDR1*, but not *MVP/LRP*.

SKOV-3 cells expressed high level of Akt1 protein, very low level of Akt2 protein, and no Akt3 protein when assayed by immunoblotting analysis (Figure 6a). We introduced siRNA targeting Akt or ILK into SKOV-3 cells at a concentration of 100 and 10 nM,

Table 1 List of genes differentially expressed in YB-1 siRNA-transfected SKOV-3 cells

Unigene	*Accession*	*Symbol*	*Description*	*Mean fold change*
Hs.489033	NM_000927	ABCB1	MDR1, ATP-binding cassette, sub-family B (MDR/TAP), member 1	2.46
Hs.369762	AB077208	TYMS	Thymidylate synthetase	1.71
Hs.198363	NM_018518	MCM10	MCM10 minichromosome maintenance deficient 10	1.70
Hs.405958	U77949	CDC6	CDC6 cell division cycle 6 homolog (*S. cerevisiae*)	1.66
Hs.442658	AB011446	AURKB	Aurora kinase B	1.65
Hs.516484	NM_005978	S100A2	S100 calcium-binding protein A2	1.48
Hs.23960	NM_031966	CCNB1	Cyclin B1	1.40
Hs.460184	AA604621	MCM4	MCM4 minichromosome maintenance deficient 4 (*S. cerevisiae*)	1.40
Hs.438720	AF279900	MCM7	MCM7 minichromosome maintenance deficient 7 (*S. cerevisiae*)	1.36
Hs.433168	NM_002960	S100A3	S100 calcium binding protein A3	1.33
Hs.115474	NM_002915	RFC3	Replication factor C (activator 1) 3, 38 kDa	1.28
Hs.122908	NM_030928	CDT1	DNA replication factor	1.28
Hs.329989	NM_005030	PLK1	Polo-like kinase 1 (*Drosophila*)	1.21
Hs.334562	NM_001786	CDC2	Cell division cycle 2, G1 to S and G2 to M	1.21
Hs.74034	NM_001753	CAV1	Caveolin 1, caveolae protein, 22 kDa	1.19
Hs.477481	NM_004526	MCM2	MCM2 minichromosome maintenance deficient 2, mitotin	1.16
Hs.284244	M27968	FGF2	Fibroblast growth factor 2 (basic)	1.10
Hs.179565	NM_002388	MCM3	MCM3 minichromosome maintenance deficient 3 (*S. cerevisiae*)	1.08
Hs.194698	NM_004701	CCNB2	Cyclin B2	1.04
Hs.506989	BC001866	RFC5	Replication factor C (activator 1) 5, 36.5 kDa	1.02
Hs.171596	NM_004431	EPHA2	EPH receptor A2	1.01
Hs.194143	NM_007294	BRCA1	Breast cancer 1, early onset	0.75
Hs.156346	NM_001067	TOP2A	Topoisomerase (DNA) II alpha 170 kDa	0.64
Hs.473163	NM_001719	BMP7	Bone morphogenetic protein 7 (osteogenic protein 1)	0.54
Hs.391464	NM_004996	ABCC1	MRP-1, ATP-binding cassette, sub-family C (CFTR/MRP), member 1	0.20
Hs.256301	NM_199249	MGC13170	Multidrug resistance-related protein	0.15
Hs.513488	NM_017458	MVP	Major vault protein	−0.05
Hs.482526	NM_014886	TINP1	TGF beta-inducible nuclear protein 1	−0.23
Hs.525557	NM_000295	SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member	−1.01
Hs.500466	BG403361	PTEN	Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	−1.05
Hs.25292	NM_002229	JUNB	Jun B proto-oncogene	−1.06
Hs.132225	AI934473	PIK3R1	Phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	−1.16
Hs.83169	NM_002421	MMP1	Matrix metalloproteinase 1 (interstitial collagenase)	−1.22
Hs.508999	NM_002742	PRKCM	Protein kinase C, mu	−1.29
Hs.326035	NM_001964	EGR1	Early growth response 1	−1.29
Hs.2256	NM_002423	MMP7	Matrix metalloproteinase 7 (matrilysin, uterine)	−1.32
Hs.197922	NM_018584	CaMKIINalpha	Calcium/calmodulin-dependent protein kinase II	−1.36
Hs.132966	AA005141	MET	Met proto-oncogene (hepatocyte growth factor receptor)	−1.39
Hs.208124	NM_000125	ESR1	Estrogen receptor 1	−1.50
Hs.73793	M27281	VEGF	Vascular endothelial growth factor	−1.53
Hs.381167	AW512196	SERPINB1	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 1	−1.70
Hs.413111	NM_002661	PLCG2	Phospholipase C, gamma 2 (phosphatidylinositol-specific)	−1.75
Hs.461086	NM_004360	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	−1.92
Hs.472793	AI631895	SGK2	Serum/glucocorticoid regulated kinase 2	−2.04
Hs.372914	NM_006096	NDRG1	*N-myc* downstream regulated gene 1	−2.34
Hs.421986	NM_001008540	CXCR4	Chemokine (C-X-C motif) receptor 4	−2.64

High-density oligonucleotide array was performed on 400 nM YB-1 siRNA-treated SKOV-3 cells and mock-treated cells. siRNA duplexes were transfected using LipofectAMINE2000 with Opti-MEM mediums. At 48 h after siRNA transfection, total RNA was prepared, and subjected to double-stranded cDNA synthesis and *in vitro* transcription. The labeled cRNA was applied to the oligonucleotide microarray.

respectively, and silencing effects of siRNA were analysed by immunoblotting (Figure 6a). In Akt siRNA almost completely silenced both Akt1 and Akt2, and siRNA for ILK, the upstream kinase for Akt, silenced ILK on protein level. Treatment with Akt siRNA and ILK siRNA resulted in a marked decrease in both pAkt expression and nuclear accumulation of YB-1 (Figure 6a). As both Akt and ILK siRNA blocked the nuclear translocation of YB-1, we examined their effects on expression of YB-1-regulated genes (Figure 6b). Treatment with Akt and ILK siRNA downregulated the expression of *CXCR4* gene, and upregulated the expression of *MDR1* gene. By contrast there appeared no marked effect on the expression of *MVP/LRP* and *YB-1* genes when treated with both siRNAs (Figure 6b).

Effect of LY294002 treatment on Akt phosphorylation and YB-1 nuclear localization in SKOV-3 xenograft

To further investigate the involvement of Akt in tumoural YB-1 nuclear localization, an *in vivo* xenograft