

Keywords: cyclin-dependent kinase; Ki-67 expression; endometrial cancer; low risk; chemo-sensitivity

Prognostic importance of CDK4/6-specific activity as a predictive marker for recurrence in patients with endometrial cancer, with or without adjuvant chemotherapy

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Background: Pathologically low-risk endometrial cancer patients do not receive postoperative treatment; however, 10–15% of these patients show recurrence with poor prognosis. We evaluated the clinical importance of cyclin-dependent kinase 4/6 (CDK4/6) activity, and its significance as a novel biomarker for the prognosis and chemo-sensitivity of endometrioid endometrial carcinoma (EEC).

Methods: Cyclin-dependent kinase 4/6 expression and enzyme activity in 109 tumour samples from patients with EEC were examined with a cell-cycle profiling (C2P) assay. CDK4/6-specific activity (CDK4/6SA) was determined, and its relationship with clinicopathological factors and expression of Ki-67 was analysed.

Results: CDK4/6-specific activity was significantly correlated with Ki-67 ($P=0.035$), but not with any other clinicopathological characteristics. CDK4/6SA was significantly higher ($P=0.002$) in pathologically low-risk patients (not receiving adjuvant chemotherapy, $n=74$) than in intermediate- or high-risk patients (receiving adjuvant chemotherapy, $n=35$). In addition, patients with high CDK4/6SA (>3.0) showed significantly ($P=0.024$) shorter progression-free survival (PFS) than those with low CDK4/6SA (<3.0). Although Ki-67 expression itself was not a marker for prognosis, the combination of high CDK4/6SA and high Ki-67 expression ($>15\%$) was robustly associated with shorter PFS ($P=0.015$), and this combination was an independent poor prognostic factor in the low-risk group. Inversely, in the intermediate-/high-risk group, patients with high CDK4/6SA had a tendency of a more favourable prognosis compared with patients with low CDK4/6SA ($P=0.063$).

Conclusions: CDK4/6-specific activity can be used as a biomarker to predict prognosis and, possibly, chemo-sensitivity. The combination of Ki-67 expression might strengthen the clinical usefulness of CDK4/6SA as a biomarker.

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Endometrial cancer is the fourth most common malignancy among women in the United States (Siegel *et al*, 2015). The main therapeutic options are surgery, chemotherapy, and radiation (Baekelandt and Castiglione, 2008). Despite recent notable developments in molecular biology, the only current indicators for the use of adjuvant chemotherapy in endometrial cancer are clinicopathological risk factors. Risk in surgically treated endometrial cancer patients is generally determined based on the patient's pathological diagnosis, including the stage, histological subtypes, tumour grade, lymph-node status, and depth of myometrial invasion, which serve to classify patients into three main risk groups: low-risk, intermediate-risk, and high-risk (Baekelandt and Castiglione, 2008; Wright *et al*, 2012). Adjuvant chemotherapy is mainly used for high-risk patients, while indicators for intermediate-risk patients remain controversial (Baekelandt and Castiglione, 2008; Wright *et al*, 2012). However, 10–15% of early-stage patients who do not receive adjuvant chemotherapy show recurrence, and their prognoses are exclusively poor with median survival of <10 months and a 5-year survival rate close to 15% (Morrow *et al*, 1991; Creutzberg *et al*, 2000). These statistics demonstrate the necessity of identifying novel biomarkers to select poor prognostic patients with indications of adjuvant treatments.

Histologically, >80% of all endometrial cancer cases are diagnosed as endometrioid endometrial carcinoma (EEC) (Baekelandt and Castiglione, 2008). Endometrioid endometrial carcinoma is frequently associated with somatic mutations in genes involved in the RAS/PI3K pathway, such as *KRAS*, *PIK3CA*, *PTEN*, *AKT1*, *CTNNB1*, and *CCND1* (Fukuchi *et al*, 1998; Moreno-Bueno *et al*, 2003; Oda *et al*, 2005, 2008; Shoji *et al*, 2009; Musgrove *et al*, 2011; Ikeda *et al*, 2012, 2013). Cyclin D1 is upregulated by the RAS/PI3K/beta-catenin pathway and cooperates with cyclin-dependent kinase 4 and 6 (CDK4/6) to promote the cell cycle from the G1 to the S phase (Musgrove *et al*, 2011). Elevated CDK4 expression is observed in 34–77% of EEC cases and is considered to be an early event of neoplastic transformation in EEC (Tsuda *et al*, 2000; Shih *et al*, 2003). Although CDK6 interacts with CDK4, little is known about the role of CDK6 activity in EEC (Shiozawa *et al*, 1997; Nakashima *et al*, 1999; Wright *et al*, 2012). Moreover, the enzymatic activities of CDK4 and CDK6 in EEC have not been analysed in detail. On basis of the high frequency of RAS/PI3K-Cyclin D1 pathway-related mutations in EEC, we hypothesised that the aggressiveness of this disease might be associated with abnormally high CDK4/6 activity.

We developed a cell-cycle profiling (C2P) assay as a novel diagnostic technique to evaluate the activities and expression levels of CDKs (Ishihara *et al*, 2005). The C2P assay can be used to determine the specific activity (SA; activity/expression) of CDKs, as kinase activity can be compensated by protein expression. Previous studies using this assay focussed on analyses of CDK1/2SA; for example, in breast cancer, high CDK1/2SA was associated with significantly poor prognosis (Kim *et al*, 2008). Furthermore, Ki-67 expression is commonly used as a marker of cell proliferation, and the combination of CDK1/2SA and Ki-67 expression was found to strongly predict the sensitivity to paclitaxel in breast cancer (Nakayama *et al*, 2009; Kim *et al*, 2012).

In the present study, we established a novel C2P method to accurately measure CDK4/6SA, and to clarify the clinical importance of CDK4/6 to EEC.

MATERIALS AND METHODS

Tumour samples. Surgical samples were obtained from 119 patients who had primary EEC and underwent tumour resection at the University of Tokyo Hospital. All tumour specimens were

freshly frozen and stored at -80°C until use. All the patients provided informed consent for the collection and research use of their samples, and the use of tissues in this study was approved by the appropriate institutional ethics committees. All patients underwent primary surgery, including hysterectomy, bilateral salpingo-oophorectomy, and systematic lymphadenectomy, and were diagnosed using the International Federation of Gynecology and Obstetrics (FIGO) 2009 staging criteria. The intermediate-/high-risk group included stage I–III patients with at least one of the following factors: deep myometrial invasion ($>1/2$), positive lymph nodes, adnexal metastases, and pelvic peritoneal metastases. Adjuvant therapy was administered to these patients as previously described (Onda *et al*, 1997; Murayama-Hosokawa *et al*, 2010). Stage IB patients received adjuvant radiation therapy (whole pelvis with 50.4 Gy in 28 fractions). Adjuvant chemotherapy with 3–6 cycles of platinum-based chemotherapy was indicated for the other intermediate-/high-risk patients. Stage IV patients (in the high-risk group) were treated post-operatively with 6–8 cycles of platinum-based chemotherapy. All other patients (low-risk group) were closely monitored, but did not receive any additional therapy.

C2P assay. The C2P assay (Sysmex Corporation, Kobe, Japan) was performed as described previously (Ishihara *et al*, 2005; Kim *et al*, 2012). Cyclin-dependent kinase 4/6 activities were determined via the C2P assay from frozen surgical tissue samples. CDK4/6-specific activity was calculated using the following formula:

$$\text{Kinase activity}(\text{CDK4CDK6})/\text{Expression}(\text{CDK4CDK6})$$

The lower detection limit of the expression assay of CDK4 and CDK6 was defined with $4\text{ eU } \mu\text{l}^{-1}$ of the lysate. A 'not informative case' was defined when the expression levels of a sample were less than the lower limits for both the CDK4 and CDK6 expression assays.

CDK4 and Ki-67 expression. For CDK4 and Ki-67 immunohistochemical analysis, $4\text{-}\mu\text{m}$ sections were cut from the tissue microarray. Immunohistochemical staining was performed according to standard techniques using a Ventana Benchmark XT autostainer (Ventana Medical Systems Inc., Tucson, AZ, USA).

Anti-CDK4 (Invitrogen, Waltham, MA, USA) and anti-Ki-67 (Dako, Carpinteria, CA, USA) monoclonal antibodies were applied at a dilution of 1:200 and 1:100, respectively. The extent of CDK4 expression was scored as follows: 0 (0%), 1+ (0–5%), 2+ (5–50%), and 3+ (>50%) along with the previous study (Shiozawa *et al*, 1997). The extent of Ki-67 staining was scored as follows: 0 (0–4%), 1+ (5–14%), 2+ (15–49%), and 3+ (>50%). The results were scored using two slides, and the average was recorded as the final expression score.

Statistical analysis. The association of variables with clinical characteristics was evaluated using the Fisher's exact test and the Student's *t*-test. In all tests, differences were considered to be significant at $P < 0.05$. Survival curves were constructed using the Kaplan–Meier method and were compared with the log-rank test. Multivariate analyses were evaluated using the Cox's proportional hazard model.

RESULTS

Characteristics of patients. Among the 119 patients enrolled in the study, 9 samples were excluded due to the lack of informative results based on the C2P assay; 7 tumours showed low CDK4/6 expression, and assay failure was noted for 2 tumours. Another sample was excluded because of a history of neo-adjuvant chemotherapy. As shown in Table 1, the remaining 109 patients were eligible for assessment in this study, and the median follow-up time was 64 months. According to their clinicopathological

Table 1. Clinicopathological background in 109 patients with endometrioid endometrial cancer

Parameter	Criteria	N (%)
Median follow-up	64 months	
Age	<60 ≥60	68 (62) 41 (38)
Stage	I II III IV	67 (61) 9 (8) 28 (26) 5 (5)
Histological grade	1 2 3	77 (71) 22 (20) 10 (9)
Tumour size	≤5 cm >5 cm	64 (59) 45 (41)
Myometrial invasion	No invasion ≤1/2 >1/2	17 (16) 70 (64) 22 (20)
Lymph-node metastasis	Positive Negative	23 (21) 86 (79)
Vascular invasion	Positive Negative	22 (20) 87 (80)
Lymphatic Invasion	Positive Negative	23 (21) 86 (79)
Adjuvant chemotherapy	Yes No	35 (32) 74 (68)
Regimen	Paclitaxel and carboplatin Adriamycin and cisplatin (with or without cyclophosphamide)	28 (80) 7 (20)
Radiotherapy (whole pelvis)	Yes No	17 (16) 92 (84)
Recurrence	Yes No	18 (17) 91 (83)
Prognosis	No evidence of disease Alive with disease Death of disease	94 (86) 9 (8) 6 (6)

characteristics, 67 patients (61%) were at stage I and 35 patients (32%) considered to be pathologically intermediate/high risk were treated with adjuvant chemotherapy. After primary treatment, 18 patients (17%) experienced recurrence. Overall, nine patients (8%) were alive with disease and six patients (6%) died from the disease.

CDK4/6SA is significantly higher in pathologically low-risk patients, and is correlated with Ki-67 expression. The median CDK4/6SA obtained via the C2P assay was 4.8 (range: 0–350.3). Analysis of CDK4 and Ki-67 expression was performed in 103 cases with samples that were suitable for immunohistochemistry. The average CDK4 expression scores determined by immunohistochemistry (CDK4-IHC) were 1.0 or higher in 53 cases (51%). The average Ki-67 expression scores were 1.5 or higher in 69 cases (67%). CDK4/6-specific activity was significantly associated with Ki-67 expression ($P=0.035$), but not with any other clinicopathological factor such as age, stage, lymph-node metastasis, histological grade, tumour size, myometrial invasion, lymph vascular invasion, and CDK4-IHC expression (Table 2).

We further focussed on variation in CDK4/6SA among patients classified based on the pathological risk, which is the main determinant for the indication of adjuvant chemotherapy. In particular, CDK4/6SA was significantly higher in low-risk patients than in intermediate-/high-risk patients ($P=0.002$; Figure 1A). Receiver operating characteristic (ROC) curve analysis in the low-risk patients revealed that the Youden Index was maximised at 3.2

for CDK4/6SA (area under the curve = 0.759, $P=0.003$; Supplementary Figure 1). Therefore, we defined the CDK4/6SA cutoff value as 3, and this value was employed for further analyses. The number of recurrent cases in each group according to pathological risk and/or CDK4/6SA is summarised in Supplementary Table 1.

As both CDK4/6 and Ki-67 are associated with cell-cycle progression, we also assessed the correlation between CDK4/6SA and Ki-67 expression using a scatter diagram with CDK4/6SA expressed on a logarithmic scale. As a result, in the low-risk group, 7 of 8 (88%) cases with progressive disease were plotted in the zone representing high CDK4/6SA (>3) and high Ki-67 expression (>1.5), whereas only 17 of 66 (26%) cases without progression were classified in the same zone, representing a statistically significant difference ($P=0.002$; Figure 1B). On the other hand, in the intermediate-/high-risk group only 2 of 10 (20%) cases with progression and 9 of 22 (41%) cases without progression were plotted in the zone of high CDK4/6SA and high Ki-67 expression, which did not reflect a significant difference ($P=0.43$; Supplementary Figure 2).

CDK4/6SA is a useful biomarker for predicting recurrence in pathologically low-risk EEC patients. Next, we explored the prognostic importance of Ki-67 expression using Kaplan–Meier survival analysis. Ki-67-High (Ki-67 average score >1.5) was not significantly associated with prognosis in both the low-risk ($P=0.191$, $N=71$) and intermediate-/high-risk patients ($P=0.392$, $N=31$) (Supplementary Figure 3). We next examined the prognostic impact of CDK4/6SA. The CDK4/6SA value was divided into CDK4/6SA-High (CDK4/6SA >3) and CDK4/6SA-Low (CDK4/6SA <3), and progression-free survival (PFS) was compared between the groups. In low-risk patients, CDK4/6SA-High was associated with a significantly worse prognosis than CDK4/6SA-Low ($P=0.024$, $N=74$; Figure 2A). However, the result tended to be inversed in intermediate-/high-risk patients, showing a more favourable prognosis in patients with CDK4/6SA-High ($P=0.063$, $N=35$; Figure 2B).

The combination of CDK4/6SA-High and Ki-67-High was significantly associated with poor prognosis, compared with other combinations of these factors (CDK4/6SA-Low and/or Ki-67-Low) in the low-risk patients ($P=0.015$, $N=71$; Figure 2C). However, this combination did not significantly influence prognosis in the intermediate-/high-risk patients ($P=0.392$, $N=32$; Figure 2D).

CDK4/6SA is an independent prognostic factor in low-risk EEC patients, and its predictive value is reinforced by Ki-67 expression. We conducted univariate and multivariate analyses in the low-risk group to more comprehensively analyse the relationship between the potential biomarkers and clinicopathological features. CDK4/6-specific activity alone was only associated with PFS in the univariate analyses (hazards ratio [HR] = 10.79, 95% confidence interval [CI]: 1.34–86.87, $P=0.026$; Supplementary Table 2). Multivariate analysis among CDK4/6SA, age, and tumour size indicated that CDK4/6SA is an independent prognostic factor in the low-risk group (HR = 10.79, 95% CI: 1.34–86.87, $P=0.036$; Supplementary Table 2). Next, we combined Ki-67 expression with CDK4/6SA in the low-risk group. As shown in Table 3, univariate analysis showed that the combination of CDK4/6SA-High and Ki-67-High was significantly associated with poor prognosis (HR = 11.80, 95% CI: 1.44–97.3, $P=0.022$). The CDK4-IHC score was not significantly associated with prognosis (HR = 1.6, 95% CI: 0.62–4.12, $P=0.33$). Multivariate analysis using the CDK4/6SA-High and Ki-67-High combination, vascular invasion, and tumour size showed that only the combination of CDK4/6SA-High and Ki-67-High was an independent poor prognostic marker (HR = 11.39, 95% CI: 1.23–105, $P=0.033$). These data suggest that CDK4/6SA could be used to effectively predict 'high-risk' patients with EEC in the pathologically low-risk

group, and the combination of Ki-67 expression could enhance the clinical significance of CDK4/6SA.

DISCUSSION

In this analysis of 109 clinical EEC samples, we demonstrated (i) a novel analysis method for examining the activity and expression of CDK4/6 using a C2P assay, (ii) an association between CDK4/6SA and Ki-67 expression, (iii) CDK4/6SA as a significant predictor of recurrence in the pathologically low-risk group, and (iv) the combination of CDK4/6SA and Ki-67 expression as a more robust predictor of recurrence in the low-risk group. In addition, our data indicated that CDK4/6SA might enhance the chemo-sensitivity in patients with adjuvant chemotherapy in the intermediate-/high-

risk group, and that CDK4/6SA is an independent prognostic factor in the low-risk group.

Various useful clinicopathological prognostic factors for EEC have been previously reported, including stage, tumour grade, depth of myometrial invasion, lymphovascular invasion, and tumour size and age (Salvesen *et al*, 2012). In addition, several biomarkers, including chromosomal instability, microsatellite instability, and TP53 mutations, have been analysed with respect to EEC (Lax *et al*, 2000; Murayama-Hosokawa *et al*, 2010). However, the status of adjuvant treatment has not yet been considered in detail, and several biomarkers might not be unveiled according to different treatment protocols. Moreover, no appropriate biomarkers have been identified to predict chemo-sensitivity in EEC. Because of the high rate of recurrence in patients with non-adjuvant chemotherapy, novel biomarkers are warranted to better identify high-risk patients who are not categorised as 'high-risk' based on the conventional pathological criteria. In our analysis, CDK4/6SA in pathologically low-risk patients (those not receiving adjuvant chemotherapy) was more broadly distributed than that in intermediate-/high-risk patients (those receiving adjuvant chemotherapy). Because CDK4/6SA indicates the activity of CDK4/6 per unit, and CDK4/6 is strongly associated with the cell cycle (Bates *et al*, 1994), we hypothesised that high CDK4/6SA might be strongly associated with poor prognosis, especially in cases diagnosed as 'low-risk' based on the pathological criteria. This hypothesis was verified, as CDK4/6SA was significantly associated with prognosis in the low-risk patients, suggesting that high CDK4/6SA promotes cell proliferation and tumour growth.

Interestingly, CDK4/6SA-High showed better prognosis in the intermediate-/high-risk patients. These data indicate that tumours with high CDK4/6SA might be more sensitive to platinum-based chemotherapy. These data are in agreement with a report demonstrating that CDK4/6 activity is required for DNA-damaging anticancer drugs to effectively suppress cell proliferation (Roberts *et al*, 2012). We recently reported that mutant cyclin D1 (T286I) promoted the cell cycle by regulating Rb function (Ikeda *et al*, 2013). As CDK4/6 cooperates with cyclin D1 to accelerate the cell cycle, CDK4/6SA-high tumours might be associated with rapid cell proliferation and promote the cell cycle from the G1 to the S phase. As platinum-DNA adducts cause various cellular responses, including cell-cycle arrest and apoptosis (Wang and Lippard, 2005; He *et al*, 2011), it is reasonable to suggest that the cell-cycle augmentation induced by high CDK4/6SA might be associated with sensitivity to platinum-based chemotherapy (He *et al*, 2011; Roberts *et al*, 2012).

Although both CDK4/6 and Ki-67 are associated with cell proliferation (Gerdes *et al*, 1984), in our study, Ki-67 expression

Table 2. Association between CDK4/6SA and various clinicopathological backgrounds, including Ki-67 expression

Parameter	Criteria	CDK4/6SA		P-value
		≤4.8	>4.8	
Age	<60	35	33	0.845
	≥60	20	21	
Stage	≤II	36	41	0.293
	≥III	19	13	
Lymph-node metastasis	No	44	44	0.760
	Yes	7	5	
Histological grade	I	34	41	0.140
	II & III	20	12	
Tumour size	<5 cm	30	30	1.000
	≥5 cm	22	23	
Myometrial invasion	≤1/2	44	43	0.798
	>1/2	10	8	
Vascular invasion	No	41	44	0.474
	Yes	13	9	
Lymphatic invasion	No	40	44	0.347
	Yes	14	9	
CDK4 IHC average score	<1.0	25	25	0.928
	≥1.0	28	25	
Ki-67 IHC average score	<1.5	12	22	0.035
	≥1.5	41	28	

Abbreviations: CDK4 = cyclin-dependent kinase 4; CDK4/6SA = CDK4/6-specific activity; IHC = immunohistochemistry. Bold entries denote $P < 0.05$ (significant).

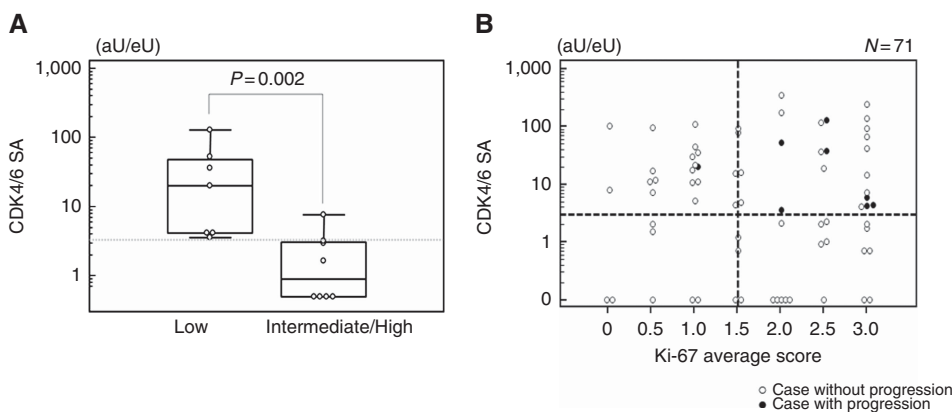


Figure 1. CDK4/6SA in low-risk and intermediate-/high-risk patients, and its association with Ki-67 expression. (A) Discordance of CDK4/6SA between low-risk and intermediate-/high-risk groups. Statistical analysis was performed using Mann-Whitney's *U*-test. **(B)** Scatter diagram of CDK4/6SA and the average Ki-67 expression score in low-risk patients.

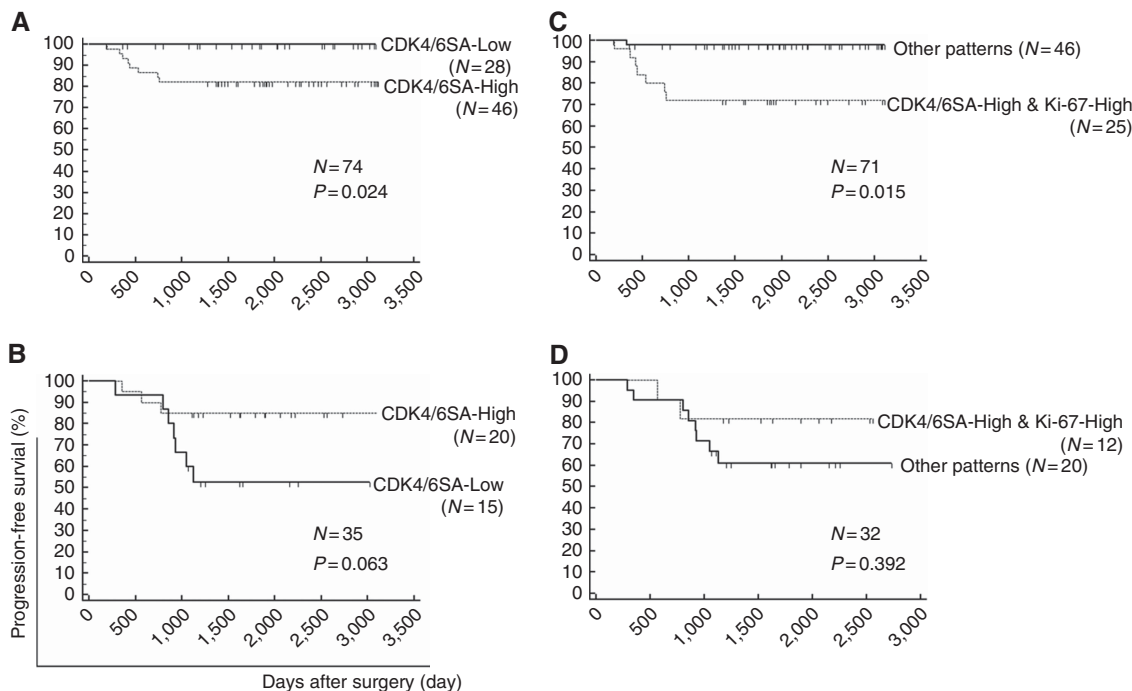


Figure 2. Prognostic impact of CDK4/6SA alone and in combination with Ki-67 expression. The prognostic value of CDK4/6SA was explored using Kaplan–Meier methods and the log-rank test. (A, B) Comparison between CDK4/6SA-High (CDK4/6SA > 3) and CDK4/6SA-Low (CDK4/6SA < 3) in (A) low-risk (those not receiving adjuvant chemotherapy) and (B) intermediate-/high-risk (those receiving adjuvant chemotherapy) patients. (C, D) Analysis of the combination of CDK4/6SA and Ki-67 expression. Cases with both CDK4/6SA-High and Ki-67-High (Ki-67 average score > 1.5) were compared with other combinations in (C) low-risk and (D) intermediate-/high-risk patients.

Table 3. Uni/Multivariate analysis of CDK4/6SA in combination with Ki-67 in low-risk patients

Parameter	Criteria	Univariate analysis			Multivariate analysis		
		HR	95% CI	P-value	HR	95% CI	P-value
Age	> 60 ≤ 60	2.00	0.150–8.85	0.336			
Stage	≤ II ≥ III	3.40	0.414–28.1	0.256			
Vascular invasion	No Yes	3.00	0.583–15.2	0.192	1.59	0.248–10.2	0.628
Histological grade	I II and III	2.30	0.209–10.0	0.287			
Tumour size	< 5 cm ≥ 5 cm	2.70	0.618–12.2	0.187	2.86	0.508–16.1	0.236
Myometrial invasion	≤ 1/2 > 1/2	0.90	0.167–4.36	0.849			
Lymphatic invasion	No Yes	1.50	0.180–12.2	0.716			
CDK4-IHC	< 1.5 ≥ 1.5	1.60	0.624–4.12	0.328			
CDK4/6SA and Ki-67	High and high Others	11.80	1.44–97.3	0.022	11.39	1.23–105	0.033

Abbreviations: CDK4 = cyclin-dependent kinase 4; CDK4/6SA = CDK4/6-specific activity; IHC = immunohistochemistry. Bold entries denote HR > 10 or P < 0.2 (significant). Italic entries indicate P < 0.05 (significant).

did not show prognostic significance on its own. This might be related to the fact that CDK4/6 activity can be directly augmented via activation of the RAS/PI3K-cyclin D1 pathway in endometrial cancer (Fukuchi *et al*, 1998; Moreno-Bueno *et al*, 2003; Oda *et al*, 2005, 2008; Shoji *et al*, 2009; Musgrove *et al*, 2011; Ikeda *et al*, 2012, 2013), which could more robustly reflect tumour

aggressiveness than the expression level of Ki-67. These data support the significance of CDK4/6SA as determined through the C2P assay. Taken together with previous reports showing that PI3K pathway-related activity is associated with chemo-sensitivity (Guinea Viniegra *et al*, 2002; Bender *et al*, 2011), CDK4/6SA might be a suitable marker for assessing the necessity of adjuvant

chemotherapy, and information on Ki-67 expression could strengthen the significance of CDK4/6SA. This additional post-operative treatment that would not be received otherwise based on the pathological criteria alone might improve the prognosis of clinicopathologically low-risk patients with high CDK4/6SA.

This study has some limitations that should be mentioned. First, the number of samples was limited, especially with respect to the number of recurrent cases, and the overall prognosis of patients was more favourable compared with the previous reports (Todo *et al.*, 2010). Further validation with a larger number of samples is warranted to strengthen our results. Second, the relationship between chemo-sensitivity and CDK4/6SA should be further clarified directly. Third, C2P analysis is not available for formalin-fixed specimens, and the requirement of freshly frozen samples might hinder its clinical application.

In conclusion, we determined the significance of CDK4/6 activity in EEC, using a new modification of the C2P assay. CDK4/6-specific activity can be used as a biomarker to predict prognosis and, possibly, chemo-sensitivity. In addition, combination with Ki-67 expression could enhance the predictive value of CDK4/6SA for pathologically diagnosed low-risk EEC patients. The selection of clinicopathologically low-risk and biologically high-risk patients based on the C2P analysis should provide useful information for establishing personalised medicine in the treatment of endometrial cancer.

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CONFLICT OF INTEREST

The funding for this study was partly provided by Sysmex Co. Ltd. (to KO and DA). HI is an employee of Sysmex Co. Ltd. C2P assay was performed at Sysmex Co. Ltd., but nobody in the company was involved in data analysis, or decision to submit.

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