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A new cancer diagnostic system based on a CDK profiling technology

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Abstract

A series of molecular pathological investigations of the molecules that stimulate the cyclin dependent kinases (CDK1, 2, 4, and 6) have led to enormous accumulation of knowledge of the clinical significance of these molecules for cancer diagnosis. However, the molecules have yet to be applied to clinical cancer diagnosis, as there is no available technology for application of the knowledge in a clinical setting. We hypothesized that the direct measurement of CDK activities and expressions (CDK profiling) might produce clinically relevant values for the diagnosis. This study investigated the clinical relevance of CDK profiling in gastrointestinal carcinoma tissues by using originally developed expression and activity analysis methods. We have established novel methods and an apparatus for analyzing the expression and activities of the CDK molecules in lysate of tumor tissue in a clinical setting, and examined 30 surgically dissected gastrointestinal carcinomas and corresponding normal mucosal specimens. We demonstrate here that remarkably elevated CDK2 activity is evident in more than 70% of carcinoma tissues. Moreover, a G1-CDK activity profiling accurately mirrored the differences in proliferation between tumor and normal colonic tissues. Our results suggest that CDK profiling is a potent molecular–clinical approach to complement the conventional pathological diagnosis, and to further assist in the individualized medications.

Keywords: Cell cycle; CDK; Kinase; Profiling; Gastrointestinal cancer

1. Introduction

A breakdown in the machinery of the cell cycle induces the uncontrolled proliferation of tumors. This is known to be mainly caused by the inactivation of RB protein. This inactivation is exerted by a variety of molecules in the cascade that activates the CDK molecules (CDK2, 4, and 6), which play a role in the G1 progression of the cell cycle by phosphorylating (inactivating) the RB protein [1,2]. One of the central players in the cascade is the p53 tumor-suppressor molecule. There is a high incidence of p53 gene mutations in various cancer tissues, and these are associated

Abbreviations: RB, retinoblastoma; CDK, cyclin dependent kinase; CDKI, CDK inhibitor; PBS, phosphate-buffered saline; RFU, relative fluorescence unit; BSA, bovine serum albumin

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with poor clinical outcomes for the individuals concerned [3–5]. The p53 protein functions as a transcription factor and regulates cell proliferation, DNA repair, differentiation, and apoptosis [6,7]. In non-cancerous cells with DNA damage, p53 transactivates the expression of a CDKI molecule, $p21^{WAF1}$, which inhibits intrinsic CDK2 activity and sustains the non-phosphorylated (active) form of RB protein, thereby arresting the cell cycle at the G1/S boundary, known as the restriction (R) point. In contrast, mutant p53 molecules are unable to function and are believed to lead to uncontrolled G1-phase CDK activity which causes RB phosphorylation (inactivation). This results in deregulated proliferation and genome instability, turning cells malignant.

A series of molecular pathological investigations of the molecules that stimulate the G1-phase CDKs (CDK2, 4, and 6) have clearly demonstrated the clinical significance of these molecules for cancer diagnosis and prognosis. For example, there is much clinical evidence indicating that the overexpression of cyclin E and cyclin D1, which bind and activate CDK2, and CDK4 and 6, respectively, correlates with tumorigenesis, prognosis or sensitivity to chemotherapy in a variety of malignancies, as does the inactivation of CDKI molecules such as $p21^{WAF1}$ and $p27^{Kip1}$, which inhibit CDK2, 4, and 6, and $p16^{INK4a}$, which inhibits CDK4 and 6 [8–24]. However, there are some contradictions among these reports. These may arise from the uncertainties inherent in immunohistochemical techniques due to inaccuracies in quantitative measurements or to variations in antibody specificity. These contradictions may also arise because of the various causes of the inactivation or activation of the molecules, which may be attributable to alterations in expression or to mutations. Several lines of evidence clearly demonstrate that the inactivation of CDKI molecules in cancer cells cannot be analyzed merely using protein expression profiles. Therefore, we hypothesized that the direct measurement of CDK activities might produce more reliable data for clinical diagnosis because such activities directly reflect the actual inactivation of the CDKI molecules or the activation of CDK-activating molecules, regardless of the cause including unknown CDK-modulating molecules.

2. Materials and methods

2.1. Clinical samples

The research plan was evaluated and approved by the ethics committee of Kyoto Prefectural University of Medicine. Thirty-seven patients with gastrointestinal tumors were diagnosed and treated surgically at the University Hospital, Kyoto Prefectural University of Medicine from 2001 to 2002. Samples of around 50 mg of tumor tissue and the surrounding normal mucosal tissues were collected during surgery with the patient's informed prior consent. The tissue samples were further dissected into 2 mm^3 pieces in ice-cold PBS containing 2% Protease Inhibitor Cocktail (Sigma, St. Louis, MO, USA), then immediately frozen in liquid nitrogen and stored at −80 °C until use.

2.2. CDK expression analysis (CPDIB method)

Lysates of pieces (2 mm^3) of surgically dissected tissues were prepared using a newly developed tissue-homogenizer (condition; 8 kg/cm^2, Sysmex, Kobe, Japan) with lysis buffer; 0.1% NP-40, 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2% Proteinase Inhibitor Cocktail (Sigma, St Louis, MO, USA). The insoluble materials were removed by centrifugation at 15,000 rpm at 4 °C, and the aliquots of supernatant were prepared and stored at −80 °C until use. Protein concentrations were analyzed (DC Kit, Pierce, Rockford, IL, USA) and 2.5 μg of total protein was applied to the well of a newly developed dot–blot device (Sysmex, Kobe, Japan) with a hydrophobic membrane (PVDF with 0.22 μm pores; Millipore, Billerica, MA, USA). The target protein in the crude sample bound to the membrane was quantitatively detected by sequential reactions with anti-CDK antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), biotinylated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and fluorescein-labeled streptavidin (Vector, Burlingame, CA, USA). Between each reaction, the well was automatically washed with TBS solution; 25 mM Tris–HCl, pH 7.4, 150 mM NaCl. Fluorescent images of the membranes were analyzed using an image analyzer (Bio-Rad, Hercules, CA, USA), and the intensity of dots quantified by 'Quantity One' (Bio-Rad, Hercules, CA, USA). RFUs and the amounts of standard recombinant proteins (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were linearly correlated in the standardized ranges: CDK1, 2.5–25 ng/dot; CDK2, 1.0–10 ng/dot; CDK4, 1.0–10 ng/dot; CDK6, 2.5–25 ng/dot.

2.3. CDK kinase analysis

Analysis of enzyme activity was performed using a non-radioisotopic method. Cell lysates were prepared as described for expression analysis. The CDK molecules were selectively precipitated from 100 μg of lysate total protein with 2 μg of the corresponding antibodies (anti-CDK1, -2, -4, or -6 antibodies; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and 20 μl of protein A beads (Amersham Pharmacia, Uppsala, Sweden) for 1 h at 4 °C. After three washes with washing buffer, 0.1% NP-40, 50 mM Tris–HCl, pH 7.4, 50 μl of the substrate mixture containing 10 μg of protein substrate, 5 mM adenosine 5′-O-(3-thiotriphosphate) (Sigma, St Louis, MO, USA), 20 mM Tris–HCl, pH 7.4, and 0.1% Triton X-100, was added to the beads and incubated under continuous shaking at 37 °C for 10 min. Histone H1 (Upstate Biotechnology, Lake Placid, NY, USA) was used as the protein substrate for CDK1 and CDK2, and recombinant RB

protein (amino acids 769–921) for CDK4 and CDK6. After the beads were removed, the introduced monothiophosphates in the substrate were further labelled by incubation with 0.6 mM 5-iodoacetamidofluorescein (Molecular Probes, Eugene, OR, USA) in coupling buffer; 150 mM Tris–HCl, pH 7.4, 5 mM EDTA, for 20 min in the dark at room temperature. The reaction was quenched with β-mercaptoethanol, and 0.4 μg of the substrate was applied to the wells of the dot–blot device (Sysmex, Kobe, Japan). After the membrane was washed, the images were evaluated using an image analyzer (Bio-Rad, Hercules, CA, USA), and the fluorescence intensity of the dots was quantified using 'Quantity One' (Bio-Rad, Hercules, CA, USA). The activity was calculated with a standard curve prepared with CDK activities corresponding to 0, 12.5, 25, 50, 100, and 150 μg of protein extracted from a K562 chronic myelogenous leukemia cell line. One unit (U) is equivalent to the kinase activity of 1 μg of total protein from the K562 cells.

3. Results and discussion

The CDK profiling system is composed of analysis of CDK1, 2, 4, and 6 expressions and activities. The analytical procedures for expression and activity were originally developed and carefully validated as follows.

The expression analysis named CPDIB method was established by modifying the dot–blot analysis. The procedure is schematically described in Fig. 1. The advantage of the CPDIB method is the allowance of direct blotting of crude cell/tissue lysate on a hydrophobic membrane. Therefore, retention of the target molecule on the membrane is critical for its quantitative performance. The retention was examined by the added-recovery analysis. The recovery was examined by blotting the recombinant CDK protein mixed into Colo205 cell lysate (total protein; 2.5 μg, 50 μl of 50 μg/ml total proteins), and more than 90% of recovery was observed in all four CDK analysis methods (data not shown). Therefore, we concluded that quantitative blotting of the target protein could be achieved for a minimum value of 2.5 μg of total protein of crude lysate. The specificity of each anti-CDK antibody was examined by blotting serial amounts (0, 2.5, 5, 7.5, 10 ng) of four recombinant CDK molecules mixed in 2.5 μg of bovine serum albumin (Fig. 2). All four antibodies showed no cross-reactivity to the other CDK isotypes except for slight cross-reactivities of both anti-CDK1 and anti-CDK4 antibodies only against CDK6, and the measurements (relative fluorescent unit) were linearly correlated to the amount of each recombinant protein. The stability of the assay was examined by evaluating the reproducibility of the values of control cell lysate (HeLa; human cervical cancer cell line) among 30 independent assays. The control variations (standard deviation divided by the average) were 21% for CDK1, 22% for CDK2, 19% for CDK4, and 34% for CDK6.

The non-radioisotopic CDK activity analysis was modified from the on-beads CDK kinase assay [25]. The assay utilizes ATP-γS instead of γ [^{32}P]-ATP. Briefly, each CDK molecule was immunoprecipitated from cell/tissue lysate. The thiophosphate of ATP-γS is transferred to the protein substrate during the on-beads kinase reaction. The introduced thiophosphate was further labeled with 5-iodoacetamidofluorescein, and blotted onto PVDF membrane. The kinase activity was calculated by measuring the fluorescence intensity of the blot as described in Materials and methods. Specificities of the antibodies to the four CDK species were secured by using same specific anti-CDK antibodies of the expression analysis. The enzyme stabilities during the reaction were examined for 120 min, and confirmed the linear increase of phosphorylated product until 15 min in all four cases (data not shown). The ATP-dependency of the enzymatic reaction in the procedure was examined in the experiments with CDK inhibitors, butyrolactone and staurosporine. The CDK2 activity was dose-dependently inhibited by both inhibitors to its background

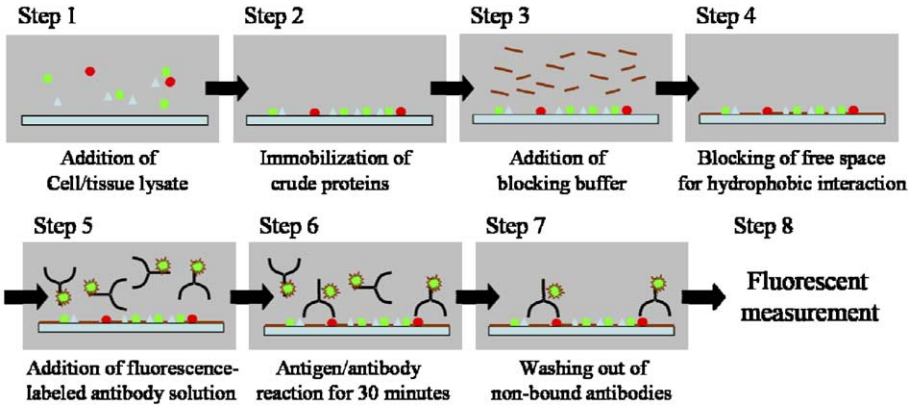

Fig. 1. Schematic figure of a procedure of CPDIB method. Step 1: Crude lysate from cells or tissue was dispensed into the well with a PVDF membrane. Step 2: The crude total proteins were blotted on the membrane by aspiration. Step 3: Blocking buffer containing 4% BSA was dispensed into the well. Step 4: Blocking solution was discarded by aspiration. Step 5: The fluorescent-labeled anti-CDK antibody solution was dispensed into the well. Step 6: The antibody/antigen reaction was allowed for 30 min. Step 7: Non-bound antibodies were washed away by 5 times of addition and aspiration of washing buffer.

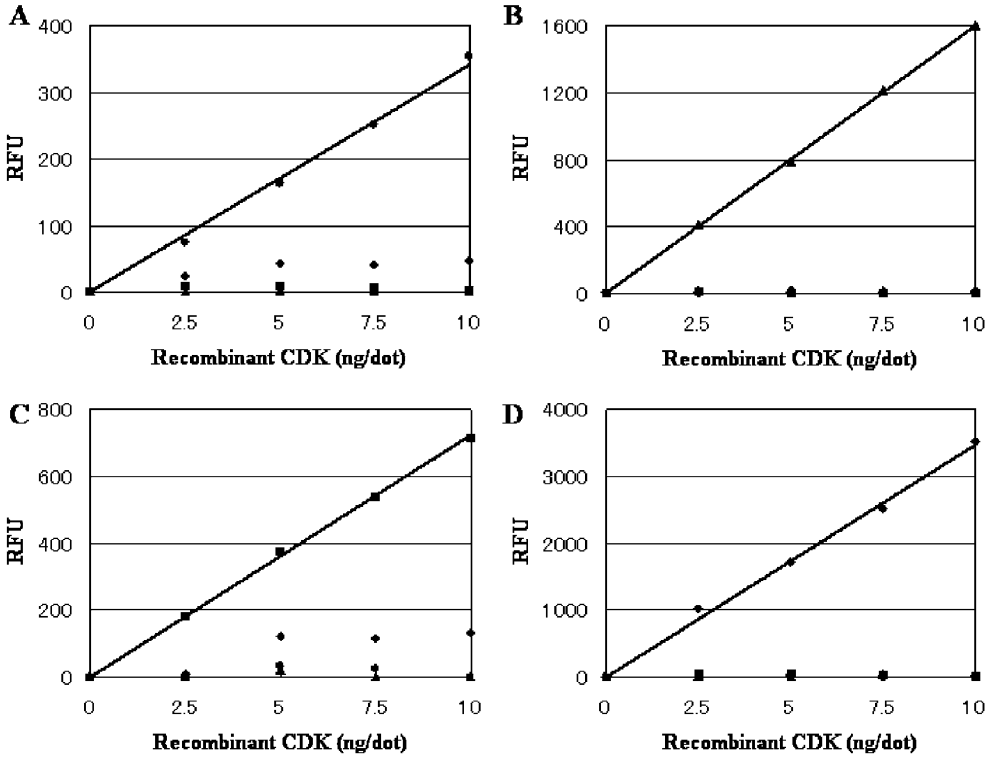

Fig. 2. Specificity and quantitative performance of CDK expression assays (CPDIB method). Serial amounts (2, 1.5, 1, 0.5 ng) of four recombinant CDK proteins (●, CDK1; ▲, CDK2; ■, CDK4; ♦, CDK6) were mixed with 2.5 μg of BSA respectively, and blotted onto the membrane. After blocking of the wells, the anti-CDK1 (A), anti-CDK2 (B), anti-CDK4 (C), and anti-CDK6 (D) antibodies were allowed to react.

level, and IC50 values were 38 μM for butyrolactone and 2 μM for Staurosporine (data not shown). The quantitative performance of the system was examined and the four assay systems illustrated a linear correlation between the measurements (relative fluorescent unit) and input amounts of cell lysate (Fig. 3). Finally, the stability was examined by

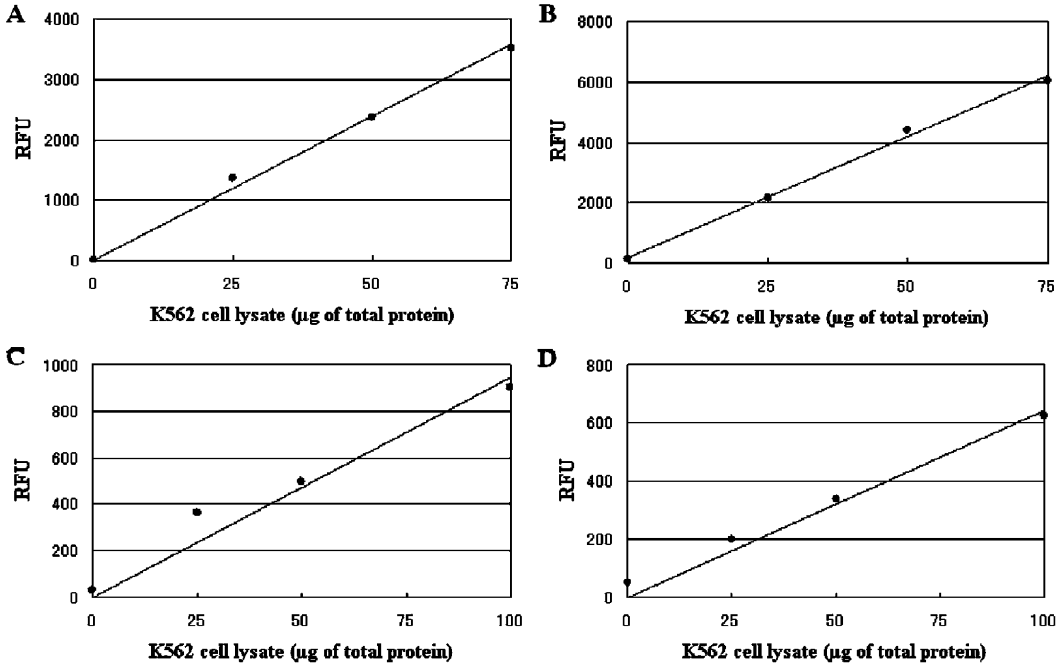

Fig. 3. Standard curves of non-radio-isotopic CDK activity assays. The each CDK molecules was immunoprecipitated from a serial amounts (0, 25, 50, 75, 100 μg of total proteins) of lysate of K562 leukemia cell lines with specific antibodies for CDK1 (A), CDK2 (B), CDK4 (C), and CDK6 (D), and non-radioisotopic assay procedure were performed as described in Materials and methods.

evaluating the reproducibility of the values of CDK2 kinase activity of the control cell lysate (K562 human leukemia cell line) among 20 independent assays. The control variation was 38%.

In order to examine the applicability of the above-established system to the clinical samples and the clinical relevance of the concept of the CDK profiling system, we analyzed clinical gastrointestinal cancer tissues and the corresponding normal mucosal tissues as a reference. Table 1 shows the clinicopathological features of patients from whom the gastrointestinal tumors used in this study were taken. The median age of the study population was 67 years (range 23–86 years). One of 22 colon carcinomas, and five of eight gastric carcinomas were at stage IV.

Table 1
Clinicopathological features of patients

Patient	Sex	Age	Stage	Histology	Site	T	N*	M
Colon carcinoma								
C001	F	53	III	well	A	3	1	0
C002	M	67	(II)	mod	R	3	X(0/5)	0
C003	M	67	(II)	mod	R	3	X(0/8)	0
C005	M	56	III	mod	A	3	1	0
C006	M	48	III	mod	R	3	1	0
C007	F	80	III	neu	R	3	1	0
C008	M	75	II	muc	A	3	0	0
C009	M	65	I	mod	R	2	0	0
C010	M	83	III	mod	T	3	1	0
C011	M	77	(II)	muc	R	3	X(0/3)	0
C012	M	86	II	mod	R	3	0	0
C013	F	71	I	por	A	2	0	0
C014	F	81	(II)	mod	R	3	X(0/8)	0
C015	F	57	III	well	R	3	1	0
C016	F	44	IV	muc	S	4	X(0/7)	1
C017	M	48	(II)	well	R	3	X(0/6)	0
C018	M	60	(II)	well	S	3	X(0/7)	0
C019	F	35	III	mod	R	3	1	0
C020	M	74	(II)	mod	A	3	X(0/9)	0
C021	M	48	III	mod	D	4	2	0
C022	M	23	II	por	C	3	0	0
C023	F	79	(II)	mod	R	3	X(0/6)	0
Gastric carcinoma								
S001	M	71	IV	tub-mod	Ca-F	2	0	1
S002	M	66	IIIA	muc	Ca-F	3	1	0
S005	M	59	II	tub-well	F-Co	3	0	0
S006	F	67	IV	tub-mod	Co-A	3	3	0
S008	M	78	IV	tub-mod	Co	2	0	1
S009	M	69	IV	tub-mod	Co	2	1	1
S010	M	73	IV	tub-mod	Co	3	1	1
S011	F	40	II	muc	Co	2	1	0

The clinical status of each patient was classified according to the rule of *World Organization. Classification of Tumors* [32]. *In the rule, definition of N0 is no metastasis in at least 12 lymph nodes for colon carcinoma. X in the table means no metastasis found in less than the above number of nodes (described in bracket). Colon carcinoma; well: well differentiated adenocarcinoma, mod: moderately differentiated adenocarcinoma, neu: neuroendocrine carcinoma, muc: mucinous adenocarcinoma, por: poorly differentiated adenocarcinoma, A: ascending colon, R: rectum, T: transverse colon, S: sigmoid colon, D: descending colon, C: cecum. Gastric carcinoma; tub: tubular adenocarcinoma, mod: moderately differentiated adenocarcinoma, muc: mucinous adenocarcinoma, well: well differentiated adenocarcinoma, Ca: cardia, F: fundus, Co: corpus, A: antrum.

Through analysis of the expressions and activities, we observed statistically significant and clear differences between the CDK2 activities of tumor and normal tissues. Statistical analysis, using both paired and non-paired tests revealed that the differences were significant in both colonic and gastric tissues (Table 2 and Fig. 4). When the cut-off value for CDK2 activity was set as the mean+two standard deviations (2 S.D.) of the value for normal tissue, 16/22 (73%) colon cancer tissues and 6/8 (75%) gastric cancer tissues were positive. Only two false positive evaluations were made in normal tissues (data not shown). These results clearly indicate that most of the tumor formation in these gastrointestinal carcinomas is associated with the dysfunction of molecule(s) in the cascade leading to CDK2 activity.

In the experiment with 18 different cell lines, we observed that CDK2 kinase activity was positively correlated to the population of S-phase ($r=0.653$, data not shown). By contrast, CDK1 activity did not show the correlation with the population of G2/M phase. This revealed that the CDK2 activity is a parameter of cell proliferation. Moreover, the level of a biologically hyperactive isoform of cyclin E, which is an assembly partner of CDK2, correlates strongly with poor survival in patients with breast cancer [26]. Combined, high CDK2 activity indicating aggressive growth of tumor tissues may be a major cause of poor clinical outcome.

Interestingly, colon cancer tissues showed slight but statistically significantly lower CDK4 and CDK6 activities than normal tissues (only in a paired statistical analysis, Fig. 4). This suggests that the accelerated G1 phase is predominantly promoted by the augmentation of CDK2 activity. Furthermore, relatively higher activities were observed for both CDK1 and CDK2 in normal gastric tissues compared with normal colonic tissues (Table 2, Fig. 4). These results might indicate active regeneration of the mucosa in gastric tissues, or hyperplastic proliferation caused by infection with *Helicobacter pylori*, which is often found in Japanese individuals [27].

Another noteworthy point in our results was observed in the analyses of CDK1. Expression analysis of the CDKs showed relatively higher CDK1 levels in both colon and gastric cancer tissues (Table 2). When the cut-off value for CDK1 expression was set at the mean+2 S.D. of the CDK1 value for the corresponding normal tissue, we found that 6/22 (27%) colon cancer tissues and 6/8 (75%) gastric cancer tissues had CDK1 expression levels above the cut-off value. Moreover, close to 50% of colon tumor tissues (9/19) showed a more than two-fold enhancement of CDK1 activity compared with the corresponding normal mucosa (Fig. 4), although the average activities between tumor tissues and normal mucosa were similar. This raises the possibility that the growth-inhibitory effects of CDK inhibitors with a relatively broad spectrum, such as CYC202 (R-roscovitine [28]), are mediated through the inhibition of CDK1 activity in a certain portion of cancer patients.

Table 2
Mean activity and expression of CDKs in normal and tumor tissues

	CDK1		CDK2		CDK4		CDK6	
	normal	tumor	normal	tumor	normal	tumor	normal	tumor
A. Activity (U/μg of total protein±S.D.)								
Colon	0.017±0.020	0.017±0.019	0.012±0.016	0.065±0.040**	0.086±0.043	0.069±0.051	0.094±0.11	0.049±0.044
	(*n*=19)	(*n*=22)	(*n*=19)	(*n*=22)	(*n*=14)	(*n*=13)	(*n*=14)	(*n*=13)
Stomach	0.034±0.036	0.027±0.038	0.040±0.031	0.18±0.17*	0.10±0.086	0.027±0.026	0.10±0.050	0.052±0.043
	(*n*=8)	(*n*=8)	(*n*=8)	(*n*=8)	(*n*=8)	(*n*=16)	(*n*=8)	(*n*=6)
B. Expression (ng/μg of total protein±S.D.)								
Colon	0.79±0.60	1.5±0.78**	0.23±0.24	0.21±0.17	0.35±0.23	0.54±0.25*	0.19±0.23	0.24±0.25
	(*n*=21)	(*n*=22)	(*n*=21)	(*n*=22)	(*n*=21)	(*n*=22)	(*n*=21)	(*n*=22)
Stomach	0.84±0.38	2.8±2.0*	0.39±0.29	0.52±0.71	0.38±0.71	0.85±0.70	0.28±0.26	0.76±1.2
	(*n*=8)	(*n*=8)	(*n*=8)	(*n*=8)	(*n*=8)	(*n*=8)	(*n*=8)	(*n*=8)

* $P<0.05$.
** $P<0.01$ in Mann–Whitney's U test.

In this study, CDK kinase activity was found to be the most dominant parameter for differentiation of cancer and normal mucosa; 5.4 times difference for colon tissue, and 4.5 times difference for gastric tissue on average. This indicates that the activity analysis provides clinically more relevant values than the expression analysis, although the assay method is thought to be difficult to apply in a clinic.

Cyclin molecules activate the CDK catalytic unit by association and regulate kinase activity by de novo synthesis and degradation, which proceeds in tandem with progression through the cell cycle. The pairing between the CDK

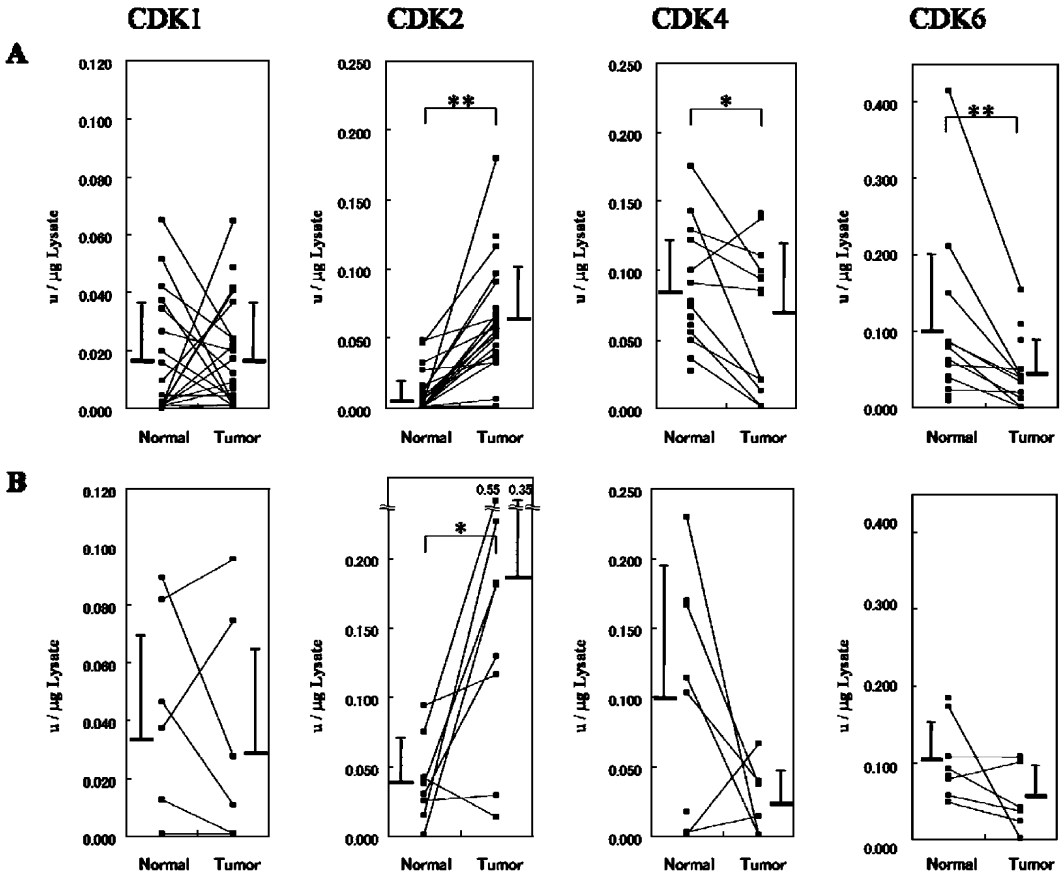

Fig. 4. CDK activities in gastrointestinal tumor tissues and normal mucosal tissues. CDK1, 2, 4, and 6 activities were measured by a non-radioisotopic kinase assay. Data from 22 patients with colon carcinomas (A), eight with gastric carcinomas (B), and the corresponding normal mucosal tissues are plotted. Data are shown as mean±standard deviation.

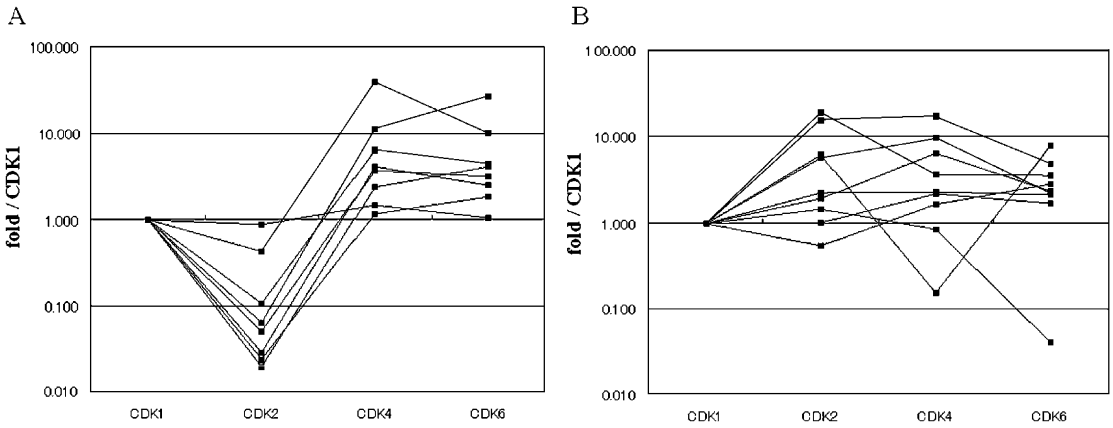

Fig. 5. G1 CDK activity profiling of colonic tissues. Profiling was performed by normalizing the values for CDK2, 4, and 6 activities to the value for CDK1 activity. (A) Eight normal colonic mucosa. (B) Nine colon carcinomas.

isotypes and cyclin isotypes is specific; CDK1 associates with cyclin B, CDK2 with cyclin E, and CDK4 or CDK6 with cyclin D. Thus, one might expect that analysis of CDK1, CDK2, and CDK4/6 activity—a tedious and time-consuming process—could be replaced by quantifying the amounts of the corresponding partner cyclins. The amount of cyclins, however, did not correspond perfectly with CDK activity in our study (data not shown), demonstrating that regulation of CDK activity is a complex event that probably includes phosphorylation of the catalytic subunit or association of CDK inhibitors ($p16^{INK4a}$, $p21^{WAF1}$, $p27^{Kip1}$, and so on) with that subunit. Therefore, accurate monitoring of the activity of particular CDKs can be performed only with conventional kinase assay systems that quantify the extent of substrate phosphorylation.

In normal cells, proliferation is strictly controlled at G1 phase by regulatory molecules, and after passing the R point, the cell-cycle machinery becomes relatively refractory to ectopic signals such as stress and growth-inhibitory signals. Therefore, we normalized the values for CDK activities at G1; CDK2, 4, and 6 to the CDK1 activity at G2/M for the purpose of this study and performed G1 CDK activity profiling for colonic tissues (this was performed for eight normal colonic mucosa and nine colon carcinomas in which CDK1 activity could be measured). It is noteworthy that normal colonic mucosal tissues shared an almost identical G1 CDK profile (Fig. 5A), although no shared profile was observed for normal gastric mucosa (data not shown). These results strongly suggest that in normal colonic tissues, the G1 phase of the mammalian cell cycle is organized via the coordination of CDK activities. On the other hand, the profiles of colon carcinoma tissues were random among the nine patients, with no common feature (Fig. 5B).

Inhibitors of CDK molecules and activators of the CDK inhibitors are being developed intensively, and some are undergoing clinical trials [29]. These may be effective in the treatment of tumors with activated CDKs. We have demonstrated here that CDK2 activity was remarkably increased in more than 70% of gastrointestinal cancers. This evidence is very much in agreement with CDK2 inhibition, showing promising growth-inhibitory effects as an anti-cancer drug [30] despite the report showing the proliferation of colon cancer cell lines in the absence of CDK2 activity [31].

According to the clinically relevant evidence with surgically resected tumor tissues in this study, the CDK profiling procedure is revealed to be clinically workable and worthwhile to apply on the automated apparatus. We believe that the concept of CDK profiling including the direct measurement of CDK activities in a clinical setting, described here, will introduce a novel molecular–clinical approach to complement morphological diagnoses for cancer, and for monitoring therapeutic stratagems currently in use. This technology might also assist in the individualized medications with novel CDK modulating drugs and conventional cell cycle modulating anti-cancer drugs.

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