Evaluation of Parameters for Cancer-Induced Sarcopenia in Patients Autopsied after Death from Colorectal Cancer

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Keywords

Colorectal cancer · Cachexia · Sarcopenia · TNF- α · Myosin light chain

Abstract

Cachexia frequently occurs in cancer patients and is correlated with reduced therapeutic responsiveness and poor prognosis. Although skeletal muscle atrophy is an important factor related to cachexia, biomarkers for its early diagnosis are not yet definitive. In this study, weight loss, body mass index, skeletal muscle index (SMI), serum carcinoembryonic antigen, serum tumor necrosis factor (TNF)- α , serum interleukin (IL)-6, serum high mobility group box (HMGB)-1, and SDS-soluble myosin light chain 1 (SDS-MYL1) of the psoas muscle were examined in 8 autopsied cases of death from colorectal cancer (CRC) as biomarkers of cachexia. SDS-MYL1 was positively correlated to SMI and TNF- α was negatively correlated, but the other factors did not show any correlations with SMI. Multivariate analysis showed that of the 3 cytokines, TNF- α and HMGB1 were correlated with SMI. Furthermore, when the biochemical skeletal muscle maturation marker, SDS-MYL1, was compared with serum cytokines, TNF- α and HMGB1 were negatively correlated but IL-6 was not. In multivariate analysis, only TNF- α was associated with

SDS-MYL1. A positive correlation was found between TNF- α and HMGB1. These findings suggest that since TNF- α was inversely correlated with SMI and SDS-MYL1, TNF- α is a serum marker of skeletal muscle atrophy in CRC. Moreover, SDS-MYL1 might be established as a biomarker linked to clinical sarcopenia in experiments in vitro and in vivo.

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Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related deaths in Japan, and its incidence has continuously risen [1]. In spite of the advancements in adjuvant therapies, patients with advanced disease still have a poor prognosis [2, 3]. One quarter of advanced cases of CRC are associated with liver metastasis, which is a life-threatening event and accounts for 30% of CRC deaths [4–6].

Cancer cachexia is a condition that impairs quality of life and physical function [7]. Cancer cachexia is found in 40% of all-stage cancer patients and in 80% of patients with advanced cancer [8, 9]. Loss of skeletal muscle vol-

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Table 1. Eight CRC cases and 5 non-cancer cases

Case No.	Age, years	Sex	Operational findings							Treatment	Survival period ^a , months
			histology	tumor progression					pStage		
				primary tumor	nodal met.	liver met.	lung met.	peritoneal met.			
1	65	M	tub2	pT4a	1	2	0	1	IVb	palliative	6
2	72	M	tub2	pT4a	3	3	1	0	IVb	palliative	8
3	78	F	tub1	pT3	2	1	0	0	IVb	curative	11
4	82	M	tub2	pT3	2	2	1	0	IVb	palliative	13
5	68	F	tub1	pT3	1	1	0	0	IVb	curative	15
6	62	M	tub2	pT4a	2	1	0	0	IVb	palliative	9
7	75	M	tub2	pT3	1	1	0	0	IVb	curative	7
8	79	M	tub2	pT3	2	1	0	0	IVb	curative	10
Non-cancer cases											
A	67	M	acute myocardial infarction								1
B	72	F	cerebral hemorrhage								2
C	74	M	acute myocardial infarction								2
D	78	F	acute myocardial infarction								4
E	81	M	cerebral hemorrhage								1

Staging of CRC was done according to the AJCC classification [32]. ; Primary tumor: pT3, primary tumor invades through the muscularis propria into the pericolorectal tissues; pT4a, primary tumor penetrates to the surface of the visceral peritoneum. Nodal metastasis: pN1, metastasis in 1–3 regional lymph nodes; pN2, metastasis in ≥4 regional lymph nodes. Liver metastasis: 1, single foci; 2, multiple foci. Lung metastasis and peritoneal metastasis: 0, none; 1, positive. pStage; IVb, any pT and any pN and metastases at >1 organ/site or the peritoneum. M, male; F, female; met., metastasis.

^a Postoperative.

ume is an important feature of cachexia [10]. Skeletal muscle atrophy affects tolerability to cancer treatments such as surgery and chemotherapy [11]. Decrease in skeletal muscle mass, i.e., sarcopenia, is associated with short-term risks of surgery for CRC [12]. For advanced CRC, standard chemotherapies such as folfiri and folfox are effective and are widely indicated [2, 3]. Skeletal muscle atrophy before or during chemotherapy is correlated with a poor response to treatment [13, 14]. Sarcopenia is significantly correlated with the poor survival of cancer patients [15–17]. Sarcopenia is also associated with poor survival in end-stage CRC and stage I–III CRC [18, 19].

The clinical diagnosis of cancer cachexia used to be based on weight loss, body composition, and physical functioning. Previous studies focused on identifying molecular biomarkers to elucidate the mechanism of cachexia [20]. The need for early diagnosis of cancer cachexia supports the search for biomarkers that reflect the process of skeletal muscle atrophy [21]. However, clinically effective and efficacious biomarkers for making a cancer cachexia diagnosis are still lacking.

In this study, we aimed to identify biomarker candidates for cachexia. We focused on the serum cytokines

known to be associated with cachexia and sarcopenia. The inflammatory process evoked by cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-6 and high mobility group box (HMGB)-1 is considered to play a pivotal role in cachexia and sarcopenia by enhancing anorexia, catabolism, and oxidative stress [11, 22–27]. To judge the significance of these cytokines as biomarkers, their levels were compared with the skeletal muscle index (SMI), a clinical marker of muscle volume [28], and SDS-soluble myosin light chain 1 (SDS-MYL1), a biochemical marker of muscle maturation [29]. SDS-MYL1 is also a candidate marker for sarcopenia and cachexia in vitro and in vivo models [30]. We investigated whether SDS-MYL correlated with clinical sarcopenia parameters as another objective of this study.

Materials and Methods

Patients

Of 42 CRC cases (ending in death) diagnosed at the Department of Molecular Pathology, Nara Medical University, from 2001 to 2010, fresh blood and skeletal muscle samples as well as analyzable image and clinical data were available for 8 only. The 8 cases

Table 2. Clinical aspects of the 8 cancer cases

Case No.	Edmonton symptom assessment scale [49] ¹								Lower leg edema ²
	total	drowsiness	nausea	appetite	shortness of breath	depression	anxiety	well-being	
1	56	5	9	10	6	8	9	9	4
2	41	2	7	7	4	6	7	8	4
3	34	3	4	8	4	4	4	7	3
4	46	6	8	8	5	5	6	8	3
5	36	3	5	6	5	7	4	6	3
6	37	4	4	6	6	5	5	7	2
7	38	6	7	6	4	4	4	7	2
8	38	4	5	7	3	5	7	7	2

¹ Evaluated from the patient complaints described in the medical record.

² Assessed according to Mosby's Guide to Physical Examination [31]. Pit depth was estimated visually and scored as: 0 = no clinical edema, 1 = slight pitting (2 mm depth) with no visible distortion, 2 = a somewhat deeper pit (4 mm) with no readily detectable distortion, 3 = a noticeably deep pit (6 mm) with the dependent extremity full and swollen, and 4 = a very deep pit (8 mm) with the dependent extremity grossly distorted.

for which data on cachexia were obtained within 2 months of death were examined (Tables 1, 2). Clinical data (Edmonton Symptom Assessment Scale, body weight, body mass index [BMI], lean body mass [LBM], serum carcinoembryonic antigen [CEA] concentration, and computed tomography [31]) were obtained from the hospital medical records.

Staging of CRC cases was done according to the American Joint Committee on Cancer Colon and Rectum Cancer Staging [32]. Lymph node metastasis and liver metastasis were observed in all cases, and the stage at the time of surgery was pStage IVb. Curative resection was performed in 4 cases, and palliative surgery was performed in the other 4. All cases underwent chemotherapy and were administered folfiri or folfox postoperatively, and the survival time after surgery was 6–15 months.

From 112 autopsies of patients who died from non-cancer causes, 5 were used as controls in this study; only these 5 had adequate samples of fresh blood and skeletal muscle as well as analyzable image and clinical data. Death had occurred within a short time (1–4 months) due to acute myocardial infarction or cerebral hemorrhage. In these cases, no cachexic or sarcopenic changes were observed clinically.

In both the CRC and control cases, autopsy diagnoses did not include severe infections such as sepsis.

Skeletal Muscle Index

The SMI was calculated as the skeletal muscle area at L3 in cm²/ (body height in m)². The skeletal muscle area was manually traced using CT images.

Protein Extraction

Psoas muscle tissue was obtained at autopsy, and then stored at –80 °C. It was then crushed with a hammer to remove the tendons and fascia. The muscle tissue was washed with cold PBS and pelleted with a sonicator (QSONICA, WakenBtech Co. Ltd., Kyoto, Japan). Whole-cell lysates were prepared as previously described

using 0.1% sodium dodecyl sulfate (SDS)-added radioimmuno-precipitation assay (RIPA) buffer (Thermo Fisher Scientific, Tokyo, Japan) [33]. The protein assay was performed using a Protein Assay Rapid Kit (Wako Pure Chemical Corp., Osaka, Japan).

Blood was obtained from the left cardiac ventricle at autopsy. The blood was incubated undisturbed at room temperature for 20 min and centrifuged at 600 g for 10 min at 4 °C. The supernatant (serum) was stored at –80 °C till used.

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) kits were used to measure the concentration of MYL1 (Cusabio Biotech Co., Ltd., Houston, TX, USA), HMGB1 (Shino-Test Co., Sagamihara, Japan), human TNF-α, and IL-6 (Abcam, Cambridge, UK), according to the manufacturers' instructions. For MYL1 measurement, whole-cell lysate was used, whereas for the measurement of HMGB1, TNF-α, and IL-6, serum was used.

Statistical Analysis

Statistical significance was calculated by Spearman R test using InStat software v3.0 (GraphPad Software, Inc., La Jolla, CA, USA). Multiple regression analysis was performed using an EZR program [34]. Data were expressed as the mean ± standard deviation of 3 independent experiments. $p < 0.05$ (two-sided) was considered statistically significant.

Results

Comparison of Cachexia-Related Markers in CRC and Non-Cancer Cases

In the 8 CRC and 5 control cases, levels of clinical markers of cachexia (weight loss, BMI, LBM, and SMI),

Table 3. Comparison of cachexia-associated parameters between CRC and non-cancer cases

	CRC cases, <i>n</i> = 8	Non-cancer cases, <i>n</i> = 5	<i>p</i> value
TNF- α , pg/mL	104 $\pm$ 15	16 $\pm$ 5	<0.0001
IL-6, pg/mL	11 $\pm$ 2	2 $\pm$ 1	<0.0001
HMGB1, pg/mL	103 $\pm$ 17	11 $\pm$ 3	<0.0001
SMI, cm ² /m ²	36 $\pm$ 5	50 $\pm$ 3	0.0002
Weight loss, kg	4.3 $\pm$ 2.3	0.36 $\pm$ 0.5	0.0039
BMI, kg/m ²	21 $\pm$ 3	28 $\pm$ 3	0.0049
LBM, kg	44 $\pm$ 5	52 $\pm$ 4	0.0210
SDS-MYL1, pg/g	18 $\pm$ 4	30 $\pm$ 3	0.0004
CEA, ng/mL	163 $\pm$ 126	1.2 $\pm$ 0.7	0.017

(1) Serum concentration. (2) Skeletal muscle index (SMI) was calculated as: skeletal muscle area at L3/body height. Skeletal muscle area was measured by computed tomography. (3) Weight loss was calculated as: body weight at operation – body weight at death. (4) Lean body mass (LBM) was calculated as: $0.32810 \times \text{body weight (kg)} + 0.33929 \times \text{height (cm)} - 29.5336$ in males, or $0.29569 \times \text{body weight (kg)} + 0.41813 \times \text{height (cm)} - 43.2933$ in females [50]. (5) SDS-soluble myosin light chain 1 (SDS-MYL1) was measured by ELISA of SDS-soluble whole lysates of the psoas muscles.

Fig. 1. Relationship between SMI and weight loss, BMI, and serum CEA. Weight loss (a), BMI (b), and serum CEA (c) were compared with SMI. Each parameter was obtained within 2 months of death. The relationship was examined by Spearman regression analysis.

inflammatory cytokines reported to be related to cachexia (TNF, IL-6, and HMGB1), SDS-MYL1 related to skeletal muscle tissue maturity, and CEA related to tumor burden, were compared (Table 3).

In CRC cases, weight loss was significantly greater and BMI, LBM, and SMI were significantly reduced compared to the controls. The 3 cytokines were increased 5- to 9-fold in CRC cases compared to controls. In contrast, SDS-MYL1 was reduced in 60% of the controls. CEA was higher in CRC cases (mean 163 ng/mL) than in controls. Thus, both SMI and SDS-MYL1 showed significant differences between CRC and control cases.

Comparison between SMI and Other Cachexia-Related Markers in CRC Cases

Next, we examined the correlation between SMI and other parameters considered to be objective markers of skeletal muscle atrophy. As shown in Figure 1, weight loss, BMI, and CEA showed no significant correlation with SMI. Figure 2 shows that an increase in TNF- α levels was significantly correlated with a decrease in SMI whereas HMGB1 and IL-6 were not correlated with SMI. In contrast, SDS-MYL1 showed a significant positive correlation with SMI.

Fig. 2. Relationship between SMI, cytokines, and SDS-MYL1. TNF- α (a), IL-6 (b), HMGB1 (c), and SDS-MYL1 (d) levels were compared with SMI. Each parameter was obtained at autopsy. The relationship was examined by Spearman regression analysis.

Among these parameters, the correlations between TNF- α , IL-6, HMGB1, and SMI were compared using a multiple regression analysis (Table 3). TNF- α was most strongly correlated with SMI, followed by HMGB1, whereas no correlation was found between SMI and IL-6.

Relationship of the Three Cytokines in CRC

Furthermore, when the relationship of the 3 cytokines was examined (Fig. 3), TNF- α and HMGB1 showed a significant positive correlation. Between TNF- α and IL-6 and between HMGB1 and IL-6, nonsignificant differences were observed.

Comparison between SDS-MYL1 and Cytokines in CRC Cases

Finally, we examined the relationship between SDS-MYL1, a biochemical marker of skeletal muscle atrophy, and the 3 cytokines (Fig. 4). Decreased SDS-MYL1 correlated significantly with increased TNF- α and HMGB1, but not with IL-6. The correlations between TNF- α , IL-6, HMGB1, and SDS-MYL1 were compared by multiple re-

gression analysis (Table 4). Only TNF- α was found to correlate with SDS-MYL1. No correlation was found between SDS-MYL1 and IL-6 or HMGB1.

Discussion

Skeletal muscle atrophy is considered an important factor in cachexia [10] and to strongly influence the formation of cachexia [15, 16, 35, 36]. However, our results showed that SMI, which is a clinical marker of skeletal muscle atrophy, was not significantly correlated with weight loss, BMI, or LBM. This could be because weight loss and BMI involve various factors such as loss of adipose tissue, fluctuation of body fluid such as edema and ascites, and reduction of skeletal muscle volume. Indeed, all cancer cases showed marked edema in the lower legs in this study.

Inflammatory cytokines are deemed to be the cause of skeletal muscle atrophy [22–24]. We compared 3 cytokines, TNF- α , HMGB1, and IL-6, all reported to be markers of skeletal muscle atrophy [23, 37], with SMI and SDS-MYL1 which are reported markers of cachexia, and then

Fig. 3. Relationship among cytokines. TNF-α and IL-6 (a), TNF-α and HMGB1 (b), and IL-6 and HMGB1 (c) were compared. The relationship was examined by Spearman regression analysis.

Fig. 4. Relationship between cytokines and SDS-MYL1. TNF-α (a), IL-6 (b), and HMGB1 levels (c) were compared with SDS-MYL1. Each parameter was obtained at autopsy. The relationship was examined by Spearman regression analysis.

Table 4. Multivariate analysis of cytokines

	Coefficient	95% CI	Standard error	<i>p</i> value
SMI				
TNF-α	-0.42634181	-0.66668295 to -0.1860007	0.08656434	0.007900247
IL-6	-0.0479042	-1.19578346 to 1.0999750	0.41343488	0.913340595
HMGB1	0.23434198	0.01337157 to 0.4553124	0.07958753	0.04220148
SDS-MYL1				
TNF-α	-0.22225742	-0.3530097 to -0.09150517	0.0470934	0.00917496
IL-6	0.22681687	-0.3976613 to 0.85129503	0.22492005	0.370295586
HMGB1	-0.03424226	-0.1544563 to 0.08597177	0.04329783	0.473278847

examined the biochemical correlations. TNF- α and HMGB1 were found to be correlated with SMI and SDS-MYL1, but IL-6 was not. In contrast, a correlation was found between TNF- α and HMGB. Both TNF- α and HMGB are involved in the activation of innate immunity and proinflammatory feedback [38]. In macrophages, both of these cytokines are known to mutually promote expression and secretion, leading to the exacerbation of inflammation in rheumatoid arthritis and severe infections [38, 39].

HMGB is also secreted from colon cancer cells and is known to promote the development of cancer [40–42]. Furthermore, it causes autocrine and paracrine hyperactivation of HMGB1 and TNF- α in inflammatory cells, particularly macrophages [43]. In end-stage cancer patients, abnormal activation of the monocytic system such as hemophagocytosis syndrome may be observed, even when the infection is not clear, suggesting excessive activation of HMGB1 or TNF- α [44, 45]. HMGB1 induces autophagy in skeletal muscle through receptors for advanced glycated end-products and Toll-like receptor 4. Its role in degradation and weight loss in mouse models has been reported [23]. It has also been reported that the inhibition of HMGB1 suppresses skeletal muscle atrophy [46]. In contrast, TNF- α induces autophagy in skeletal muscles through the activation of NF- κ B [47]. The induction of autophagy by inflammatory cytokines is emphasized as a cause of skeletal muscle atrophy. The promotion of autophagy in skeletal muscle atrophy has also been supported by the decrease in SDS-MYL1. Our data did not show any correlation between HMGB1 and SDS-MYL1 in univariate or multivariate analysis. HMGB1 has a marked cytokine-activating reaction on the disulfide form [45]. The action of HMGB1 on skeletal muscle is also considered to be more accurate by measuring the disulfide HMGB1.

IL-6 has been found to be a factor that promotes skeletal muscle atrophy, and TNF- α promotes the expression of IL-6 [47]. However, in our study, no correlation was found between IL-6 and SMI or SDS-MYL1. This could be because of IL-6's bidirectional action on skeletal muscle [48], i.e., IL-6 promotes satellite cell activation and myotube regeneration, but also causes skeletal muscle wasting depending on differences in concentration and duration of action. This could be the reason why no clear association was found between IL-6 and cachexia or skeletal muscle atrophy in this study.

Cachexia and sarcopenia are extremely clinical concepts, and how to extrapolate the results of animal models and cell experiments to humans is an important issue. In

this study, although the number of cases was small, it was possible to clearly show the associations among SDS-MYL1, SMI, and cytokines. SDS-MYL1 is associated with skeletal muscle maturation [29] and reflects sarcopenia in mouse cachexia models [30]. The fact that SDS-MYL1 correlated well with SMI in this study is an important finding, suggesting that the results of SDS-MYL1 can be extrapolated to clinical sarcopenia in animal models and culture cell experiments.

In conclusion, our study results showed that serum TNF- α levels can be considered effective biomarkers for skeletal muscle atrophy. Furthermore, the interaction between TNF- α and HMGB1 is thought to induce skeletal muscle atrophy, so targeting this interaction could be useful for the prevention and amelioration of cancerous skeletal muscle atrophy and thus cancerous cachexia. In this study, a small number of cases had to be examined due to the lack of biosamples and clinical data. In the future, it is hoped that the interest in cachexia and sarcopenia in cancer patients will deepen and studies using more cases will be possible.

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Statement of Ethics

Written informed consent was obtained from the patients' families for their participation in the study, and all identifying information was removed from patient samples prior to their analysis to ensure strict privacy protection (unlinkable anonymization). All procedures were performed in accordance with the Ethics Guidelines for Human Genome/Gene Research enacted by the Japanese Government and with the approval of the Ethics Committee of Nara Medical University (approval No. 937).

Disclosure Statement

The authors have no conflict of interest to declare.

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