



Original article

CDK1 and CDK2 activity is a strong predictor of renal cell carcinoma recurrence

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Received 19 March 2014; received in revised form 12 May 2014; accepted 13 May 2014

Abstract

Background: In renal cell carcinoma (RCC), the prediction of metastasis via tumor prognostic markers remains a major problem. The objective of our study was to evaluate the efficacy of cyclin-dependent kinase (CDK)1 and CDK2 activity as a prognostic marker in human RCC.

Methods: Surgical specimens were obtained from 125 patients with RCC without metastasis. Protein expression and kinase activity of CDKs were analyzed using a newly developed assay system named C2P (Sysmex, Kobe, Japan). We then examined the specific activities (SAs) of CDK1 and CDK2 and calculated CDK2SA-CDK1SA ratio in RCC. Also, risk score (RS) was examined.

Results: A total of 125 cases were tested, though 34 cases were excluded because of low sample quality (25 cases) and assay failure (9 cases). In total, 91 cases were analyzed. They included 68 male and 23 female patients, ranging in age from 19 to 83 years. At a median follow-up of 36 months (1–109M), tumor with low CDK2SA-CDK1SA ratio showed significantly better 5-year recurrence-free survival than those with high CDK2SA-CDK1SA ratio (88.7% vs. 54.7%, $P = 0.00141$). Also, RS enabled the classification of RCCs into high-risk and low-risk groups, and patients with tumors classified as low RS showed better recurrence-free survival than patients with tumors with high RS (88.7% vs. 54.7%, $P = 0.0141$).

Conclusion: CDK1SA of tumors and the CDK2SA are both associated with recurrence and prognosis.

Impact: CDK-based risk demonstrated is strongly associated with clinical outcome. CDK-based risk should be an accurate system for predicting recurrence and survival for planning follow-up. © 2014 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/3.0/>).

Keywords: Renal cell carcinoma; Biomarker; Cyclin-dependent kinase

1. Introduction

Renal cell carcinoma (RCC) accounts for approximately 3% of adult malignancies and 90% of neoplasm arising from the kidney. Approximately 40% of human RCCs are currently diagnosed incidentally. It is estimated that there

were 36,000 new cases of RCC in the United States in 2006 with almost 13,000 deaths [1]. Of patients after radical nephrectomy for localized RCC, 30% experience local or distant tumor recurrence [2].

Clinical and pathological staging according to the TNM system has served as a standard for predicting prognosis, but predicting value is not accurate enough for localized cancer [3,4]. Some nomograms based on clinical and pathological parameters have been discussed for predicting

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patient survival [5,6]. The investigation of additional molecular markers that reflect the individual tumor behavior should improve patient management after surgery.

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 [7–9].

We have been focusing on CDKs (CDK1 and CDK2) and investigating their prognostic significance in breast cancers because CDKs play a pivotal role in cell cycle regulation [10,11]. 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 activities themselves 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 [12]. The clinical utility of the technology was first evaluated in breast cancer, and combination analysis of CDK1 and CDK2 activity was shown to be a significant prognostic indicator for relapse [13,14]. The objective of this study was to clarify the prognostic implications of CDKSA in RCCs.

2. Material and methods

2.1. Patients

For this study, 158 patients who had undergone radical nephrectomy between September 1999 and August 2009 were recruited. These patients were selected randomly for this study. The following data were available: age at diagnosis, histological type, TNM stage, local and systematic therapy, recurrence, and overall survival. No tyrosine kinase inhibitor (TKI), mammalian target of rapamycin inhibitor, or immunotherapy (interferon alfa alone or with IL-2) was administered before recurrence.

2.2. Assay for CDKSA

Tumor tissue was dissected from the surgical resection and stored at -80°C in the Department of Urology, Kyoto Prefectural University of Medicine. The system to measure the CDKSA is called “C2P” (for “Cell Cycle Profiling”; Sysmex, Kobe, Japan). In brief, lysates of frozen material were applied to a well of 96-well polyvinylidene fluoride filter plate (Millipore, MA). Expression of CDKs was detected quantitatively by sequential reactions with primary anti-CDK antibodies, biotinylated antirabbit antibodies, and fluorescein-labeled streptavidin. To measure the kinase activity, CDK molecules were immunoprecipitated from the tissue lysate using protein beads, as reported in detail earlier [1,2]. The thiophosphate of adenosine 5'-[γ -thio] triphosphate was transferred to the protein substrate during the on-bead kinases reaction. The introduced thiophosphate

was labeled further with 5-iodoacetamidofluorescein and blotted onto a well of the filter plate. The kinase activity was determined by measuring the fluorescence intensity of the well. CDKSA was calculated as CDK activity units (aU/ μl lysate) divided by its corresponding CDK expression units (eU/ μl lysate). Both aU (CDK activity unit) and eU (CDK expression unit) were defined as the expression and activity equivalent to 1 ng of recombinant active CDK1 and CDK2, respectively.

The blood contamination is visually determined by using a color bar of redness. The ranges from dark to faint and is graded 1 to 10. Only qualified technicians who passed the technical examination conduct the C2P assay with patient's samples.

Affection of the blood contamination was examined with lysates of human cell line spiked with various amount of human blood. In results, blood contamination of score 4 and more of redness showed significant error ($>10\%$) in expression assays of both CDK1 and CDK2. By contrast, the activity assay was quite robust against the blood contamination, as a step of bound/free separation is in the activity assay, but not in the expression assay.

According to the aforementioned results, the samples with blood contamination of score 4 and more of redness were excluded from the C2P analysis.

2.3. Statistical methods

Association between clinicopathological characteristics and the C2P parameters were examined in the chi-square test. Recurrence-free survival (RFS) according to C2P parameters was analyzed with the Kaplan-Meier plot, and the differences of RFS in each category 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 at $P < 0.05$.

3. Results

3.1. Patients

Of 158 patients, 25 were excluded because of poor sample quality with severe blood contamination. In total, 11 patients were excluded from our study because of clinical exclusion (benign: 1, nonrenal cancer: 2, metastatic tissue: 2, and treatment with TKIs: 5). Of the cases, 9 were non-informative cases of the assay. A total of 26 patients developed recurrence (the lung, liver, bone, etc.). Of these 113 patients, 22 patients with metastases were excluded for analysis of RFS and overall survival. Of the 91 cases, the median follow-up period was 37 months (1–121M), and the RFS rate at 5 years was 84.6 % (77/91). They included 68 male and 23 female patients, ranging in age from 19 to 83 years. The median follow-up period was 37 months (1–121M) (Table 1).

Table 1
Patient background

Parameters	Category	n	%
Age, y	>60	67	59.3
	≤60	46	40.7
Sex	Male	84	74.3
	Female	29	25.7
T	1a	29	25.7
	1b	30	26.5
	2	15	13.3
	3a	16	14.2
	3b	13	11.5
	3c	2	1.8
	4	8	7.1
N	N0	98	86.7
	N1	12	10.6
	N2	3	2.7
Stage	1	55	48.7
	2	13	11.5
	3	19	16.8
	4	26	23.0
Grade	1	7	6.2
	2	67	59.3
	3	39	34.5
T size, cm	<4	33	29.2
	4–7	39	34.5
	>7	41	36.3
Histology	Belini	1	0.9
	Chromophobe	3	2.7
	Clear cell	91	80.5
	Oncocytoma	2	1.8
	Papillary	10	8.8
	Papillary + sarcomatoid	1	0.9
	Sarcomatoid	4	3.5
	Unclassified	1	0.9
Event	Recurrence	26	23.0
	Dead	11	9.7
	Median RFS, mo	20	
	Median OS, mo	29	

Surgical specimens were obtained from 147 patients with renal cell carcinoma. These patients were selected randomly for this study. The median patient age was 64 years (range: 19–85). Overall, 72.5% had stage I or II disease and 61.5% were diagnosed as clear cell carcinoma. The median follow-up period was 37 months (range: 1–121M), and the recurrence-free survival (RFS) rate in all patients tested at 5 years after surgery was 84.6% (77/91). Metastatic cases were excluded from our analysis (22 cases). OS = overall survival.

3.2. Cutoff value

The cases were then categorized into 2 groups by the optimal cutoff point determined in receiver operating characteristic (ROC) analysis of respective parameter: CDK1SA, CDK2SA, CDK2SA-CDK1SA ratio, or C2P risk score (C2P-RS). The cutoff values for CDK1SA, CDK2SA, and CDK2SA-CDK1SA ratio were defined as

the points that gave the best discrimination in RFS by receiver operating characteristic curve (Fig. 1). The optimal cutoff values of CDK1SA and of CDK2SA for recurrence prediction are 4.8 and 7.2, respectively. The cutoff value CDK2SA-CDK1SA ratio is 1.3. C2P-RS was given by the following equations:

$$\text{C2P-RS} = \text{Equation 1} \times \text{Equation 2} \times 3,000$$

$$\text{Equation 1} = 0.25 / (1 + \text{Exp}(-(x - 1.0) \times 6)),$$

$$x = \log(\text{CDK2SA} / \text{CDK1SA})$$

$$\text{Equation 2} = 0.15 / (1 + \text{Exp}(-(y - 1.6) \times 7)),$$

$$y = \log(\text{CDK1SA})$$

3.3. Correlation analysis of C2P parameters with clinicopathological characteristics

The correlation of various clinicopathological parameters and C2P parameters is shown in Table 2. High stage and high grade showed significantly poor prognosis but tumor size showed no correlation with 5-year RFS in this study. The C2P parameters did not show any statistically significant correlations with tumor size. By contrast, the categories showed significant correlations with clinical stage and tumor grade (Tables 3).

3.4. Significance of each C2P parameters for recurrence prediction

Next, we studied the relationship of the combination of CDK1SA and CDK2SA with prognosis. Patients with high CDK1 or high CDK2SA tumors or both showed poor prognosis (5-y RFS rate was 84.6% [77/91]), whereas patients with tumors in which both CDK1SA and CDK2SA were less than lower measurement limits showed good prognosis.

Tumors with low CDK2SA-CDK1SA ratio showed significantly better 5-year RFS than those with high CDK2-CDK1 ratio (88.7% vs. 54.7%, $P = 0.00141$). Also, RS enabled the classification of RCCs into high-risk and low-risk groups, and patients with tumors classified as low RS showed better RFS than patients with tumors with high RS (88.7% vs. 54.7%, $P = 0.0141$) (Fig. 2).

The Cox proportional hazards model revealed that clinical staging and tumor grade were significant prognostic factors for RFS in all patients tested (stage: $P = 0.041$, hazard ratio [HR] = 3.025; grade: $P = 0.009$, HR = 4.06). Among the C2P parameters, CDK2SA, CDK2SA-CDK1SA ratio, and C2P-RS showed significance in the analysis, but CDK1SA did not (CDK1SA: $P = 0.051$, HR = 2.98; CDK2SA: $P = 0.044$, HR = 2.98; CDK2SA-CDK1SA ratio: $P = 0.021$, HR = 3.47; CDK-RS: $P = 0.041$, HR = 3.12, respectively) (Fig. 3).

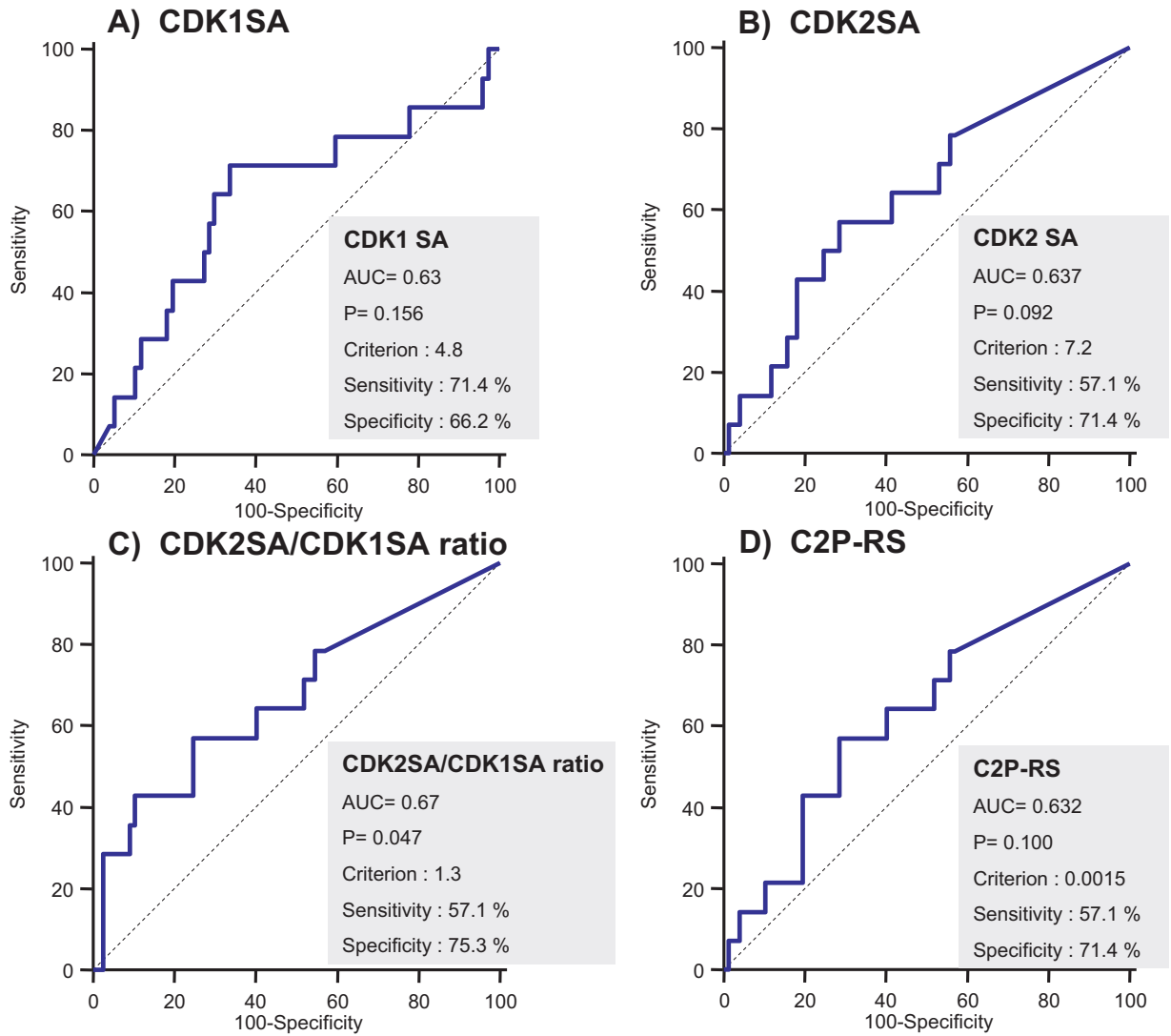


Fig. 1. Setting of an optimal cutoff value for recurrence prediction in renal cancer by the ROC analysis: (A) CDK1-specific activity, (B) CDK2-specific activity, (C) CDK2SA-CDK1SA ratio, and (D) C2P-RS. ROC = receiver operating characteristic.

Table 2
 Correlation analysis of C2P parameters with clinicopathological characteristics

Parameters	Category	C2P-RS		Significance ^a (P value)	CDK2SA-CDK1SA ratio		Significance ^a (P value)
		Low	High		Low	High	
Stage	1	32	20	52 (57.1%)	40	12	52 (57.1%)
	2	11	2	13 (14.3%)	8	5	13 (14.3%)
	3	12	8	20 (22.0%)	13	7	20 (22.0%)
	4	1	5	6 (6.6%)	2	4	6 (6.6%)
				0.0450			0.1326
Grade	1	6	1	7 (7.7%)	7	0	7 (7.7%)
	2	39	24	63 (69.2%)	45	18	63 (69.2%)
	3	11	10	21 (23.1%)	11	10	21 (23.1%)
				0.2899			0.0485
Size	<4	19	10	29 (32.2%)	24	5	29 (32.2%)
	4–7	19	12	31 (34.4%)	20	11	31 (34.4%)
	>7	18	12	30 (33.3%)	18	12	30 (33.3%)
				0.9010			0.1363

The C2P parameters did not show any statistically significant correlations with tumor size. By contrast, the categories showed significant correlations with clinical stage and tumor grade.

^aChi-square test.

Table 3
Cox regression hazards model analysis

Parameters	Criteria	Hazard ratio	95% CI of HR	Significance
Univariate				
Age, y	>60 vs. ≤60	0.7433	0.2560–2.1584	0.5874
Stage	1 vs. 2, 3, 4	1.6665	0.5870–4.7315	0.3399
	1, 2 vs. 3, 4	3.0254	1.0516–8.7041	0.04107
Grade	1 vs. 2, 3	10.8898	0.000–2.38E + 180	0.9553
	1, 2 vs. 3	4.0609	1.4294–11.5368	0.008866
T size	<4 vs. ≥4 cm	3.1899	0.7191–14.1508	0.1289
	≤7 vs. >7 cm	1.214	0.4088–3.6052	0.7283
	<4 vs. 4–7 vs. >7 cm	1.4629	0.7570–2.8271	0.2601
C2P-RS	L vs. H	3.1193	1.0507–9.2606	0.04149
CDK2-CDK1 ratio	L vs. H	3.4733	1.2102–9.9680	0.02128
Multivariate				
Stage	1, 2 vs. 3, 4	1.218	0.3280–4.5234	0.7694
Grade	1, 2 vs. 3	3.8954	1.0747–14.119	0.03948
C2P-RS	L vs. H	3.2519	1.0692–9.8901	0.0387
Stage	1, 2 vs. 3, 4	1.4803	0.4437–4.9386	0.5255
Grade	1, 2 vs. 3	2.7201	0.8204–9.0187	0.1035
CDK2-CDK1 ratio	L vs. H	2.6623	0.9039–7.8417	0.07714

Univariate and multivariate analyses were performed against clinical staging, tumor grade, and C2P-RS. C2P-RS was shown to be an independent and significant predictor against clinical staging and tumor grade (hazard ratio = 3.25, $P = 0.0387$).

4. Discussion

In this study, we applied an original method enabling simultaneous analysis of protein expressions and kinase activities of the CDK molecules (C2P) to predict outcomes in the patients with RCC. CDK1- and CDK2-SAs of tumors are both associated with recurrence and prognosis. 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 nonmetastatic RCCs.

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 [2,13,15,16]. Some studies have shown that CDK1 may be required for apoptosis that is independent of the regulation of the cell cycle [17,18]. However, the prognostic effect of activity of CDK1 or CDK2 has not been investigated.

In RCC, the prediction of metastasis via tumor prognostic markers remains a major problem. Various conventional parameters, such as a tumor size, stages, and grades, have been studied to identify subsets of patients with a prognosis. Several molecular markers have been appeared

to refine the prognosis and prediction of RCC. Carbonic anhydrase IX is one of studied markers in RCC. High carbonic anhydrase IX expression in metastatic cases was associated with better disease-specific survival [19], but not in nonmetastatic cases.

The mammalian cell cycle is driven by a variety of molecules regulating activities of CDKs [20]. Von Hippel Lindau (VHL) is also supposed to be a key molecule of RCC, because mutations of the VHL gene are a critical event leading to development of RCC in both sporadic and hereditary forms. This lack of VHL function leads to the stabilization of both HIF1a and HIF2a proteins as well as an increase in DNA damage. Furthermore, cyclin D1 is overexpressed and remains inappropriately high during contact inhibition in pVHL-deficient RCC cell lines [21]. Contrarily, in a study in a mouse RCC cell line Renca, the overexpression of one of CDK inhibitors p21^{WAF1/CIP1} is a more potent growth suppressor than p53 [22], though there are no clinical studies of the significance of p21 in RCC.

Other cell cycle-based markers were reported in RCC. Ki-67 has been suggested as a tissue-based marker for tumor aggressiveness in a lymph node-negative RCC, and Ki-67 labeling index is predictive when used with other factors [23]. Furthermore, p53 is an independent predictor

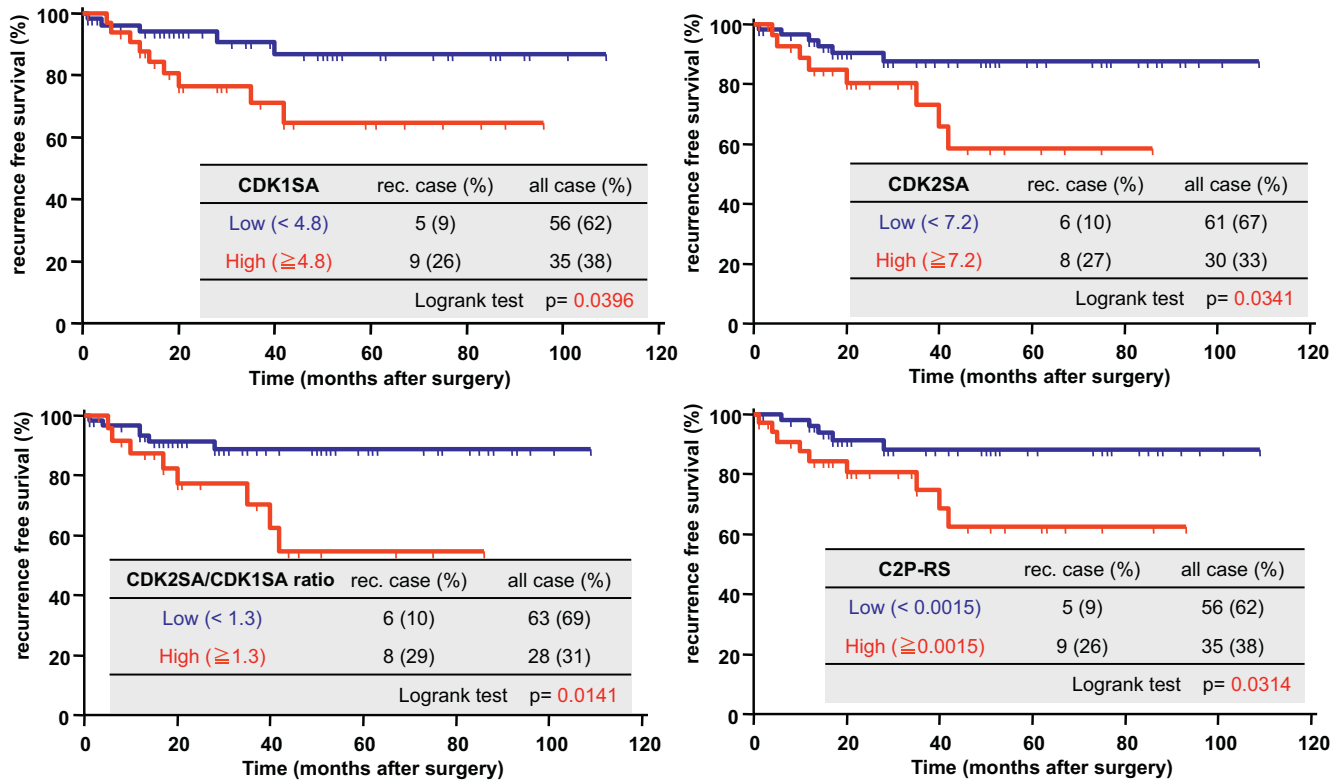


Fig. 2. Significance of each C2P parameters for recurrence prediction. Progression-free survival (RFS) rates according to C2P parameters in renal cell carcinoma ($n = 91$) tested in this study: A strong prognostic significance was observed in all C2P parameters using log-rank test. (A) CDK1-specific activity, (B) CDK2-specific activity, (C) CDK2SA-CDK1SA ratio, and (D) C2P-RS.

of tumor recurrence and progression after nephrectomy in patients with localized RCC [24]. However, further studies are required in this regard.

On the one hand, molecular targeting therapies, such as TKIs and mammalian target of rapamycin inhibitors, have been approved for RCC. However, the effectiveness is

limited and the biomarkers have been investigated to improve the effectiveness. On the other hand, CDK inhibitors have been developed intensively, and some are undergoing clinical trials [25].

5. Conclusion

We have shown that CDK-based risk demonstrated by evaluating CDK1SA and CDK2SA is strongly associated with clinical outcome especially for patients with non-metastatic RCC. We consider that the CDK-based risk should be a new prognostic factor and a routine laboratory test. An accurate system for predicting recurrence and survival is useful for planning follow-up. However, our results need to be validated in a multicenter study with a larger number of patients.

6. Conflict of interest

Fumiya Hongo, Natsuki Takaha, Masakatsu Oishi, Takashi Ueda, Terukazu Nakamura, Yasuyuki Naitoh, Yoshio Naya, Kazumi Kamoi and Koji Okihara have no conflict of interest.

Tomoko Matsushima, Satoshi Nakayama, and Hideki Ishihara are employee of Sysmex Corporation.

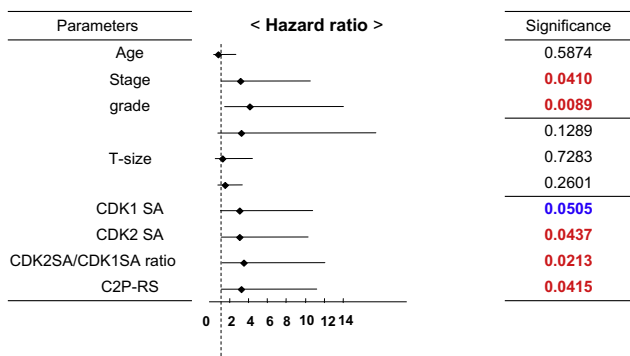


Fig. 3. Forest-plot of each parameter for recurrence prediction. The Cox proportional hazards model revealed that clinical staging and tumor grade were significant prognostic factors for RFS in all patients tested (stage: $P = 0.041$, hazard ratio [HR] = 3.025; grade: $P = 0.009$, HR = 4.06). Among the C2P parameters, CDK2SA, CDK2SA-CDK1SA ratio, and C2P-RS showed significance in the analysis, but CDK1SA did not (CDK1SA: $P = 0.051$, HR = 2.98; CDK2SA: $P = 0.044$, HR = 2.98; CDK2-CDK1 ratio: $P = 0.021$, HR = 3.47; CDK-RS: $P = 0.041$, HR = 3.12). (Color version of figure is available online.)

Tsuneharu Miki and Toshiyuki Sakai have received research grant from Sysmex Corporation.

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