Phosphorylated retinoblastoma protein is a potential predictive marker of irinotecan efficacy for colorectal cancer

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Abstract. Irinotecan has been used in the first-line treatment of metastatic colorectal cancer. However, no clear predictive marker of irinotecan efficacy has been identified. It is controversial whether the response to irinotecan could be predicted by the expression level of topoisomerase-I, a direct target of irinotecan. The present study aimed to identify a feasible predictive marker of irinotecan efficacy. We hypothesized that the efficacy of SN38 (an active metabolite of irinotecan) is related to the cell proliferation and the phosphorylation status of RB in colorectal cancer cells. Indeed, the IC_{50} of SN38 was positively correlated with the doubling time of each cell line (R^2=0.9315). Moreover, the phosphorylation level of RB was related to SN38 sensitivity. Consistent with the *in vitro* data, colorectal cancer tissues of irinotecan responders showed a significantly higher rate of phosphorylated RB (serine 780) expression using immunohistochemistry (P=0.0006), although a generally used proliferative marker, Ki-67, showed no significance. Finally, we investigated whether the phosphorylation of RB plays a crucial role in the efficacy of irinotecan. To suppress the expression of phosphorylated RB, we performed the knockdown of CDKs, which are known to phosphorylate RB. Intriguingly, the knockdown of both CDK4 and CDK6, but not CDK2, allowed RB to become the most hypophosphorylated form and converted the SN38-sensitive cells to a resistant state. Taking together the above findings from *in vitro* and clinical research, the immunohistochemistry of phosphorylated RB protein might be feasible to predict the irinotecan efficacy of colorectal cancer in clinical practice.

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Abbreviations: RB, retinoblastoma gene; CDK, cyclin-dependent kinase

Key words: colorectal cancer, immunohistochemistry, irinotecan, phosphorylated RB, predictive marker

Introduction

Irinotecan has been used for around two decades in chemotherapies against several advanced cancers. Especially in the first-line treatment of metastatic colorectal cancers, irinotecan has been one of the mainstay drugs, with longer progression-free survival and overall survival (1-3). However, no clear predictive marker of irinotecan efficacy has been identified, despite its widespread use.

Irinotecan directly inhibits topoisomerase I (Top I) to prevent re-ligation of the nicked DNA strand during DNA replication (4,5), and then it has been suggested that a high expression level of Top I correlates with its efficacy (6). For instance, in a large prospective study, the UK MRC FOCUS trial for advanced colorectal cancer patients, moderate or high Top I expression was associated with longer survival in patients treated with irinotecan in addition to 5-fluorouracil (7). Conversely, in the CAIRO study, in which advanced colorectal cancer patients were also prospectively interrogated, no association was found between the response to irinotecan and Top I expression (8,9). Additionally, another report mentioned that the cells that acquired resistance to SN38 (an active metabolite of irinotecan) maintained Top I expression levels (10). Therefore, Top I expression is supposed to be insufficient for the prediction of irinotecan efficacy clinically.

On the contrary, it has been supposed that cytotoxic chemotherapies show susceptibility in highly proliferative cells. Actually, a recent meta-analysis study suggested that the response to Top I inhibitors is associated with the cell growth rate (11). One of the critical points in the regulation of cellular proliferation is the G1/S transition, which is controlled by the phosphorylation status of RB protein. In malignant tumor cells, RB inactivation with its phosphorylation leads to cellular proliferation.

Considering the above, we investigated whether the efficacy of SN38 is related to the cell proliferation rate and the phosphorylation status of RB in colorectal cancer cells. We then validated the relationship between the levels of phosphorylated RB and the responses to irinotecan in clinical samples

of patients suffering from advanced colorectal cancers. The present study suggests a feasible predictive marker of irinotecan efficacy for colorectal cancer in a clinical setting.

Materials and methods

Cell culture and reagents. HCT116, SW480 and SW620 cells were obtained from the American Type Culture Collection. CCK-81, CoCM-1 and SW837 cells were obtained from the Health Science Research Resources Bank. LIM1215 cells were obtained from the European Collection of Cell Cultures. Caco-2 cells were obtained from RIKEN BioResource Center. The authenticity of each cell line was confirmed by short tandem repeat profiling at each cell bank. All the cells purchased from each cell bank were immediately expanded after receipt, and stocks of each cell line were prepared within 3 passages and stored in liquid nitrogen. For experiments, cells were used for fewer than 3 months after resuscitation. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM). Medium was supplemented with 10% fetal bovine serum (FBS), 4 mM glutamine, 50 U/ml penicillin and 100 μg/ml streptomycin. All cells were incubated at 37˚C in a humidified atmosphere of 5% CO_2. SN38 was obtained from Sigma (St. Louis, MO, USA). It was dissolved in the solvent dimethyl sulfoxide (DMSO) as stock and stored at -20˚C.

Cell viability assay. The cell viability was measured by a Cell Counting Kit-8 assay according to the manufacturer's instructions (Dojindo Laboratories, Kumamoto, Japan) to determine the IC_{50} values of each cell line. After the incubation of cells for 72 h with the indicated concentrations of SN38, kit reagent WST-8 was added to the medium and incubated for a further 4 h. The absorbance of samples (450 nm) was measured using a multi-plate reader (DS Pharma Biomedical Co., Ltd., Osaka, Japan). Cell numbers were measured using the ViaCount Assay according to the manufacturer's instructions (EMD Millipore, Billerica, MA, USA) to determine the doubling time of each cell line.

Protein isolation and western blotting. Cells were lysed with a buffer containing 50 mM Tris-HCl, 1% SDS, 1 mM DTT, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride and phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). The lysate was sonicated and centrifuged at 20,400 x g for 20 min at 4˚C and the supernatant was collected. Equal amounts of lysate were boiled for 5 min and loaded onto a 12% (for CDK2, CDK4, CDK6 and GAPDH detection) or 7% (for phospho-RB, pRB, topoisomerase-I, BCRP and α-tubulin detection) polyacrylamide gel, subjected to electrophoresis and transferred to PVDF membranes (EMD Millipore). The following primary antibodies were used: mouse anti-human pRB (BD Biosciences, San Jose, CA, USA), mouse anti-human α-tubulin (EMD Millipore), mouse anti-human CDK6, rabbit anti-human phospho-RB (Ser780), rabbit anti-human phospho-RB (Ser807/811) (Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-human CDK2, rabbit anti-human CDK4 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit anti-human topoisomerase-I, mouse anti-human BCRP (Abcam, Cambridge, UK), and mouse anti-human GAPDH (HyTest Ltd., Turku, Finland). The blots were incubated with the appropriate HRP-conjugated secondary antibody (GE Healthcare, Piscataway, NJ, USA), and the signals were detected with Chemi-Lumi One (Nacalai Tesque) or Western Chemiluminescent HRP Substrate (EMD Millipore).

Small interfering RNA transfection. Small interfering RNAs (siRNA) were obtained from Ambion (Carlsbad, CA, USA) for CDK2 (UAAGUACGAACAGGGACUCca), CDK4 (UGUGG GUUAAAAGUCAGCAtt), CDK6 (UUCUACGAAACAUU UCUGCaa), and Silencer® Select Negative Control #2 siRNA. Cells were transfected with 50 nM of each siRNA using Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA, USA). At 24 h after the transfection, cells were incubated with 5 nM SN38, 0.1% DMSO or DMEM for 72 h, and harvested for cell cycle analysis and western blotting.

Cell cycle analysis. Cells were exposed to SN38 at the indicated concentrations for 72 h and then harvested. They were permeabilized with 0.1% Triton X-100 and the nuclei were stained with propidium iodide. The DNA contents were measured using a FACSCalibur (Becton-Dickinson, Franklin Lakes, NJ, USA) and analyzed with ModFit LT (Verity Software House, Topsham, ME, USA).

Human tissue samples. Primary tumor samples were obtained from 23 patients with clinical Stage IV colorectal cancer, who underwent a colectomy/rectectomy at Kyoto Prefectural University of Medicine between 2008 and 2013. The samples were embedded in paraffin after 24 h of formalin fixation. The patient eligibility criteria were: no synchronous tumors, and not having received a preoperative chemotherapy or a radiation therapy. All the patients gave their written informed consent. Relevant clinicopathological and survival data were obtained from the hospital database. After operations, 22 patients underwent second- or third-line chemotherapies with irinotecan, which targeted metastasis or recurrence, and only 1 patient underwent treatment with irinotecan as the first-line therapy. The following treatments with irinotecan were applied: FOLFIRI (5-FU+leucovorin+irinotecan), FOLFIRI+BEV (FOLFIRI+bevacizumab), FOLFIRI +C-mab (FOLFIRI+cetuximab), FOLFIRI+P-mab (FOLFIRI +panitumumab), CPT-11+C-mab (irinotecan+cetuximab), IRIS (irinotecan+S-1), and IRIS+BEV (IRIS+bevacizumab). Computed tomography (CT) was performed after the treatment with irinotecan for approximately 5 courses. The curative effects were evaluated by RECIST v1.1 (12), referring to the pictures of CT. A total of 5 patients were classified with partial response (PR), 10 with stable disease (SD) and 8 with progressive disease (PD). We classified PR patients into a responder group, and SD and PD patients into a non-responder group. Disease staging was principally based on The Union for International Cancer Control/TNM Classification of Malignant Tumours (7th edition) (13).

Immunohistochemistry. Paraffin sections (3 mm thick) of tumor tissue were subjected to immunohistochemical staining for phosphorylated RB (serine 780) and Ki-67 (MIB-1) using the avidin-biotin-peroxidase method. Briefly, paraffin sections were dewaxed in xylene and hydrated through a graded series

Table I. The IC_{50} values and the doubling times of eight colorectal cancer cell lines.

Cell name	SN38 IC_{50} (nM) Ave.	SD	Doubling time (h)
Sensitive			
HCT116	3.54	2.74	18.55
LIM1215	1.22	0.55	31.27
SW480	3.97	3.30	38.16
SW620	3.19	0.77	19.47
Resistant			
CCK-81	166.46	51.23	55.18
CoCM-1	415.10	48.03	103.41
SW837	89.64	28.69	52.01
Caco-2	86.99	19.56	41.65

Ave., average (n=3); SD, standard deviation.

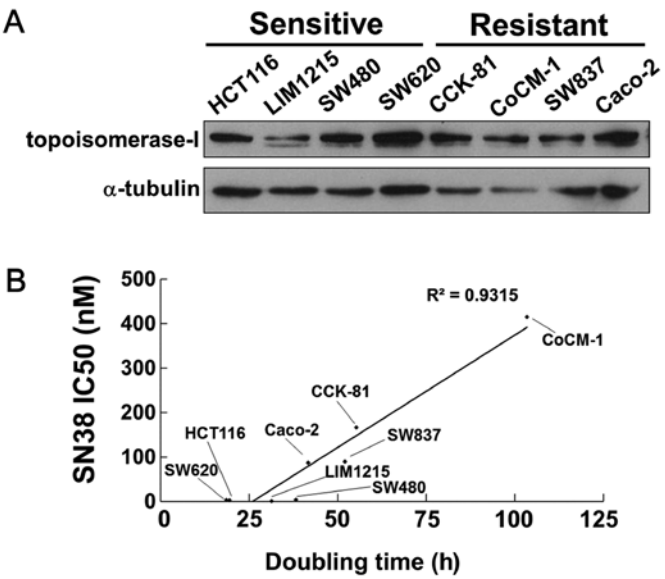

Figure 1. SN38 sensitivity is related to the proliferation rate of cells. (A) The expression levels of topoisomerase-I were analyzed by western blotting in eight colorectal cancer cell lines. α-tubulin was used as a loading control. (B) The IC_{50} values and the doubling times of cell lines were plotted.

of alcohol. Endogenous peroxidase activity was quenched by incubating the sections for 30 min in 0.3% H_2O_2. The sections were then treated with a protein blocker and incubated with antibodies. Sections were incubated for 1 h at 37˚C with a mouse anti-RB (phospho S780) antibody (Abcam), and were incubated for 1 h at room temperature with Ki-67 (Santa Cruz Biotechnology). The avidin-biotin-peroxidase complex system (Vectastain ABC Elite kit; Vector Laboratories, Burlingame, CA, USA) was used for color development with diaminobenzidine tetrahydrochloride. The sections were counterstained with hematoxylin. Finally, the sections were dehydrated through a graded series of alcohol, cleared in xylene and mounted. Tumor cells with immunohistochemical expression in the cytoplasm were counted as phosphorylated RB-positive. For scoring the phosphorylated RB expression, the percentage of the total cell population that expressed phosphorylated RB (serine 780) was evaluated for each case. The labeling index (LI) of immunohistochemistry for Ki-67 was determined by counting positive tumor cells in the most intensely stained region. Severely keratinized portions in the nest of colorectal cancers were excluded.

Statistical analysis. Significance was assessed by Student's t-test or Mann-Whitney U test for comparisons between two groups. Fisher's exact test was performed to investigate the correlations between clinicopathological parameters and phosphorylated RB expression level. Differences were considered significant when the P-value was <0.05.

Results

The sensitivity to SN38 is inversely correlated with the doubling time of cells. We examined each IC_{50} value of SN38 using eight human colorectal cancer cells as shown in Table I. The HCT116, LIM1215, SW480 and SW620 cells were sensitive to SN38, with IC_{50} values of <4 nM. The CCK-81, CoCM-1, SW837 and Caco-2 cells were resistant to SN38, with IC_{50} values of >80 nM. We found no association between the sensitivity to SN38 and the expression level of Top I in these cell lines (Fig. 1A).

We next measured the doubling times of the cell lines (Table I) and plotted them against the IC_{50} values of SN38. As shown in Fig. 1B, the doubling times of the cell lines and the IC_{50} values of SN38 showed an appreciable positive correlation (R^2=0.9315) (Fig. 1B). These results indicate that highly proliferative cells are more sensitive to SN38.

Phosphorylation status of RB is relevant to the sensitivity to SN38 of cell lines. We then performed western blotting to detect the phosphorylated RB of each cell line, which is unable to bind to the transcription factor E2F, resulting in proliferation of the cells. The SN38-sensitive cell lines showed hyperphosphorylation of RB without alteration of the total amount of RB protein, while the resistant cell lines showed hypophosphorylation of RB, with the exception of the phosphorylated RB at serines 807/811 in Caco-2 cells (Fig. 2A). Caco-2 cells are known to express BCRP (breast cancer resistance protein) (14), which transports SN38 (15). Indeed, we confirmed that only Caco-2 cells expressed BCRP among these cell lines (Fig. 2B), suggesting that these cells show resistance to SN38 despite hyperphosphorylation of RB at serines 807/ 811.

Taken together, these results suggest that the phosphorylation status of RB could predict the efficacy of irinotecan.

Phosphorylation status of RB protein detected by immunohistochemistry may predict the response to irinotecan in patient specimens. We then examined whether the expression levels of phosphorylated RB could predict the response to irinotecan in the patient specimens. The 23 patients with colorectal cancers were classified into responder and non-responder groups, according to the responses to the treatments including irinotecan, as described in Materials and methods (Table II).

Table II. Clinicopathological characteristics of 23 cases of colorectal cancer.

Case no.	Age (years)	Gender	Location	cStage (UICC 7)	pStage (UICC 7)	Metastasis/ Recurrence	1st therapy	Therapeutic response	2nd therapy	Therapeutic response	3rd therapy	Therapeutic response
1	66	Female	T	IVb	IVb	Peritoneum	mFOLFOX6	PD	**FOLFIRI**	**PD**		
2	55	Female	Ra	IVa	IVa	Liver	FOLFOX	SD	**FOLFIRI+BEV**	**SD**		
3	60	Female	A	IVb	IVb	Peritoneum	mFOLFOX6	PD	**CPT-11+C-mab**	**PD**		
4	54	Male	D	IVb	IVb	Peritoneum	mFOLFOX6	PD	**CPT-11+C-mab**	**PD**		
5	53	Female	Rs	IVa	IIIc	Lung	FOLFOX	PD	FOLFOX+BEV	PR	**FOLFIRI+BEV**	**PR**
6	52	Female	Ra	IVa	IVa	Lung	FOLFOX+BEV	SD	**FOLFIRI**	**PD**		
7	68	Male	Rs	IVa	IIb	Peritoneum	**FOLFIRI**	**PD**				
8	67	Male	T	IVa	IVa	Liver	mFOLFOX6+BEV	PR	**FOLFIRI+BEV**	**PD**		
9	64	Male	Rs	IVa	IVa	Liver	mFOLFOX6	PR	mFOLFOX6+BEV	PR	**FOLFIRI+BEV**	**SD**
10	67	Male	Rs	IVb	IVb	Liver/lymph node	mFOLFOX+BEV	PR	**FOLFIRI+BEV**	**PR**		
11	74	Male	T	IVa	IVa	Liver	FOLFOX+BEV	SD	**FOLFIRI+BEV**	**PD**		
12	61	Male	Rs	IVa	IVa	Liver	FOLFOX	SD	**FOLFIRI+BEV**	**SD**		
13	64	Male	T	IVa	IVa	Liver	FOLFOX+C-mab	SD	**FOLFIRI+C-mab**	**SD**		
14	60	Female	Rb	IVa	IVa	Liver	XELOX	SD	**IRIS**	**SD**		
15	39	Male	S	IVb	IVb	Peritoneum	XELOX+BEV	PD	**FOLFIRI+P-mab**	**SD**		
16	67	Female	Ra	IVa	IVa	Lung	XELOX	SD	**FOLFIRI**	**PR**		
17	55	Female	T	IVb	IVb	Peritoneum	XELOX+BEV	PD	**FOLFIRI+P-mab**	**SD**		
18	64	Female	Rb	IVa	IVa	Lymph node	XELOX	PD	**FOLFIRI**	**SD**		
19	72	Female	Rb	IVa	IVa	Lung	XELOX	PD	**FOLFIRI+P-mab**	**SD**		
20	62	Female	Rs	IVa	IVa	Liver	XELOX+BEV	PD	**FOLFIRI+P-mab**	**PR**		
21	55	Female	A	IVb	IVb	Liver/lung	FOLFOX BEV	PD	**IRIS+BEV**	**PR**		
22	74	Male	T	IVb	IVb	Liver/lung	XELOX	PD	**FOLFIRI**	**PD**		
23	45	Female	T	IVa	IVa	Liver	XELOX BEV	PD	**IRIS+BEV**	**SD**		

A, ascending colon; T, transverse colon; D, descending colon; S, sigmoid colon; Rs, rectosigmoid; Ra, rectum above the peritoneal reflection; Rb, rectum below the peritoneal reflection; cStage, clinical stage; pStage, pathological stage; UICC, The Union for International Cancer Control; FOLFIRI, 5-FU+leucovorin+irinotecan; FOLFIRI+BEV, FOLFIRI+bevacizumab; FOLFIRI+C-mab, FOLFIRI+cetuximab; FOLFIRI+P-mab, FOLFIRI+panitumumab; CPT-11+C-mab, irinotecan+cetuximab; IRIS, irinotecan+S-1; IRIS+BEV, IRIS+bevacizumab. Therapies including irinotecan are indicated in bold.

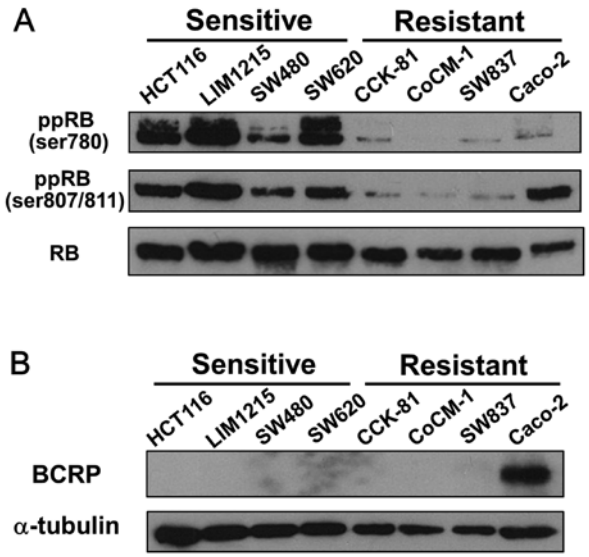

Figure 2. RB protein is highly phosphorylated in SN38-sensitive cell lines. (A) The phosphorylated RB at serine 780, serines 807/811 and total RB were analyzed by western blotting in eight colorectal cancer cell lines. (B) Caco-2 cells express BCRP, the transporter protein of SN38. The expression levels of BCRP were analyzed by western blotting in eight colorectal cancer cell lines. α-tubulin was used as a loading control.

Table III. Clinicopathological characteristics according to the response to the treatment with irinotecan.

Variables	Responder (n=5)	Non-responder (n=18)	P-value
Gender			
Male	1	9	0.339
Female	4	9	
Age (years)			
≥65	2	6	1
<65	3	12	
Location			
Colon	1	10	0.317
Rectum	4	8	
pT			
pT0-2	0	4	0.539
pT3-4	5	14	
pN			
pN0-1	1	13	0.056
pN2	4	5	
pM			
M0	1	1	0.395
M1	4	17	
pStage			
I-III	1	1	0.395
IV	4	17	

pT, pathological T stage; pN, pathological N stage; pM, pathological M stage; pStage, pathological stage. Fisher's exact test was used for two-group comparisons.

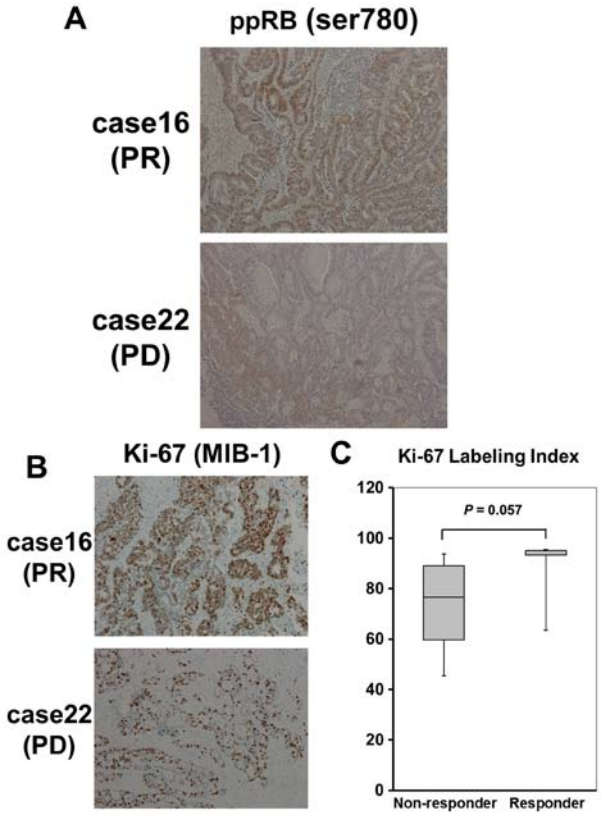

Figure 3. Immunohistochemical staining of primary colorectal cancer samples with phosphorylated RB or Ki-67. (A) Two representative cases stained with phosphorylated RB (serine 780) (X100) are shown. PR, partial response; PD, progressive disease. (B) Two representative cases stained with Ki-67 (x100) are shown. PR, partial response; PD, progressive disease. (C) Ki-67 LI was evaluated in 23 cases. The grouped data of Ki-67 LI are shown as box plots. The median value is shown by a horizontal line in the box plot. The gray box denotes the 75th (upper margin) and 25th (lower margin) percentiles of the values. The upper and lower bars indicate the 90th and 10th percentiles, respectively. The statistical analysis was performed by Mann-Whitney U test.

The patient characteristics of the irinotecan responders and non-responders are shown in Table III. There was no significant difference in the overall survival between the responders and non-responders (data not shown). Next, we analyzed the relationships between various clinicopathological parameters and the phosphorylated RB expression levels determined by immunohistochemistry (IHC). Representative phosphorylated RB expression levels of the tumors are shown in Fig. 3A. We then evaluated the phosphorylated RB expression levels in 23 samples, as described in Materials and methods, and defined the appropriate IHC cut-off values of stained tumor cells. As a consequence, we found that the colorectal cancer tissues of irinotecan responders showed a significantly higher positivity rate of the phosphorylated RB (serine 780) at a cut-off value of 25% (P=0.0006; Table IV), while the labeling index (LI) of Ki-67 (MIB-1), which is clinically used as a diagnostic marker of tumor proliferation, showed no statistically significant difference between responders and non-responders (Fig. 3B and C).

Taking these findings together, IHC of the phosphorylated RB (serine 780) in tumor might be useful to predict the response to irinotecan, rather than Ki-67.

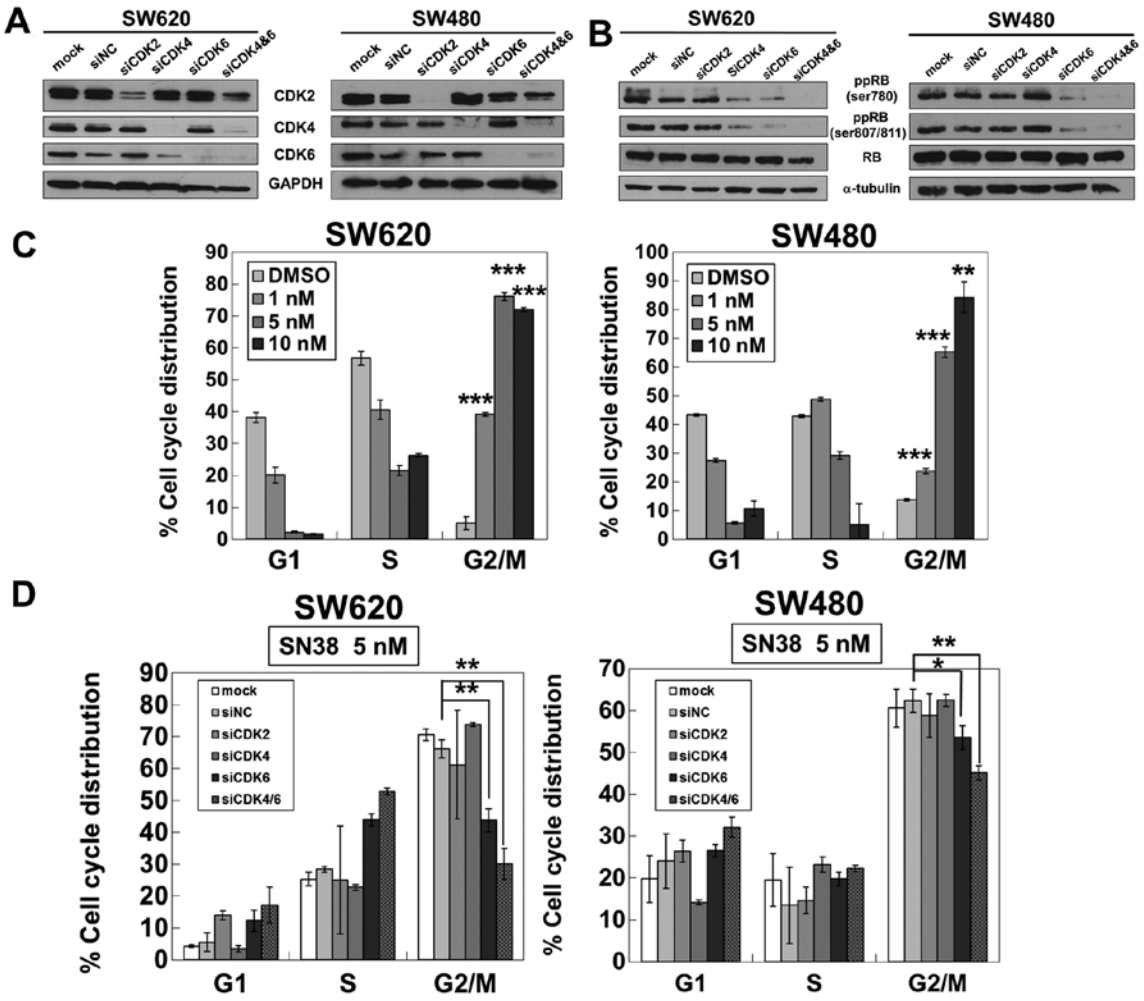

Figure 4. The simultaneous knockdown of CDK4 and CDK6 affects the phosphorylation status of RB with a decrease of G2/M accumulation induced by SN38. (A) The knockdown efficacies of siCDK2, siCDK4, siCDK6, and siCDK4/6 were validated by western blotting in SW620 and SW480 cells. GAPDH was used as a loading control. (B) The phosphorylated RB at serine 780, serines 807/811 and total RB were analyzed by western blotting in SW620 and SW480 cells transfected with siCDK2, siCDK4, siCDK6, siCDK4/6, or negative control (siNC). α-tubulin was used as a loading control. (C) SW620 and SW480 cells were treated with SN38 at the indicated concentrations for 72 h. DNA contents of the cells were analyzed by flow cytometry. The percentages of cells in the G1, S and G2/M phases of the cell cycle are shown. Columns, means (n=3); bars, standard deviation (SD). $^{**}P<0.01$; $^{***}P<0.001$, compared with siNC by Student's t-test. (D) Cell cycle distribution was analyzed in SW620 and SW480 cells transfected with siCDK2, siCDK4, siCDK6, siCDK4/6 or siNC. Cells were exposed to 5 nM SN38 for 72 h following the treatment of each siRNA. DNA contents of the cells were analyzed by flow cytometry. The percentages of cells in the G1, S, and G2/M phases of the cell cycle are shown. Columns, means (n=3); bars, standard deviation (SD). $^{*}P<0.05$; $^{**}P<0.01$, compared with siNC by Student's t-test.

Knockdown of both CDK4 and CDK6 reduces G2/M accumulation induced by SN38 with RB dephosphorylation. We next investigated whether the phosphorylation of RB plays a crucial role in the efficacy of irinotecan. We performed the knockdown of CDKs, which are well known to phosphorylate RB protein, to suppress the expression of phosphorylated RB. CDK2, CDK4, CDK6 and CDK4/6 were silenced by siRNA in the SN38-sensitive cell lines (Fig. 4A). RB protein was converted to the unphosphorylated form most strikingly after the simultaneous knockdown of CDK4 and CDK6 in SW620 and SW480 cells, while the knockdown of CDK2 did not affect the phosphorylation status of RB protein (Fig. 4B).

SN38 treatment for 72 h induced dose-dependent G2/M arrest in SW620 and SW480 cells (Fig. 4C). However, the G2/M arrest induced by 5 nM SN38 for 72 h in these cells was restored most by siCDK4/6 (Fig. 4D), which most markedly reduced the level of the phosphorylated form of RB protein (Fig. 4B).

The sensitivity to SN38 is dampened by the knockdown of both CDK4 and CDK6. Finally, we investigated whether CDK4/6 activities affected the sensitivity to SN38. As shown in Fig. 5A, SW620 cells exhibited resistance to SN38 after both CDK4 and CDK6 were silenced. The transfection of siCDK4/6 gave an increased median IC_{50} value of SN38 of 58.4 nM, while that of a negative control gave a median IC_{50} value of 1.4 nM (Fig. 5B). Thus, the sensitivity to irinotecan might be governed by the activities of CDK4/6 in colorectal cancer.

Discussion

We showed for the first time that the efficacy of irinotecan could be predicted by phosphorylated RB expression, which is known to be crucial for G1/S progression and cell proliferation. We proved the positive correlation between the phosphorylated status of RB and the efficacy of SN38 in colorectal cancer cell lines. In line with the *in vitro* study, our clinical retrospective

Table IV. Associations between the clinicopathological characteristics of colorectal cancer patients and phosphorylated RB (serine 780) (cut-off value, 25%).

Variables	ppRB (ser780) Negative (<25) (n=19)	Positive (≥25) (n=4)	P-value
Gender			
Male	9	1	0.604
Female	10	3	
Age (years)			
≥65	6	2	0.589
<65	13	2	
Location			
Colon	10	1	0.59
Rectum	9	3	
pT			
pT0-2	4	0	1
pT3-4	15	4	
pN			
pN0-1	13	1	0.26
pN2	6	3	
pM			
M0	2	0	1
M1	17	4	
pStage			
I-III	2	0	1
IV	17	4	
Treatment response			
Responder	1	4	**0.0006**[a]
Non-responder	18	0	

pT, pathological T stage; pN, pathological N stage; pM, pathological M stage; pStage, pathological stage. [a]P<0.01, Fisher's exact test was used for two-group comparisons.

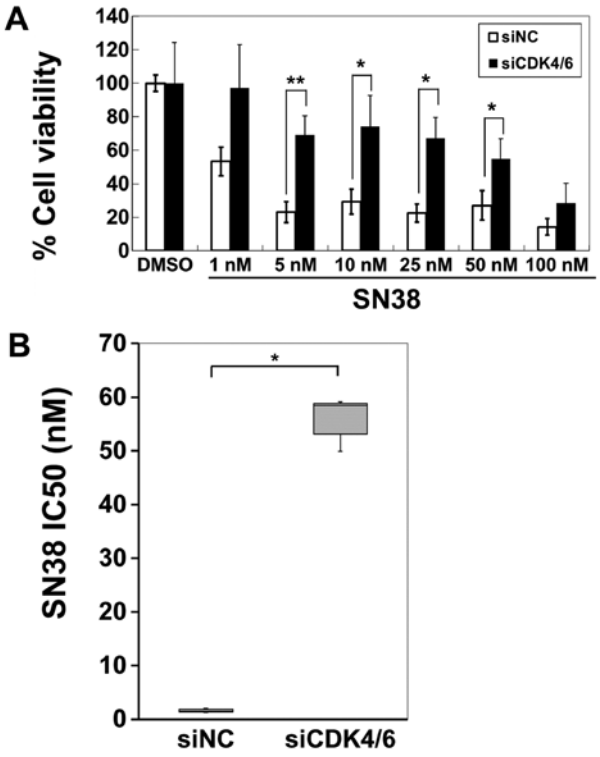

Figure 5. The simultaneous knockdown of CDK4 and CDK6 impairs the antitumor activity of SN38. (A) SW620 cells were exposed to SN38 at the indicated concentrations for 72 h following the treatment of siCDK4/6 or negative control (siNC). Cell viability was measured by a Cell Counting Kit-8 assay. The value obtained with the solvent dimethyl sulfoxide (DMSO) was taken as 100%. Columns, means (n=3); bars, standard deviation (SD). *P<0.05; **P<0.01, compared with siNC by Student's t-test. (B) The IC_{50} values of SW620 cells treated with SN38 were calculated with or without the transfection of siCDK4/6. The grouped data of IC_{50} values are shown as box plots. The median value is shown by a horizontal line in the box plot. The gray box denotes the 75th (upper margin) and 25th (lower margin) percentiles of the values. The upper and lower bars indicate the 90th and 10th percentiles, respectively. *P<0.05, compared with siNC by Mann-Whitney U test.

study demonstrated that the positivity rate of the phosphorylated RB (serine 780) was significantly higher in colorectal cancer tissues of irinotecan responders.

The phosphorylation of RB protein endows the cells with the ability to pass through the restriction point at the late G1 phase, enabling the cell cycle to progress (16). In most cancer cells, RB protein is hyperphosphorylated, resulting in uncontrolled cell proliferation (17-20). Indeed, colorectal cancer tissues were shown to express the phosphorylated RB at a high level compared with adenomas or normal colonic mucosa (21). Thus, RB phosphorylation is considered to play a crucial role in colorectal cancer progression.

Irinotecan targets proliferative cells and works during S phase with the inhibition of Top I (4,5,22-24). In our clinical study, colorectal cancer tissues of irinotecan responders showed a significantly higher positivity rate of phosphorylated RB at serine 780, rather than that of Ki-67 clinically used as a proliferative marker. The expression of Ki-67 increases in S phase but reaches a maximum in M phase (25-27), suggesting its efficacy as an M-phase marker. On the contrary, the phosphorylation of RB occurs at the end of G1 phase, with cells progressing to S phase (28,29), suggesting its efficacy as an S-phase marker. Given that irinotecan attacks cancer cells in S phase, it is rational that the phosphorylated RB affects the susceptibility to irinotecan, rather than Ki-67.

RB protein is phosphorylated by CDKs, resulting in cell cycle progression. However, there seems to be a difference in terms of which kinds of CDKs are essential for the proliferation of cancer cells, depending on the cellular context (30). For instance, CDK2 activity was shown to be related to the prognosis of breast cancer (31,32) and renal cell carcinoma (33), while CDK4 contributed to cell proliferation in colorectal cancer (34) and non-small cell lung cancer (35). We performed knockdown experiments of CDK2, CDK4, CDK6 and CDK4/6 to convert the phosphorylated RB to the unphosphorylated form. Of particular interest, our data showed that silencing of both CDK4 and CDK6, but not CDK2, led RB to adopt the most hypophosphorylated form (Fig. 4B) and rendered cells resistant to SN38 (Fig. 5), suggesting that CDK4/6 activities

are indispensable for the proliferation of colorectal cancer cells. Thus, the role of CDK4 and CDK6 could be crucial in not only RB phosphorylation and proliferation of colon cancer cells but also sensitivity to irinotecan.

In conclusion, we found that the phosphorylation status of RB protein could be a novel and promising predictive marker of irinotecan efficacy for colorectal cancer, based on the rationale that cells with highly phosphorylated RB would be more exposed to irinotecan through proceeding to S phase. In the chemotherapies for colorectal cancer, which have been more complicated and diverse, it has become increasingly necessary to stratify the treatment responders. Now we are planning larger cohort studies to examine whether this novel predictive marker of irinotecan efficacy could improve the outcome of colorectal cancer.

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