Minichromosome Maintenance Protein 7 Is a Risk Factor for Recurrence in Patients with Dukes C Colorectal Cancer

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Abstract. *Background: It has been hypothesized that minichromosome maintenance (MCM) proteins, which are replicative control factors, can be used to detect tumor proliferation. The aim of the present study was to investigate the expression of MCM in colorectal cancer tissues and correlate it to clinical outcomes. Patients and Methods: The study included 145 patients with colorectal cancer who underwent curative surgery, from January 2002 until December 2004, at the Kurume University Hospital in Fukuoka, Japan. The median follow-up duration was 87 months. The expression of MCM7 in tissues was studied by immuno-histochemical staining. The labeling index (LI) of MCM7 was calculated by dividing the number of positively-stained cells by the total number of cells counted. We divided samples into two groups: positive (MCM7 LI 76% or higher) and negative (MCM7 LI less than 76%). Results: In patients with Dukes A and B, there were no significant differences in either overall survival (OS) or recurrence-free survival (RFS) between patents with MCM7-positive and those with MCM7-negative disease. On the other hand, in patients with Dukes C, there was significantly worse OS and RFS for patients with MCM7-positive compared to those with MCM7-negative disease. Conclusion: We found that the expression of MCM7 is an independent risk factor for RFS in patients with Dukes C colorectal cancer. Further studies are required to investigate the validity of MCM7 protein expression for its potential clinical use in colorectal cancer therapy and prognosis.*

In Japan, there has been an alarming increase in the incidence of colorectal cancer, which is the third most common cancer and the leading cause of cancer death, with more than 100,000 new cases and 36,000 deaths per year (1).

There is increasing recognition that tumor biology is modulated by cell proliferation, the immune system, and the tumor microenvironment (2). Conventional proliferative indices, such as Ki-67, Proliferating Cell Nuclear Antigen (PCNA) and topoisomerase, have been generally used as markers of tumor cell proliferation. There are many reports that show correlations between these markers and tumor grade, tumor proliferation, and prognosis (3, 4).

The cell cycle consists of four phases – G_1, S, G_2, and mitosis, with checkpoints at each of them. In addition, cell proliferation is controlled by numerous proteins (5). Recently, it has been proposed that minichromosome maintenance (MCM) proteins, which are replicative control factors, are potential markers of tumor proliferation (6).

Genotoxic stress evokes multiple responses, including cell-cycle arrest, enhanced DNA repair, changes in transcription, and apoptosis. The coordination of these responses is achieved through signal transduction pathways that sense DNA lesions and stalled replication forks. MCM association with chromatin, nuclear localization, and activity are all tightly regulated. MCM proteins are also substrates of at least two cell cycle-regulated kinases.

MCM proteins are attractive candidates for regulation by cell-cycle checkpoints. MCM proteins must be retained at stalled replication forks to resume DNA replication. If they dissociate, it is unclear how they could be re-assembled because replication licensing is not allowed once the S phase has begun (7). MCM proteins have been suggested to play a role in plasmid replication and cell-cycle progression (8), and activated MCM proteins appear to play a key role as DNA helicases (9). The MCM 2-7 helicase complex has a role in both the initiation and elongation phases of eukaryotic DNA replication, specifically the formation and elongation of the replication fork. MCM proteins are also tightly-bound to chromatin in late mitosis and G_1 phase, while being removed in S and G_2 phases, while they remain as a soluble nuclear pool during G_2 phase and early mitosis (10).

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Because of the above, we considered that MCM proteins have potential as cell-cycle markers. According to analyses of MCM protein expression using immunohistochemistry, MCM proteins are expressed in all phases of the cell cycle, but they are degraded in cells that have abandoned the cell cycle (11). Furthermore, MCM proteins have been reported to be correlated with the TNM classification or histological grade in other types of cancer, including prostate cancer, lung adenocarcinoma, and renal cell carcinoma (12-14). Many researchers have reported that high expression of MCM is significantly associated with poor prognosis in several tumor types, including glioma, renal cell, prostate, breast, and urothelial cancer (15-19).

Therefore, it is important to investigate the expression of MCM for its potential clinical use in colorectal cancer therapy and prognosis. The aim of the present study was to investigate the expression of MCM in colorectal cancer tissues and relate it to clinical outcomes.

Patients and Methods

Patients and tissue samples. This study included 145 patients with colorectal cancer who underwent curative surgery, from January 2002 until December 2004, at the Kurume University Hospital in Fukuoka, Japan. The median follow-up duration was 87 months (range=3-128 months). Informed consent was obtained from each of the patients before performing surgical resection, and we also received approval from the Institutional Review Committee for Research on Human Subjects at the Kurume University Hospital (Approval number 12338). Tumor differentiation and the degree of invasion were examined by pathologists, and histopathological classification was performed according to the General Rules for Colorectal Cancer Study.(Citation; Japanese Society for Cancer of the Colon and Rectum: Generarl Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus. Kanehara-shuppan, 2009) Patients who had synchronous and multiple types of cancer were excluded. The pathological evaluation was established according to the Japanese Classification of Colorectal Carcinoma and Dukes' classification. (Citation; Japanese Society for Cancer of the Colon and Rectum: Generarl Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus. Kanehara-shuppan, 2009)

Immunohistochemical staining. The expression of MCM7 in tissues was evaluated by immunohistochemical staining. Resected colorectal cancer specimens were fixed in formalin and embedded in paraffin wax. Paraffin-embedded tissue samples were cut at 4-μm and examined on a coated slide glass, and labeled with anti-MCM7 (diluted ×100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) using a Bond-Max autostainer (Leica Microsystems, Newcastle, UK). Immunostaining was performed on the same fully-automated Bond-Max system using onboard heat-induced antigen retrieval with ER2 for 20 min and a Refine polymer detection system (Leica Microsystems, Newcastle, UK). Diaminobenzidine (DAB) was used as the chromogen.

Evaluation of immunohistochemical staining. Cells were counted at high-power magnification (×400). Nuclei from at least 1,000 tumor cells from 10 randomized fields throughout the entire section were counted. The labeling index (LI) of MCM7 was calculated by dividing the number of positively-stained cells by the total number of cells counted (20, 21). MCM7 expression was evaluated using the following criteria because the median LI was 76%: positive (MCM7 LI was higher than 76%) and negative (MCM7 LI was less than 76%)

Table I. *Patients' characteristics*.

Clinicopathological factor		Value
Age, years	Median (range)	67 (range/29-88)
Gender, n	Male	92 (63.4%)
	Female	53 (36.6%)
Tumor location, n	Rectum	71 (49.0%)
	Colon	74 (51.0%)
Histological type, n	Well	93 (64.1%)
	Moderate	42 (29.0%)
	Other	10 (6.9%)
Maximum tumor diameter, cm	Median (range)	50 (12-125)
Depth of invasion, n	T1	9 (6.2%)
	T2	22 (15.2%)
	T3	106 (73.1%)
	T4	8 (5.5)
Lymphatic invasion, n	Positive	73 (50.3%)
	Negative	72 (49.7%)
Venous invasion, n	Positive	112 (77.2%)
	Negative	33 (22.8%)
Budding, n	Positive	91 (62.8%)
	Negative	54 (37.2%)
Perinerual invasion, n	Positive	19 (13.1%)
	Negative	126 (86.9%)
Lymph node metastasis, n	Present	56 (38.6%)
	Absent	89 (61.4%)
Preoperative CEA, mg/dl	Median (range)	4.1 (0.5-129.3)
Dukes' stage, n	A	26 (17.9%)
	B	63 (43.4%)
	C	56 (38.7%)
Recurrence	Present	20 (13.8%)
	Absent	125 (86.2%)

Data analysis. The relationships of MCM7 expression and clinicopathological factors (age, gender, tumor location, histological type, maximum tumor diameter, depth of invasion, lymphatic invasion, venous invasion, budding, perineural invasion, lymph node metastasis, preoperative carcinoembryonic antigen (CEA), Dukes stage, and recurrence) were evaluated. The age and maximum tumor diameter were stratified at the median values, and the preoperative CEA was set at a reference value of 5.0 mg/dl for analysis of Cox proportional hazard models. Overall survival (OS) and recurrence free survival (RFS) were used as the survival time analysis.

Statistical analysis. Statistical analysis was performed using JMP version 10.0.0 (SAS Institute, Cary, NC, USA). Correlations between MCM7 expression and clinicopathological factors were analyzed using Fisher's exact test, the chi-square test, or Student's t-test, depending on the type of data. Kaplan-Meier survival curves were compared using a log-rank test. Univariate and multivariate

Table II. *Correlation between MCM7 expression and clinichopathological profiles.*

	MCM7 expression				
	Negative (n=73)		Positive (n=72)		*p*-Value
Age, years	64.6±1.37		65.9±1.38		0.497
Gender, n					
Male	46	63%	46	64%	0.913
Female	27	37%	26	36%	
Location, n					
Rectum	35	48%	36	50%	0.805
Colon	38	52%	36	50%	
Tumor differentiation, n					
Well	49	67%	44	61%	0.450
Others	24	33%	28	39%	
Maxmum tumor diameter, (mm)	51.7±2.75		53.9±2.78		0.554
Depth of invasion, n					
T1, T2	15	21%	16	22%	0.806
T3,T4	58	79%	56	78%	
Lymphatic invasion, n					
Positive	42	57%	31	43%	0.081
Negative	31	43%	41	57%	
Venous invasion, n					
Positive	57	78%	55	76%	0.808
Negative	16	22%	17	24%	
Budding, n					
Positive	49	67%	42	58%	0.274
Negative	24	33%	30	42%	
Perineural invasion, n					
Positive	12	16%	7	10%	0.231
Negative	61	84%	65	90%	
Lymph node metastasis, n					
Present	28	38%	28	39%	0.948
Absent	45	62%	44	61%	
Preoperative CEA, mg/dl	10.9±2.08		8.2±2.10		0.355
Dukes					
A	11	15%	15	21%	0.605
B34	47%	29	40%		
C28	38%	28	39%		
Recurrence, n					
Present	9	12%	11	15%	0.607
Absent	64	88%	61	85%	

associations between risk factors and RFS were analyzed with Cox proportional hazard models (for Dukes C). In each analysis, *p*-values smaller than 0.05 were considered statistically significant.

Results

Clinicopathological characteristics. The clinical characteristics of patients are summarized in Table I. A total of 145 patients with colorectal cancer underwent curative surgery. The tumors were located in the rectum in 71 and in the colon in the other 74. Out of 145 patients, the Dukes classification was Dukes A in 26 (17.5 percent), Dukes B in 63 (43.4 percent), and Dukes C in 56 patients (38.7 percent). Tumor depth of invasion was identified in 9 patients for T1, in 22 patients for T2, in 106 patients for T3, and in 8 patients for T4. In this group, 20 patients (13.8%) had a recurrence of colorectal cancer.

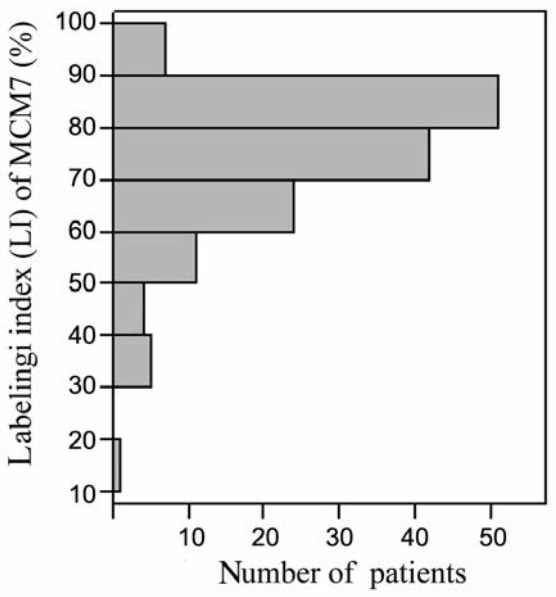

Figure 1. *Labeling index (LI) of minichromosome maintenance protein 7 (MCM7) in colorectal cancer.*

Immunohistochemical staining. Figure 1 shows the labeling indexes (LI) of MCM7 expression for which the median value was 76% (range=18.9-97.4%) in colorectal cancer. Given the LI of MCM7 that was calculated, we divided patients into two groups for MCM7 expression: positive (MCM7 LI of 76% or higher) and negative (MCM7 LI less than 76%) (Figure 2).

In normal colorectal mucosa, MCM7-positive cells were confined to the basal proliferative compartment (Figure 3). This suggests that MCM7 expression reflects cell proliferation. We showed that for MCM7 in colorectal cancer, MCM7 expression was higher in colorectal cancer cells than in normal colorectal mucosa (Figure 2).

Next, we analyzed the expression of MCM7 in relation to clinicopathological findings. There were no statistical differences in clinicopathological factors according to the expression of MCM7 (Table II).

Kaplan–Meier OS and DFS curve analysis The average follow-up time was 87 months (range=3-128 months). We analyzed the relationship between the expression of MCM7 and prognosis using statistical analysis. Although there were no

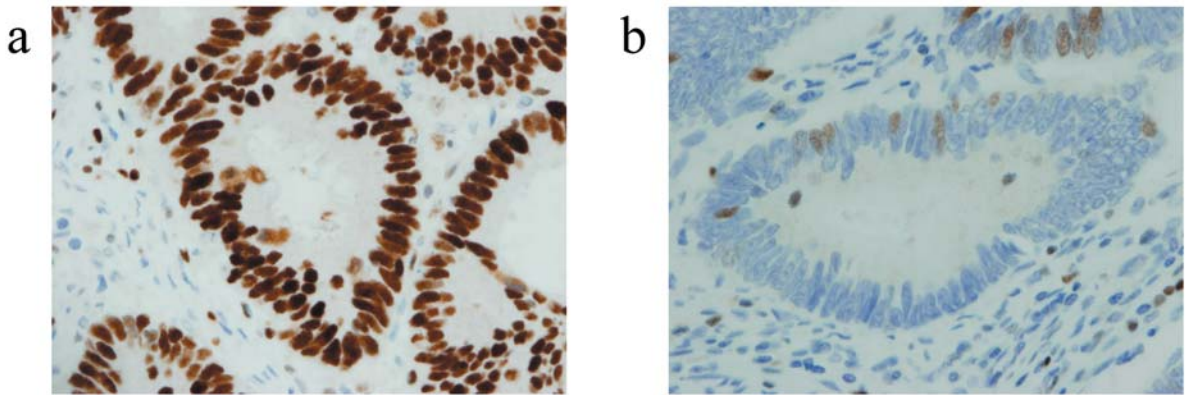

Figure 2. *Representative immunostaining showing positive (a) and negative (b) expression of minichromosome maintenance protein 7 (MCM7) in tumor tissue (×400).*

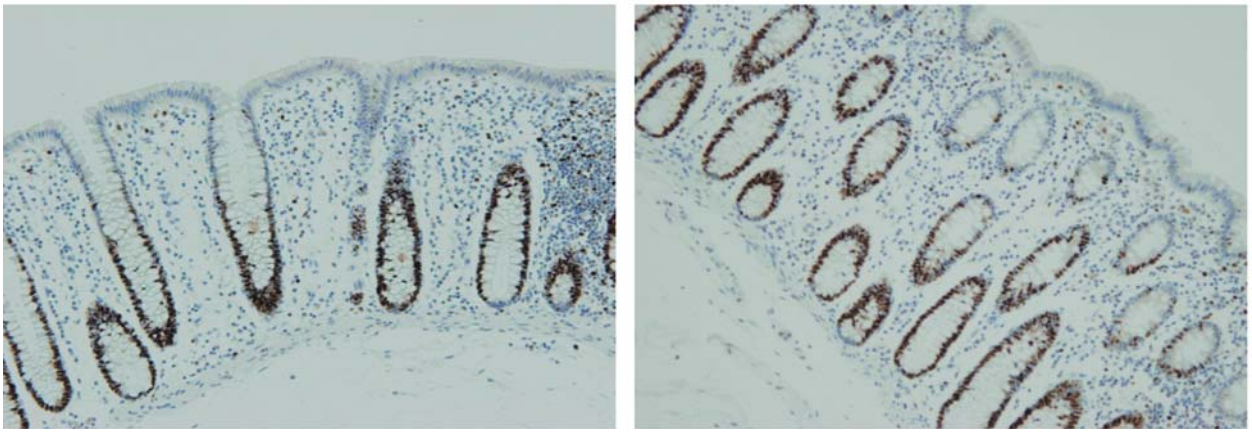

Figure 3. *Immunohistochemical staining in normal colorectal mucosa (×100).*

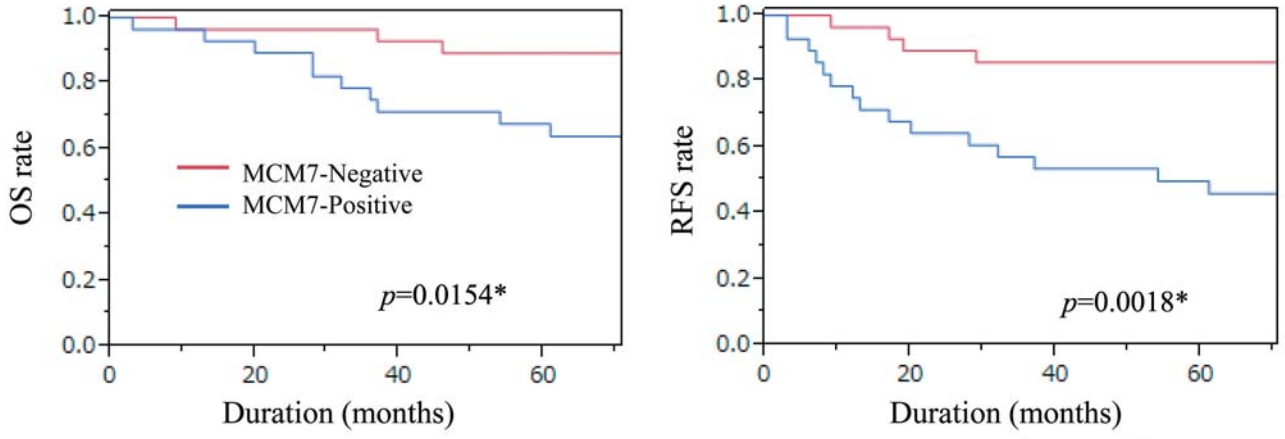

Figure 4. *Kaplan–Meier curves for overall survival (OS) and recurrence free survival (RFS) according to minichromosome maintenance protein 7 (MCM7) expression in patients with Dukes C colorectal cancer.* Significant at p<0.05.*

Table III. *Univariate and multivariate analysis Cox proportional hazard models for RFS in Dukes C patients.*

Variable		Univariate analysis			Multivariate analysis		
		HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Age	≥60 *vs.* <60 years	1.28	0.5-4.0	0.628			
Gender	Male *vs.* Female	1.22	0.5-3.3	0.674			
Tumor location	Rectum *vs.* Colon	2.05	0.8-5.3	0.120	2.24	0.9-5.8	0.082
Tumor differenciation	Well *vs.* Others	0.98	0.4-2.4	0.971			
Maxmum tumor diamater	≥50 *vs.* <50 cm	2.12	0.8-6.0	0.116	1.51	0.6-4.8	0.438
Depth of invasion	T3, T4 *vs.* T1, T2	3.78	1.1-23.8	0.036*	2.05	0.5-14.4	0.362
Lymphatic invasion	Positive *vs.* Negative	1.08	0.4-2.9	0.872			
Venous invasion	Positive *vs.* Negative	0.65	0.2-2.8	0.513			
Budding	Positive *vs.* Negative	1.16	0.4-3.6	0.771			
Perineural invasion	Positive *vs.* negative	1.25	0.4-3.3	0.677			
Preoperative CEA	≥5.0 *vs.* <5.0 mg/dl	1.24	0.5-3.1	0.638			
MCM7	Positive *vs.* Negative	4.87	1.8-17.1	0.002*	4.12	1.5-14.7	0.006*

significant differences overall, patients who had MCM7-positive disease tended to have shorter OS and RFS than negative cases (5-year OS: negative=85.6%, positive=80.1%; p=0.112; 5-year RFS: negative=83.1%, positive=71.6%, p=0.060).

Furthermore, we analyzed prognosis using Dukes' classification. In patients with Dukes A and B, there were no significant differences for both OS and RFS in MCM7-positive and -negative cases (data not shown). On the other hand, in patients with Dukes C, there were significantly worse OS and RFS results for positive cases compared to negative cases (Figure 3).

Table III shows the results of univariate and multivariate COX proportional hazard models of various factors for RFS in Dukes C cases. Multivariate analysis was performed for factors that gave a value of $p<0.20$ in univariate analysis. In the univariate Cox proportional hazard model, we found that depth of tumor invasion and positive expression of MCM7 were significant risk factors for poorer RFS. Furthermore, in the multivariate COX proportional hazard model, we found that positive expression of MCM7 is an independent risk factor for poorer RFS in Dukes C.

Discussion

The present study demonstrated that the expression of MCM7 was related to prognosis, especially that of RFS for patients with Dukes C disease. In addition, we determined that the expression of MCM7, a replicative control factor, is useful for detecting tumor cell proliferation. Therefore, it is likely that measuring the expression of MCM7 has potential clinical use in colorectal cancer therapy and prognosis.

Ki-67 has been used to evaluate the proliferative activity of cancer cells. Although it has been reported that Ki-67 is correlated with clinicopathological factors in several types of cancer such as gastric (22) and breast (23), Ki-67 has not been related to factors in colorectal cancer such as Dukes' stages, lymph node metastasis, histological type, venous invasion, depth of invasion, or lymphatic invasion in (24). Thus, Ki-67 is not thought to be a colorectal cancer marker, and the function of Ki-67 remains unknown (25). For this reason, we considered that MCM proteins may be a marker for cell proliferation because they have helicase activity and other functions in DNA replication (26).

The convergence point for the growth regulatory pathways that control cell proliferation is the initiation of genome replication. The basis of this is the assembly of pre-replicative complexes resulting in chromatin being made available for DNA replication in the subsequent S phase. There are many proteins that regulate this process, including pre-replicative complex proteins origin recognition complex (ORC), CDC6, and MCM in cycling and non-proliferating quiescent, differentiated, and replicative senescent human cells. In brief, in early G_1 phase, the ORC recruits CDC6, which in turn promotes loading of MCM proteins onto chromatin. Activation of CDC7/DBF4 kinase and S phase-promoting cyclin-dependent kinases induces a conformational change in the MCM complex that is required for unwinding DNA, which then recruits CDC45 to the pre-recognition complex. The initiation of DNA replication occurs when replication protein A (RPA) and the DNA polymerase are recruited to the unwound replication origin. Stoeber *et al*. reported that down-regulation of CDC6 and MCM constituents of the replication initiation pathway is a common downstream mechanism for the loss of proliferative capacity in human cells (11).

There have been some reports concerning MCM7 function (27-29). Cortez *et al*. reported that the MCM7 subunit is essential for regulation of the intra-S-phase checkpoint response. They also reported that one possible explanation for

defects in MCM7-depleted cells is that the lack of MCM7 decreases damage signaling. The MCM complex may unwind DNA in advance of replicative polymerases. Reduced MCM function could lead to a reduced number of replication forks, a reduced amount of single-stranded DNA exposed after damage, and therefore, reduced checkpoint signaling (30).

There have been few reports that refer to correlations between colorectal cancer and the expression of MCM proteins (31). Nishihara *et al*. studied the expression of MCM7 in colorectal cancer tissues (20), and found mean positive tumor LIs for MCM7, MCM2, and Ki67 of 58.1%, 57.1%, and 40.6%, respectively. The mean LI for MCM7-positive but Ki67-negative tumor cells was 17.6%, and was significantly correlated with N status, distant metastasis, and UICC stage. The high LI of >58.1% for MCM7 was an independent prognostic factor according to multivariate Cox regression analysis. Therefore, MCM7 expression is an independent prognostic factor for human colorectal cancer, and MCM7-positive Ki67-negative tumor cells are correlated with tumor metastasis.

In the present study, we evaluated the cell proliferative activity of colorectal cancer with MCM7 expression using immunohistochemistry, and we examined the correlation between clinicopathological factors and MCM7 expression. Although there were no differences between clinicopathological factors and MCM7 expression in patients with colorectal cancer overall, patients with Dukes C disease with MCM7-positive expression had a significantly poorer prognosis than those who had MCM7-negative in regards to survival time, including OS and RFS. Being an independent risk factor for poorer RFS in Dukes C colorectal cancer suggests that MCM7 may be a predictor of recurrence for patients with Dukes C colorectal cancer.

Although there were significant differences in OS and RFS by MCM7 expression in patients with Dukes C disease, there were no significant differences in patients with Dukes A and B. This may be explained by the small number of recurrent cases in Dukes A (n=2, 7.7%) and B (n=6, 9.5%).

We conclude that MCM7 is a predictor of recurrence in patients with colorectal cancer who have undergone curative resection and have lymph node metastasis. We speculate that MCM7 may be a good candidate for monitoring prognosis, and perhaps as a pharmacological target, for treating colorectal cancer. Future studies should continue to investigate the validity of MCM7 protein expression for its potential clinical use in colorectal cancer therapy and prognosis.

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