Prognostic impact of sarcopenia and its correlation with circulating miR-21 in colorectal cancer patients

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Abstract. Severe malnutrition accompanied by sarcopenia and cachexia, is strongly associated with the surgical and oncological outcomes in cancer patients. The aim of the present study was to clarify the clinical significance of sarcopenia and its correlation with sarcopenia-associated miRNA in colorectal cancer (CRC). A total of 167 CRC patients were enrolled in the present study. We evaluated psoas muscle mass index (PMI) and intramuscular adipose tissue content (IMAC). The expression of miR-21 in CRC tissues and preoperative serum was evaluated using quantitative PCR. Despite the lack of significant correlation between IMAC and disease-correlated factors, decreased PMI was significantly associated with well-established clinicopathological factors for disease progression. Decreased PMI was an independent prognostic factor for both overall survival and disease-free survival and was an independent risk factor for various types of metastasis. In contrast to the expression of tissue miR-21, the expression of serum miR-21 was significantly increased in CRC patients with low PMI. Furthermore, postoperative PMI was drastically improved compared with preoperative PMI in CRC patients with potentially curative resections. In conclusion, skeletal muscle mass may be a prognostic and predictive biomarker for distant metastasis in CRC patients and quantification of serum miR-21 expression could help clinicians make decisions regarding nutrition intervention strategies in CRC patients.

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Introduction

Cachexia is a complex metabolic and behavioral syndrome associated with underlying illness and characterized by the loss of skeletal muscle (1). Whereas malnutrition is reversible with conventional nutrition intervention, cachexia is recognized as irreversible by conventional nutrition support (2). In particular, cancer cachexia occurs in approximately 80% of patients with advanced cancer and is one of the most critical aspects affecting their quality of life and mortality (3,4). There is accumulating evidence that cancer cachexia is the direct cause of cancer-related death in approximately 20% of all cancer patients (5,6). Based on this background, it is critical to evaluate the clinical significance of cancer cachexia and clarify the factors associated with cachexia in cancer patients.

MicroRNAs are an abundant class of small non-coding RNA molecules 18-25 nucleotides in length that regulate gene expression at the post-transcriptional level by promoting messenger RNA degradation or blocking translation (7). Since a single miRNA molecule can target hundreds of diverse mRNAs, miRNAs possess an enormous regulatory potential concerning the protein-expression status and represent critical regulators of various biological processes, including cell differentiation, proliferation and apoptosis (8). Besides the crucial biological role of miRNAs in cancer development (9-13), emerging evidence reveals that microvesicle-encapsulated miRNAs stably exist in body fluids and are associated with the regulation of cellular processes involved in cell communication,

angiogenesis, extracellular matrix remodeling and biological mechanisms in cancer cachexia (14-16).

Colorectal cancer (CRC) is one of the most common cancers worldwide and the second leading cause of cancer-related death in the US (17,18). Despite advances in diagnostic and therapeutic techniques in the last decade, the prognosis of CRC patients with distant metastasis remains poor (19,20), thus it is essential to identify new predictive biomarkers for distant metastasis and poor outcome in order to improve both the early detection of recurrence, as well as the prognosis of CRC patients.

Previous studies by our research group revealed that several serum markers reflecting systemic inflammatory response, including interleukin (IL)-1b, IL-1ra, IL-6, IL-10, C-reactive protein and albumin, are differentially expressed in serum from patients with advanced CRC and can be used as predictive biomarkers for postoperative nutritional status, morbidity and mortality in CRC patients (21-24). Furthermore, we successfully revealed that several circulating miRNAs could be used as biomarkers for diagnosis, prognosis and metastasis prediction in CRC patients (25-29). In the present study, we systematically investigated the preoperative body composition status of CRC patients and the expression profile of sarcopenia-associated miRNAs in CRC tissues and matched serum to clarify the clinical significance of preoperative body composition and its correlation with cachexia-associated miRNA expression in CRC patients.

Materials and methods

Patients and methods. Recently, our research group demonstrated that serum miR-21 is a promising biomarker for the early detection and prognosis in CRC patients (25). From the validation cohort of this previous study, a total of 167 patients with CRC, who underwent primary resection at our Mie University Hospital between 2005 and 2010 and had available preoperative images of plain computed tomography (CT) were enrolled in the present study. A written informed consent was obtained from each patient and the study was approved by the review board of Mie University Hospital. Patients who were treated with radiotherapy or chemotherapy before surgery were excluded. No perioperative mortalities were observed. The diagnosis of CRC was confirmed for all patients based on clinicopathological findings. The tumor-node-metastasis (TNM) staging system from the American Joint Committee on Cancer was used for the pathological tumor staging of CRC (30). All patients were classified according to the UICC stage classification of resected specimens: 33 patients were diagnosed with stage I disease, 54 with stage II, 41 with stage III and 39 with stage IV. All CRC patients diagnosed with stage III and IV disease received 5-fluorouracil-based chemotherapy, whereas no adjuvant chemotherapy was given to patients with stage I and II disease. The locations of tumors and distant metastases were determined by barium enema, colonoscopy, CT and magnetic resonance imaging. Resection of the primary tumor was performed in all patients and all patients were followed up for tumor recurrence at regular intervals for up to 5 years. Patients were observed at 3-month intervals for 24 months after the completion of surgery, followed by every 6 months for the next 3 years and then, yearly. A history was taken and a physical examination was performed in each visit and chest X-ray, colonoscopy and CT were performed annually.

Image analysis. Using preoperative plain CT at the superior aspect of the fourth lumbar vertebra (L4) as previously described (31), we assessed the cross-sectional area of the bilateral psoas muscles by manual tracing and calculated the psoas muscle mass index (PMI) as follows: PMI = cross-sectional area of bilateral psoas muscle/height^2 (cm^2/m^2). Low PMI was considered as a proxy for low muscle volume as previously described (32-34). Subfascial muscular tissue in the multifidus muscle was estimated by manual tracing at the same level on preoperative plain CT images and mean CT values [Hounsfield units (HU)] for these areas were determined with the Aquarius NET server (TeraRecon, Inc., San Mateo, CA, USA). We placed four circles on areas of subcutaneous fat away from major vessels in CT images, which were used as the regions of interest (ROIs) for subcutaneous fat. The mean CT values (HU) for the ROI of subcutaneous fat were assessed. IMAC was calculated by the ratio of these CT values, as previously reported by Kitajima *et al* (35,36), as follows: IMAC = mean CT value of ROI of multifidus muscle (HU)/mean CT value of ROI of subcutaneous fat (HU). High IMAC was considered as a proxy for low muscle quality. For the assessment of postoperative PMI or IMAC, we assessed these values on a postoperative plain CT image obtained 6 months after the resection of the primary tumor.

Sample collection, RNA isolation and quantitative reverse-transcription polymerase chain reaction. The present study included the analysis of 287 serum samples and matched surgical tissue specimens (serum, 153; cancerous tissue, 134) obtained from the enrolled CRC patients. All of these patients were previously described as the validation cohort in a biomarker study of serum miR-21 (25). Blood samples were obtained by venipuncture before surgery. Each sample was centrifuged at 3,000 x g for 5 min and stored at -80˚C until analysis.

RNA isolation and qRT-PCR from serum. RNA extraction and miRNA enrichment from all sera were performed using the Qiagen miRNeasy kit (Qiagen, Valencia, CA, USA). Briefly, 250 μl of serum or medium was thawed on ice and centrifuged 1,700 x g for 15 min at room temperature to remove cell debris and then, 200 μl of supernatant was lysed in 5 volumes of Qiazol solution. To normalize any inadvertent sample-to-sample variations during the RNA isolation procedure, reverse transcription (RT) and PCR reaction, 25 fmol of synthetic *C. elegans* miRNA (cel-miR-39) was added to each denatured sample. Small RNAs were enriched and purified according to the manufacturer's protocol, with the exception that the enriched small RNAs were eluted in 40 μl of nuclease-free water. For the miRNA-based RT-PCR assays, 1.67 μl of enriched small RNAs from serum or cell-culture medium was reverse-transcribed using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, San Diego, CA, USA) in a total volume of 5.0 μl. reverse transcription (RT) products (1:15 dilution) were used as template for the PCR. PCR reactions for the quantification

of miR-21 and cel-miR-39 were performed in duplicate with TaqMan 2X Universal PCR Master Mix under previously described conditions (25,37).

RNA isolation and qRT-PCR from formalin-fixed, paraffin-embedded (FFPE) tissues. Total RNA was isolated from FFPE samples using the RecoverAll Total Nucleic Acid Isolation kit (Ambion Inc.; Thermo Fischer Scientific, Austin, TX, USA). Briefly, tissue sections were microdissected to enrich for neoplastic cells, followed by deparaffinization and RNA extraction according to the manufacturer's protocol. Total RNA was eluted in the appropriate buffer and quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Reverse transcription reactions were carried out using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) in a total reaction volume of 15 μl. miR-16 was chosen as an endogenous normalizer for miRNAs based on the previous finding that miR-16 was one of the most suitable reference genes for relative quantification of small ncRNAs in colonic tissues including CRC and normal colonic mucosa in microarray analysis of large cohorts. Furthermore, we previously demonstrated that miR-16 is a reliable normalizer for tissue samples (25-27,29,38-40). miR-21 and miR-16 were quantified in duplicate by qRT-PCR using MicroRNA Assay kits (Applied Biosystems). qRT-PCR was performed on an Applied Biosystems 7000 Sequence Detection system with TaqMan Universal PCR Master Mix 2X under the following cycling conditions: 95˚C for 10 min, followed by 45 cycles at 95˚C for 15 sec and 60˚C for 1 min. Cycle threshold (Ct) values were calculated using the same threshold cut-off values for each assay to prevent plate-to-plate variations and data were analyzed with Sequence Detection Software v.1.4.1 (Applied Biosystems).

Calculation of the expression of miRNA. The expression levels of serum or tissue miRNAs were normalized using cel-miR-39 (for serum samples) and miR-16 (for tissue samples) using the $2^{-\Delta Ct}$ method as previously described (25-27,29,38,39). The differences between groups are presented as ΔCt, indicating differences between the Ct values of the miRNAs of interest and the Ct values of the normalizer miRNAs.

Statistical analysis. Statistical analysis was performed using MedCalc software version 16.8.4 (Mariakerke, Belgium). The results are expressed as the means ± standard deviation (SD). The differences between groups were estimated using Chi-square and Mann-Whitney U test. F-tests were conducted to assess the equality of variance for comparable groups. Statistical differences in PMI between different times (pre- and postoperative) from the same group of patients were compared using the Wilcoxon test for paired samples. For time-to-event analyses, survival estimates were calculated using Kaplan-Meier analysis and groups were compared with the log-rank test. Receiver operating characteristic (ROC) curves were established to determine the cut-off values for analyzing prognosis and various types of metastasis by Youden's index. Overall survival (OS) was determined from the date the patient underwent surgery until the date of death resulting from any cause (i.e., cancer-unrelated deaths were not censored) or the last known follow-up for patients that were still alive. Disease-free survival (DFS) was derermined from the date the patient underwent curative surgery to the date of disease recurrence, death from any cause (i.e., cancer-unrelated deaths were not censored), or last contact with the patient. The Cox's proportional hazards models were used to estimate hazard ratios (HR) for death and recurrence. Assumption of proportionality was confirmed for the Cox proportional hazards analyses by generating Kaplan-Meier survival curves (e.g., high vs. low PMI groups) and ensuring that the two curves did not intersect each other. Multivariate logistic regression models were used to predict factors influencing hepatic, peritoneal, distant metastasis and low PMI. Forced-entry regression was used to include these variables in all multivariable equations to analyze whether each of the predictors affected the outcome after adjusting for known confounding factors. For the univariate and multivariate analyses for patient prognosis, in addition to target PMI and IMAC status, we included the following previously identified confounding clinical factors that impact the prognosis of patients with rectal cancer: sex, age at diagnosis, pathological differentiation (well-moderate or poor), T stage (T1/2 or T3/4), venous invasion (present or absent), lymphatic vessel invasion (present or absent), lymph node metastasis (present or absent) and UICC stage classification (stages I/II or stages III/IV). All P-values were two-sided and $P<0.05$ was considered to indicate a statistically significant difference.

Results

In contrast to the IMAC status, preoperative PMI is significantly decreased with disease progression in CRC patients. Firstly, to determine whether body composition status had clinical significance in CRC patients, we analyzed the association between preoperative PMI/IMAC and various clinicopathological factors (Table I). With the exception of a significant correlation with sex (female) and older age, preoperative IMAC status was not significantly correlated with other clinicopathological factors in this cohort (Fig. 1A). In contrast, decreased preoperative PMI was significantly associated with all well-established clinicopathological factors for disease progression, including advanced T stage (P=0.05), presence of venous invasion (P=0.021), lymphatic vessel invasion (P=0.036), lymph node metastasis (P=0.001), hepatic metastasis (P=0.028), peritoneal metastasis (P=0.003), distant metastasis (P=0.0004) and advanced TNM stage classification (P=0.0001) in CRC patients (Table I; Fig. 1B).

Decreased preoperative PMI is associated with poor outcome in CRC patients. Subsequently, we performed time-to-event analysis to evaluate the potential of preoperative PMI and IMAC levels as a prognostic biomarker and generated Kaplan-Meier survival curves subdivided by PMI and IMAC score. Although there was no significant correlation between preoperative IMAC status and recurrence or survival (log-rank test, P=0.15, P=0.36, respectively; Fig. 1C and D), patients with decreased preoperative PMI had a significantly poor prognosis compared with those with high PMI in terms of both OS and DFS (log-rank test, P=0.003, P=0.04, respectively; Fig. 1E and F). To determine the potential of

Table I. Correlation between clinicopathological variables and PMI/IMAC in colorectal cancer patients.

		PMI			IMAC		
Variable	n	High (n=112)	Low (n=55)	P-value	High (n=82)	Low (n=85)	P-value
Sex				<0.0001[b]			**<0.0001[b]**
sMale	99	79	20		35	64	
Female	68	33	35		47	21	
Age (years)				0.83			**0.004[b]**
≥67[a]	84	57	27		32	52	
>67	83	55	28		50	33	
Histological type				0.13			0.33
Differentiated	151	104	47		76	75	
Undifferentiated	16	8	8		6	10	
Pathological T category				**0.05**			0.39
pT1/2	50	39	11		22	28	
pT3/4	117	73	44		60	57	
Vessel invasion				**0.021[b]**			0.61
Present	70	40	30		36	34	
Absent	97	72	25		46	51	
Lymphovascular invasion				**0.036[b]**			0.68
Present	126	79	47		63	63	
Absent	41	33	8		19	22	
Lymph node metastasis				**0.001[b]**			0.91
N0	97	75	22		48	49	
N1	70	37	32		34	36	
Hepatic metastasis				**0.028[b]**			0.16
H0	142	100	42		73	69	
H1	25	12	13		9	16	
Peritoneal metastasis				**0.003[b]**			0.77
P0	158	110	48		78	80	
P1	9	2	7		4	5	
Distant metastasis				**0.0004[b]**			0.25
M0	128	95	33		66	62	
M1	39	17	22		16	23	
UICC TNM classification				**0.0001[b]**			0.36
Stage I	33	29	5		18	15	
Stage II	54	41	13		26	28	
Stage III	41	26	15		22	19	
Stage IV	39	17	22		16	23	

[a]The median age of patients at surgery was 67 years in this cohort. [b]P<0.05.

preoperative PMI as a predictive biomarker of recurrence and prognosis in CRC patients, multivariate Cox's regression analysis was performed. In addition to advanced T stage, presence of vascular invasion, lymphatic vessel invasion and decreased IMAC, we revealed that decreased preoperative PMI was an independent prognostic factor for OS in CRC patients (HR: 2.99, 95%; CI: 1.37-6.55, P=0.006) (Table IIA). Furthermore, decreased preoperative PMI was also an independent prognostic factor for poor DFS in these patients (HR: 2.62, 95%; CI: 1.04-6.63, P=0.042; Table IIB).

Decreased PMI is an independent risk factor for metastasis in CRC patients. Notably, low PMI status was significantly correlated with metastasis-related clinicopathological factors such as presence of hepatic, peritoneal and distant metastasis in CRC patients. Based on these findings, we performed multivariate logistic analysis to determine the clinical significance of low PMI status as a predictive biomarker for metastasis (Table IIIA-C). Notably, low PMI status was an independent predictive factor for hepatic metastasis (OR: 13.6, 95%; CI: 3.03-60.6, P=0.0006), peritoneal metastasis (OR: 5.73, 95% CI:

Table II. Multivariate analyses.

A, predictors of overall survival

	Univariate			Multivariate		
Variables	HR	95% CI	P-value	HR	95%CI	P-value
Sex (male)	1.29	0.67-2.49	0.45	1.5	0.67-3.39	0.33
Age (>67 year-old)[a]	1.07	0.58-1.97	0.84	1.3	0.63-2.67	0.48
Histological type (undifferentiated)	2.2	0.97-5.0	0.06	1.19	0.49-2.03	0.69
Location (left)	0.81	0.42-1.54	0.51	0.67	0.33-1.36	0.27
T classification (pT3/4)	15.8	2.17-114.8	**0.006**[b]	8.76	1.07-71.4	**0.043**[b]
Vessel involvement (present)	4.31	2.11-8.81	**0.0001**[b]	2.21	1.02-4.78	**0.045**[b]
Lymphatic vessel involvement (present)	12.8	1.76-93.1	**0.012**[b]	1.99	0.23-17.1	0.53
Lymph node metastasis (present)	6.78	3.12-14.7	**<0.0001**[b]	0.57	0.16-2.01	0.39
Stage classification (stage III/IV)	10.3	4.03-26.4	**<0.0001**[b]	9.67	2.13-44.0	**0.003**[b]
PMI (low)	2.51	1.35-4.68	**0.004**[b]	2.99	1.37-6.55	**0.006**[b]
IMAC (low)	1.77	0.93-3.38	**0.084**	2.85	1.34-6.08	**0.007**[b]

B, predictors of disease-free survival

	Univariate			Multivariate		
Variables	HR	95% CI	P-value	HR	95%CI	P-value
Sex (male)	1.09	0.5-2.38	0.83	1.15	0.41-3.21	0.78
Age (>67 year-old)[a]	1.96	0.88-4.37	0.1	1.52	0.61-3.78	0.37
Histological type (undifferentiated)	1.69	0.58-4.96	0.34	0.42	0.1-1.74	0.23
Location (left)	0.64	0.29-1.38	0.25	0.4	0.15-1.07	0.07
T classification (pT3/4)	4.29	1.29-14.2	**0.017**[b]	2.06	0.51-8.25	0.31
Vessel involvement (present)	4.4	1.98-9.81	**0.0003**[b]	2.64	1.09-6.41	**0.032**[b]
Lymphatic vessel involvement (present)	11.5	1.56-84.7	0.017[b]	2.22	0.24-20.3	0.48
Lymph node metastasis (present)	3.68	1.72-7.88	**0.0008**[b]	3.55	1.4-8.99	**0.008**[b]
PMI (low)	2.18	1.02-4.68	**0.045**[b]	2.62	1.04-6.63	**0.042**[b]
IMAC (low)	1.43	0.65-3.12	**0.37**	2.1	0.81-5.44	0.13

[a]Median age of CRC patients at surgery was 67 years. Cut-off thresholds of PMI/IMAC were determined by ROC analysis using Youden's index for overall survival (OS) and disease-free survival (DFS) in CRC patients. HR, hazard ratio; PMI, psoas muscle mass index; IMAC, intramuscular adipose tissue content. [b]P<0.05.

1.1-29.7, P=0.038) and distant metastasis (OR: 4.26, 95%; CI: 1.61-11.2, P=0.003), indicating that the presence of distant metastasis was intimately correlated with sarcopenia in CRC patients.

Serum miR-21 expression is significantly increased in CRC patients with low PMI compared with those with high PMI. miR-21 is one of the most representative oncogenic secretory miRNAs (25,41) and a recent study revealed its pivotal function in the pathogenesis of sarcopenia (42). Considering these findings, we subsequently assessed serum and tissue miR-21 expression levels to examine the correlation between miR-21 expression levels and preoperative body composition status in CRC patients. Indeed, despite the lack of significant correlation between the IMAC status and miR-21 expression in cancer tissues or serum, serum miR-21 expression, but not tissue miR-21 expression, was significantly increased in patients with low PMI compared with those with high PMI (P=0.031; Fig. 2). Furthermore, multivariate logistic regression analysis, adjusted by age, sex, tissue miR-21 and serum albumin level, clearly demonstrated that increased serum miR-21 level was an independent risk factor for decreased PMI in CRC patients (Table IIID).

Changes in PMI in patients with CRC before and after resection of primary tumors. In a previous study, we evaluated serum miR-21 expression levels in paired pre- and postoperative serum samples and separately analyzed the data based on potentially curative (Stages I-III) vs. non-curative surgeries (Stage IV) (25). Our study revealed postoperative reductions

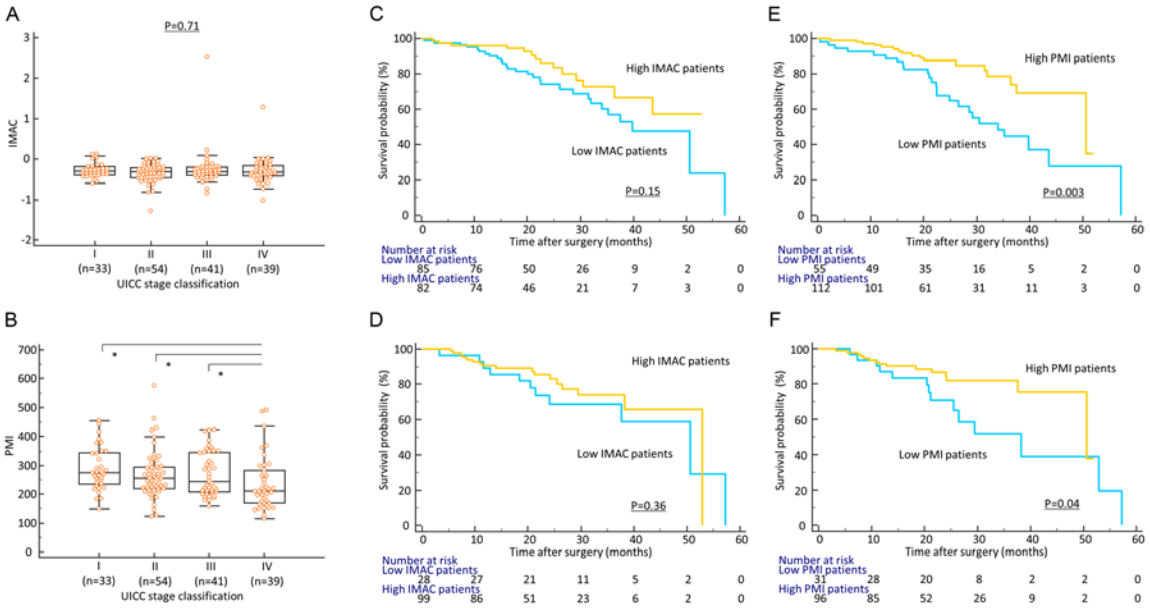

Figure 1. Clinical significance and prognostic impact of PMI and IMAC in CRC patients. (A and B) Changes in IMAC and PMI according to the TNM stage classification. (C and D) Kaplan-Meier survival curves for overall survival (OS) and disease-free survival (DFS) in CRC patients based on the IMAC status. The IMAC status was not significantly correlated with poor prognosis in (C) OS and (D) DFS. (E and F) Kaplan-Meier survival curves for OS and DFS in CRC patients based on the PMI status in CRC patients. Decreased PMI was significantly correlated with poor prognosis for (E) OS (P=0.003; log-rank test) and (F) DFS (P=0.04; log-rank test). All statistical tests were two-sided.

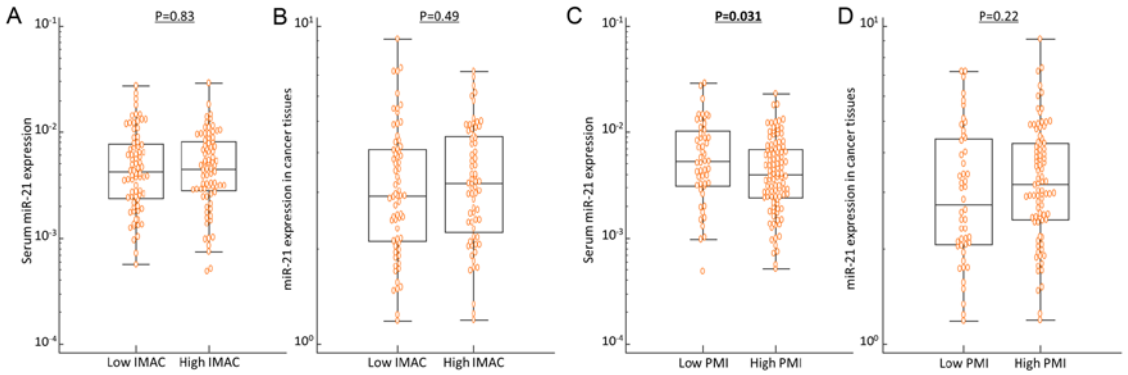

Figure 2. Dysregulation of miR-21 expression in preoperative serum specimens and CRC tissues according to IMAC and PMI status. (A and B) miR-21 expression in preoperative serum specimens (P=0.83) and CRC tissues (P=0.49) did not significantly correlate with IMAC status in CRC. (C and D) Despite no significant correlation between PMI status and miR-21 expression in CRC tissues, serum miR-21 expression was significantly increased in patients with lower PMI compared with those with higher PMI (P=0.031). Statistically significant differences were determined using the Mann-Whitney U test, cut-off thresholds of PMI/IMAC were determined by ROC analysis with Youden's index for OS and all statistical tests were two-sided.

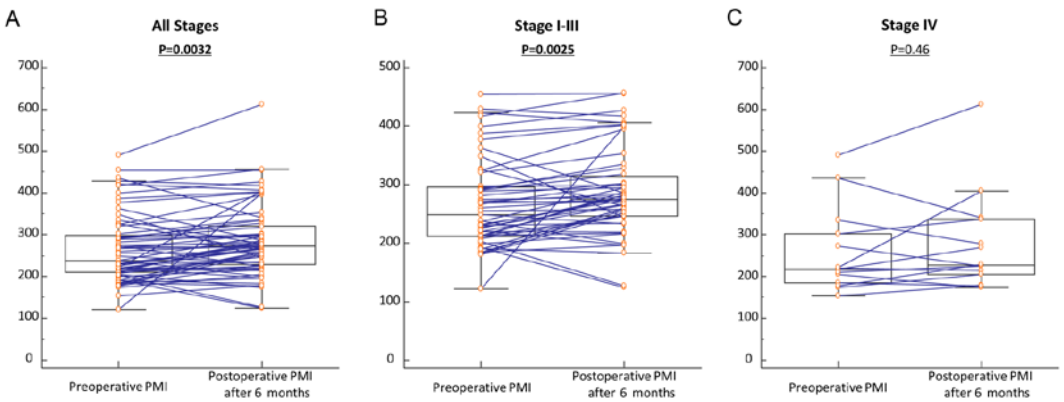

Figure 3. Changes in PMI in CRC patients before surgery and 6 months after surgical removal of primary tumors. (A) Comparison of PMI in all CRC patients (n=65). (B) Comparison of PMI in 51 CRC patients who underwent potentially curative surgeries. (C) Comparison of PMI in 14 CRC patients who underwent non-curative surgeries. Statistically significant differences were determined using the Wilcoxon test and all statistical tests were two-sided.

Table III. Multivariate analyses.

A, predictors of hepatic metastasis

	Univariate			Multivariate		
Variables	OR	95% CI	P-value	OR	95% CI	P-value
Sex (male)	2.45	0.92-6.51	0.07	9.35	1.88-46.3	**0.006**[b]
Age (>67 years-old)[a]	0.63	0.27-1.5	0.3	0.43	0.12-1.54	0.19
Histological type (differentiated)	1.26	0.27-5.91	0.77	8.68	0.96-78.7	0.05
Location (left)	0.46	0.19-1.08	0.07	0.21	0.06-0.77	**0.018**[b]
T classification (pT3/4)	3.63	1.03-12.7	**0.044**[b]	0.68	0.13-3.61	0.65
Vessel involvement (present)	3.57	1.44-8.83	**0.006**[b]	2.77	0.77-10.0	0.12
Lymphatic vessel involvement (present)	4.35	0.98-19.3	0.05	0.55	0.06-4.71	0.58
Lymph node metastasis (present)	14.4	4.09-50.4	**<0.0001**[b]	24.6	4.35-139	**0.0003**[b]
PMI (low)	4.78	1.94-11.8	**0.0007**[b]	13.6	3.03-60.6	**0.0006**[b]
IMAC (low)	2.43	0.94-6.31	0.07	3.96	0.95-16.5	0.06

B, predictors of peritoneal metastasis

	Univariate			Multivariate		
Variables	OR	95% CI	P-value	OR	95% CI	P-value
Sex (male)	0.32	0.08-1.34	0.12	0.6	0.11-3.25	0.55
Age (>67 year-old)[a]	0.8	0.21-3.09	0.75	0.81	0.16-4.1	0.8
Histological type (differentiated)	0.18	0.04-0.8	**0.025**[b]	0.37	0.06-2.26	0.28
Location (Left)	0.58	0.15-2.25	0.43	0.8	0.17-3.84	0.78
T classification (pT3/4)	-	-	0.99	-	-	0.99
Vessel involvement (present)	5.28	1.06-26.2	**0.042**[b]	1.7	0.29-10.0	0.56
Lymphatic vessel involvement (present)	-	-	0.99	-	-	0.99
Lymph node metastasis (present)	5.28	1.06-26.2	**0.042**[b]	2.03	0.35-11.9	0.43
PMI (low)	11.2	2.61-47.6	**0.001**[b]	5.73	1.1-29.7	**0.038**[b]
IMAC (low)	0.31	0.08-1.2	0.09	0.72	0.14-3.78	0.69

C, predictors of distant metastasis

	Univariate			Multivariate		
Variables	OR	95% CI	P-value	OR	95% CI	P-value
Sex (male)	1.3	0.62-2.74	0.48	2.1	0.77-5.96	0.15
Age (>67 year-old)[a]	0.73	0.35-1.49	0.38	0.59	0.24-1.47	0.26
Histological type (differentiated)	0.47	0.16-1.38	0.17	0.97	0.25-3.71	0.96
Location (left)	0.81	0.38-1.72	0.59	0.71	0.28-1.8	0.48
T classification (pT3/4)	2.88	1.12-7.4	**0.028**[b]	0.8	0.23-2.82	0.73
Vessel involvement (present)	5.21	2.37-11.5	**<0.0001**[b]	3.37	1.24-9.16	**0.017**[b]
Lymphatic vessel involvement (present)	5.07	1.47-17.5	**0.01**[b]	1.36	0.28-6.7	0.7
Lymph node metastasis (present)	6.15	2.74-13.8	**<0.0001**[b]	3.74	1.44-9.71	**0.007**[b]
PMI (low)	4.14	1.95-8.77	**0.0002**[b]	4.26	1.61-11.2	**0.003**[b]
IMAC (low)	1.53	0.74-3.16	0.25	1.79	0.68-4.71	0.24

Table III. Continued.

D, predictors of decreased PMI

	Univariate			Multivariate		
Variables	OR	95% CI	P-value	OR	95% CI	P-value
Sex (female)	4.19	2.12-8.3	**<0.0001**[b]	5.02	2.12-11.9	**0.0003**[b]
Age (>67 year-old)[a]	0.93	0.49-1.77	0.83	1.24	0.54-2.84	0.62
Tissue miR-21 expression (high)	0.8	0.39-1.62	0.53	0.81	0.38-2.02	0.76
Serum miR-21 expression (high)	2.18	1.02-4.66	**0.044**[b]	2.68	1.07-6.74	**0.036**[b]
Serum albumin level (low)	3.17	1.62-6.19	**0.0007**[b]	5.69	2.32-13.9	**0.0001**[b]

[a]Median age of CRC patients at surgery was 67 years. Cut-off thresholds of tissue/serum miR-21 and albumin level were determined by ROC analysis with Youden's index for hepatic metastasis, peritoneal metastasis, distant metastasis and prognosis in CRC patients. OR, odds ratio. [b]P<0.05.

in serum miR-21 levels among patients with potentially curative surgeries (Stages I-III), but no significant differences in patients with non-curative resections (Stage IV). These data highlighted the feasibility of serum miR-21 as a disease-specific biomarker for CRC. Based on these findings, we analyzed paired pre- and postoperative PMI values in a subset of 65 CRC patients who underwent surgical resection of their tumors. Among the 65 CRC patients, 51 underwent potentially curative resection whereas 14 had multiple hepatic metastases and underwent primary resection to prevent bleeding and bowel obstruction (non-curative resection). Postoperative PMI was significantly increased compared with preoperative PMI for total CRC patients (P=0.0032; Fig. 3A). Interestingly, postoperative PMI was drastically improved compared with preoperative PMI in CRC patients with potentially curative resection (P=0.0025; Fig. 3B), whereas PMI levels did not change between pre- and postoperative status in CRC patients with non-curative resection (P=0.46; Fig. 3C). These findings were consistent with previous findings of a dysregulated pattern of serum miR-21 expression. Collectively, these results revealed an intimate correlation between serum miR-21 and skeletal muscle mass and highlighted the expression of serum miR-21 as a clinically feasible biomarker for monitoring cancer cachexia in CRC patients.

Discussion

Cancer cachexia is recognized as severe malnutrition accompanied by sarcopenia and is one of the major obstacles affecting various clinical aspects in patients with malignancies. Accumulating evidence has revealed that cancer cachexia is intimately associated with the deterioration of functional status and quality of life, low tolerance to chemotherapy and poor prognosis in patients with gastrointestinal cancer (43,44). However, its prognostic impact and clinical significance remain unclear and the underlying pathological mechanism has not been elucidated. We made several novel and interesting discoveries during the course of our investigation. Firstly, despite no significant correlation between IMAC and disease-correlated factors, loss of PMI was significantly correlated with well-established factors for disease progression, including advanced T stage, presence of venous invasion, lymphatic vessel invasion, lymph node, hepatic, peritoneal and distant metastasis as well as advanced TNM stage classification in CRC patients. Secondly, loss of PMI was significantly correlated with poor prognosis and was an independent prognostic factor for both OS and DFS in CRC patients by multivariate analysis. Furthermore, we revealed that loss of PMI was an independent risk factor for various types of metastasis in patients with CRC, including hepatic, peritoneal and distant metastasis, indicating that sarcopenia may be intimately associated with distant metastasis in CRC. Thirdly, although tissue miR-21 expression was not correlated with preoperative PMI status, serum miR-21 expression was significantly increased in CRC patients with low PMI compared with those with high PMI. Finally, we compared pre- and postoperative PMI status. Although there was no significant change between pre- and postoperative PMI in patients with non-curative resection, postoperative PMI was drastically improved compared with preoperative PMI in CRC patients with potentially curative resection. These findings successfully validated previous results revealing patterns of dysregulation between pre- and postoperative serum miR-21 expression.

Currently, various components of body composition are recognized as factors influencing surgical and oncological outcome in patients with malignancies. Skeletal muscle mass is one of the representative body composition components and loss of skeletal muscle mass, called sarcopenia, is associated with aging, inactivity and chronic benign and malignant disease (2,45). Several studies revealed that sarcopenia frequently occurred in patients with various malignancies and was a significant risk factor for surgical complications in CRC patients (46-49). Muscle steatosis, an increase in intramuscular adipose tissue, has also attracted increasing attention as an important body composition component in the field of oncological research (32,33,50). Okumura *et al* (32) investigated 230 patients who underwent resection of pancreatic cancer to assess the prognostic impact of muscle quantity and quality in these patients. They demonstrated that low PMI and high IMAC were independent prognostic factors of poor OS

and recurrence-free survival and suggested that low quality and quantity of skeletal muscle may be closely related to mortality after resection of pancreatic cancer. In the present study, we performed matched analysis of preoperative PMI and IMAC to directly compare the quality and quantity of skeletal muscle and to clarify the clinical significance of these components in CRC patients. One of the major findings in the present study is the clinical and prognostic impact of low PMI in CRC patients. In contrast to IMAC, low PMI status was an independent prognostic factor for both OS and DFS in CRC patients. Furthermore, low PMI status was an independent risk factor for various type σ of metastasis. These findings, combined with previous evidence, indicated that assessment of PMI status could be a promising prognostic marker for disease progression in patients with CRC and that sarcopenia is closely associated with disease progression, especially distant metastasis, in CRC patients.

Another major finding of the present study is the significant correlation between quantity of skeletal muscle mass and circulating miR-21 expression in CRC patients. A recent study (42) clearly demonstrated that cancer-derived microvesicles containing miR-21 activated the TLR7 receptor on murine myoblasts and promoted muscle cell death through c-Jun N-terminal kinase (JNK) activity in cancer cachexia. Furthermore, our research group previously revealed several specific features of serum miR-21 in CRC patients (25). Firstly, using supernatants from cultured CRC cell lines, miR-21 was identified as a novel secretory miRNA and serum expression of miR-21 in CRC patients correlated positively with the expression status in tumor tissues. Secondly, serum miR-21 expression was significantly increased in patients with adenoma and CRC and quantification of serum miR-21 could robustly discriminate the patients with adenoma and CRC from healthy volunteers. Furthermore, we also demonstrated that serum miR-21 expression decreased significantly in postoperative serum samples from patients who underwent curative CRC surgery. Consistent with these findings, the present study revealed that serum miR-21 was significantly increased in patients with low PMI compared with those with high PMI regardless of the lack of a significant correlation between tissue miR-21 expression and PMI status. Furthermore, postoperative PMI was significantly improved in CRC patients who underwent curative surgery, supporting a significant correlation between serum miR-21 and PMI status. Collectively, our data revealed that circulating miR-21 was more directly correlated with sarcopenia compared to tissue miR-21 expression in CRC patients and indicated that assessment of serum miR-21 level may be used to evaluate the risk of sarcopenia and cancer cachexia in CRC patients.

In conclusion we presented novel findings on the clinical significance of sarcopenia and its intimate correlation with circulating miR-21 expression in CRC. Skeletal muscle mass may be used as a prognostic and predictive biomarker for various types of distant metastasis in CRC patients and quantification of the expression of serum miR-21 could be beneficial in planning a nutrition intervention strategy for CRC patients.

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Competing interests

The authors declare that they have no competing interests.

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