Expression of DNA double-strand break repair proteins predicts the response and prognosis of colorectal cancer patients undergoing oxaliplatin-based chemotherapy

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Abstract. DNA intrastrand cross-linking agents such as oxaliplatin induce DNA double-strand breaks (DSBs) during DNA repair and replication. In the present study, we hypothesized that DNA intrastrand cross-linking agents may significantly benefit colorectal cancer patients with deficiencies in DSB repair. Seventy-eight patients with metastatic or recurrent colorectal cancer who had measurable target lesions and who underwent resection for primary colorectal cancer in our institution between April 2007 and March 2013 were included in the present study. The median age was 64.5 years, and the cohort consisted of 49 males and 29 females. The median progression-free survival (PFS) was 10.9 months. The expression of DSB repair proteins such as RAD51 and MRE11 was investigated by immunohistochemistry, and associations between RAD51 and MRE11 expression and clinicopathological factors or chemotherapeutic effect were assessed. MRE11-negative cases and RAD51-negative cases achieved significantly better tumor reduction compared with cases with positive expression. Cases with negative expression of both proteins or negative expression of either protein had significantly longer PFS than cases with positive expression for both proteins. In conclusion, DSB repair protein expression-negative colorectal cancer cases may be more highly sensitive to chemotherapy, and thus DSB repair protein expression may be a useful prognostic indicator for colorectal cancer patients.

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Introduction

Remarkable progress has been made in chemotherapy for colorectal cancer during the last decade. Currently, standard first-line treatments for unresectable advanced or recurrent colorectal cancer include fluorouracil with irinotecan or oxaliplatin, alone or in combination with molecular-targeted agents, such as the monoclonal antibody against vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) (1,2). Combinations of drug therapies in unresectable advanced or recurrent colorectal cancer patients has prolonged the survival time to more than 30 months (3-5), but the therapeutic effects vary depending on each case. Thus, it is important to predict the chemotherapeutic effect and select patients who will benefit from cancer chemotherapy. Several studies have shown that various biomarkers predict the sensitivity to chemotherapy or chemoradiation therapy. The presence of microsatellite instability (MSI) and mutations of the *KRAS* gene are reliable biomarkers for sensitivity to fluorouracil and anti-EGFR monoclonal antibodies, respectively (5,6). However, there is no reliable biomarker for oxaliplatin and irinotecan.

DNA intrastrand cross-linking agents such as oxaliplatin induce DNA double-strand breaks (DSBs) during the process of DNA replication and repair (7). BRCA1, 2, the MRE11-RAD50-NBS1 (MRN) complex and RAD51 play an important role in homologous recombination during DSB repair. A previous study showed that DNA damage repair competence varies among individual breast tumors, and is closely correlated with chemosensitivity (8). The Fanconi anemia-*BRCA* pathway plays an important role in restoring cytotoxic damage by anticancer agents and radiation (9,10). Furthermore, previous studies have shown that BRCA-associated cancer is particularly sensitive to DNA interstrand cross-linking agents such as mitomycin C or platinum-based drugs (11,12). Differences in the expression of DNA DSB repair proteins (DDRPs) among individual colon cancer cases may also be related to the sensitivity to treatment, as well as breast cancer.

MRE11 forms the core of the MRN complex, which has essential roles in detection, signaling, protection and repair of

DSBs (13,14). RAD51 is an important factor in homologous recombination as well as MRE11, and is a predictive factor for chemoradiotherapy response in a variety of human cancers. Moreover, overexpression of RAD51 confers resistance to DNA interstrand cross-linking agents such as cisplatin in non-small cell lung cancer (15-17).

In the present study, we hypothesized that DNA intrastrand cross-linking agents may significantly benefit colorectal cancer patients with deficiencies in DSB repair. We investigated the expression of MRE11 and RAD51 by immunohistochemistry. Associations between expression and therapeutic effect in colorectal cancer patients were also explored.

Materials and methods

Patients. Seventy-eight patients with metastatic or recurrent colorectal cancer who had measurable target lesions such as hepatic, pulmonary, lymphatic and peritoneal metastases, underwent resection for primary colon and rectal cancer at our institution between April 2007 and March 2013. All patients underwent combination chemotherapy including oxaliplatin.

Assessments of therapeutic effect. Descriptions of the therapeutic effects were evaluated using the best overall response to first-line chemotherapy using RECIST version 1.1. Changes in tumor size were expressed as the relative change in the sum of the longest diameters of the target lesions. Non-target lesions and newly occurring lesions were not considered in the measurement of tumor size changes (18). The endpoints of the long-term outcome study were progression-free survival (PFS). PFS was calculated by progression of target lesions as the only events for survival analyses.

Immunohistochemistry. Five-micrometer sections were deparaffinized with xylene and rehydrated with alcohol, and placed in 0.1 M NaOH citrase buffer (pH 7.0) for RAD51 immunostaining or 0.01 M NaOH citrate buffer (pH 6.0) for MRE11 immunostaining, and heated in an autoclave at 121˚C for 15 min. Sections were then preincubated with 3% H_2O_2 in methanol for 30 min at room temperature to quench endogenous peroxidase activity. After blocking with normal goat serum, the sections were incubated with mouse anti-RAD51 3C10 monoclonal antibody (1:800; clone 51RAD01; Thermo Scientific, Fremont, CA, USA) and mouse anti-MRE11 12D7 monoclonal antibody (1:1600; ab214; Abcam, Cambridge, UK) for 60 min at 4˚C. Thereafter, the sections were incubated with a secondary antibody (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA) for 30 min, washed with phosphate-buffered saline (PBS) and treated with peroxidase-conjugated streptavidin for 30 min. The sections were visualized by incubation with diaminobenzidine tetrahydrochloride (Liquid DAB+ Substrate Chromogen System; Dako, Carpinteria, CA, USA) and counterstained with hematoxylin.

Evaluation of immunohistochemical staining. Immunohistochemistry (IHC) scores for <10% of nuclear staining in cancer cells were negative, whereas those cases with IHC scores for >10% stained cells were deemed positive.

Table I. Clinicopathological characteristics of the patients (n=78).

Factors	Date
Median age (years)	64.5
Gender, n	
Men	49
Female	29
Location, n	
Proximal	20
Distal	58
Median tumor size (mm)	50
Histology, n	
Well differentiated	25
Moderately differentiated	44
Poorly differentiated	4
Others	5
Site of metastases, n	
Liver	44
Lung	21
Peritoneum	11
Lymph node	8
Bone	1
Regimen, n	
mFOLFOX6 + bevacizumab	50
CapeOX + bevacizumab	20
mFOLFOX6	7
CapeOX	1
Treatment cycles, mean number	10
Median overall survival (months)	32.5
Median progression-free survival (months)	10.9

Statistical analysis. Categorical analysis of variables was performed using either the Chi-square or Fisher's exact test, as appropriate. Continuous data were compared with the Mann-Whitney U test. Survival curves were plotted according to the Kaplan-Meier method, and any differences were analyzed using the log-rank test. A multivariate analysis with Cox proportional hazards model was adopted to clarify the independent prognostic factors. Differences were considered to be significant if the P-value was <0.05. All statistical analyses were carried out using the R software (version 3.1.1).

Results

Patient clinicopathological characteristics. Patient clinical characteristics are detailed in Table I. The median age was 64.5 years, and the cohort consisted of 49 males and 29 females. Most patients received combination chemotherapy in addition to bevacizumab (n=70, 90%). Fifty patients received mFOLFOX6 + bevacizumab and 20 patients received CapeOX + bevacizumab. The median PFS was 10.9 months.

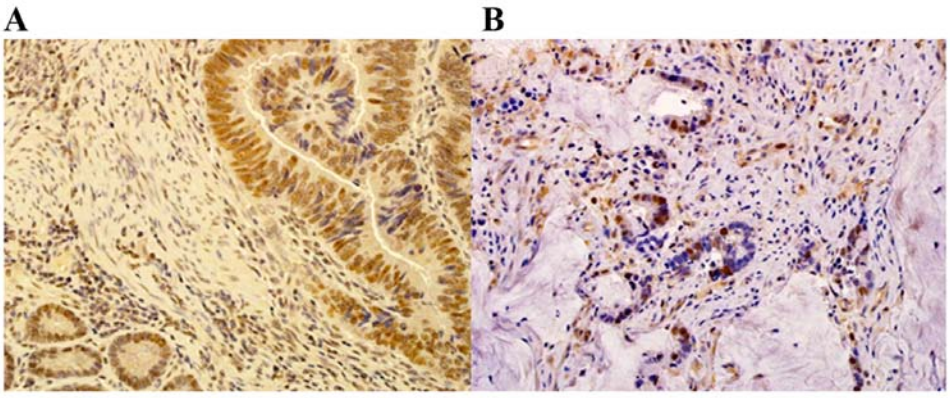

Figure 1. MRE11 and RAD51 expression in colorectal cancer tissues as assessed by immunohistochemistry. (A) MRE11 was stained in the nucleus in the cancer cells (magnification, x400). (B) RAD51 was stained in the nucleus in the cancer cells (magnification, x400).

Table II. Relationship between DDRP expression and clinicopathological characteristics of the patients (n=90).

	MRE11			RAD51		
Factors	Negative (n=30)	Positive (n=48)	P-value	Negative (n=38)	Positive (n=40)	P-value
Median age (years)	64	65.5	0.2	64	67	0.3
Gender						
Male	15	34	0.064	26	23	0.3
Female	15	14		12	17	
Location						
Proximal	10	10	0.2	9	11	0.7
Distal	20	38		29	29	
Median tumor size (mm)	55	50	0.4	55	50	0.2
Median treatment cycles	11.5	9.5	0.2	11.5	8	0.1
Histological type						
Differentiated	29	40	0.080	35	34	0.3
Undifferentiated	1	8		3	6	
Median serum CEA level (ng/ml)						
Before treatment	6.6	21.05	0.045	7.25	22.35	0.07
3 months later	5.25	10.1	0.047	4.45	12.65	0.003
CEA reduction ratio	0.70	0.61	0.4	0.50	0.76	0.033
Relative change	0.65	0.92	0.029	0.48	1.01	<0.001

DDRP, DNA double-strand break repair protein; CEA, carcinoembryonic antigen.

MRE11 expression and clinical outcome. Positive nuclear staining of MRE11 was observed in 48 (61.5%) of the 78 cases (Fig. 1A). The association between MRE11 expression and clinicopathological characteristics is shown in Table II. There was no significant association between MRE11 expression and age, tumor location or tumor size. Male gender and undifferentiated type tended to be associated with MRE11 positivity. There was no significant association between MRE11 expression and CEA reduction ratio. MRE11-negative cases had significantly better relative change compared with MRE1-positive cases. Thus, MRE11-negative cases achieved better size reduction of the target lesion when compared with MRE11-positive cases. The association between MRE11 expression and prognosis is shown in Fig. 2A. MRE11-positive cases exhibited poorer PFS when compared with MRE11-negative cases, but no significant association was identified between MRE11 expression and PFS.

RAD51 expression and clinical outcome. Positive nuclear staining of RAD51 was observed in 40 (51.2%) of the 78 cases (Fig. 1B). There was no significant association between RAD51 expression and age, gender, tumor location, tumor

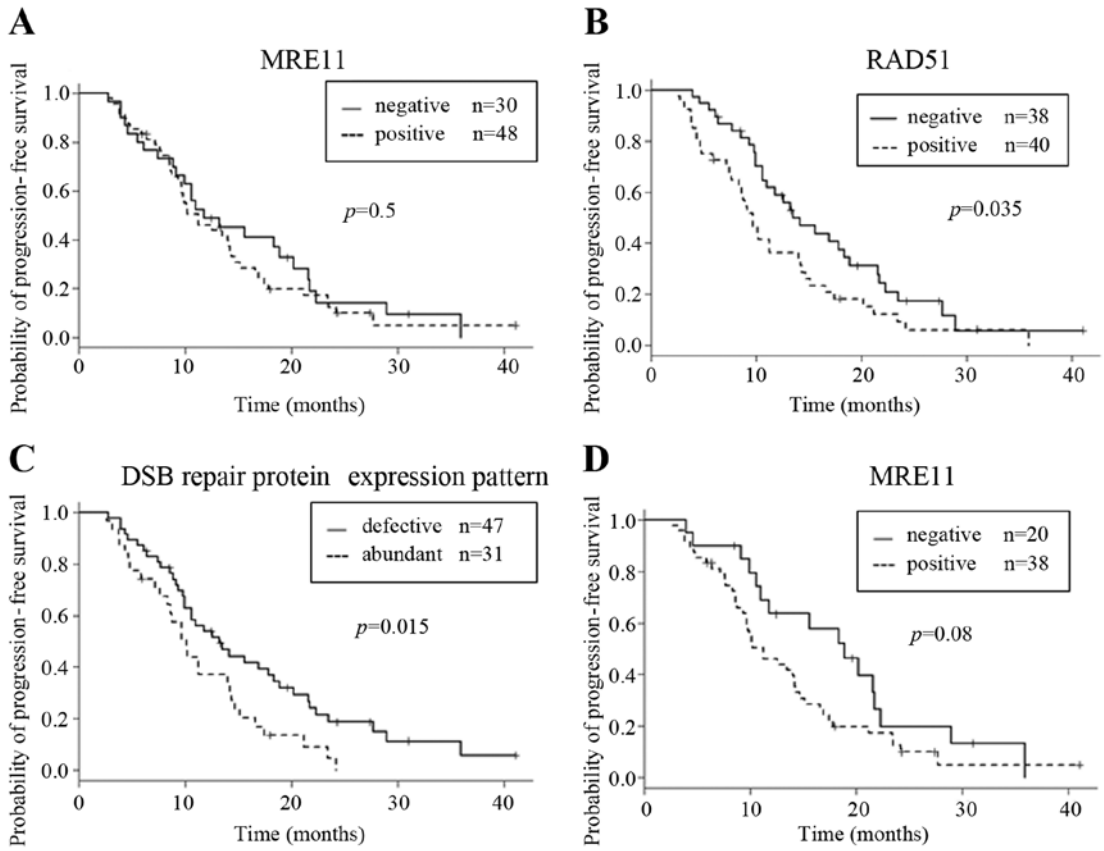

Figure 2. Kaplan-Meier progression-free survival curves of colon cancer patients. (A) MRE11 expression status in all cases (P=0.5). (B) RAD51 expression status in all cases (P=0.035). (C) DSB repair protein expression pattern in all cases (P=0.015). (D) MRE11 expression status in distal colon (P=0.077).

size or histological type (Table II). RAD51-negative cases had significantly better CEA reduction ratios and relative change. RAD51-positive cases had significantly poorer PFS when compared with RAD51-negative cases (Fig. 2B). However, for multivariate analysis, RAD51 was not an independent prognostic factor (Table III).

DSB repair protein expression pattern. In the DSB repair pathway, the role of MRE11 and RAD51 are sequential. When one of either is defect, it may be impossible to recovery from DSBs. Therefore, we defined two groups. The 'defective pattern' is described as the state when expression of both MRE11 and RAD51 expression is negative or expression of either one of these protein is negative. The 'abundant pattern' is described as the state when expression of both proteins is positive. In addition, we investigated the association between the two groups and therapeutic effect. The association between expression patterns and clinical characteristics or chemotherapeutic effect is shown in Table IV. None of the examined clinicopathological characteristics correlated with the expression pattern. For the therapeutic effects, there was no significant difference between expression pattern and CEA reduction ratio. The defective pattern had significantly better relative change compared with the abundant pattern. As shown in Fig. 2C, the median PFS for the defective pattern and abundant pattern was 13.2 and 10.1 months, respectively, and there was a significant difference between the defective pattern and abundant pattern for PFS (Fig. 2C). Nonetheless, for the multivariate analysis, the expression pattern or RAD51 expression alone were not independent prognostic factors (Table III).

Distal colon cancer patients benefit more from these ex vivo tests. MRE11 mutations occur in 83.7% of MMR-defective primary colorectal cancers. MSI is displayed in ~15% of colorectal cancer cases. We reviewed all subjects in the present study with the exception of one case of tumor localization to a site proximal to the splenic flexure due to the association of high-frequency MSI with this tumor site. In these cases, MRE11-negative cases exhibited longer PFS than the positive cases (P=0.077) (Fig. 2D). Moreover, by multivariate analysis, DSB repair protein expression pattern was an independent prognostic factor (P=0.036) (Table V).

Discussion

The recent development of chemotherapies such as FOLFOX and FOLFIRI along with several molecular-targeting agents has markedly improved the survival of unresectable colorectal cancer patients. Previous studies have shown that the median survival time was prolonged to 11-26 months in unresectable advanced or recurrent colorectal cancer (19,20). In the present study, we calculated the curative effect of chemotherapy by determining the correlations between DSB repair protein expression pattern and chemotherapeutic effect; those patients exhibiting better relative changes in

Table III. Univariate and multivariate analyses for progression-free survival.

	Univariate		Multivariate	
Factor	Median PFS	P-value	HR (95% CI)	P-value
Age (years)				
>60	11.8	0.8		
≤60	10.6			
Gender				
Male	13.2	0.070		
Female	10			
Location				
Proximal	9.3	0.002	0.56 (0.31-1.00)	0.051
Distal	13.5			
Tumor size (mm)				
<50	11.3	0.5		
≥50	11.3			
Histology				
Undifferentiated	11.8	0.1		
Differentiated	9.7			
Treatment cycles				
<10	6.4	0.005	0.49 (0.30-0.80)	0.005
≥10	14.2			
CEA reduction ratio				
<0.6	11.3	0.4		
≥0.6	11.3			
Relative change				
<0.7	18.4	<0.001	2.53 (1.39-4.62)	0.002
≥0.7	9,0			
MRE11				
Negative	11.8	0.5		
Positive	11.3			
RAD51				
Negative	13.5	0.035	0.80 (0.35-1.83)	0.6
Positive	9.7			
DSB repair protein expression pattern				
Defective	13.2	0.015	1.39 (0.58-3.34)	0.5
Abundant	10.1			

HR, hazard ratio; PFS, progression-free survival; CI, confidence interval; DSB, double-strand break.

tumor size had longer survival times. According to a previous study, the chemotherapeutic effect by first-line treatment may be a prognostic factor. Therefore, it is necessary to detect biomarkers that predict the effect of first-line treatment to obtain further chemotherapeutic effects.

Oxaliplatin is a DNA intrastrand cross-linking agent and is frequently used to treat colorectal cancer that has spread.

Table IV. Relationship between the DSB protein expression pattern and clinicopathological characteristics.

Factors	Defective (n=47)	Abundant (n=31)	P-value
Median age (years)	64	68	0.3
Gender, n			
Male	30	19	0.8
Female	17	12	
Location, n			
Right	13	7	0.8
Left	34	24	
Median tumor size (mm)	55	50	0.6
Median treatment cycles	11	8	0.1
Histological type, n			
Differentiated	44	25	0.08[a]
Undifferentiated	3	6	
Median serum CEA level (ng/ml)			
Before treatment	7	24	0.009
3 months later	4.7	12.8	0.001
CEA reduction ratio	0.55	0.68	0.1
Relative change	0.5	1.02	<0.001

DSB, DNA double-strand break.

Table V. Univariate and multivariate analyses for PFS in the distal colon.

	Univariate		Multivariate	
Factor	Median	P-value	HR (95% CI)	P-value
Treatment cycles				
<10	8.8	0.009	0.46 (0.27-0.78)	0.004
≥10	14.5			
CEA reduction ratio				
<0.6	13.5	0.2		
≥0.6	12.6			
Relative change				
<0.7	18.9	<0.001	2.23 (1.17-4.24)	0.014
≥0.7	9.7			
MRE11				
Negative	18.9	0.077		
Positive	11.3			
RAD51				
Negative	15.6	0.036	0.44 (0.15-1.37)	0.2
Positive	10.1			
DSB repair protein expression pattern				
Defective	16.9	0.001	3.62 (1.09-11.99)	0.036
Abundant	10.1			

DSB, double-strand break.

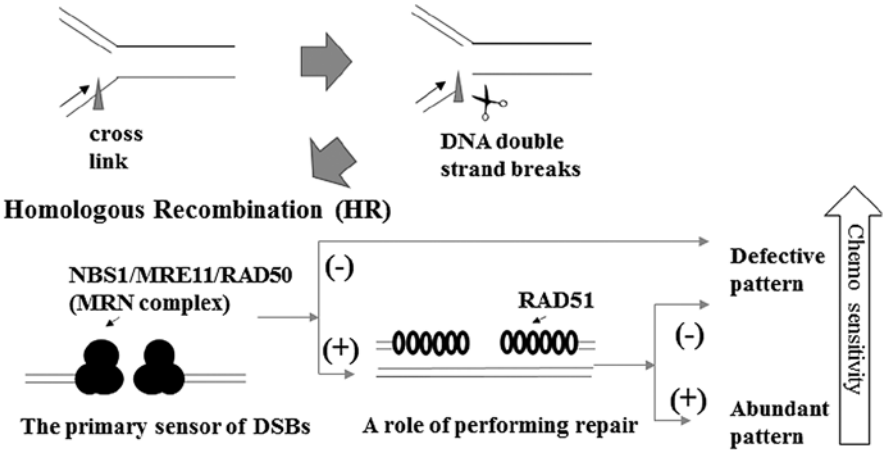

Figure 3. Schema of DNA double-strand breaks (DSBs) by cross-linking agents, the role of DSB repair proteins for homologous recombination and definition of DSB repair protein expression pattern. In the DSB repair pathway, the role of MRE11 and RAD51 are sequential. When expression of either is defective, it may be impossible to repair DNA DSBs. Therefore, we defined two groups. The 'defective pattern' is a state when levels of expression of MRE11 and RAD51 are negative or a state when expression of one of these proteins is negative. The 'abundant pattern' is the state when expression levels of both proteins are positive. We hypothesized that a defective pattern possesses higher sensitivity to chemotherapy than the abundant pattern.

The cytotoxic reaction of oxaliplatin is dependent on DSBs. In DSBs defective cases, the chemotherapeutic effect of oxaliplatin may be greater. MRE11 and RAD51 are important components of homologous recombination, which functions in the repair of DSB. In the present study, we examined the correlation between the expression of these proteins and the chemotherapeutic effect in colorectal cancer patients. RAD51 protein forms a helical nucleoprotein filament to promote DNA strand exchange and stimulate DNA-pairing activity, the basic steps of homologous recombination (21,22). In the present study, patients negative for MRE11 or RAD51 expression obtained better size reduction of target lesions. We showed that RAD51-negative cases achieved longer survival times than positive cases. Several previous studies demonstrated that RAD51 expression is correlated with resistance to chemotherapy and survival in various types of cancer such as lung, breast and esophageal cancer (23-25).

MRE11 is the core component of the MRN complex, the primary sensor of DSBs (12,13). In the present study, MRE11 expression was not an independent prognostic factor. The combined evaluation of MRE11, which acts as a sensor, and RAD51, which functions in repair, may lead to a better indication of the chemotherapeutic effect (Fig. 3). In fact, the relative change and PFS were significantly different between the defective pattern and abundant pattern, the expression pattern of DSB repair proteins.

We investigated the reason for the difference between MRE11 and RAD51 despite it also being a DSB repair protein, and postulated that some factors that may affect MRE11 alone intervened in the result. MRE11 mutations occur in 83.7% of MMR-defective primary colorectal cancers (26,27). Microsatellite instability (MSI) is displayed by ~15% of colorectal cancer cases, and high levels of MSI may be a predictive marker for lack of efficacy of fluorouracil-based therapy (28,29). This raises the possibility that some MRE11-negative patients have poor prognosis as a consequence of their MSI status. We reviewed the subjects of our study with the exception of one case of tumor localization to a site proximal to the splenic flexure due to the association of high-frequency MSI with this tumor site (6). In this instance, MRE11 expression tended to be related to PFS, and DSB repair protein expression pattern is a factor that independently predicts PFS. In cases of tumor development in the distal colon, DSB repair protein expression may predict prognosis.

In the present study, we only examined cases with a targeted lesion to clarify associations with chemotherapeutic effect. Therefore, it is necessary to confirm expression in other cases. Moreover, we only investigated cases receiving oxaliplatin-based chemotherapy regimen as first-line treatment since there were few cases undergoing other regimens as first-line treatment. In future studies, we will investigate the association between the expression of DSB repair proteins and the chemotherapeutic effect for other regimens, and compare these findings with the results of the present study.

In conclusion, cases with a defective pattern of DSB repair protein expression may possess higher sensitivity to chemotherapy for colorectal cancer. The expression pattern of DSB repair proteins may be a useful prognostic indicator for colorectal cancer patients.

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