Clinical significance of microRNA-21 as a biomarker in each Dukes' stage of colorectal cancer

YOSHIHISA FUKUSHIMA, HISAE IINUMA, MITSUO TSUKAMOTO, KEIJI MATSUDA and YOJIRO HASHIGUCHI

Department of Surgery, Teikyo University School of Medicine, Itabashi-ku, Tokyo 173-0003, Japan

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Abstract. The potential value of microRNAs (miRNAs) as prognostic biomarkers are of interest. It is known that microRNA-21 (miR-21) is implicated in the promotion, proliferation and progression of several types of human cancers. However, the prognostic significance of miR-21 in each tumor stage of colorectal cancer (CRC) remains elusive. The objective of this study was to clarify the prognostic value of miR-21 for CRC patients at each tumor stage. The expression levels of miR-21 in the tumor tissues and normal adjacent tumor tissues of 306 CRC patients were determined by TaqMan microRNA assays. In order to clarify the miRNA profile in CRC tissues, miRNA arrays were examined. In this analysis, miR-21, miR-224, miR-96, miR-31 and miR-155 showed marked upregulation, and miR-21 showed the highest level. Upon comparison of clinicopathological factors, miR-21 expression showed significant association with depth of invasion, lymphatic and venous invasion, liver metastasis and Dukes' stage. In the Kaplan-Meier survival curve analysis of all patients, overall survival (OS) and disease-free survival (DFS) rates of the patients with high miR-21 expression were significantly worse than these rates in patients with low miR-21 expression. In the Kaplan-Meier analysis of each tumor stage, the DFS of patients with high miR-21 expression was significantly worse than patients with low miR-21 levels in Dukes' stage A tumors. In Dukes' stage B and C, patients with high miR-21 expression showed a significantly worse OS and DFS than patients with low miR-21 expression. In Dukes' stage D, patients with high miR-21 expression showed a significantly worse OS than patients with low miR-21 expression. In the Cox multivariate analysis, it was shown that miR-21 expressions in CRC tissues is an independent prognostic factor in Dukes' stage B, C and D. In conclusion, miR-21 expression may be a valuable biomarker for prediction of poor prognosis in CRC patients with Dukes' stage B, C and D.

Correspondence to: Dr Hisae Iinuma, Department of Surgery, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-0003, Japan
E-mail: iinuma@med.teikyo-u.ac.jp

Abbreviations: miR-21, microRNA-21; RT-PCR, reverse transcription-polymerase chain reaction; CRC, colorectal cancer

Key words: microRNA-21, biomarker, prognostic factor, colorectal cancer

Introduction

Colorectal cancer (CRC) is the third most common cause of cancer-related mortality worldwide (1). Despite the fact that recent advances in chemotherapeutic regimens in combination with molecular-targeted compounds have improved the survival rates of CRC patients, the risk of metastasis and recurrence still remain (2). To improve prognosis, adjuvant chemotherapy such as 5-fluorouracil (5-FU) plus leucovorin (LV) (5-FU/LV) and tegafur plus uracil (UFT)/LV has been established as a generalized regimen. For the treatment of advanced-stage CRC patients, 5-FU/LV plus irrinotecan (FOLFIRI) and 5-FU/LV plus oxaliplatin (FOLFOX), combined with molecular-targeted compounds such as bevacizumab, cetuximab and panitumumab have been approved (3). Although these combination therapies of chemotherapy and molecular-targeted agents have demonstrated survival benefits, the substantial financial cost involved in these treatments remains an issue (4). Furthermore, chemotherapy without certain selection tends to lead to overtreatment of patients with toxic agents that exert severe side-effects. To facilitate individually tailored treatment for CRC, useful biomarkers for the selection of patients at high risk of recurrence are required. Particularly, markers for the selection of high-risk patients in Duke's stage B are desirable, since the role of adjuvant chemotherapy in these patients remains controversial (5).

MicroRNAs (miRNAs) are small 21-25 nucleotide non-coding RNAs that participate in the regulation of cell differentiation, cell cycle progression, apoptosis and tumorigenesis (6-8). They target protein-coding mRNAs at the post-transcriptional level by direct cleavage of the mRNAs or by inhibition of protein synthesis (9). Recent evidence indicates that some miRNAs function as oncogenes (10,11) or tumor-suppressor genes (12,13) and play a central role in carcinogenesis. Accumulating evidence shows that microRNA-21

(miR-21) is an oncogenic miRNA, and overexpression of this miRNA has been reported in several types of human malignant solid tumors, including those of the breast, lung and colon (14). It was demonstrated that miR-21 promotes carcinogenesis through inhibition of apoptosis, proliferation, invasion, migration and metastasis (15). Additionally, high expression of miR-21 in CRC tissues may be associated with poor prognosis (16). However, no other studies have investigated the prognostic value of miR-21 at each tumor stage.

In the present study, we investigated the clinicopathological characteristics and the prognostic value of miR-21 mRNA levels in CRC patients of each tumor stage.

Patients and methods

Patients. A total of 306 CRC patients were studied from September 2000 to April 2012 at Teikyo University Hospital (Tokyo, Japan). The median follow-up period was 48 months (range, 24-90 months). All samples were derived from patients who did not receive any chemotherapy or radiotherapy prior to surgery. Immediately following surgical resection, primary CRC tissues and normal adjacent tumor tissues (normal tissue) were mounted by Tissue-Tek O.C.T. Compound (Sakura Finetechnical Co., Tokyo, Japan) and were frozen in liquid nitrogen. After surgery, patients with Dukes' stage A and B were not treated with chemotherapy. Dukes' stage C patients were treated with a standard regimen based on 5-FU/LV. Dukes' stage D patients were treated with a standard regimen based on FOLFIRI and FOLFOX, combined with molecular-targeted compounds (bevacizumab, cetuximab). The study protocol conformed to the guidelines of the Ethics Committee and was approved by the Review Board of Teikyo University. Written informed consent was obtained from all patients.

Follow-up of patients. Postoperative follow-up was performed along the guidelines published by the Japanese Society for Cancer of the Colon and Rectum. Confirmation of recurrence in all patients was required by evaluating imaging or pathological diagnosis. Physical examination and tumor marker (CEA and CA19-9) testing was conducted every 3 months for 3 years, and then every 6 months for 5 years. Computed tomography (CT) or magnetic resonance imaging (MRI) scans were repeated every 3 months for 3 years, and then 6 months for up to 5 years after surgery. Colon evaluation, including colonoscopy or colon radiography was performed every 2 years or annually for 3 years.

LCM and RNA isolation. Frozen tissues were used for laser capture microdissection (LCM). Ten micrometer-thick frozen sections of CRC and normal tissues were prepared using a Leica CM 1900 cryostat (Leica, Germany). The sections were placed on Membrane slides (Leica), fixed in 75% alcohol for 30 sec and stained with 0.5% violet-free methyl green (Sigma, USA). After staining, the sections were air-dried and microdissected using a Leica AS LMD system (Leica). Total RNAs were extracted by miRNeasy Mini kit (Qiagen Inc., Valencia, CA, USA) and treated with DNase I according to the manufacturer's instructions (Qiagen Inc.). Then, total RNA was reverse transcribed to complementary DNA (cDNA) using SuperScript II reverse transcriptase system with random hexamer primers according to the manufacturer's protocol (Invitrogen Corporation, Carlsbad, CA, USA).

Real-time PCR-based miRNA array. The miRNA expression profiling was performed using Cancer microRNA qPCR array with the QuantiMir system (System Biosciences, Mountain View, CA, USA). This system is a real-time PCR-based array containing a panel of 95 cancer-related mature miRNA assays and the U6 transcript as a normalization signal. In brief, total RNA containing small RNA extracted from tissue samples was first polyadenylated by poly(A) polymerase and then reverse transcribed to cDNA using a mixture of oligo(dT) adaptor. The cDNA then served as the template for SYBR real-time PCR using Power SYBR Master Mix (Life Technologies, Carlsbad, CA, USA), using miRNA-specific primers provided by the manufacturer. SYBR PCR was performed using a LightCycler 480 real-time PCR system (Roche Applied Science, Indianapolis, IN, USA). ΔCt was calculated by subtracting the Ct values of U6 from the Ct values of the gene of interest. ΔΔCt was then calculated by subtracting ΔCt of the control from ΔCt of the sample. The fold change of the gene was calculated by the equation: $2^{-\Delta\Delta Ct}$.

TaqMan microRNA assay for miR-21. Total RNAs, including the miRNAs were extracted from 306 primary CRC tissues and normal tissues by miRNeasy Mini kit (Qiagen Inc.). The miR-21 and RNU6B-specific cDNA were synthesized using gene-specific primer sets according to the TaqMan microRNA assays protocol (Life Technologies). RNU6B was used as the internal control. In brief, reverse transcriptase reactions contained 10 ng of total RNAs, 50 nmol/stem-loop RT primer, 1X RT buffer, 0.25 mmol/l each of deoxynucleotide triphosphate (dNTP), 3.33 U/μl MultiScribe reverse transcriptase, and 0.25 U/μl RNase inhibitor. The 7.5 μl reaction samples were incubated in a GeneAmp PCR System 9700 (Life Technologies) for 30 min at 16˚C, 30 min at 42˚C, 5 min at 85˚C and then folded at 4˚C. Quantitative real-time PCR was performed using Step One (Life Technologies). The 20 μl PCR samples included 1.33 μl of RT products, 10 μl of TaqMan Universal PCR Master Mix, 1 μl of primers and probe mix, and 7.67 μl of nuclease-free water and were incubated in optical plates. The PCR conditions were 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 6 sec. Each sample was analyzed in duplicate. Relative quantification of miRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = \Delta Ct\ (Ct_{miR21} - Ct_{RNU6B})_{tumor\ tissues} - \Delta Ct\ (Ct_{miR21} - Ct_{RNU6B})_{normal\ tissues}$.

Statistical analysis. Relationships between miR-21 expression and clinicopathological factors were analyzed using the Student's t-test, the Chi-square test and ANOVA. The cut-off level of miR-21 was determined as the mean level of relative quantity according to a previously published study (11). Overall survival (OS) and disease-free survival (DFS) curves were analyzed using the Kaplan-Meier survival curve method, and the differences were examined using log-rank tests. Cox proportional-hazards regression analysis was used to estimate the univariate and multivariate hazard ratios for OS and DFS. All p-values are two-sided, and $p<0.05$ was considered to indicate a statistically significant result. Statistical analyses were

Table I. Five most highly upregulated miRNAs in the CRC samples based on the miRNA profiling array.

	microRNA	MirBase no.	Fold-change
1	miR-21	MIMAT0000076	5.10
2	miR-224	MIMAT0000281	4.82
3	miR-96	MIMAT0000095	4.76
4	miR-31	MIMAT0000089	4.53
5	miR-155	MIMAT0000646	3.16

CRC, colorectal cancer.

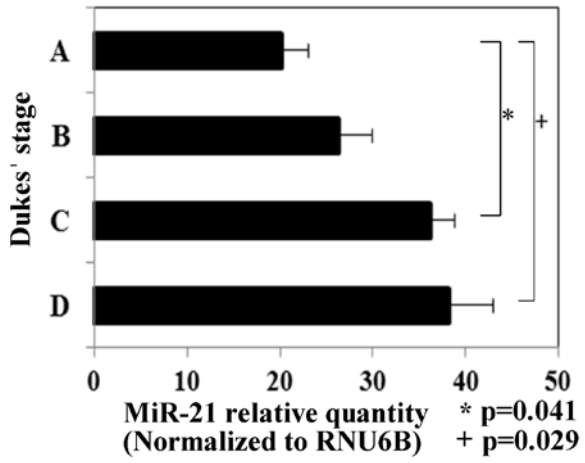

Figure 1. Comparison of miR-21 expression in each Dukes' stage of CRC. Relative miR-21 levels were determined in 306 CRC patients (Dukes' stage A, 44 cases; Dukes' stage B, 106 cases; Dukes' stage C, 94 cases and Dukes' stage D, 62 cases). Data are expressed as the mean ± SE. Expression of miR-21 in Dukes' stage C (*p=0.041) and Dukes' stage D (+p=0.029) was significantly higher than that in Dukes' stage A. CRC, colorectal cancer.

performed using JMP 9.0 software (SAS Institute, Inc., Cary, NC, USA).

Results

miRNA profile of CRC. First, we examined the profiling of miRNA expression in the CRC tissues using the real-time PCR-based miRNA arrays containing a panel of 95 cancer-related mature miRNAs. In this assay, we used pooled RNA samples of primary CRC tissues and matched normal tissues adjacent to the tumor tissues from 5 patients. Table I shows the top five miRNAs which were upregulated in this assay. miR-21, miR-224, miR-96, miR-31 and miR-155 were included in this list. Our results showed the highest upregulation of miR-21 as compared with other miRNAs. On the basis of these profiling results and other information from published studies, we selected miR-21 as a marker for CRC and for use in the following study.

Expression of miR-21 in each stage of CRC tissues. The miR-21 expression levels of the primary CRC tissues were compared for each Dukes' stage (Fig. 1). These samples were collected from 306 CRC patients. miR-21 levels were normalized by RNU6B levels. The means (± SE) expression level of miR-21 showed a significant increase according to the level of advancement of the tumor stage (p=0.041, Dukes A vs. C; p=0.029, Dukes A vs. D).

Table II. Relationship between clinicopathological factors and high miR-21 expression in the tumor tissues.

Variables	No. of patients (n=306)	miR-21 high (n=140)	Positive rate (%)	P-value
Tumor size (cm)				0.606
<5	171	76	44.4	
≥5	135	64	47.4	
Histological type				0.502
Well	210	93	44.3	
Not well	95	46	48.4	
Depth of invasion				0.015[a]
pT1	26	6	23.1	
>pT2	280	134	47.9	
Lymphatic invasion				0.034[a]
Ly (-)	188	77	41.0	
Ly (+)	118	63	53.4	
Venous invasion				0.010[a]
V (-)	118	43	36.4	
V (+)	188	97	51.6	
Lymph node metastasis				0.067
N (-)	166	68	41.0	
N (+)	140	72	51.4	
Liver metastasis				0.033[a]
H (-)	269	117	43.5	
H (+)	37	23	62.2	
Peritoneal dissemination				0.107
P (-)	289	129	44.6	
P (+)	17	11	64.7	
Dukes' stage				0.036[a]
A	44	14	31.8	
B	106	46	43.4	
C	94	43	45.7	
D	62	37	59.7	

[a]Significant association with high miR-21 expression.

Relationship between clinicopathological factors and miR-21 expression of the tumor tissues. This study included 306 CRC patients (190 men and 116 women) with a mean age of 65 years (range, 25-86). To evaluate the correlation between the miR-21 expression levels and the clinicopathological characteristics, patients were divided into two groups (high and low expression). The cut-off level (15 as miR-21) was determined as the mean level of the relative quantity. As shown in Table II, a

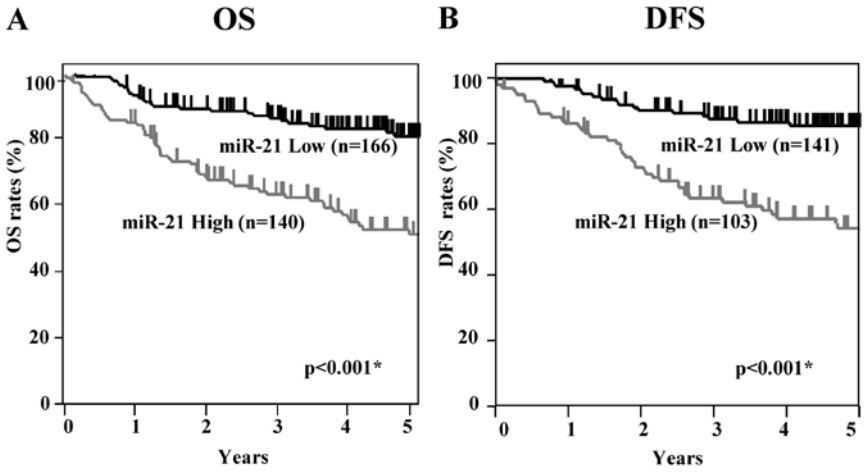

Figure 2. Kaplan-Meier survival curves of OS and DFS based on miR-21 expression in all patients. Patients were divided into two groups: those with low and those with high miR-21 expression. The significance of these groups for OS (A) and DFS (B) was demonstrated (*statistical significance). OS, overall survival; DFS, disease-free survival.

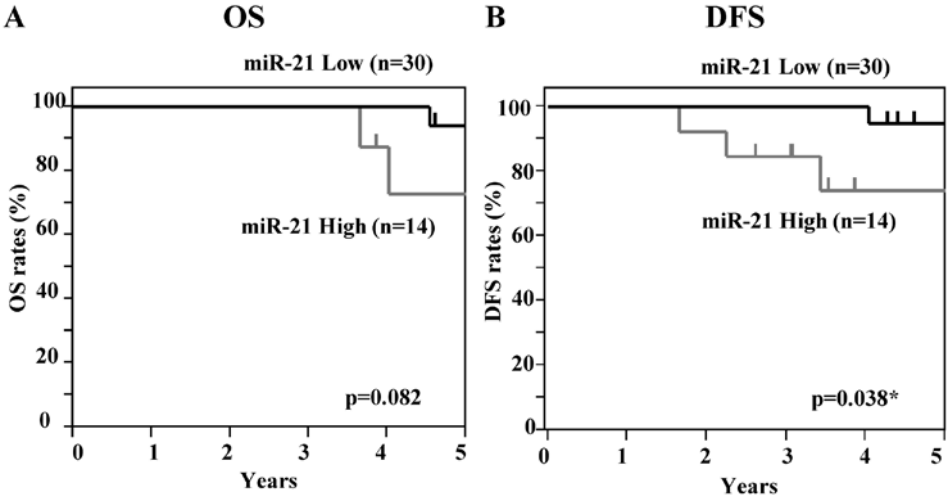

Figure 3. Kaplan-Meier survival curves of (A) OS and (B) DFS based on miR-21 expression in Dukes' stage A patients. Patients were divided into two groups: those with low and those with high miR-21 expression. The significance of these groups for DFS (B) was demonstrated (*statistical significance). OS, overall survival; DFS, disease-free survival.

statistically significant association was observed between high miR-21 expression and depth of invasion, lymphatic and venous invasion, liver metastasis and Dukes' stage.

Correlation between miR-21 levels and overall survival and disease-free survival. Kaplan-Meier OS and DFS curves of the CRC patients according to the levels of miR-21 expression were examined (Fig. 2). All patients were included for OS analysis (n=306), and patients who had undergone curative surgery were included for DFS analysis (n=244). The patients in the high miR-21 expression group showed significantly worse OS rates than patients in the low miR-21 group (log-rank p<0.001). DFS analysis revealed that patients in the high miR-21 group had significantly worse survival rates than those in the low expression group (log-rank p<0.001). These results suggest that high expression of miR-21 is associated with poorer prognosis in CRC patients.

Next, OS and DFS curves at each tumor stage were examined according to the levels of miR-21. In patients with Dukes' stage A, significant differences between the high and low miR-21 expression groups were apparent in regards to DFS but not OS (OS p=0.082, DFS p=0.038) (Fig. 3). In Dukes' stage B, patients in the high miR-21 expression group showed significantly worse OS and DFS rates than those in the low expression group (OS p=0.003, DFS p=0.002) (Fig. 4). In Dukes' stage C, patients in the high miR-21 expression group showed significantly worse OS and DFS rates than patients with low miR-21 expression (OS, DFS p=0.003) (Fig. 5). In Dukes' stage D, OS analysis revealed that patients with high miR-21 expression had significantly worse survival rates than those with low miR-21 expression (p=0.015) (Fig. 6).

These results suggest that high expression of miR-21 is associated with poor prognosis in CRC patients with Dukes' stage A, B, C and D.

Univariate and multivariate Cox analyses for overall survival and disease-free survival. Table III shows the results of the univariate and multivariate Cox proportional hazard regression

Table III. Univariate and multivariate analyses of the prognostic factors for OS of all patients.

Variables	Univariate analysis			Multivariate analysis		
	Regression coefficient	Hazard ratio (95% CI)	P-value	Regression coefficient	Hazard ratio (95% CI)	P-value
Tumor size	0.411	1.508 (0.984-2.312)	0.059		-	
Depth of invasion	2.273	9.711 (2.161-171.168)	0.001[a]	1.104	3.017 (0.579-55.791)	0.223
Lymph node metastasis	1.369	3.932 (2.482-6.434)	<0.001[a]	0.134	1.143 (0.445-3.268)	0.789
Lymphatic invasion	1.056	2.875 (1.871-4.467)	<0.001[a]	0.288	1.334 (0.781-2.291)	0.292
Venous invasion	1.128	3.088 (1.860-5.429)	<0.001[a]	0.629	1.875 (1.005-3.735)	0.048[a]
Histological type	0.767	2.153 (1.394-3.307)	0.001[a]	0.158	1.171 (0.675-2.007)	0.571
Liver metastasis	2.122	8.351 (4.334-14.942)	<0.001[a]	1.082	2.950 (1.177-6.879)	0.022[a]
Peritoneum dissemination	1.648	5.194 (3.103-8.426)	<0.001[a]	0.279	1.321 (0.624-2.68)	0.456
Serum CEA	1.841	6.303 (3.710-11.453)	<0.001[a]	0.496	1.643 (0.475-5.41)	0.425
Serum CA19-9	0.851	2.343 (1.658-3.337)	<0.001[a]	-0.081	0.922 (0.503-1.662)	0.789
Dukes' stage	1.140	3.127 (2.190-4.587)	<0.001[a]	0.716	2.046 (1.121-3.765)	0.020[a]
miR-21	1.125	3.080 (1.970-4.937)	<0.001[a]	1.058	2.880 (1.696-5.084)	<0.001[a]

[a]Significant association with OS. OS, overall survival; CI, confidence interval.

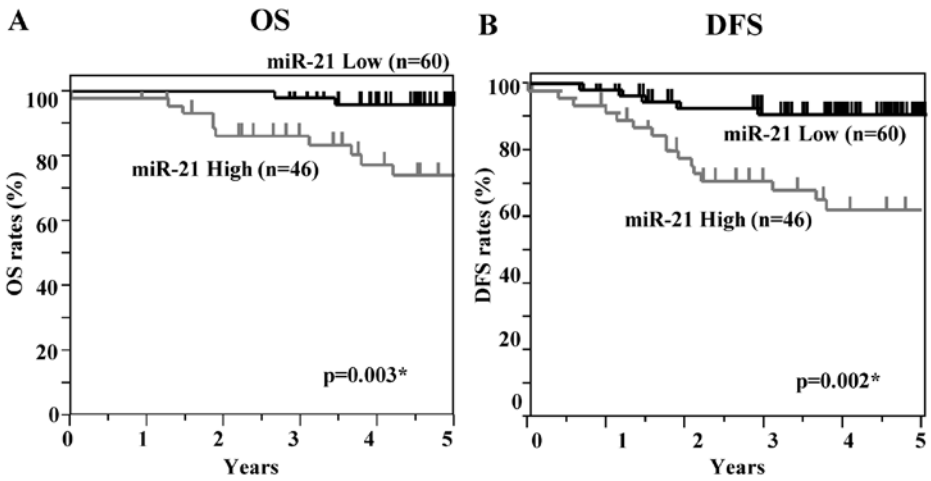

Figure 4. Kaplan-Meier survival curves of OS and DFS based on miR-21 expression in Dukes' stage B patients. Patients were divided into two groups: those with low and those with high miR-21 expression. The significance of these groups for OS (A) and DFS (B) was demonstrated (*statistical significance). OS, overall survival; DFS, disease-free survival.

analyses for OS in all of the CRC patients (n=306). Multivariate analysis was performed for factors that showed significance in the univariate analysis. In the univariate analysis of all patients, depth of invasion, lymph node metastasis, lymphatic and venous invasion, histological type, liver metastasis, peritoneum dissemination, serum CEA, serum CA19-9, Dukes' stage

Table IV. Univariate and multivariate analyses of prognostic factors for DFS of all patients.

	Univariate analysis			Multivariate analysis		
Variables	Regression coefficient	Hazard ratio (95% CI)	P-value	Regression coefficient	Hazard ratio (95% CI)	P-value
Tumor size	-0.110	0.896 (0.519-1.503)	0.683		-	
Depth of invasion	1.342	3.825 (1.196-23.331)	0.020[a]	0.449	1.566 (0.261-12.422)	0.630
Lymph node metastasis	0.943	2.567 (1.550-4.307)	0.001[a]	-1.015	0.363 (0.039-2.828)	0.339
Lymphatic invasion	0.544	1.723 (1.027-2.854)	0.040[a]	-0.239	0.788 (0.440-1.382)	0.408
Venous invasion	1.250	3.490 (1.434-5.131)	0.004[a]	0.762	2.143 (0.532-3.142)	0.303
Histological type	0.337	1.401 (0.804-2.360)	0.227		- (0.675-2.007)	
Serum CEA	0.595	1.814 (1.246-2.660)	0.002[a]	0.522	1.686 (0.937-3.041)	0.081
Serum CA19-9	0.703	2.020 (1.361-2.993)	0.001[a]	0.521	1.684 (0.902-3.094)	0.101
Dukes' stage	0.782	2.186 (1.283-3.885)	0.004[a]	0.706	2.026 (1.110-3.877)	0.021[a]
miR-21	1.275	3.578 (2.098-6.359)	<0.001[a]	1.078	2.940 (1.682-5.360)	<0.001[a]

[a]Significant association with DFS. CI, confidence interval. DFS, disease-free survival.

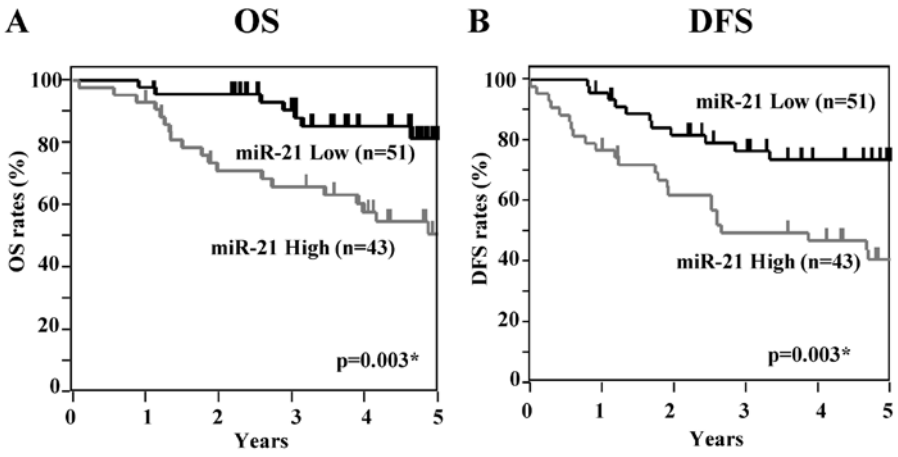

Figure 5. Kaplan-Meier survival curves of OS and DFS based on miR-21 expression in Dukes' stage C patients. Patients were divided into two groups: those with low and those with high miR-21 expression. The significance of these groups for OS (A) and DFS (B) was demonstrated (*statistical significance). OS, overall survival; DFS, disease-free survival.

and miR-21 showed a significant association with OS. In the multivariate analysis, venous invasion, liver metastasis, Dukes' stage and miR-21 level showed a significant association with OS. Table IV shows the results of the univariate and multivariate Cox analyses for DFS in CRC patients who underwent curative surgery (n=244). In the univariate analysis, depth of invasion, lymph node metastasis, lymphatic and venous invasion, serum CEA, serum CA19-9, Dukes' stage and miR-21 showed a significant association with OS. In the multivariate analysis, Dukes' stage and miR-21 showed a significant association for DFS. Next, we examined the univariate and multivariate Cox analyses in patients with Dukes' stage A, B, C and D, respectively. In the univariate analysis of Dukes' stage A patients, no factor showed a significant association

Table V. Univariate and multivariate analyses of prognostic factors for OS and DFS in Dukes' stage B patients.

Variables	Univariate analysis			Multivariate analysis		
	Regression coefficient	Hazard ratio (95% CI)	P-value	Regression coefficient	Hazard ratio (95% CI)	P-value
For overall survival (OS)						
Tumor size	-0.436	0.647 (0.173-2.053)	0.467		-	
Lymphatic invasion	0.094	0.910 (0.202-3.051)	0.887		-	
Venous invasion	1.893	6.641 (1.291-121.369)	0.020[a]	1.625	5.078 (0.968-93.408)	0.056
Histological type	0.001	1.001 (0.222-3.355)	0.999		-	
Serum CEA	1.296	3.655 (1.149-13.720)	0.028[a]	0.964	2.621 (0.818-9.906)	0.106
Serum CA19-9	0.337	1.401 (0.311-4.698)	0.623		-	
miR-21	0.982	7.125 (1.876-46.376)	0.003[a]	1.813	6.130 (1.607-39.991)	0.006[a]
For disease-free survival (DFS)						
Tumor size	-0.726	0.484 (0.174-1.177)	0.112		-	
Lymphatic invasion	-0.248	0.780 (0.256-1.972)	0.618		-	
Venous invasion	0.503	1.653 (0.679-4.610)	0.278		-	
Histological type	-0.141	0.868 (0.285-2.195)	0.778		-	
Serum CEA	0.971	2.641 (1.137-6.413)	0.024[a]	0.723	2.060 (0.852-5.164)	0.108
Serum CA19-9	0.941	2.562 (1.023-5.985)	0.045[a]	0.671	1.957 (0.757-4.752)	0.159
miR-21	0.659	3.734 (1.532-10.422)	0.003[a]	1.215	3.369 (1.373-9.448)	0.007[a]

[a]Significant association with OS or DFS. OS, overall survival; DFS, disease-free survival; CI, confidence interval.

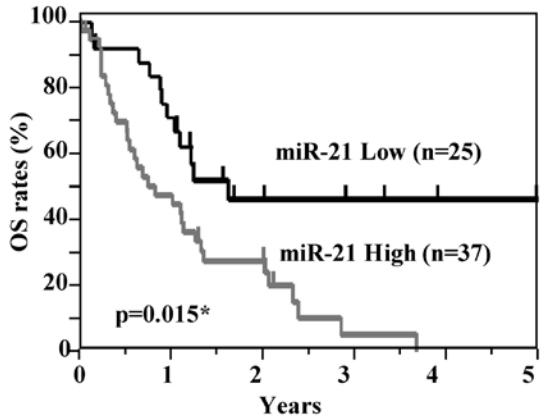

Figure 6. Kaplan-Meier survival curves of OS based on miR-21 expression in Dukes' stage D patients. Patients were divided into two groups: those with low and those with high miR-21 expression. The significance of these groups for OS was demonstrated (*statistical significance). OS, overall survival.

with OS and DFS (data not shown). In the univariate analysis of Dukes' stage B patients, venous invasion, serum CEA and miR-21 showed a significant association with OS (Table V). In the univariate analysis for DFS, serum CEA, serum CA19-9 and miR-21 showed significance. In the multivariate analysis of Dukes' stage B patients, only miR-21 showed significance for OS and DFS. In the univariate analysis of Dukes' stage C patients, lymphatic invasion, serum CEA and miR-21 showed a significant association with OS (Table VI). In the univariate analysis for DFS, serum CEA, serum CA19-9 and miR-21 showed significance. In the multivariate analysis of Dukes' stage C patients, miR-21 showed a significant association with OS and DFS. Table VII shows the Cox analysis in patients with Dukes' stage D. In the univariate and multivariate analyses, liver metastasis and miR-21 showed a significant association with OS.

Table VI. Univariate and multivariate analyses of prognostic factors for OS and DFS in Dukes' stage C patients.

	Univariate analysis			Multivariate analysis		
Variables	Regression coefficient	Hazard ratio (95% CI)	P-value	Regression coefficient	Hazard ratio (95% CI)	P-value
For overall survival (OS)						
Tumor size	-0.432	0.649 (0.277-1.425)	0.286		-	
Lymphatic invasion	0.828	2.288 (1.039-5.390)	0.040[a]		-	
Venous invasion	0.388	1.475 (0.662-3.595)	0.351		-	
Histological type	0.010	1.010 (0.413-2.257)	0.981		-	
Serum CEA	0.803	2.233 (1.025-5.106)	0.043[a]	0.727	2.069 (0.949-4.736)	0.068
Serum CA19-9	0.737	2.089 (0.949-4.531)	0.067		-	
miR-21	1.228	3.413 (1.499-8.743)	0.003[a]	1.179	3.251) (1.425-8.340)	0.005[a]
For disease-free survival (DFS)						
Tumor size	-0.054	0.947 (0.477-1.842)	0.874		-	
Lymphatic invasion	0.521	1.683 (0.862-3.390)	0.128		-	
Venous invasion	0.662	1.940 (1.943-4.385)	0.073		-	
Histological type	0.137	1.147 (0.551-2.267)	0.703		-	
Serum CEA	0.817	2.264 (1.160-4.557)	0.017[a]	0.434	1.544 (0.663-3.571)	0.312
Serum CA19-9	0.940	2.560 (1.308-4.989)	0.007[a]	0.562	1.754 (0.775-4.067)	0.179
miR-21	1.030	2.801 (1.405-5.952)	0.003[a]	0.921	2.512 (1.250-5.372)	0.009[a]

[a]Significant association with OS or DFS. OS, overall survival; DFS, disease-free survival; CI, confidence interval.

These results suggest that miR-21 levels of tumor tissues have an independent prognostic value for OS and DFS in CRC patients with Dukes' stage B, C and D.

Discussion

In the present study, we examined the prognostic value of miR-21 in CRC patients at each tumor stage. Our results demonstrated that the high expression of miR-21 in CRC tissues indicated a significantly poor prognosis for OS and DFS in CRC patients with Dukes' stage B and C, and for OS in patients with Dukes' stage D.

It has been reported that miR-21 is one of the prominent miRNAs implicated in the genesis and progression of human cancer (17). Not only has it been implicated in the promotion of tumor growth, proliferation and response to chemotherapy, but studies have also shown that miR-21 is overexpressed in several human malignant solid tumors such as glioblastoma, hepatocellular and pancreatic cancer and CRC (9,18-20). In the present study, we demonstrated a significantly increased expression of miR-21 in the CRC tissues, along with the progression of tumor stage. It has been reported that the expression of miR-21 is associated with more advanced stages of CRC (16). Furthermore, we examined the association of miR-21 expression and clinicopathological factors, and found that high miR-21 expression had a significant association with depth of invasion, lymphatic and venous invasion, liver metastasis and Dukes' stage. Another study revealed that CRC patients overexpressing miR-21 are more likely to have lymph node and distant metastasis (17).

The prognostic value of miR-21 has been reported in many types of cancer, including CRC. In the present study, we demonstrated that miR-21 expression in tumor tissues was significantly associated with the poor prognosis of patients

Table VII. Univariate and multivariate analyses of prognostic factors for OS in Dukes' stage D patients.

	Univariate analysis			Multivariate analysis		
Variables	Regression coefficient	Hazard ratio (95% CI)	P-value	Regression coefficient	Hazard ratio (95% CI)	P-value
Tumor size	0.090	1.094 (0.590-2.130)	0.781		-	
Lymph node metastasis	0.642	1.900 (0.933-4.307)	0.079		-	
Lymphatic invasion	0.443	1.558 (0.839-3.037)	0.163		-	
Venous invasion	0.205	1.228 (0.558-3.240)	0.633		-	
Histological type	0.534	1.705 (0.932-3.185)	0.084		-	
Liver metastasis	-0.756	0.470 (0.258-0.860)	0.015[a]	-0.796	0.451 (0.246-0.831)	0.011[a]
Peritoneum dissemination	0.422	1.525 (0.768-2.864)	0.218		-	
Serum CEA	-0.722	0.486 (0.215-1.306)	0.141		-	
Serum CA19-9	-0.551	0.577 (0.278-1.194)	0.136		-	
miR-21	0.860	2.363 (1.276-4.524)	0.006[a]	0.895	2.448 (1.316-4.712)	0.005[a]

[a]Significant association with OS. OS, overall survival; CI, confidence interval.

with CRC. Similar results were reached independently by Schetter *et al* (16). However, the significance of miR-21 expression in each tumor stage has not been previously reported. In the present study, the prognostic significance of miR-21 levels was investigated using the Kaplan-Meier method and Cox multivariate analysis on 306 patients with CRC at each Dukes' stage. In these analysis, our data demonstrated the existence of an independent prognostic impact of miR-21 for OS and DFS in CRC patients with Dukes' stage B and C, and for OS in patients with Dukes' stage D. In Dukes' stage A patients, miR-21 was associated with a significantly worse prognosis for DFS in the Kaplan-Meier analysis. However, a significant prognostic impact was not demonstrated in the Cox analysis of Dukes' stage A patients. This may be due to the low number of patients who suffered recurrence and death. To the best of our knowledge, this is the first study to clarify the significance of the prognostic value of miR-21 at each stage in CRC patients. At present, the role of adjuvant chemotherapy for Dukes' stage B patients remains controversial. Many Dukes' stage B patients will benefit from therapy. However, if surgery is curative, additional therapy may harm the quality of life with little therapeutic benefit. Therefore, it is important to develop novel biomarkers to identify high-risk patients who may be suitable for therapeutic intervention. In the present study, we established that miR-21 has potential for the selection of high-risk patients in need of adjuvant chemotherapy among Dukes' stage B patients.

miRNAs target protein-coding mRNAs at the post-transcriptional level by direct cleavage of the mRNAs in two different ways: one is direct cleavage of the target mRNAs and the other is inhibition of protein synthesis (9,21,22). Bioinformatically predicted targets for miR-21 that have been experimentally validated include the tumor-suppressor genes: programmed cell death 4 (PDCD4), phosphatase and tensin homologue (PTEN), tropomyosin 1 (TPM1), reversion-inducing cysteine-rich protein (RECK), ras homing gene family member B or maspin (23-28). PDCD4 has been characterized as a novel tumor-suppressor gene that acts as a suppressor of transformation, tumorigenesis and progression, invasion and metalloproteinase activation and as an inducer of apoptosis. We previously demonstrated a significant inverse relationship between miR-21 and PDCD4 mRNA in CRC samples (29). miR-21 targets the 3'-UTR of the PDCD4 gene at nucleotides 228-249 with perfect complementarity, thereby post-transcriptionally regulating its expression (15). Through the inhibition of PDCD4 tumor-suppressor gene by miR-21, tumor growth may be promoted, leading to a poor prognosis. We will await further study to clarify the tumor regulation mediated by miR-21.

In conclusion, the present study demonstrated that miR-21 may be a valuable biomarker for identifying high-risk CRC patients with Dukes' stage B or C. To clarify the prognostic value of miR-21 in Dukes' stage A patients, further study with a large number of cases is required.

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