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Cell Cycle Kinetic Analysis of Colorectal Neoplasms Using a New Automated Immunohistochemistry-Based Cell Cycle Detection Method

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Abstract: We have recently developed a new method called the immunohistochemistry-based cell cycle detection (iCCD), which allows the determination of cell cycle phases on a cell-by-cell basis. This automated procedure can be performed on tissue sections and involves triple immunostaining for geminin, cdt1, and γ H2A.X, which are nuclear proteins expressed sequentially, with a few overlaps, during the cell cycle. In the current study, we applied this technique to resected specimens of colorectal neoplasm to determine the usefulness of iCCD for the pathological examination of colorectal cancers.

We examined 141 cases of colorectal cancers. Normal mucosa and adenomas were analyzed as controls.

In nonneoplastic mucosa, we observed a pattern of distribution of the cells positive for these cell cycle markers. Adenomas showed a slight distortion in this pattern, the geminin-positive cells, indicative of S/G2/M phase, were localized in the upper one-third region of the crypts. In neoplastic mucosa, the marker expression pattern was disorganized. Compared with normal mucosa, colorectal neoplasms showed an increased proportion of geminin-positive cells and decreased percentages of cdt1-positive cells (G1 phase). However, we did not find significant difference in the expression pattern between adenomas and carcinomas. Cellular proportions were correlated with clinicopathological parameters such as microscopic vascular invasion and pT stages. In cases of preoperative adjuvant therapy, the proportion of geminin-positive cells decreased, whereas that of γ H2A.X-positive cells (indicative of apoptosis/degeneration) increased significantly.

We believe that this novel method can be applied to clinical samples to evaluate cell cycle kinetics and the effects of preoperative adjuvant therapy in colorectal cancers.

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Abbreviations: Ag–Ab = antigen–antibody, iCCD = immunohistochemistry-based cell cycle detection, mTRG = modified tumor regression grade, RFS = recurrence-free survival, TNM = tumor–lymph node–metastasis.

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INTRODUCTION

Colorectal cancers are one of the most common malignancies observed world over. A multidisciplinary approach is crucial for management of patients with colorectal neoplasms. Pathologists play a central role in the preoperative diagnosis and tumor-stage identification based on resected specimens. Recent advances in technology have enabled us to examine tissue samples at the molecular level. Immunostaining for drug targets (eg, epidermal growth factor receptor) and gene (*KRAS*) mutation analysis are new part of routine practice. Assuming a continuation in this trend, many investigators are developing new molecular pathology based techniques that provide valuable information to supplement conventional histological findings.

Cell growth is tightly regulated by cell cycle related genes. Uncontrolled cell growth leads to tumorigenesis or aggressive behavior of cancer cells.[1] How the cell cycle is regulated in malignant neoplasms remains a topic in the field of cancer research. A gene, cell-cycle-related and expression-elevated protein in tumor (*CREPT*), was recently identified as a novel regulator of cell cycle by a database homology screening.[2] *CREPT* promotes the G1 to S phase transition by regulating expressions of cell cycle related genes including cyclin D1. *CREPT* is overexpressed in various malignant neoplasms including colorectal cancers, suggesting that this gene is involved in tumor development and progression.[2]

We recently developed a new method called immunohistochemistry-based cell cycle detection (iCCD) method, which allows us to determine cell cycle phases on a cell-by-cell basis.[3] This tissue section based approach is applicable to formalin-fixed and paraffin-embedded specimens. The triple immunostaining is an automated process and used 3 primary antibodies against geminin (a marker for S, G2, and M phases),[4] cdt1 (G1),[5,6] and γ H2A.X (cellular apoptosis and degeneration),[7] which are nuclear proteins expressed sequentially, with a few overlaps, during the cell cycle. Impaired expression of cdt1 and geminin indicates tumor development and poor prognosis for several types of cancer, including colorectal carcinoma.[6,8–10]

In this study, we analyzed resected specimens of colorectal neoplasms by the iCCD method to validate its usefulness in the pathological evaluation of colorectal tumors.

MATERIALS AND METHODS

Patients

We included 141 patients with colorectal carcinoma in this study. These patients underwent surgical resection at the

Division of Gastrointestinal Surgery, Kobe University Hospital, between January 2009 and September 2013. Cases of special histological types (ie, mucinous adenocarcinoma, squamous cell carcinoma) were excluded because of their particularity. All patients provided their informed consent to the pathological examination of resected specimens, and the Clinical Ethics Committee of Kobe University Graduate School of Medicine approved this study.

Specimen Preparation

The tissue specimens were fixed in 10% buffered neutral formalin and embedded in paraffin. We used 4-μm-thick sections for the iCCD. Pathological specimens were evaluated based on the Dukes system[11] and tumor–lymph node–metastasis (TNM)-based classification.[12] Effects of preoperative therapy were assessed based on the modified tumor regression grade (mTRG).[13] Nonlesional mucosa were sampled from 3 cases as controls. Nine resected specimens showed adenomas localized outside the carcinoma. These benign tumors were examined separately.

Single Immunostaining

Immunostaining was performed using an autostainer Bond Max (Leica Microsystems, Wetzlar, Germany) according to the preinstalled protocol. Primary antibodies were against geminin (GMNN, ×100; Proteintech Group Inc, Chicago, IL), cdt1 (CDT1, ×500; GeneTex, Irvine, CA), and γ H2A.X antibodies (P-Histone H2A.X, ×20; Cell Signaling Technology, Danvers, MA). Deparaffinized sections were heat-treated for 10 or 20 minutes.

Immunohistochemistry-Based Cell Cycle Detection

The iCCD procedure was performed using an automated immunostainer (BOND-MAX, Leica Microsystems, Wetzlar, Germany) by a method described previously.[3] All incubations were at room temperature, unless specified otherwise. The sections were deparaffinized and pretreated on a hot plate with citric acid buffer (pH 6.0, 100°C) for antigen retrieval. After a 10-minute incubation with 3% peroxidase blocking agent, the sections were incubated with antigeminin rabbit antibody

TABLE 1. Relationship Between Clinicopathological Characteristics and Cell Cycle Kinetics in Colon Cancer Without Preoperative Therapy

Variables		n	Geminin Average, %	*P* Value	Cdt1 Average, %	*P* Value	γ H2A.X Average, %	*P* Value
Patients total		101						
Sex	Male	47	34.049	NS	64.660	NS	1.291	NS
	Female	54	34.004		61.794		1.202	
Location	C	12	37.680	NS	61.894	NS	0.426	NS
	A	21	37.310		61.774		0.917	
	T	18	36.234		62.529		1.237	
	D	5	34.717		64.816		0.467	
	S	45	34.157		64.140		1.703	
Histology	G1	25	34.869	NS	63.972	NS	1.159	NS
	G2	69	36.245		62.367		1.387	
	G3	7	32.268		67.606		0.125	
pT category	pT1	5	34.483	NS	64.758	NS	0.759	NS
	pT2	26	34.578		63.738		1.684	
	pT3	49	37.020		62.129		0.851	
	pT4a	18	32.263		65.720		2.017	
	pT4b	3	44.121		55.880		0.000	
pN category	0	61	35.458	NS	63.358	NS	1.184	NS
	1	28	35.041		63.847		1.111	
	2	12	37.869		60.278		1.853	
cM category	0	83	36.286	NS	62.475	NS	1.216	NS
	1a	12	33.113		65.412		1.475	
	1b	6	31.572		67.264		1.163	
Dukes system	A	23	35.076	NS	63.169	NS	1.755	NS
	B	38	35.689		63.472		0.838	
	C	40	35.890		62.776		1.334	
ly	Negative	55	34.224	NS	64.413	NS	1.363	NS
	Mild	43	36.747		62.096		1.157	
	Extended	4	45.373		54.335		0.292	
v	Negative	52	34.444	0.007	64.178	0.008	1.379	NS
	Mild	47	35.756	0.010	63.098	0.012	1.146	
	Extended	2	63.479		36.521		0.000	
Recurrence	(+)	16	35.455	NS	63.198	NS	1.347	NS
	(−)	85	35.742		63.640		0.618	

G1 = well-differentiated adenocarcinoma, G2 = moderately differentiated adenocarcinoma, G3 = poorly differentiated adenocarcinoma, H2A.X = ??, ly = lymphatic infiltration, NS = not significant, v = venous infiltration.

(GMNN, ×100; Proteintech Group Inc) for 40 minutes followed by the secondary antibody MACH 2 Double Stain 2 (Biocare Medical, Concord, CA) for 30 minutes. A blue staining pattern was obtained using the PermaBlue/AP chromogen system (Diagnostic Biosystems, Pleasanton, CA). We heated the slides in citric acid buffer (pH 6.0) for 3 minutes at 100°C to stop the antigen–antibody (Ag–Ab) reaction and for additional antigen retrieval. The sections were incubated with anti-cdt1 rabbit antibody (CDT1, ×500; GeneTex) for 60 minutes followed by the secondary antibody MACH 2 Double Stain 2 (40 minutes) and the alkaline phosphatase coloring agent PermaRed/AP (Diagnostic Biosystems) (10 minutes). The tissues were incubated for 5 minutes with a denaturing solution (Biocare Medical) to terminate the cdt1 Ag–Ab reaction. Subsequently, they were incubated for 80 minutes with rabbit antibody against γ H2A.X antibodies (P-Histone H2A.X, ×20; Cell Signaling Technology) followed by 40 minutes with the secondary antibody MACH 2 Double Stain 1 (Biocare Medical). Finally, brown-colored staining pattern was obtained using 3-3?-diaminobenzidine4 hydrochloride (Invitrogen, Grand Island, NY). After completion of the staining process, the specimens were sealed with Marinol (Muto Pure Chemicals, Tokyo, Japan).

Statistical Analysis

Positive cells were counted in randomly selected high-power fields (200×). Cell counts were totaled and converted into ratios. The proportion of positive cells for each marker were correlated with clinicopathological parameters (tumor locations, degrees of tumor differentiation, TNM classification, Dukes stages, lymphatic infiltration, venous infiltration, and recurrence) based on the Student *t* tests (2 groups) and Tukey–Kramer tests (multiple groups) using JMP 11 (SAS Institute Inc, Cary, NC). The iCCD results from patients with or without preoperative adjuvant therapy were also compared. Significance was defined as $P < 0.05$.

RESULTS

Patient Characteristics

Of the 141 patients examined in this study, 129 were treatment-naïve (colon cancer, n = 101; rectal cancer, n = 28); the remaining 12 received preoperative adjuvant chemotherapy or chemoradiotherapy (colon cancer, n = 4; rectal cancer, n = 8). Patients' characteristics are presented in Tables 1 and 2. Of the 101 cases of colon cancers without

TABLE 2. Relationship between clinicopathological characteristics and cell cycle kinetics in rectal cancer without preoperative therapy

Variables		n	Geminin average (%)	*P* Value	Cdt1 average (%)	*P* Value	γ H2A.X average (%)	*P* Value
Patients total		28						
Sex	Male	20	29.663	NS	66.952	NS	3.326	NS
	Female	8	29.722		66.597		3.740	
Location	Rs	8	32.592	NS	65.506	NS	1.902	NS
	Ra	11	32.043		64.428		3.529	
	Rb	9	23.016		70.893		6.090	
Histology	G1	5	21.030	NS	68.739	NS	10.231	0.045
	G2	22	31.708		66.008		2.285	
	G3	1	28.311		71.690		0.000	NS
pT category	pT1	2	45.016	0.0052	53.428	NS	1.556	NS
	pT2	8	27.410	0.0084	68.332		4.259	
	pT3	15	11.795		65.324		1.821	
	pT4a	3	8.972		78.066		12.308	
	pT4b	0	N/A		N/A		N/A	
pN category	0	10	27.678	NS	68.741	NS	3.581	NS
	1	12	30.641		64.889		4.469	
	2	6	31.093		66.913		1.995	
cM category	0	24	28.998	NS	67.234	NS	3.768	NS
	1a	3	38.801		58.131		3.069	
	1b	1	18.667		79.556		1.777	
Dukes system	A	4	25.923	NS	68.617	NS	5.460	NS
	B	6	28.857		68.824		2.329	
	C	18	30.792		65.564		3.645	
ly	negative	19	26.511	NS	68.758	NS	4.730	NS
	mild	8	38.580		60.200		1.220	
	extended	1	18.667		79.556		1.778	
v	negative	8	25.303	NS	67.528	NS	7.169	NS
	mild	19	31.221		66.687		2.092	
	extended	1	35.407		60.287		4.306	
Recurrence	(+)	5	29.348	NS	66.858	NS	3.794	NS
	(−)	23	32.560		65.212		2.228	

G1 = well-differentiated adenocarcinoma, G2 = moderately differentiated adenocarcinoma, G3 = poorly differentiated adenocarcinoma, ly = lymphatic infiltration, N/A = not available, NS = not significant, v = venous infiltration.

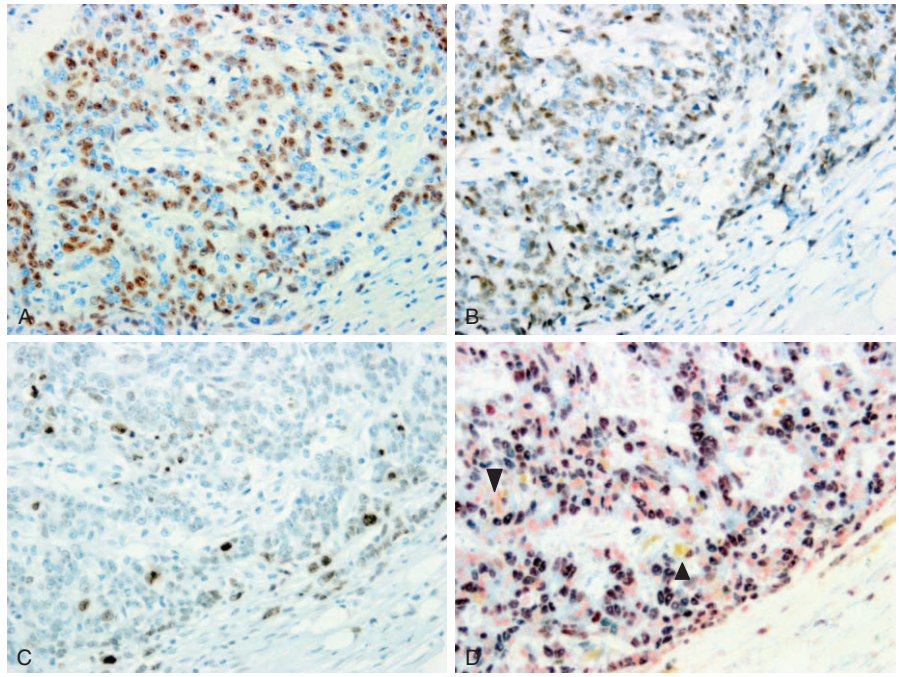


FIGURE 1. Comparison of conventional immunohistochemistry with single antibody and iCCD method. Single immunostaining for geminin (A, ×200), cdt1 (B, ×200), and γ H2A.X (C, ×200) shows randomly distributed positive cells. iCCD (D, ×200) on the same case reveals the cells immunoreactive for geminin (blue), cdt1 (red), and γ H2A.X (brown, arrow head). The immunoreactive cells appear to be similar in number and distribution between the 2 methods. cdt1 = ??, H2A.X = ??, iCCD = immunohistochemistry-based cell cycle detection.

preoperative adjuvant chemotherapy, 5 were classified as pT1 (submucosal layer invasion), 26 as pT2 (muscularis propria invasion), 49 as pT3 (subserosal invasion), 18 as pT4a (involvement of the visceral peritoneum), and 3 as pT4b (invasion of other organs or structures). Rectal cancers without preoperative treatment were classified as pT1 with lymph node metastasis (n = 2), pT2 (n = 8), pT3 (n = 15), and pT4a (n = 3). Of the 12 patients who had received preoperative chemotherapy, 6 patients with rectal cancer received preoperative chemoradiotherapy, whereas the remaining 6 received only chemotherapy.

Correlation Between Single Immunostaining and iCCD

To validate findings of iCCD, its staining pattern was compared with those of single immunostaining. As shown in Figure 1, the tumor cells positive for geminin, cdt1, or γ H2A.X

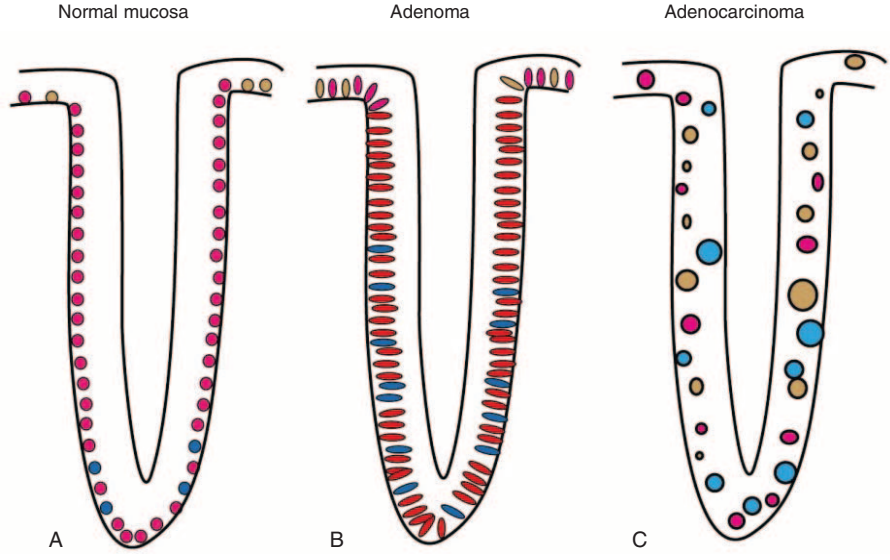


FIGURE 2. Pattern diagram of typical distribution in different tissue type. These figures were views showing a frame format of 3 kinds of cells. Their polarity was more disturbed in adenoma than in normal mucosa, and even worse in adenocarcinoma.

were similar in numbers and distribution between the 2 methods, suggesting that the triple immunostaining could be a useful tool to assess cell cycles on tissue sections.

iCCD Findings in Nonlesional and Neoplastic Epithelia

The iCCD method stains geminin blue, cdt1 red, and γ H2A.X brown. The staining patterns of these nuclear markers clearly differed between healthy and neoplastic tissues (Figure 2). In the nonneoplastic mucosa, iCCD highlighted a well-organized distribution of positive cells. Geminin-positive epithelial cells (S/G2/M phases) were noted mainly at the bottom of crypts (Figure 3A and B), whereas cdt1-positive cells (G1 phase) were distributed widely throughout the crypts. Cells undergoing apoptosis or degeneration (positive for γ H2A.X) were confined to the epithelial surface.

In colorectal tumors, all 3 types of cells were also observed, but without a specific distribution pattern. In adenomas (Figure 3A), the geminin-positive cells were localized to the upper one-third region of the crypts. However, 3 cell-types were almost randomly distributed in carcinomas (Figure 3B and C). Cells positive for each marker appeared to vary in number and distribution among cases. Furthermore, their counts varied with location even within a single tumor. Colorectal neoplasms showed an increase in the proportion of geminin-positive cells and a decrease in percentage of cdt1-positive cells compared with nonneoplastic mucosa (Figure 4). The proportion of cdt1-positive cells in colon cancers was significantly lower than that in colonic adenomas ($P = 0.048$). We did not observe any other significant differences in the cellular proportions between adenomas and carcinomas.

Associations Between iCCD Findings and Clinicopathological Features in Colon Cancer

We correlated the iCCD results with clinicopathological parameters in patients with colon cancers who did not receive preoperative therapy (Table 1). The percentage of geminin-positive cells was significantly higher in patients with extensive venous infiltration compared with those with no or mild venous infiltration ($P = 0.007$ and $P = 0.010$, respectively). Conversely, extensive venous infiltration was associated with decreased percentages of cdt1-positive cells. Approximately, 15% of the patients with colon cancers reported recurrence at 0.5 to 36 months after surgery (median 6 months). The percentage of γ H2A.X-positive cells in such patients was slightly higher compared with those without a relapse (Figure 5).

Associations Between iCCD Findings and Clinicopathological Features in Rectal Cancer

We performed similar association analyses for 28 patients with rectal cancers who did not receive preoperative adjuvant chemotherapy (Table 2). Percentages of geminin-positive cells showed negative correlations with depth of tumor invasion (pT1 vs pT4, $P = 0.0052$; pT2 vs pT4, $P = 0.0084$). In addition, the percentages of γ H2A.X-positive cells in well-differentiated cancers were significantly lower than that in moderately differentiated tumors ($P = 0.045$). There was a weak association

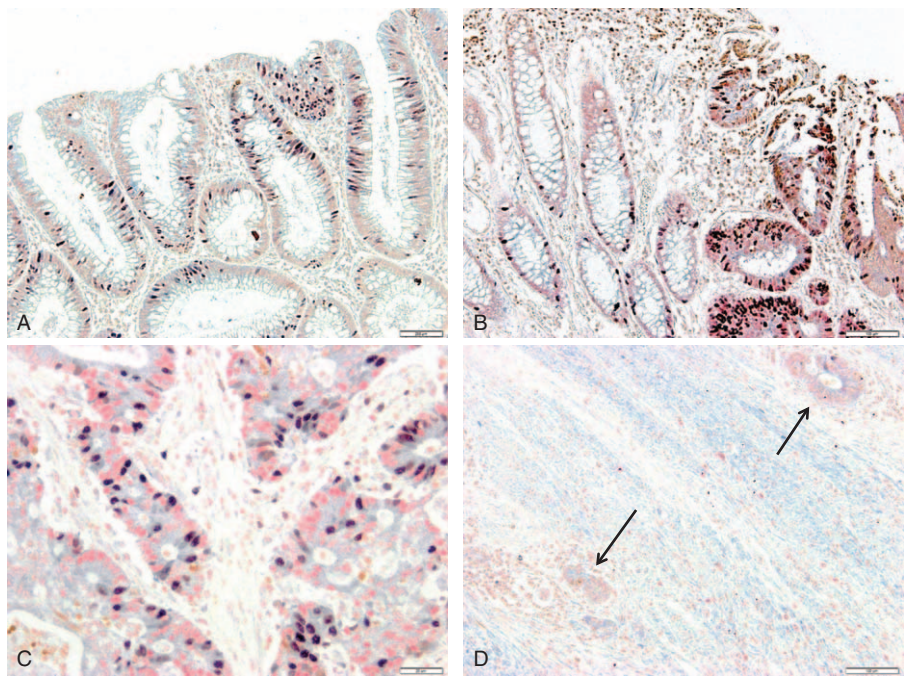


FIGURE 3. Histology and immunostaining of colorectal mucosa. (A) Tumor margin showing healthy (left half) and adenomatous (right half) tissue expressing geminin in the lower and upper areas of the crypts, respectively (triple staining, ×100). (B) Colon cancer (right) was clearly distinguished from healthy epithelium (left), based on the poor organization and the presence of γ H2A.X-positive cells (triple staining, ×100). (C) High-magnification image of adenocarcinoma showing higher numbers of blue-stained and brown-stained nuclei. The atypical glands in the cancer tissue showed a loss of cell arrangement and apoptosis (brown) (triple staining, ×200). (D) Resected specimen stained after preoperative therapy. As the mTRG grade of this case was Grade 2, few abnormal glands remained (arrow). The geminin-positive cells were probably viable (triple staining, ×100). H2A.X = ??, mTRG = modified tumor regression grade.

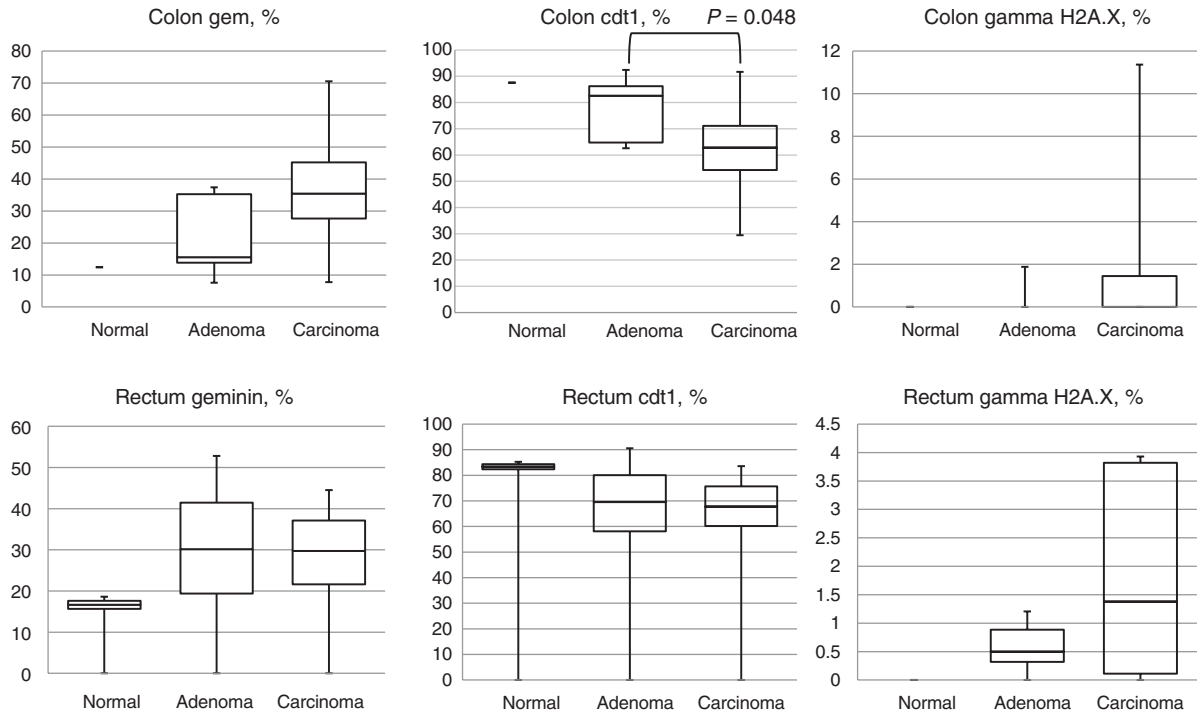
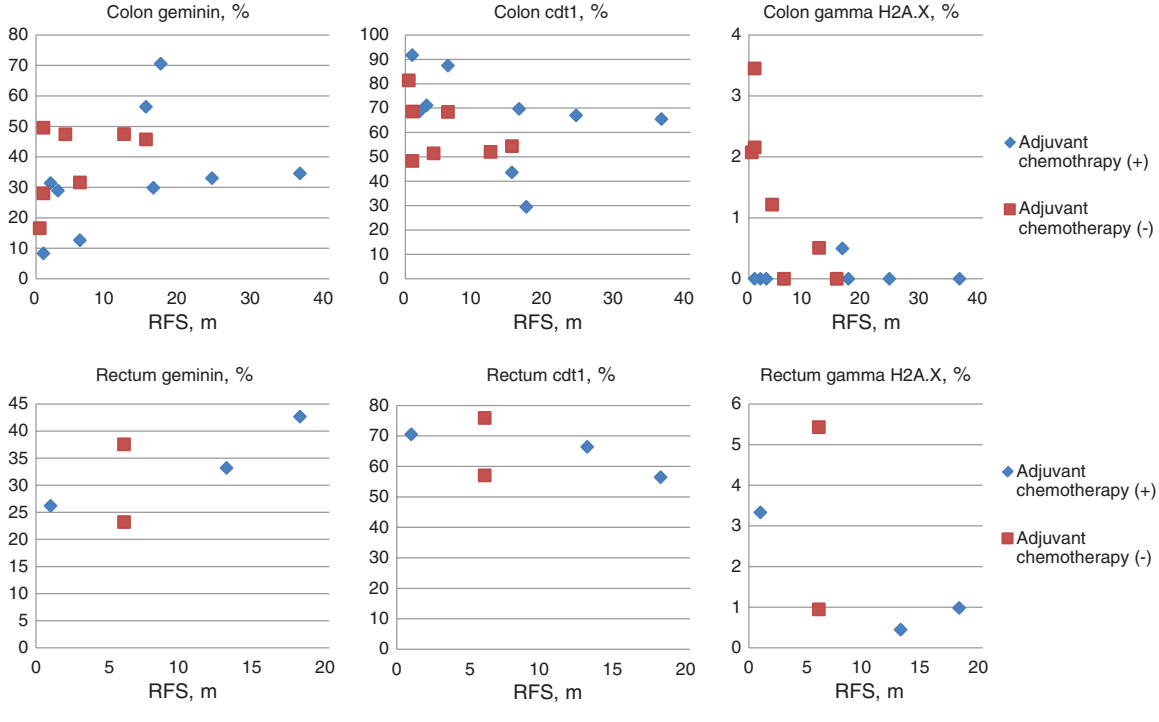


FIGURE 4. Expression of cell cycle markers in different tissue types. Data are expressed as the percentage of cells positive for geminin, cdt1, or γ H2A.X in normal and abnormal tissue specimens from the colon (N = 101) and rectum (N = 28). All 3 markers were more commonly detected in adenoma and carcinoma than in normal tissue, except γ H2A.X in colon tumors. H2A.X = ??, RFS = recurrence-free survival.



RFS; recurrence free survival

FIGURE 5. Relationship between the RFS and cell cycle kinetics. This graph plotted about the RFS and 3 phases of cells, in the patients having a recurrence. No strong correlation was found, but between the rates of γ H2A.X and the term to relapse, there may be negative correlation. H2A.X = ??, RFS = recurrence-free survival.

TABLE 3. Demographics and Tumor Characteristics of Patients Who Received Preoperative Therapy

No.	Age	Sex	Location	Histology	pT	pN	cM	Dukes System	ly	v	Preoperative Therapy	Regimen	mTRG
1	49	Female	A	G2	3	0	1a	B	(+)	(−)	CT	XEROX	4
2	63	Male	Peritoneal dissemination of colon cancer	G2	N/A	N/A	N/A	N/A	(−)	(+)	CT	N/A	4
3	57	Male	S	G1	3	1	1a	C	(−)	(−)	CT	mF6	2
4	63	Female	C	G2	3	0	1a	B	(−)	(−)	CT	mF6, 5-FU/LV	4
5	59	Male	Rb	G2	3	0	0	B	(−)	(−)	CRT	UFT/LV	3
6	76	Male	Rb	G2	3	0	0	B	(−)	(+)	CRT	UFT/LV	3
7	63	Male	Rb	G2	3	0	0	B	(−)	(+)	CRT	UFT/LV	2
8	64	Female	RbP	G2	3	0	0	B	(−)	(+)	CRT	N/A	4
9	63	Male	Local recurrence of rectal cancer	G1	N/A	N/A	N/A	N/A	(−)	(−)	CT	XEROX	4
10	62	Male	RbP	G2	4b	2	1a	C	(−)	(+)	CT	mF6	4
11	67	Male	Rb	G2	3	2	0	C	(−)	(+)	CRT	UFT/LV	3
12	70	Male	Rb	G2	2	2	1a	C	(−)	(−)	CRT	UFT/LV	2

5-FU = fluorouracil, CRT = chemoradiotherapy, CT = chemotherapy, G1 = well-differentiated adenocarcinoma, G2 = moderately differentiated adenocarcinoma, H2A.X = ??, LV = leucovorin, ly = lymphatic infiltration, mF6 = modified FOLFOX6, mTRG = modified tumor regression grade, N/A = not available, UFT = uracil-tegafur, v = venous infiltration, XEROX = capecitabine and oxaliplatin.

between the increasing of the ratio of γ H2A.X-positive cells and shortening of recurrence-free survival (RFS) (Figure 4).

Impact of Preoperative Therapy on Cell Cycle Kinetics in Colorectal Cancer

The preoperative chemotherapy was either pyrimidine fluoride or platinum-based (Table 3). Additional radiotherapy was for patients suspected with a locally advanced tumor (T4) or metastasis in the lateral pelvic lymph node. Irradiation schedule involved 25 sessions of 1.8 Gy each. The patients subsequently underwent radical surgery with lymph node dissections. Representative iCCD findings for colorectal cancer with preoperative therapy are shown in Figure 3D. Preoperative therapy significantly reduced the proportion of geminin-positive cells (colon cancers, $P = 0.006$; rectal cancers, $P = 0.047$). As anticipated, the percentage of γ H2A.X-positive cells increased significantly (colon cancers, $P < 0.0001$; rectal cancers, $P = 0.039$) (Table 4). However, mTRGs did not correlate with the proportion of cells positive for cell cycle markers, with the exception of reduced proportion of cdt1-positive (G1 phase) cells in mTRG4 rectal cancers (mTRG2 vs mTRG4, $P = 0.0098$). No discernible difference was found in the iCCD results between the chemotherapy and chemoradiotherapy groups.

Discussion and Conclusion

Various molecules are involved in the development and progression of neoplasms.[14,15] Researchers have made several

TABLE 4. Impact of Preoperative Therapy of Cell Cycle Kinetics

Variables		n	Geminin Average, %	*P* Value	Cdt1 Average, %	*P* Value	γ H2A.X Average, %	*P* Value
Colon cancer preoperative therapy	None	101	35.6291		63.1276		1.2433	
	CT	4	16.5567	0.006	74.8206	0.0795	8.62268	<0.0001
mTRG	2	1	27.6596	NS	65.9574	NS	6.38298	NS
	3	0	N/A		N/A		N/A	
	4	3	12.8558		77.775		9.36925	
Rectal cancer preoperative therapy	None	28	29.6795	0.0469	66.6986	0.3155	3.62188	0.039
	CT	2	20.8915		66.5665		2.379	
	CRT	6	19.1946		72.6276		5.74902	
mTRG	2	2	9.663	NS	80.2116		10.1254	
	3	4	24.1454		71.3266		4.528	
	4	4	21.9907		64.2388	0.0098	13.7706	0.048

CRT = chemoradiotherapy, CT = chemotherapy, H2A.X = ??, mTRG = modified tumor regression grade, N/A = not available, NS = not significant.

efforts to visualize these key molecular events on resected tissue sections. Two immunohistochemical markers commonly used in routine practice are Ki-67 and p53.[16,17] Increased number of Ki-67-labeling indices represent increased proliferation and can indicate neoplastic transformation under appropriate settings.[18] Diffused nuclear expression of p53 usually suggests a malignant transformation.[17] However, these immunostaining results are often difficult to interpret, because similar patterns are observed even under inflammatory conditions. Recently, multicolor immunostaining has found acceptance in routine analysis. Availability of automated immunostainers with reproducible staining protocols has made it theoretically possible to stain 2 or 3 different molecules in a single section.[19,20,21]

The present study was based on a multicolor immunostaining procedure and highlighted differences in the cell cycle kinetics between healthy and neoplastic colorectal tissues. The iCCD method demonstrated that, in normal mucosa, geminin-positive cells (S/G2/M phase cells) were localized at the bottom of crypts, whereas cdt1-positive cells (G1 phase cells) were localized in the middle and upper portions. In contrast, the geminin-positive cells in adenomatous mucosa were found in the middle and upper regions, suggesting disorganized cell cycle kinetics in these crypts. This finding supports a mode of colorectal tumorigenesis, in which, stem cells with adenomatous polyposis coli mutation are proposed to proliferate in lower crypt and move toward the surface of the mucosa, as they lose their capacity to divide and differentiate.[22] In cancerous tissue, cell cycle kinetics seemed strikingly distorted as indicted by the randomly distributed immunomarker-positive cells. Furthermore, the numbers of cells positive for each marker varied with location, even within a single tumor, indicating their very heterogeneous cell cycle activities.

We correlated the iCCD results with clinicopathological parameters. We found the proportion of geminin-positive cells to be associated with microscopic vascular invasion in colon cancers and negatively correlated with depth of tumor invasion in rectal cancers. Venous infiltration is a well-known predictive factor of metastasis or recurrence in patients with colorectal carcinoma. All the patients showing extensive venous infiltration presented with tumor recurrence within 17 months after surgery, in spite of postoperative chemotherapy. Although we noted several differences among tissues, interpretation of iCCD results was not as easy as we previously expected. One possible reason is the significant variability in iCCD findings among cases.

Preoperative adjuvant chemotherapy is increasingly used for patients with advanced colorectal cancer. Therapeutic effects are currently assessed only based on the area of residual tumors and degree of necrosis. Distinguishing viable cancer cells from those undergoing the apoptotic or necrotic process is also challenging.[11,23,24] The iCCD method may help estimate the effects of treatment in a more objective way, as we observed that preoperative therapy decreased the proportion of geminin-positive cells and increased those of γ H2A.X-positive cells.

We also present a couple of interesting findings, which could not be proven statistically. First, increased proportions of γ H2A.X-positive cells were weakly associated with shorter RFS. Second, some patients with long RFS showed high percentages of geminin-positive cells. These findings appear paradoxical and require validation using larger cohorts.

The iCCD method is superior to conventional single-color immunostaining, because it allows examination of multicell populations at a glance. Unlike multicolor immunofluorescence techniques, which necessitate the use of fluorescence microscopes, iCCD requires only light microscopes. Western blotting also examines quantitative expression of proteins but cannot determine the cellular origin of expressed proteins. A multicolor immunostaining method like iCCD is preferred when examining the location and interaction of several molecules on a single section.

As described above, *CREPT* was recently found to be a novel regulator of the cell cycle. In the original study, the overexpression of *CREPT* was confirmed in various tumors including colorectal cancers, and its expression was negatively correlated with prognosis of patients with gastric cancer. *CREPT* seems to promote the G1 to S phase transition by regulating expressions of cyclins and cyclin-dependent kinases, eventually leading to shortening of the cell cycle in malignant cells. As expected by the *CREPT* study, iCCD nicely demonstrated that the proportion of geminin-positive cells (S, G2, and M phases) is increased, and the percentage of cdt1-positive cells (G1 phase) was decreased in colon cancers.[14]

One limitation of this study is that some groups, particularly the preoperative therapy group, had small numbers of patients. We could not examine more cases, because we found that the iCCD method frequently fails to work on tissues stored for >3 years. A similar problem has been previously reported for another nuclear antigen.[25] Although iCCD is automated, it requires nearly thrice the number of steps as in conventional immunostaining. This limitation makes the procedure longer and can induce tissue damage. A potential solution is to use cocktail antibodies, wherein tissue sections are incubated with multiple antibodies at the same time. However, to make this approach feasible, the antibodies should be derived from different species. Currently, all antibodies used for iCCD are rabbit-derived and polyclonal. The cocktail treatment can be considered when a mouse monoclonal antibody becomes available for at least 1 of the 3 markers.

In conclusion, we examined the cell cycle kinetics of colorectal neoplasms using a fully automated, section-based procedure. Our iCCD findings correlated with some clinicopathological features of colorectal cancer. This novel method might also help us to evaluate the effects of preoperative therapy. We recommended this automated multicolor immunostaining as a reliable tool to assess tissue samples in a clinical oncology setting.

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