Minichromosome maintenance protein 7 in colorectal cancer: Implication of prognostic significance

KEISUKE NISHIHARA[1,2], KOHEI SHOMORI[1], SHINJI FUJIOKA[1], NARUO TOKUYASU[1],
AIKO INABA[1], MITSUHIKO OSAKI[3], TOSHIHIDE OGAWA[2] and HISAO ITO[1]

[1]Division of Organ Pathology, Department of Microbiology and Pathology, and [2]Division of Radiology,
Department of Pathophysiological and Therapeutic Science, Faculty of Medicine, Tottori University;
[3]Division of Molecular Genetics and Biofunction, Department of Biomedical Science,
Tottori University Graduate School of Medical Science, 86 Nishi-cho, Yonago, Tottori 683-8503, Japan

Abstract. Minichromosome maintenance (MCM) proteins are essential components for DNA replication, and also prognostic markers for various human tumors. MCM-positive but Ki67-negative cells (e.g. primary oocytes) are thought to be licensed non-proliferating populations, and are significantly correlated with the clinicopathological profiles of some human tumors. In the present study, we evaluated the expression levels of MCM7, MCM2 and Ki67 in colorectal cancer to clarify their pathobiological significance. We carried out Western blot analyses of 5 human colorectal cancer cell lines and performed immunohistochemistry on 202 surgically removed colorectal cancers of Dukes' B and C stages. Double-labeling immunofluorescence was also carried out on the cancer specimens to identify MCM-positive but Ki67-negative tumor cells. MCM proteins were detected in all the 5 cell lines examined. MCM7 and MCM2 were coexpressed in almost the same populations of tumor cells, whereas MCM7-negative but Ki67-positive tumor cells were absent in the double-labeled specimens, except for mitotic cells. The mean positive tumor labeling indexes (LIs) for MCM7, MCM2 and Ki67 were 58.1, 57.1 and 40.6%, respectively. The mean LI for MCM7-positive but Ki67-negative tumor cells was 17.6%, and significantly correlated with the N status (P=0.01), distant metastasis (P=0.01) and UICC stage (P=0.02). The high LI of >58.1% for MCM7 were independent prognostic factors in multivariate Cox regression analysis (relative risk = 2.12; P=0.02). Our results indicate that MCM7 expression is an independent prognostic factor for human colorectal cancer, and MCM7-positive but Ki67-negative tumor cells are correlated with tumor metastases.

Introduction

Colorectal cancer is one of the most common cancers around the world. In Japan, the incidence of colorectal cancer has doubled over the past two decades, and colorectal cancer has become the third cause of death from neoplastic disease (1), while the current TNM classification system has some limitation, and does not necessarily predict the prognosis of individual patients accurately (2,3). Therefore, it is important to identify new molecular markers of biological and prognostic significance and predictive value in patients with colorectal cancer.

The most crucial point for genomic stability is DNA replication, since this process must ensure that the entire genome is precisely duplicated once per cell cycle. DNA replication licensing involves the stepwise assembly of initiator proteins, origin recognition complex (ORC), Cdt1, Cdc6 and minichromosome maintenance (MCM) protein complex consisting of MCM2-7, on origins of replication (4). The prereplicative complex formed by these licensing factors is required for subsequent initiation of DNA replication. The activated MCM complex appears to play a key role in the DNA unwinding step, acting as a DNA helicase (4). Additionally, DNA helicase activity is found in the trimeric complex of MCM4, -6 and -7, and the trimeric complex of MCM2, -3, and -5 play role of regulator in an *in vitro* study (5).

Individual MCM proteins are known to contribute to the regulation of transcription, chromatin remodeling and checkpoint responses (6-8). MCM proteins have also been reported to be prognostic factors for various human tumors, including prostate and breast cancer and bronchial adenocarcinoma (9-15). In addition, MCMs have been suggested to contribute to carcinogenesis. Specifically, overexpression of MCM7 was reported to actively contribute to tumor formation, progression and malignant conversion in the skin carcinogenesis process in a mouse model (16), while nude mice that received

injections of HEK293 cells transfected with MCM3 developed tumors (17).

Ki67 is a proliferation antigen which is expressed during all phases of the cell cycle except for G0 phase and recognized a cell cycle entry marker (18). Previous studies have shown that MCM proteins are expressed not only within rapidly cycling cells but also in slowly cycling populations, such as primary oocytes and premenopausal breast cells (19,20). These cells exhibit an MCM-positive but Ki67-negative phenotype, characterized as licensed but non-proliferating cells, and significantly increase with tumor grade in renal cell carcinoma, prostate cancer and oligodendroglioma (13-15).

Overexpression of MCM has been reported in colorectal cancer, but its prognostic potential has not been investigated (21-23). We picked the MCM7 out from complex with DNA helicase activity, and MCM2 from regulator complex. Here, we focused on the expression of MCM7 in human colorectal cancer. Furthermore, we assessed the correlations between the licensed but non-proliferating tumor cell population and clinicopathological factors.

Materials and methods

Cell culture and Western blot analysis. The human colon carcinoma cell lines Colo201, Colo320, DLD-1, LoVo and WiDr were routinely cultured in RPMI-1640 supplemented with 10% FBS at 37˚C in a 10% CO_2-containing atmosphere.

Cells were lysed in SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% ß-mercaptoethanol, 0.005% bromophenol blue). The resulting protein samples were separated by SDS-PAGE using 10 or 12% gels and transferred onto nitrocellulose membranes. The membranes were blocked for 2 h in TBS containing 0.01% Tween-20 and 5% skim-milk and then incubated overnight with primary antibodies at 4˚C at the following dilutions: mouse anti-MCM7 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-MCM2 [1:100; clone 2H10; developed in our laboratory, as previously reported (24)]; and mouse anti-ß-actin (1:2000; Sigma, St. Louis, MO). Next, the membranes were incubated for 1 h with HRP-conjugated anti-mouse IgG (1:2000; MBL, Nagoya, Japan). The antibody-antigen complexes were detected using a chemiluminescent detection system (ECL detection system; Amersham Pharmacia Biotech, Amersham, UK).

Surgical specimens. A total of 202 colorectal carcinoma specimens from patients, diagnosed as Dukes' B stage or Dukes' C stage, who underwent radical en bloc resection between January 2000 and December 2005 were selected from the files of Yonago Medical Center Hospital (Yonago, Japan). No patients received preoperative chemotherapy and/or radiation therapy. Among these, 141 carcinomas were in colon and 61 were in rectum. One hundred and one carcinomas were classified as Dukes' B stage and 101 were classified as Dukes' C stage. Overall, 113 patients were male and 89 were female. The median age of the patients was 68.0 years, with a range of 33-94 years. The mean follow-up period was 30.8 months, with a range of 0.1-82.9 months.

After routine processing, the surgical specimens were fixed in 10% buffered formalin and embedded in paraffin
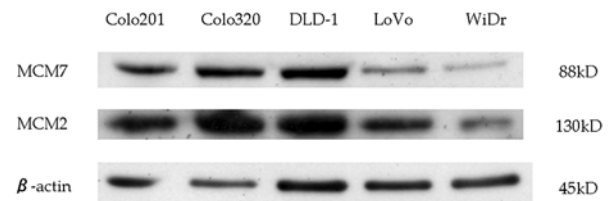

Figure 1. Western blot analysis for MCM7 and MCM2 expressions in the 5 human colorectal cancer cell lines. MCM7 and MCM2 proteins are expressed in all the 5 cell lines with specific bands of 88 and 130 kD, respectively.

wax. All tumors were routinely stained with hematoxylin and eosin. A histological diagnosis was established according to the TNM staging system (3) and the 7th Edition of the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus (25). The study protocols were approved by the respective Institutional Review Boards of Tottori University Faculty of Medicine.

Immunohistochemistry. Immunohistochemistry was performed using a standard streptavidin-biotin-peroxidase complex (SAB) protocol. Sections (4 µm thick) prepared from the formalin-fixed paraffin wax-embedded specimens were deparaffinized and subjected to microwave pretreatment in 10 mM sodium citrate buffer for 20 min at 95˚C for antigen retrieval. After cooling to room temperature, endogenous peroxidase activity was blocked by incubation with 0.6% hydrogen peroxide in methanol for 30 min. Non-specific binding sites were blocked by incubation with 2% FBS for 15 min. Next, the sections were incubated with one of the following antibodies at 4˚C overnight: mouse anti-MCM7 antibody (1:100); mouse anti-MCM2 antibody (1:100); and mouse anti-Ki67 antibody (1:50; DAKO, Glostrup, Denmark). Incubation with biotinylated anti-mouse IgG (Nichirei, Tokyo, Japan) as the secondary antibody was carried out for 30 min, followed by incubation with a streptavidin-biotinylated-HRP complex for 30 min. Peroxidase staining was performed for 10 min using a solution of 3,3'-diamino-benzidine tetrahydrochloride in 50 mM Tris-HCl containing 0.02% hydrogen peroxide. Finally, the sections were counter-stained with Mayer's hematoxylin.

Quantitative analysis was undertaken by two of the authors, who were unaware of the clinicopathological variables. Counts were performed in high-magnification fields from the most intensely stained areas using the FLOVEL Image Filing System FlvFs (FLOVEL Inc., Tachikawa, Japan). Cells were identified as positive if there was any nuclear staining present and any stromal or inflammatory cells in the field were excluded. An average of 730 nuclei was counted for each case. The labeling index (LI) was calculated by dividing the number of positive cells by the total number of cells counted.

Double-labeling immunofluorescence. Paraffin-embedded tissue sections were deparaffinized, immersed in 10 mM sodium citrate buffer, microwaved at 95˚C for 20 min and blocked with 10% FBS for 15 min. Next, the sections were incubated overnight at 4˚C with a mixture of the rabbit anti-
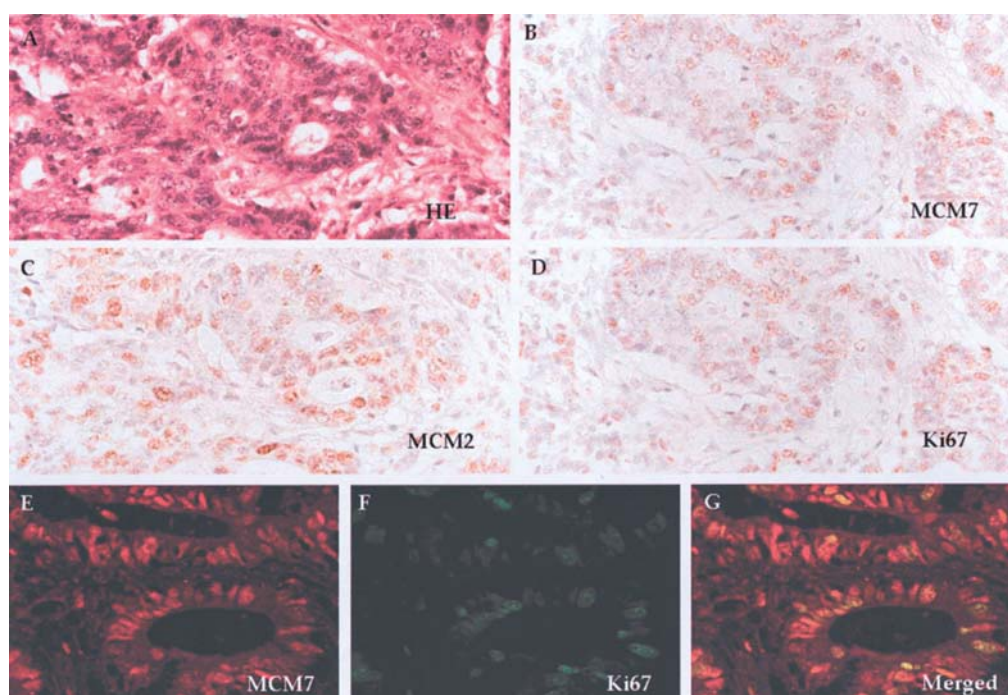
Figure 2. Immunohistochemistry for MCM7, MCM2 and Ki67, and double-labeling immunofluorescence for MCM7 and Ki67 in human colorectal cancer specimens. Serial sections stained with hematoxylin and eosin (A) and immunohistochemically stained for MCM7 (B), MCM2 (C) and Ki67 (D) are shown. Double-labeling immunofluorescence for MCM7 (E) and Ki67 (F) was performed. All tumor cells positive for Ki67 are found to coexpress MCM7 in the merged image (yellow) (G). A large number of cells show MCM7 expression (red) alone, but no cells show Ki67 expression (green) alone.

MCM7 polyclonal antibody (1:50; Santa Cruz Biotechnology) and anti-Ki67 antibody (1:50). The sections were then incubated for 6 h at 4°C with a mixture of Alexa Fluor 546-conjugated goat anti-rabbit IgG (1:200; Molecular Probes Inc., Eugene, OR) and Alexa Fluor 488-conjugated rabbit anti-mouse IgG (1:200; Molecular Probes Inc.), before being washed and mounted. The sections were observed using a fluorescence microscope (Eclipse E800; Nikon, Tokyo, Japan).

Statistical analysis. The correlations between the LIs and clinical variables were analyzed using a bivariate analysis with a paired t-test, Pearson's correlation coefficient test, Student's t-test or the Mann-Whitney U test. One-factor factorial ANOVA or the Kruskal-Wallis rank test were used for analyses of more than two values. Kaplan-Meier cumulative survival curves were constructed for the LIs (26). A log-rank test was used to assess the statistical significance of these curves. Univariate and multivariate Cox regression models for survival were used to evaluate the contributions of various factors (27). Values of $P<0.05$ were considered to indicate statistical significance. The analyses were carried out using Statcel Ver. 2 for Windows (OMS Publishing Inc., Saitama, Japan) and Dr. SPSS II for Windows (SPSS Inc., Tokyo, Japan).

Results

MCM protein expressions in human colorectal cancer cell lines. Initially, we examined the expressions of MCM7 and MCM2 in 5 human colorectal cancer cell lines by Western blotting. As shown in Fig. 1, MCM7 and MCM2 proteins

Table I. The labeling indicies for MCM7, MCM2, Ki67 and MCM7-Ki67.

	Mean ± SD	Median	Range %
MCM7	58.1±9.2	58.3	36.0-85.0
MCM2	57.1±9.0	56.5	36.6-86.4
Ki67	40.6±10.2	40.8	12.3-67.8
MCM7-Ki67[c]	17.6±10.4	17.0	0.2-57.0

[a]$P<0.05$, [b]$P<0.001$; paired t-test. [c]MCM7-positive but Ki67-negative tumor cells.

were detected at various levels in all the 5 colorectal carcinoma cell lines as clear single bands of 88 and 130 kD, respectively.

Double-labeling immunofluorescence and immunohistochemistry. Immunoreactivities for MCM7, MCM2 and Ki67 were noted exclusively in the nucleus of tumor cells and a few lymphocytes (Fig. 2A-D). In non-neoplastic mucosa, positive cells were observed in the basal third of crypts corresponding to the proliferative zone and in the germ center of lymphoid follicles.

Double-labeling confocal immunofluorescence microscopy was conducted to identify MCM-positive but Ki67-negative cells (Fig. 2E-G). All Ki67-positive cells (green) also expressed MCM7 (red), producing a yellow signal

Table II. Correlation between labeling indices and clinicopathological profiles.

		N	MCM7	MCM2	Ki67	MCM7-Ki67[a] %
Age						
<68		100	58.8	56.9	40.1	18.8
≥68		102	57.5	57.2	41.0	16.5
	P[c]		0.15	0.59	0.75	0.16
Sex						
Male		113	58.0	56.9	40.9	17.1
Female		89	58.4	57.3	40.1	18.3
	P[c]		0.76	0.80	0.56	0.75
Tumor location						
Colon		141	57.4	56.2 ⎤	39.9	17.5
Rectum		61	59.9	59.2 ⎦	42.1	17.8
	P[c]		0.08	0.02	0.16	0.85
Dukes' stage						
B		101	59.1	58.1 ⎤	41.6	17.5
C		101	57.2	56.1 ⎦	39.5	17.7
	P[c]		0.08	0.03	0.08	0.71
Histological differentiation						
Well		90	57.3	55.6 ⎤	39.1 ⎤	18.2
Moderately		94	59.6	58.7 ⎦	42.7 ⎥	16.9
Poorly		18	54.9	56.2	36.3 ⎦	18.6
	P[d]		0.06	0.04	0.01	0.28
T stage						
T1+2		12	56.1	55.9	34.8 ⎤	21.3
T3		163	58.5	57.3	41.4 ⎦	17.1
T4		27	57.2	56.6	38.2	19.0
	P[d]		0.77	0.53	0.02	0.22
N status						
N0		101	59.1	58.1	41.6 ⎤	17.5 ⎤⎤
N1		72	56.8	55.4	40.3 ⎥	16.5 ⎥⎥
N2		23	57.6	57.9	39.8 ⎥	17.9 ⎥⎥
N3[b]		6	60.5	56.9	29.7 ⎦	30.9 ⎦⎦
	P[d]		0.41	0.26	0.04	0.01
Distant metastasis						
M0		165	58.2	57.3	41.2 ⎤	16.9 ⎤
M1		37	58.1	56.1	37.5 ⎦	20.7 ⎦
	P[c]		0.50	0.22	0.02	0.01
UICC stage						
II		99	58.9	57.9	41.3	17.7
III		66	57.0	56.4	41.2	15.8 ⎤
IV		37	58.1	56.1	37.5	20.7 ⎦
	P[d]		0.43	0.42	0.12	0.02

Mean percentage positive cells. [a]MCM7-positive but Ki67-negative tumor cells. [b]The N3 presents to metastatize to the lateral area, and/or other areas (superior mesenteric artery areas and inferior mesenteric artery areas). [c]Student's t-test or Mann-Whitney's U-test. [d]One-factor ANOVA or Kruskal-Wallis rank test.

when the images were merged. No cells that were only Ki67-positive (green fluorescent signal alone) were detected, indicating that MCM7 was expressed in all cells expressing Ki67, except for mitotic cells which have no MCM complexes
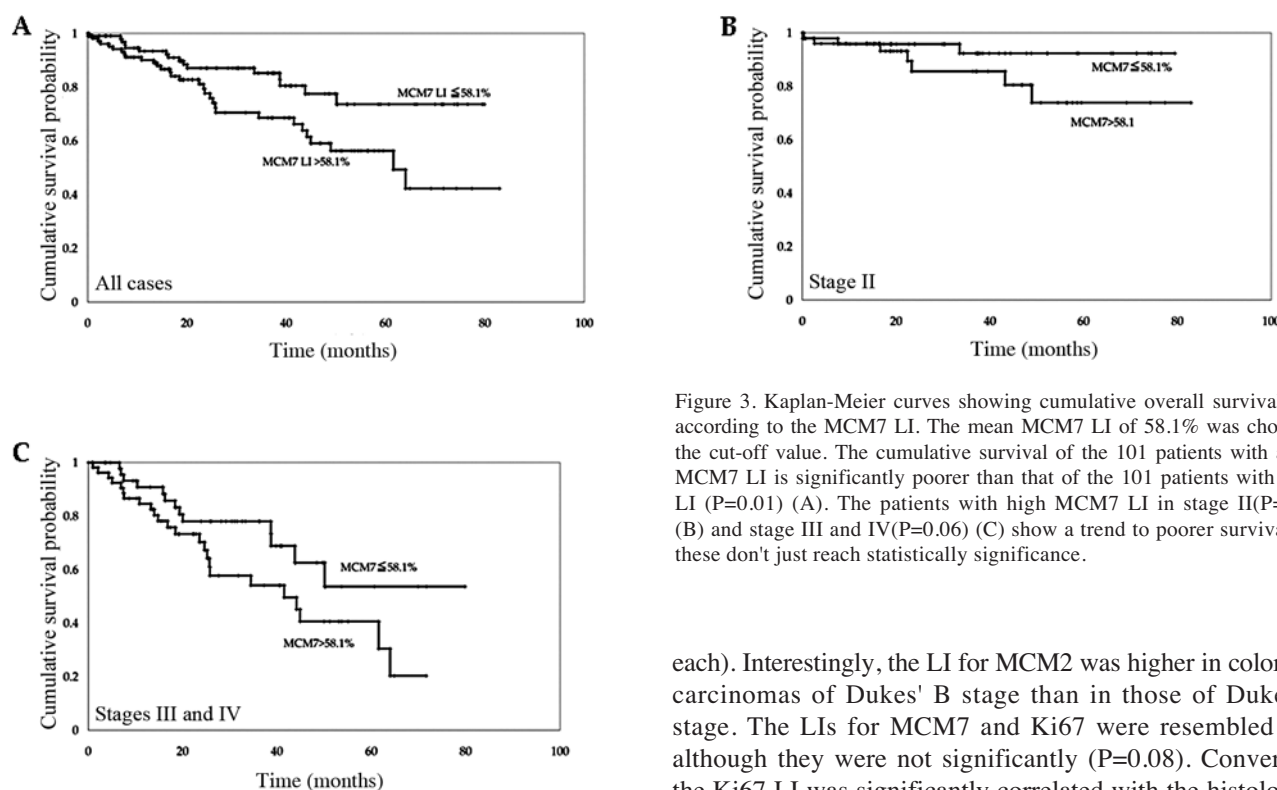
Figure 3. Kaplan-Meier curves showing cumulative overall survival rates according to the MCM7 LI. The mean MCM7 LI of 58.1% was chosen as the cut-off value. The cumulative survival of the 101 patients with a high MCM7 LI is significantly poorer than that of the 101 patients with a low LI (P=0.01) (A). The patients with high MCM7 LI in stage II(P=0.15) (B) and stage III and IV(P=0.06) (C) show a trend to poorer survival, but these don't just reach statistically significance.

binding to their chromatin and are not licensed cells. MCM-positive but Ki67-negative cells were characterized as licensed non-proliferating populations in both tumor cells and normal cells according to a previous report (13).

MCM7, MCM2 and Ki67 were more frequently expressed in the marginal area than in the center of the tumor. Therefore, we mainly evaluated the marginal areas. Table I shows the LIs for MCM7, MCM2 and Ki67 in the 202 colorectal cancer specimens. The mean LIs were 58.1±9.2%, 57.1±9.0% and 40.6±10.2%, respectively. In addition, we analyzed the LI for MCM7-positive but Ki67-negative tumor cells on the same area of serial sections. The mean LI for MCM7-positive but Ki67-negative tumor cells was 17.6±10.4%. Both the MCM7 and MCM2 LIs were significantly higher than the Ki67 LI (P<0.001). The MCM7 LI was significantly higher than the MCM2 LI (P<0.05). There was a significant positive correlation between MCM7 and MCM2 (r=0.58, P<0.001).

Correlations between MCM protein and Ki67 expressions and clinicopathological profiles. We evaluated the correlations between the LIs for MCM7, MCM2, Ki67 and MCM7-positive but Ki67-negative tumor cells and the clinico-pathological profiles (Table II). Although N3 is not provided in the TNM staging system (3), it is defined as lymph nodes metastasized to the lateral area and/or other areas (superior mesenteric and inferior mesenteric artery areas) according to the 7th Edition of the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus (25).

No significant correlations were noted between the MCM7 LI and any of the clinicopathological profiles examined, whereas the MCM2 LI was correlated with tumor location, the Dukes' stage and histlogical differentiation (P<0.05 for

each). Interestingly, the LI for MCM2 was higher in colorectal carcinomas of Dukes' B stage than in those of Dukes' C stage. The LIs for MCM7 and Ki67 were resembled this, although they were not significantly (P=0.08). Conversely, the Ki67 LI was significantly correlated with the histological differentiation, T stage, N status and distant metastasis (P<0.05 for each). Interestingly, the LI for MCM7-positive but Ki67-negative tumor cells was significantly correlated with the N status (P=0.01), distant metastasis (P=0.01) and UICC stage (P=0.03).

Prognosis analysis. We examined the cumulative survival of two groups of patients with high and low LIs using the Kaplan-Meier method and log-rank test. We chose the mean LI as the cut-off value through trial and error. A significantly poorer prognosis was found for the 104 patients with a high MCM7 LI (LI>58.1%) compared to the 98 patients with a low MCM7 LI (P=0.01) (Fig. 3A). No correlation was detected between the MCM2 LI (P=0.67; cut-off value = 57.1%) and the Ki67 LI (P=0.70; cut-off value = 40.6%) and cumulative survival. The high MCM7 LI group stratified by stages showed a trend to poorer survival but this did not reach significance (stage II, P=0.15, Fig. 3B; stage III and IV, P=0.06, Fig. 3C), probably due to the number of cases and follow-up time.

We performed univariate Cox regression analyses to evaluate the contributions of potential prognostic markers to the overall survival. High LIs for MCM7 (cut-off value = 58.1%), MCM2 (cut-off value = 57.1%), Ki67 (cut-off value = 40.6%) and MCM7-positive but Ki67-negative tumor cells (cut-off value = 17.6%) were noted in 99, 104, 106 and 94 patients, respectively. The overall survival was significantly correlated with a high LI for MCM7 (relative risk (RR)=2.11; P=0.02), but not with a high LI for MCM2 (RR=1.13; P=0.67), Ki67 (RR=1.12; P=0.70) or MCM7-positive but Ki67-negative tumor cells (RR=0.88; P=0.65).

Subsequently, a multivariate Cox regression analysis was undertaken to determine whether the markers examined offered prognostic information compared with age, sex, tumor location, histological differentiation, T stage, N status

Table III. Multivariate Cox regression analysis of the contribution of various parameters to the overall survival.

	RR	95% CI	P-value
MCM7 LI			
≤58.1%	1		
>58.1%	2.39	1.25-4.59	0.01
Histological differentiation			
Well	1		
Moderately	1.39	0.72-2.67	0.33
Poorly	4.01	1.62-9.88	<0.01
UICC stage			
II	1		
III	2.36	1.04-5.37	0.04
IV	7.12	3.34-15.19	<0.001
Tumor location			
Colon	1		
Rectum	0.45	0.21-0.94	0.03

LI, labeling index; RR, relative risk; CI, confidence interval.

and UICC stage. Significant and independent prognostic factors detected in this analysis included the UICC stage, showing the highest RR value, followed by histological differentiation, the MCM7 LI (LI>58.1%; RR=2.12, P=0.02), and turmor location (Table III).

Discussion

The present study clearly demonstrated that MCM7, MCM2 and Ki67 were expressed in both non-neoplastic mucosa and colorectal carcinomas, including the 5 human colorectal carcinomas cell lines, and appeared as specific bands on Western blot analysis, indicating the specificities of the antibodies used.

The LIs for MCM7, MCM2 and Ki67 tended to be lower in Dukes' C stage tumors than in Dukes' B stage tumors. A lower LI for Ki67 was reported to be associated with a poorer prognosis for colorectal cancers (28,29). Thus, tumor proliferation may not necessarily be associated with the patient prognosis. The present study also showed that the Ki67 LI was correlated with the current TNM classification, such as the histological differentiation, T stage, N status and distant metastasis, whereas the MCM7 LI was not correlated with any of the clinicopathological profiles. Paradoxically, a high LI for MCM7 was found to be an independent prognostic factor. These results may suggest that MCM7 is a prognostic factor, regardless of the tumor stage or N status, while MCM proteins have been reported to be correlated with the TNM classification or histological grade in other cancers, including prostate cancer, lung adenocarcinoma and renal cell carcinoma (11,13,14). This discrepancy may partly be due to the pathobiological characteristics of colorectal cancer.

Although most colorectal cancer cells showed expression of MCM2 and MCM7, MCM7 was confirmed as an independent prognostic marker, while MCM2 was not. This may be partly explained by biochemical differences between MCM7 and MCM2. The trimeric complex of MCM4, MCM6 and MCM7 was found to possess DNA helicase activity in an *in vitro* study, and this complex also interacts with and is regulated by another trimeric complex comprising MCM2, MCM3 and MCM5 to form a hexameric complex (5). Furthermore, MCM7 is the only family member whose promoter contains an E-box sequence, which is the binding site for members of the MYC transcription factor family, and MCM7 is a direct target of both MYCC and MYCN (30-32). In addition, MCM7 was reported to be expressed at higher levels in cervical intraepithelial neoplasia (CIN) III, corresponding to a higher grade of cervical dysplasia than CIN II, despite a lack of differences in MCM2 and MCM5 (10,21,33). We consider that MCM proteins may play different roles as prognostic factors. We conclude that MCM7 is more effective than MCM2 as a prognostic marker for advanced colorectal cancer.

Higher LIs were noted for MCM2 and/or MCM7 than for Ki67, as a typical cell cycle entry marker. It was reported that all cells expressing Ki67 also expressed MCM2 in laryngeal squamous cell carcinoma as evaluated by double-labeling immunofluorescence observations (34). We also confirmed that no cells expressed Ki67 alone without expressing MCM7 in both cancer cells and normal colorectal mucosa, except for mitotic cells that possess no MCM complexes binding to their chromatin. MCM-positive but Ki67-negative cells have been identified as licensed non-proliferating populations, meaning that they have proliferative potential but are not undergoing the cell cycle. Although increased MCM7-positive but Ki67-negative tumor cells were correlated with metastases, but not correlated with overall survivals in present study. This may be partly explained by small number of cases examined. Almost a half of number was without metastases (stage II), and their LIs were high. This may affect good prognosis and no correlation between MCM7-positive but Ki67-negative tumor cells and overall survival. We should observe more cases with metastasis (stage III and IV) in order to conclude.

Recently, a cancer stem cell theory has been postulated (35,36). Cancer cell subpopulations with tumorigenic potential as cancer stem cells have been prospectively identified from selected types of human solid cancers, including colorectal cancer (37-39). One of the characters of cancer stem cells is slow cycling, which mimics non-neoplastic stem cells. This character resembles the behavior of licensed non-proliferating cell populations. A previous transgenic mouse study revealed that the MCM-positive cell fraction contains a non-neoplastic stem/progenitor population, and concluded that MCM proteins may be markers of stem/progenitor cells (40). In the present study, an increased population of MCM7-positive but Ki67-negative tumor cells was significantly correlated with lymph node and distant metastases. Therefore, we speculate and hypothesize that our licensed non-proliferating population may contain cancer stem cells and may be correlated with tumor metastases. More studies are required for proof of this hypothesis.

In conclusion, a high LI for MCM7 was found to be an independent prognostic factor for colorectal cancer. The population of MCM-positive but Ki67-negative tumor cells, as a licensed non-proliferating population, was significantly correlated with metastasis and may represent a useful biomarker for the existence of metastatic tumors.

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