Concurrent Expression of CD47 and CD44 in Colorectal Cancer Promotes Malignancy

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Keywords

CD47 · CD44 · Epithelial-mesenchymal transition · Metastasis · Nivolumab

Abstract

CD47 activates signal regulatory protein alpha expressed on macrophages and suppresses its phagocytic ability; therefore, CD47 is drawing attention as an immune checkpoint in the innate immune system. Expression of CD47 in cancer is thought to allow cancer cells to escape antitumor immunity of the innate immune system. In this study, expression of CD47 was examined by immunostaining in colorectal cancer (CRC) and compared with the expression of CD44, which is a marker for cancer stem cells. In 95 cases of stage II–IV CRC, CD47 and CD44 showed overexpression in 82 and 80 cases, respectively. Both expression levels correlated with distant metastasis. Moreover, the expression of CD47 and CD44 in each case showed a significant correlation. In stage III cases, disease-free survival of cases showing high expression of CD47 and CD44 was worse than that of the cases with low expression. Furthermore, 3 of the stage IV cases were admin-

istered nivolumab, a checkpoint inhibitor of the acquired immune system, and 2 patients showed recurrence thereafter. All recurrent tumors highly expressed CD47 and CD44 and showed the epithelial-mesenchymal transition (EMT) phenotype. Our results suggest that CD47 promotes the malignancy of CRC in association with EMT and enhances the stemness of cancer cells. Moreover, our study suggests that CD47 and CD44 are involved in imparting resistance to programmed cell death (PD)-1/PD-ligand 1 inhibitors.

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Introduction

Cancer treatment has greatly advanced owing to the development of an immune checkpoint inhibitor. A currently developed immune checkpoint inhibitor promotes the cytotoxic action of T cells and brings about an antitumor effect. In addition, the innate immune system is largely involved in imparting antitumor effects. Cancer cells have a mechanism to escape antitumor immunity conferred by the innate immune system, and it has be-

come clear that it promotes the progression of cancer and resistance to antitumoral treatment [1]. We have focused on high-mobility group B-1 (HMGB1) as a factor to suppress the antitumor immunity of the innate immune system. HMGB1 is secreted from cancer cells and induces apoptosis in monocyte lineage cells at high concentrations [2]. In colorectal cancer (CRC), HMGB1 reduces the number of antitumoral macrophages [3], lymph node internal sinus macrophages, dendritic cells [4, 5], and Kupffer cells [6] in the tumor, resulting in lymph node metastasis and thus promoting liver metastasis.

HMGB1, which induces cell death in macrophages, is suppressed by CD47. CD47 is widely expressed in normal cells and acts as an immunity checkpoint in the innate immune system [7]. Signal regulatory protein α (SIRP α) that binds to CD47 is expressed in myeloid cells such as macrophages, and when phagocytes undergo immunosurveillance, their phagocytosis is suppressed when bound to CD47 expressed by other cells [8]. For this reason, CD47 is said to be a “don’t eat me” signal, and tumor cells expressing CD47 can escape the antitumor effect of the innate immune system. In particular, since cancer cells that disseminate in body fluids during metastasis are vulnerable to the host’s immune system, those cells that can escape from the immune system via CD47 have a high metastatic potential [9].

On the other hand, thrombospondin-1, which is a ligand of CD47, suppresses the expression of stem cell-related transcription factors through CD47 in normal cells. On the contrary, in cancer cells it helps to maintain stemness [10]. CD44 is a transmembrane glycoprotein that is involved in cell movement as a hyaluronan receptor and is also considered a stem cell marker in CRC together with CD133 and leucine-rich orphan G-protein-coupled receptor 5 [11, 12].

In this study, we examined the relationship between CD47 expression in CRC and malignancy of CRC. Additionally, we report the relationships between CD47 expression and immunity checkpoint inhibitors, and expressions of CD44 and CD47 using CD44 as a stem cell marker.

Materials and Methods

Patients

A total of 95 cases of invasive CRC, which were histopathologically diagnosed by the Department of Molecular Pathology, Nara Medical University (Kashinara, Japan), during 2006–2017, were randomly selected for analysis. As written informed consent was not obtained from patients for their participation in the pres-

Table 1. Expression of CD44 and CD47 in 95 colorectal carcinomas

Parameter	<i>n</i>	CD44 ²	<i>p</i> value ³	CD47 ²	<i>p</i> value ³
Normal mucosa	10	80±11		22±5	
Cancer	95	180±7	<0.0001	122±6	<0.0001
Age					
<60 years	47	173±11		128±8	
≥60 years	48	186±10	NS	116±9	NS
Sex					
Female	26	172±13		141±10	
Male	69	183±9	NS	114±8	NS
Grade ¹					
1	31	158±12		118±11	
2	42	188±10		135±8	
3	22	195±16	NS	103±13	NS
pT ¹					
2–3	56	173±10		120±8	
4	39	189±9	NS	125±8	NS
pN ¹					
0	55	169±10		108±8	
1–2	40	194±10	NS	141±9	0.0072
pM ¹					
0	83	170±7		115±6	
1	12	254±47	0.0002	167±15	0.0062
pStage ¹					
I–IIa	29	152±14		94±11	
I Ib	25	188±13		126±10	
III	29	171±10		128±11	
IV	12	250±13	0.0005	166±15	0.0041

¹ Histological grade and clinicopathological parameters are according to the UICC-TNM classification [26]. Pathological T factor (pT): pT2 (tumor invades muscularis propria), pT3 (tumor invades through the muscularis propria into the pericorectal tissues), and pT4 (tumor penetrates to the surface of the visceral peritoneum or invades other organs); pathological N factor (pN): pN0 (no regional lymph node metastasis), pN1 (metastasis in 1–3 regional lymph nodes), pN2 (metastasis in 4 or more lymph nodes); pathological M factor (pM): pM0 (no distant metastasis), pM1 (distant metastasis); pathological stage: stage IIa (cases invaded into the subserosal layer or below with no metastasis), stage IIb (cases invaded into the visceral peritoneum with no metastasis), stage III (any cases with lymph node metastasis), and stage IV (any case with or without lymph node metastases but with distant metastases); G1 (well-differentiated), G2 (moderately differentiated), G3 (poorly differentiated). ² The expression index was calculated as the staining strength score multiplied by the staining area. Normal colon mucosa = 100. ³ The *p* value was calculated by an ordinary ANOVA test. NS, not significant.

ent study, 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 Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government, and with the ap-

Fig. 1. Expression of CD47 and CD44 in CRC. **a** Expressions of CD47 and CD44 in colon cancer were examined by immunostaining. Both proteins show an immune response in the cell membrane of cancer cells. Bar, 100 μm; HM, high magnification. **b** Expressions of CD47 and CD44 in 95 cases of CRC were compared, and the correlation was examined by Spearman R. **c** Disease-free survival (DFS) of stage III cases. Disease-free survival was examined by dividing cases into groups with a high CD47 × CD44 index (CD47 × CD44: high) and a low index (CD 47 × CD44: low) in 29 stage III colon cancer cases. The survival curve was tested for significant difference by the Kaplan-Meier method.

proval of the Ethics Committee of Nara Medical University (Approval No. 937).

Immunohistochemistry

Consecutive 4-μm sections were immunohistochemically stained using the immunoperoxidase technique described previ-

ously [13], with primary anti-CD47 (Abcam, Cambridge, UK), anti-CD44 (Santa-Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-CK20 (DAKO Corp, Glostrup, Denmark), antivimentin (DAKO), anti-programmed cell death ligand (PD-L) 1, anti-human mutL homolog (hMLH)-1 (Abcam), and appropriate secondary antibodies (Medical and Biological Laboratories, Nagoya,

Table 2. Concurrent expression of CD44 and CD47 in 95 colorectal carcinomas

Parameter	<i>n</i>	CD44 × CD47 ²	<i>p</i> value ³
Normal mucosa	10	1,785±591	
Cancer	95	23,690±1,649	<0.0001
Age			
<60 years	47	24,104±2,462	
≥60 years	48	23,285±2,224	NS
Sex			
Female	26	25,330±2,953	
Male	69	23,072±1,986	NS
Grade ¹			
1	31	20,163±2,705	
2	42	26,763±2,376	
3	22	22,796±3,880	NS
pT ¹			
2–3	56	23,316±2,360	
4	39	24,228±2,190	NS
pN ¹			
0	55	19,657±1,853	
1–2	40	29,236±2,768	0.0036
pM ¹			
0	83	2,095±1,533	
1	12	42,616±5,046	<0.0001
pStage ¹			
I–IIa	29	16,368±2,594	
IIb	25	23,860±2,546	
III	29	20,360±2,650	
IV	12	42,616±5,046	<0.0001

¹ Histological grade and clinicopathological parameters are according to the UICC-TNM classification [26]. Pathological T factor (pT): pT2 (tumor invades muscularis propria), pT3 (tumor invades through the muscularis propria into the pericorectal tissues), and pT4 (tumor penetrates to the surface of the visceral peritoneum or invades other organs); pathological N-factor (pN): pN0 (no regional lymph node metastasis), pN1 (metastasis in 1–3 regional lymph nodes), pN2 (metastasis in 4 or more lymph nodes); pathological M factor (pM): pM0 (no distant metastasis), pM1 (distant metastasis); pathological stage: stage IIa (cases invaded into the subserosal layer or below with no metastasis), stage IIb (cases invaded into the visceral peritoneum with no metastasis), stage III (any cases with lymph node metastasis), and stage IV (any case with or without lymph node metastases but with distant metastases); G1 (well-differentiated), G2 (moderately differentiated), G3 (poorly differentiated). ² The expression index was calculated as the staining strength score multiplied by the staining area. Normal colon mucosa = 100. ³ The *p* value was calculated by an ordinary ANOVA test. NS, not significant.

Japan) (all 0.2 µg/mL). The tissue sections were then color-developed with diamine benzidine hydrochloride (DAKO, Glostrup, Denmark) and counterstained with Meyer's hematoxylin (Sigma). We counted cells that exhibited immunoreactivity at the cytoplasmic membrane and scored staining strength between 0 to 3 (where

Table 3. Relation of CD47 × CD44 expression with colorectal carcinoma metastasis

CD47 × CD44 index	<i>n</i>	ly+	N+	v+	M+
1st–25th percentile	24	20	8	12	0
26th–50th percentile	23	23	8	14	2
51st–75th percentile	24	24	7	23	1
76th–100th percentile	24	24	9	24	9
<i>p</i> value		0.0063	NS	<0.0001	0.0003

ly, lymph vessel infiltration; N, nodal metastasis; v, venous infiltration; M, distant metastasis. The *p* value was calculated by the χ^2 test. NS, not significant.

a score of 1 was used to describe the expression level in a normal colonic epithelium). The staining index was then calculated as the staining strength score multiplied by the staining area (%).

Statistical Analysis

Statistical significance was calculated using an ordinary ANOVA, Kaplan-Meier test, and Spearman correlation test (InStat software, GraphPad, Los Angeles, CA, USA). A two-sided *p* value of <0.05 was considered to indicate statistical significance.

Results

Immunostaining Study of Expression of CD47 and CD44 in CRC

Expression levels of CD47 and CD44 were examined by immunostaining for 95 cases of CRC (Fig. 1a, Table 1). In 95 cases of stage II–IV CRC, CD47 and CD44 showed overexpression in 82 and 80 cases, respectively. Both CD47 and CD44 showed expression on the cell membrane of cancer cells (Fig. 1a). Although heterogeneity was observed in the tumor, certain characteristics were not observed in the distribution of cancer cells showing high expression.

As shown in Table 1, the expressions of CD47 and CD44 were compared with clinicopathological factors. Both CD47 and CD44 showed higher expression than normal tissues; especially, the expression level of CD47 increased 5.5-fold compared to normal tissues. Both CD47 and CD44 showed no correlation with tissue grade and local invasion (pT), whereas distant metastasis showed a significant correlation between both. Furthermore, correlation was found in lymph node metastasis in CD47. As a result, there was a positive significant correlation between the CD47 or CD44 and metastasis of CRC.

Table 4. Expression of immune checkpoint proteins before chemotherapy and molecular targeting therapy in stage IV colorectal carcinoma

Case	Age, years	Sex	Histological grade ¹	pT ¹	pN ¹	pM	CD44 ²	CD47 ²	PD-L1, %	MLH1	Nivolumab treatment
1	62	M	2	3	1	H	180	200	3	Positive	
2	68	M	3	4	0	H	200	50	64	Negative	+
3	62	M	3	4	2	H	200	200	45	Negative	+
4	55	M	1	3	2	HL	200	90	12	Positive	
5	22	F	3	4	2	P	210	180	28	Positive	
6	50	M	2	2	1	HL	270	100	33	Positive	
7	69	M	2	4	2	HP	270	180	25	Positive	
8	71	F	2	3	1	L	270	200	53	Positive	+
9	34	M	3	3	1	HL	300	200	0	Positive	
10	44	M	1	3	1	HLP	300	200	36	Positive	
11	48	F	2	3	1	HL	300	200	44	Positive	
12	54	M	3	3	1	HL	300	200	0	Positive	

¹ Histological grade and clinicopathological parameters are according to the UICC-TNM classification [26]. H, liver metastasis; L, lung metastasis; P, peritoneal metastasis. ² The expression index was calculated as the staining strength score multiplied by the staining area. Normal colon mucosa = 100.

Concurrent Expression of CD47 and CD44

Since both CD47 and CD44 were correlated with distant metastasis of CRC, the expression levels of CD47 and CD44 were compared in each case (Fig. 1b). We observed a significant correlation between the expressions of both proteins and confirmed coexpression.

To examine the significance of coexpression, the value (CD47 × CD44 index) obtained by integrating the expression of CD47 and CD44 in each case was compared with clinicopathological factors (Table 2). The CD47 × CD44 index correlated with lymph node metastasis, distant metastasis, and stage and was significantly higher in comparison with CD47 or CD44 alone.

Furthermore, 95 cases were divided into 4 groups according to the value of the CD47 × CD44 index, and the association with metastasis and histological vascular invasion was examined (Table 3). Lymphatic invasion correlated with lymphoid metastasis, and this correlation was significantly greater in the high index group. However, there was no significant difference in lymph node metastasis. On the other hand, venous invasion and distant metastasis correlated with hematogenous metastasis and were frequently observed in the high index group.

In order to confirm the effect of coexpression of CD47 and CD44 on the prognosis, disease-free survival (DFS) was examined in the case of stage III. All 29 cases of stage III underwent curative resection and received 5-fluorouracil-based chemotherapy after surgery. 29 patients

were divided into two groups with CD 47 × CD44 index values, and DFS was compared between 14 high index and 15 low index groups (Fig. 1c). Incidentally, the number of stage IIIb cases included in each group was not significant in 9 high index and 7 low index groups. The number of the recurrent cases was only 1 in the low index group, whereas in the high index group there was a significant recurrence rate in 7 cases. Furthermore, DFS was significantly shorter in the high index group. Thus, coexpression of CD47 and CD44 was strongly associated with poor prognosis of CRC.

Expression of CD47 and CD44 in Nivolumab-Refractory Cases

In 2 of the nivolumab-refractory cases, both CD47 and CD44 were highly expressed, and an epithelial-mesenchymal transition (EMT) phenotype was observed (Fig. 2, Table 4). Of the 95 cases of CRC examined in this study, 3 cases of multiple liver metastasis received an anti-PD-1 antibody, nivolumab, as a third line (cases 2, 3, and 8 in Table 4). Metastatic lesion shrinkage was observed in all 3 cases; however, in 2 of them (cases 2 and 8), regrowth was observed. When examining the tumor after regrowth, the cancer cells showed unicellular proliferation lacking cell adhesion. In immunostaining as well as cell morphology analyses, EMT phenotypes such as decreased cyto-keratin 20 (CK20, epithelial marker) expression and enhanced vimentin (mesenchymal marker) expression were

Fig. 2. Expression of CD47 and CD44 and the EMT phenotype in nivolumab-resistant cases. **a** Histological examination of the tumor tissue before nivolumab administration (PRE, HE) and gene expressions (PD-L1, hMLH1). **b** Tissue type after administration (POST, HE) and gene expressions (CD47, CD44, CK20, vimentin, PD-L1) after administration in nivolumab-resistant colon cancer investigated by immunohistochemistry. Bar, 100 μm. PD-L1, programmed cell death ligand-1; hMLH1, human MutL homolog 1; CK20, cytokeratin 20. **c** Scatter plot analysis between CD47 expression and PD-L1 positivity in stage IV CRC tumors. Notably no correlation was found between them.

found in cancer cells. Furthermore, CD47 and CD44 were diffusely expressed in cancer cells, whereas PD-L1 expression was reduced remarkably. These results suggested that resistance to PD-1 inhibitor, enhancement of stemness, and linkage of EMT phenotype with high expression of CD47 could all be linked.

In the above case, expressions of CD47 and PD-L1 showed an inverse correlation. We then finally examined the relationship between CD47 expression and PD-L1 positivity before treatments other than resection of the primary tumor in the stage IV tumors (Fig. 2c). There was no correlation between them.

Discussion

In our study, both CD44 and CD47 were highly overexpressed in CRC compared to that in normal tissues. In particular, the expression level for CD47 was higher than in normal tissues. High expression of CD47 has been reported in many cancers [8], which is consistent with our results.

Our study on the relationship between the expression of CD44 and CD47 and clinicopathological parameters indicated that their expressions were correlated with distant metastasis. Additionally, CD47 expression correlated with lymph node metastasis. Expression of CD47 was examined in 12 cases of intramucosal cancer; however, no overexpression was observed. Even in individual cases and in cases in which both CD44 and CD47 were highly expressed (high CD44 $\times$ CD47 values), distant metastasis was observed at a higher frequency than in other cases.

CD44 is a known stem cell marker; however, it is known to provide stemness to colon cancer cells [14]. Furthermore, stemness of cancer cells correlates with metastatic potential [15]. On the other hand, CD47 inhibits phagocytosis of macrophages by activating SIRP α protein expressed on the membrane surface of macrophages. These phenotypes are thought to lead to deviation from surveillance and high survivability, especially during migration in the blood which is likely to be trapped in the surveillance of the host antitumor immune system, among other processes of metastasis formation. It also suggests that the degree of venous invasion in the primary tumor is high.

Based on the DFS examination in stage III patients undergoing postoperative chemotherapy, the recurrence rate was significantly higher and the DFS was poor in half the cases with high CD44 $\times$ CD47 expression compared to half the cases with a lower expression index. Based on these results, it is clear that not only metastasis is observed in CD44 and CD47 coexpression cases, but also prognosis is poor in comparison with stages. It is thought that recurrent metastasis is likely to occur due to the high metastatic potential, and it supports the fact that both recurrent cases are distant metastases. Furthermore, low responsiveness to anticancer agents due to high stemness is also considered to be a cause of poor prognosis.

It was shown that the coexpression of CD44 and CD47 correlates with the malignancy of CRC. However, when compared with each other, a significant correlation was found between the expression of CD44 and CD47. Myc and nuclear respiratory factor 1 have been reported to promote CD47 expression [16–18]. CD44 regulates gene

expression as nuclear CD44/acetylated-STAT3 and induces CD47 expression [14]. In addition, activation of the β -catenin transcription factor pathway leads to overexpression of CD47 [19], as the catenin transcription factor 4 binding site is present in the CD47 promoter region. Since β -catenin abnormality is frequently observed in colon cancer, CD47 overexpression is likely to occur in CRC. On the other hand, CD44 is induced to be expressed via Wnt5a, a noncanonical pathway [20].

More interestingly, it was suggested that resistance to PD-1 inhibitors, enhancement of stemness, and the high possibility of linking EMT phenotype with high CD47 expression may be derived from a case study in nivolumab administration. In triple-negative breast cancer, it has been reported that cancer cells expressing CD47 and PD-L1 selectively remain by anticancer drug treatment [21].

Expression of both CD47 and PD-L1 is promoted by Myc [16], expression of checkpoint molecules of both acquired and innate immunities were induced in recurrent cancer cells after multiple chemotherapeutic regimens. In our data, no correlation was detected in the expressions of CD47 and PD-L1 in stage IV tumors. In such advanced cases, imbalance might have occurred in checkpoints between innate and acquired immunities. Such immune checkpoint molecules inhibit host antitumor immunity; however, they also acquire the EMT phenotype, stemness, enhance viability, promote drug resistance, and promote cancer malignancy [22].

In CRC cells, mutator phenotypes, such as the microsatellite instability-high phenotype in Lynch syndrome or in patients with sporadic promoter methylation of the MLH1 promoter region, are categorized in different molecular subtypes with high sensitivity to immunoreactivity [23]. The cases of the mutator phenotype show a good response to the immune checkpoint inhibitors [24]. We then examined the expression of hMLH1 in the stage IV tumors by immunohistochemistry. Nivolumab was administered to the 3 cases with high PD-L1 positivity in the primary tumors. In the 3 cases, 2 cases showed no nMLH1 expression, which suggests that these might be the mutator phenotype. Indeed, tumor repression was provided by nivolumab. However, 3 cases of the mutator phenotype showed no association with expressions of CD47 or PD-L1. The molecular subtypes of CRC should be examined in further studies.

Our results suggest that cancer cells can survive by means of checkpoint molecules of innate immunity, even when the checkpoint molecules of acquired immunity are inhibited. Anti-PD-1/PD-L1 antibody drugs are also effective [25] in mismatch repair gene abnormal cases of

CRC that are resistant to standard therapy. An increase in cases administered anti-PD-1/PD-L1 antibody drugs might induce refractoriness to the immune checkpoint inhibitors with high CD47/CD44 expressions. It is important to establish novel molecular target therapies targeting CD47 in the future.

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