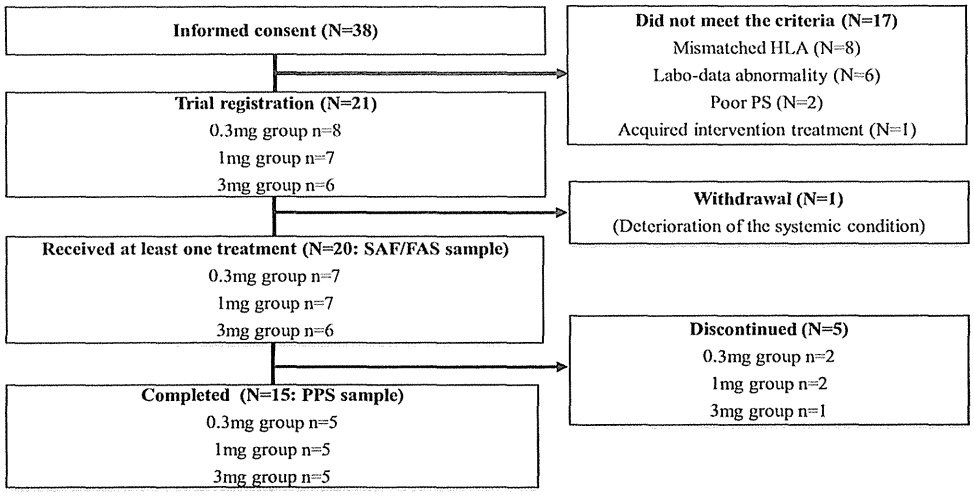

Figure 2: Enrollment of patients

Thirty-eight patients were assessed for eligibility and 21 were initially enrolled in this trial. One patient was withdrawn before the first vaccination due to deterioration of the systemic condition. Twenty patients who received at least one vaccination were evaluated for safety as the full analysis set. Five patients discontinued halfway through the protocol due to progression of the disease. Fifteen patients received the complete regimen and were evaluated for efficacy of the vaccine as the per protocol set. SAF: Safety Analysis Set, FAS: Full Analysis Set, PPS: Per Protocol Set, HLA: Human Leukocyte Antigen, PS: Performance Status.

Table 2: Profiles and clinical outcomes of patients who completed the regimen

Clinical Background						Immunological Response		Antitumor Response		
Dose	Age	Gender	Origin	Status	HLA class I	Tetramer increase	ELISPOT increase	RECIST	CEA	CA19-9
0.3 mg	63	Woman	Pancreas	Inoperable	+	35	-17	**SD**	**Decreased**	**Decreased**
0.3 mg	69	Woman	Pancreas	Inoperable	+	5	-31	**SD**	WNL	Increased
0.3 mg	53	Woman	Pancreas	Post-op	-	7	6	PD	Increased	Increased
0.3 mg	68	Man	Pancreas	Post-op	+	8	17	PD	Increased	Increased
0.3 mg	78	Man	Colon	Post-op	+	-4	2	PD	Increased	Increased
1.0 mg	61	Man	Pancreas	Inoperable	+	21	-1	**SD**	Increased	Increased
1.0 mg	84	Woman	Colon	Post-op	+	28	14	**SD**	Increased	Increased
1.0 mg	69	Man	Stomach	Post-op	+	7	26	**SD**	Increased	Increased
1.0 mg	59	Man	Colon	Post-op	+	29	16	PD	Increased	Increased
1.0 mg	62	Man	Colon	Post-op	+	15	2	PD	Increased	WNL
3.0 mg	41	Woman	Pancreas	Post-op	+	12	158	**SD**	WNL	**Stable**
3.0 mg	66	Man	Pancreas	Inoperable	+	9	19	**SD**	**Decreased**	Increased
3.0 mg	64	Man	Pancreas	Post-op	+	2	-16	**SD**	WNL	**Decreased**
3.0 mg	50	Man	Pancreas	Post-op	+	9	21	PD	WNL	Increased
3.0 mg	64	Woman	Pancreas	Inoperable	+	0	10	PD	Increased	Increased

Post-op: Post-Operative, SD: Stable Disease, PD: Progressive Disease, WNL: Within the Normal Limit

CR. The disease control rate was 53.3%. Among the 8 patients who were defined as having SD, the CEA levels and the CA19-9 levels were decreased or at least stable during vaccination in 2 patients and 3 patients, respectively. The CEA levels stayed within the normal range (0~5.9ng/ml) throughout the study in 4 patients, and the CA19-9 level stayed within the normal range (0~37 U/ml) in one patient. It was noted that all three patients who had undergone immunotherapy before the registration had PD. Moreover, the result for one patient who had HLA class I-negative cancer tissue was also PD.

Tetramer assay and ELISPOT assay

We investigated whether the SVN-2B peptide vaccination could actually induce specific immune responses in the enrolled patients. The peptide-specific CTL frequencies in PBLs before the first vaccination (CTLpre) and after the fourth vaccination (CTLpost) were assessed using the HLA-A24/SVN-2B tetramer, and the tetramer increase (CTLpost-CTLpre) was calculated (Table 2). The HLA-A24/HIV peptide (RYLRDQQLL) tetramer was used as a negative control. SVN-2B-specific CTL frequencies were increased after the vaccination in all patients except two who had undergone immunotherapy before the registration. We compared the tetramer increases between the PD group (non-responders) and SD group (responders). The mean tetramer increase of the SD group was higher than that of the PD group (Figure 3A), although there was no statistical significance

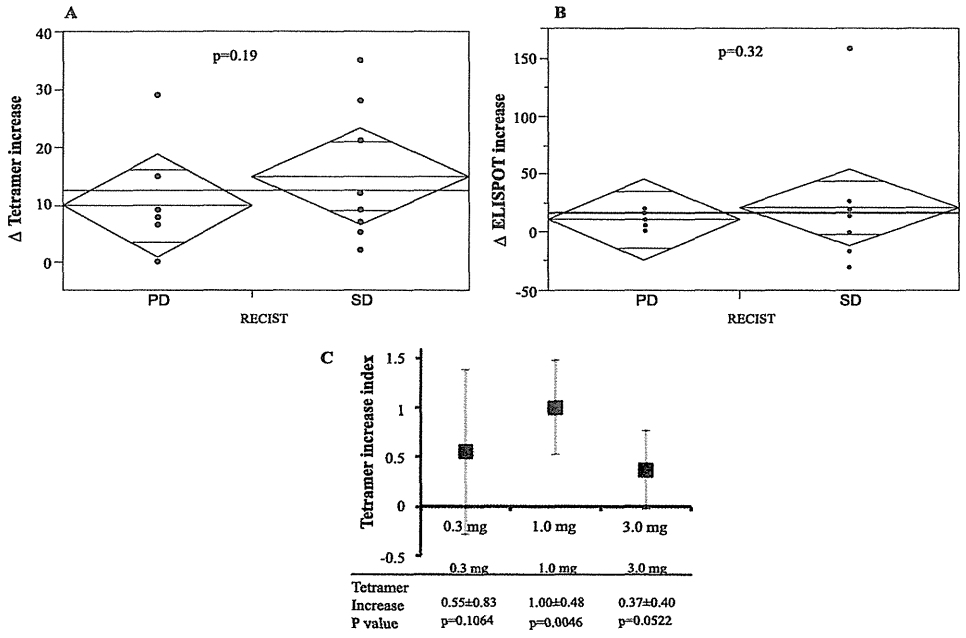

Figure 3: Tetramer assay and ELISPOT assay

(A) The tetramer increase (CTLpost-CTLpre) was calculated from the peptide-specific CTL frequency in PBLs before the first vaccination (CTLpre) and after the fourth vaccination (CTLpost) using the HLA-A24/SVN-2B tetramer. The mean tetramer increases of the PD (non-responders) and SD groups (responders) were compared. (B) The ELISPOT increase was calculated from the numbers of the peptide-specific IFN-γ spots before the first vaccination and after the fourth vaccination. The mean ELISPOT increases of the PD and SD groups were compared. (C) The mean tetramer increase index was calculated according to the following formula: Tetramer increase index=Log10(1+CTLpost)-Log10(1+CTLpre). The mean tetramer indices of the three groups (0.1mg dose, 1.0mg dose, and 3.0mg dose) were compared.

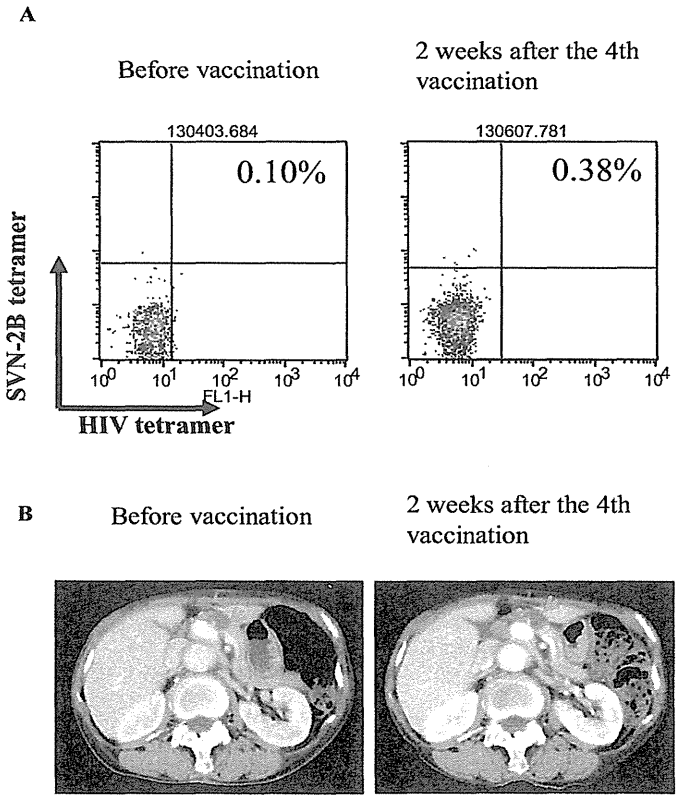

Figure 4: Tetramer assay and CT scan images of the patient with a metastatic pancreatic tumor

An 84-year-old woman with primary colon cancer and metastatic pancreatic tumor. (A) Tetramer assay before vaccination (left panel) and 2 weeks after the 4th vaccination (right panel). (B) CT scan images before vaccination (left panel) and 2 weeks after the 4th vaccination (right panel). The arrowhead indicates the metastatic pancreatic tumor. The tumor grew slightly from 16 mm to 17mm during the 8 weeks of the study.

(p=0.19). To determine the optimal dose of the peptide to induce specific CTLs in patients, the mean tetramer increase indices of the three groups (0.1mg dose, 1.0mg dose, and 3.0mg dose) were compared (Figure 3C). It was found that 1.0mg was the most effective dose for the induction of peptide-specific T cells after the fourth vaccination (p=0.0046).

We also analyzed the peptide-specific IFN-γ responses of CD8-positive T cells by ELISPOT assay. The HIV peptide (RYLRDQQLL) was used as a negative control. The numbers of peptide-specific IFN-γ spots before the first vaccination and after the fourth vaccination were counted, and the ELISPOT increase was calculated (Table 2). There was no significant difference in the mean ELISPOT increase between the SD group and PD group (Figure 3B).

Overall, this study suggests that the immunological response of the vaccine is well represented by tetramer assay rather than ELISPOT assay and that the immunological responses, at least in some patients, appropriately reflect the antitumor responses.

A Case Study

An 84-year-old woman who had primary colon cancer and metastatic liver and pancreatic tumors received the 1.0 mg dose of the SVN-2B vaccine. CT images and tetramer staining data are shown in Figure 4. In this case, the clinical response was defined as SD, and the peptide-specific CTL frequency was increased after the vaccination (Figure 4A). The metastatic pancreatic tumor barely changed from 16 mm to 17 mm during the 8 weeks of the study (Figure 4B). She continued the vaccination after the study. After 6 months, the pancreatic tumor size had increased by 31%, and a new lesion appeared in the caudate lobe of the liver. The time to progression was 267 days. There was no treatment-related AE and she could maintain high quality of daily life for almost one and a half years.

Discussion

The present study demonstrated the safety and clinical efficacy of the survivin-2B peptide vaccine for patients with advanced gastrointestinal cancer. However, the efficacy of vaccination with the SVN-2B peptide plus oil adjuvant Montanide ISA51VG was limited and not sufficient to elicit overt clinical responses. It is obvious that superior clinical and immunological responses are necessary for cancer immunotherapy. It should be considered that vaccination in combination with immunostimulatory adjuvants or cytokines may lead to greater immune and clinical responses. We have reported that type I interferon (IFN) can enhance the antitumor and immunological responses of the peptide vaccine [19,20]. On the basis of the results in this phase I study, we have started a phase II study of the SVN-2B peptide vaccine in combination with IFN-γ.

Immunomonitoring revealed that the tetramer increases were well correlated with antitumor responses as compared with ELISPOT analysis. Therefore, we used the tetramer increase as an index of vaccine-specific immune responses and determined the optimal peptide dose. A significantly higher frequency of tetramer-positive CTLs was induced in the 1mg dose group. However, the optimal dose may vary depending on conditions such as the vaccination interval and combination with distinct adjuvants and/or cytokines, and may have to be reevaluated in combination with IFN. It is enigmatic why the 3mg dose vaccination caused less induction of the peptide-specific CTLs. It was reported previously that persistent vaccine depots could induce sequestration, dysfunction and depletion of antigen-specific CTLs [27]. That may explain, at least in part, the mechanism of the bell-shaped dose effect of the antigenic peptide.

Three patients with a history of immunotherapy such as a dendritic cell vaccine and certain peptide vaccine failed to respond to the SVN-2B peptide vaccine clinically and immunologically. It is possible that their cancers may have had immunoescape phenotypes, thereby maintaining resistance to the vaccine as well as the prior immunotherapy. Alternatively, prior immunotherapy might have affected the immune system, thereby inducing tolerance against the vaccination. In any case, a history of immunotherapy was considered to be a predictive factor for a worse response, and was therefore added to the exclusion criteria in the ongoing phase II clinical study.

In conclusion, we demonstrated the safety and clinical efficacy of the SVN-2B peptide vaccine for patients with advanced gastrointestinal cancer, although clinical interpretation of the results was limited due to this being a phase I study with a small number of patients. At present, a phase II study (UMIN000012146) of the SVN-2B peptide vaccine for advanced pancreatic cancer is ongoing in combination with IFN-γ.

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Prognostic value of HLA class I expression in patients with colorectal cancer

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Abstract

Background: Prognostic factors are useful for determination of the therapeutic strategy and follow-up examination after curative operation in cancer treatment. The immunological state of the host can influence the prognosis for cancer patients as well as the features of the cancer. Human lymphocyte antigen (HLA) class I molecules have a central role in the anti-cancer immune system. Therefore, we focused on the HLA class I expression level in cancer cells to investigate its prognostic value in patients with colorectal cancer.

Methods: We reviewed the clinical pathology archives of 97 consecutive patients with stage II colorectal cancer who underwent curative operation at the Sapporo Medical University, Japan, from February 1994 to January 2005. Fifty-six high-risk patients had adjuvant chemotherapy. The cancer cell membrane immunoreactivity level for HLA class I expressed by EMR8-5 was classified into three categories (positive, dull, and negative). In this study, the cases were divided into two groups: "positive" and "dull/negative". HLA class I expression level and clinicopathological parameters were evaluated with the Pearson χ^2 test. Survival analysis was assessed by the Kaplan-Meier methods, and the differences between survival curves were analyzed using the log-rank test.

Results: Immunohistochemical study of HLA class I revealed the following. There were 51 cases that were positive, 40 were dull, and six negative. The HLA class I expression level had no significant correlation with other clinicopathological parameters, except for gender. Univariate and multivariate analyses related to disease-free survival (DFS) revealed that tumor location, HLA expression level, and venous invasion were significant independent prognostic factors ($P < 0.05$). The 5-year DFS rates in HLA class I positive group and in the dull/negative group were 89% and 70%, respectively. For high-risk patients with adjuvant chemotherapy, the 5-year DFS rates in the HLA class I positive group and in the dull/negative group were 84% and 68%, respectively. For low-risk patients without the chemotherapy, the
5-year DFS rates in the HLA class I positive group and in the dull/negative group were 100% and 71%, respectively.

Conclusions: Our study concluded that the HLA class I expression level might be a very sensitive prognostic factor in colorectal cancer patients with stage II disease.

Keywords: HLA class I, Colorectal cancer, Prognostic factor, Relapse, Disease-free survival

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Background

Recently, the number of patients with colorectal cancer is increasing. Colorectal cancer is the third most common cancer and the fourth most frequent cause of cancer death worldwide [1]. In Japan, colorectal cancer is the third leading cause of cancer-related death.

The prognosis is related to many histopathological and clinical parameters, with the most important prognostic factor affecting survival for patients undergoing curative operation being the presence or absence of regional lymph node involvement [2]. Therefore, it is generally recommended that patients with stage III colorectal cancer, which includes regional lymph node metastases, should undergo adjuvant chemotherapy. However, controversy still exists regarding the necessity of adjuvant chemotherapy for node-negative patients with stage II disease [3]. The QUASAR trial demonstrated that adjuvant chemotherapy with fluorouracil/leucovorin (FU/LV) could improve survival of patients with stage II colorectal cancer, although the absolute improvements were small [4]. Pooled analysis (IMPACT B2) of randomized trials comparing groups with adjuvant chemotherapy receiving FU/LV and those with surgery alone demonstrated that there was no significant difference in event-free and overall survival [5]. Meanwhile, O'Connor et al. reported that no 5-year survival benefit from adjuvant chemotherapy was observed for patients with stage II disease, although a benefit was observed for those with stage III disease [6]. In the present situation, adjuvant chemotherapy is conducted for patients categorized into a high-risk group among those with stage II disease on the basis of various histopathological or clinical parameters such as poorly differentiated histology, lymphovascular invasion, perineural invasion, T4 tumor stage, bowel obstruction or perforation, and an elevated preoperative plasma level of carcinoembryonic antigen (CEA) [7]. These parameters are indicated in some guidelines such as the National Comprehensive Cancer Network (NCCN), European Society for Medical Oncology (ESMO), etc. [8], although they are not based on conclusive evidence.

The immune system discriminates between self and nonself, targeting, for example, cancer cells. However, cancer cells can escape from the immune system and grow, metastasize, and finally cause death. One mechanism of the immune escape by cancer development is the downregulation of human lymphocyte antigen (HLA) class I molecules, which are cancer antigen-presenting molecules for cytotoxic T lymphocytes (CTLs) [9-12]. The immune state is of great importance in the prognosis of cancer patients. Therefore, we focused on the HLA class I expression level in cancer cells to investigate its prognostic value in patients with colorectal cancer. Since most anti-HLA class I antibodies recognize the allele-specific native structure of HLA class I molecules, these antibodies have been unable to react with denatured HLA class I molecules in formalin-fixed paraffin-embedded tissue sections. However, we created a novel monoclonal pan-HLA class I antibody, EMR8-5, suitable for the immunostaining of formalin-fixed tissue specimens [13]. Therefore, we are now able to retrospectively investigate HLA class I expression levels in cancer specimens that were surgically resected and stored for a long time.

In this study, we investigated the prognostic value of HLA class I expression in patients with stage II colorectal cancer.

Methods

Patients

The study was approved by the Clinical Institutional Ethical Review Board of the Medical Institute of Bioregulation, Sapporo Medical University, Japan. We reviewed the clinical pathology archives of 97 consecutive patients with stage II (TNM classification [UICC]) colorectal cancer (61 men and 36 women; age range: 31–83 years) who underwent curative operation, defined as the removal of all of the tumoral masses, the absence of microscopic residual tumors, histology-negative resection margins, and lymphadenectomy extended beyond the involved nodes at the postoperative pathologic examination, at the Sapporo Medical University Hospital, Sapporo, Japan, from February 1994 to January 2005. Written informed consent was obtained from each patient according to the guidelines of the Declaration of Helsinki. Fifty-six patients with poorly differentiated histology or positive lymphovascular invasion had adjuvant chemotherapy. These patients were randomly assigned to receive 5-FU plus daily divided dose cisplatin (5-FU, 320 mg/m^2 daily for 21 days; CDDP, 3.5 mg/m^2 daily for 21 days) followed by oral 5-FU (200 mg/body daily for 2 years) or oral 5-FU therapy (200 mg/body daily for 2 years) exclusively as randomized trial [14]. No patients with rectal cancer had radiotherapy. Patients whose medical reports were incomplete were excluded. The median follow-up time was 54 months. Patients' characteristics were assessed by tumor stage (stage IIA, stage IIB, and stage IIC), age, gender, tumor size, tumor location, histological type, and lymphovascular invasion.

Antibody

The monoclonal anti-pan-HLA class I antibody EMR8-5 was established at our laboratory [13]. This mouse mAb (currently commercially available from Hokudo Co., Ltd., Japan) reacts with extracellular domains of HLA-A*2402, A*0101, A*1101, A*0201, A*0207, B*0702, B*0801, B*1501, B*3501, B*4001, B*4002, B*4006, B*4403, Cw*0102, Cw*0801, Cw*1202, and Cw*1502 [15]

and shows strong reactivity in Western blots and conventional light microscopic analysis of formalin-fixed, paraffin-embedded sections.

Immunohistochemistry

Immunohistochemical staining with the antibody was performed on formalin-fixed, paraffin-embedded tissues after steam heat-induced epitope retrieval. Subsequent incubations with a secondary biotinylated antibody, avidin-conjugated peroxidase complex, and chromogen were carried out on a Ventana NexES (Ventana Medical Systems, Inc., Tucson, AZ) [16]. Slides were then counterstained with hematoxylin, rinsed, dehydrated through graded alcohols into nonaqueous solution, and coverslipped with mounting media. Positive reactivity to EMR8-5 was confirmed by staining of vascular endothelial cells and lymphocytes in sections of tumor specimens [15].

Evaluation of HLA class I expression

The cancer cell membrane immunoreactivity level for HLA class I expressed by EMR8-5 was classified into three categories (positive, dull, and negative). Positive was defined as complete and heterogeneous membrane staining in more than 80% of the tumor cells (Figure 1a). Dull was defined as faint, incomplete, and heterogeneous membrane staining in 20% ~ 80% of the tumor cells (Figure 1b). Negative was defined as membrane staining in less than 20% of the tumor cells (Figure 1c). All specimens were reviewed independently using light microscopy in at least five areas at × 200 magnification by two investigators who were blinded to the clinicopathological data (TT and YI).

Statistical analysis

We investigated the relationships between HLA class I expression levels and the other parameters (age, gender, tumor location, tumor size, depth, histological type, lymphovascular invasion, budding, number of lymph nodes analyzed after surgery (<12), HLA class I expression level, and adjuvant chemotherapy) and clinical outcome (disease-free survival: DFS). Some of these parameters (depth, histological type, lymphovascular invasion, budding, number of lymph nodes analyzed after surgery (<12)) were recommended as potential prognostic factors for curatively resected colorectal cancer by ESMO guidelines [8] or NCCN Guidelines Version 2 (2014). Statistical analysis was performed using SPSS Statistics 17.0. Deviation between the HLA class I expression level and clinicopathological parameters was evaluated with the Pearson χ^2 test. Survival analysis was assessed by the Kaplan-Meier method, and the differences between survival curves were analyzed using the log-rank test. To evaluate the correlations between the survival rate and clinicopathological parameters, univariate and multivariate regression analyses according to the Cox proportional hazards regression model were used. A P value <0.05 was considered to indicate statistical significance.

Results

HLA class I expression level and patient characteristics in patients with stage II colorectal cancer

Immunohistochemical study of HLA class I in cancer cells revealed the following. There were 51 cases (53%) that were positive, which was defined as complete and heterogeneous membrane staining in more than 80% of the tumor cells, as well as 40 (41%) that were dull, which was defined as faint, incomplete, and heterogeneous membrane staining in 20% ~ 80% of the tumor cells, and six (6%) that were negative, which was defined as membrane staining in less than 20% of the tumor cells. In this study, the cases were divided into two groups, those that were "positive" ($n = 51$) and those that were "dull and negative" ($n = 46$). The relationships between HLA class

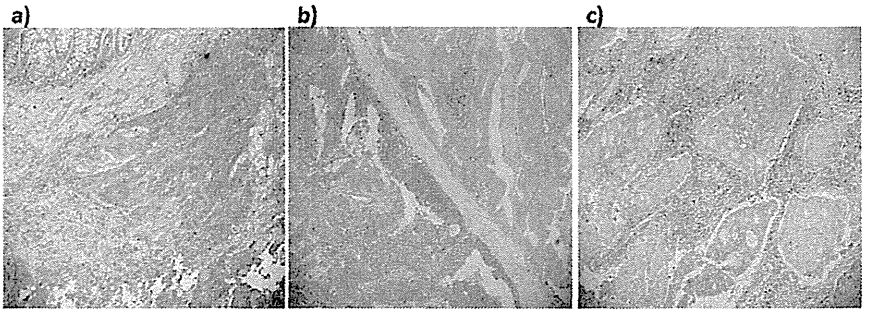

Figure 1 Representative picture of immunostaining with the antibody EMR8-5. The cancer cell membrane immunoreactivity level for HLA class I, which was expressed by EMR8-5, was classified into three categories (positive, dull, and negative). Positive was defined as complete and heterogeneous membrane staining in more than 80% of the tumor cells. Dull was defined as faint, incomplete, and heterogeneous membrane staining in 20% ~ 80% of the tumor cells. Negative was defined as membrane staining in less than 20% of the tumor cells. **(a)** Positive, **(b)** dull, and **(c)** negative.

I expression level and patients' characteristics, i.e., tumor stage (stage IIA, stage IIB, and stage IIC), age, gender, tumor size, tumor location, histological type, and lymphovascular invasion, were assessed. The HLA class I expression level had no significant correlation with other clinicopathological parameters, except for gender (Table 1).

Prognostic factors in patients with stage II colorectal cancer

Univariate analysis related to DFS revealed that the tumor location ($P = 0.01$) and HLA class I expression level ($P = 0.02$) might be significant prognostic factors among age, gender, tumor location, tumor size, depth, histological type, lymphovascular invasion, budding, number of lymph nodes analyzed, HLA class I expression level, and adjuvant chemotherapy. It also suggested that venous invasion might be a prognostic factor ($P = 0.05$). Moreover, multivariate analysis revealed that tumor location, HLA expression level, and venous invasion were significant independent prognostic factors ($P < 0.05$) (Table 2).

HLA class I expression and 5-year DFS

Univariate and multivariate analyses revealed that the HLA class I expression level might be a useful prognostic factor related to DFS. Therefore, survival analysis was conducted using the Kaplan-Meier method. The 5-year DFS rates in the HLA class I positive group and in the dull and negative (dull/negative) group were 89% and 70%, respectively ($P = 0.01$) (Figure 2).

HLA class I expression and adjuvant chemotherapy

Fifty-six stage II colorectal cancer patients with poorly differentiated histology or positive lymphovascular invasion had adjuvant chemotherapy. For patients with this

Table 1 HLA class I expression levels and characteristics of the patients (stage II colorectal cancer)

	Positive ($n = 51$; 53%)	Dull and negative ($n = 46$; 47%)	Total ($n = 97$)	*p* value
Stage				0.54
Stage IIA	46 (90)	42 (91)	88	
Stage IIB	2 (4)	0 (0)	2	
Stage IIC	3 (6)	4 (9)	7	
Age (years)				0.11
Mean ± SD	64 ± 9.7	60 ± 12.3		
Range	42 ~ 80	31 ~ 83		
Gender—no. of patients (%)				0.03
Male	27 (53%)	34 (74%)	61	
Female	24 (47%)	12 (26%)	36	
Diameter of primary tumor (mm)—no. (%)				0.87
≦30	11 (22%)	12 (26%)	23	
31–50	21 (41%)	17 (37%)	38	
≧51	19 (37%)	17 (37%)	36	
Location—no. of patients (%)				0.84
Right	16 (31%)	13 (28%)	29	
Left	15 (30%)	16 (35%)	31	
Rectum	20 (39%)	17 (37%)	37	
Histological type—no. (%)				0.23
Well/mod	48 (94%)	40 (87%)	88	
Por/muc	3 (6%)	6 (13%)	9	
Lymphatic invasion—no. of patients (%)				0.55
Negative	45 (88%)	40 (87%)	85	
Positive	6 (12%)	6 (13%)	12	
Venous invasion—no. of patients (%)				0.33
Negative	44 (86%)	42 (91%)	86	
Positive	7 (14%)	4 (9%)	11	

Table 2 Univariate and multivariate analyses related to disease-free survival in 97 colorectal cancer patients

Variables	Univariate		Multivariate	
	Hazard ratio	*P* value	Hazard ratio	*P* value
Age	0.98 (0.94–1.02)	0.38		
Gender (F)	1.42 (0.50–4.04)	0.51		
Tumor location (colon vs rectum)	4.23 (1.49–12.01)	0.01	4.11 (1.42–11.91)	0.009
Tumor size (≦5 cm)	0.64 (0.24–1.73)	0.38		
Tumor invasion (SI)	0.52 (0.12–2.28)	0.39		
Differentiation (por or muc)	1.50 (0.20–11.35)	0.70		
Lymphatic invasion (ly0, 1 vs ly2, 3)	1.10 (0.25–4.83)	0.90		
Venous invasion (v0, 1 vs v2, 3)	3.10 (1.00–9.56)	0.05	3.85 (1.15–12.92)	0.03
Budding	0.52 (0.19–1.41)	0.20		
Number of lymph nodes analyzed (<12)	1.32 (0.51–3.43)	0.57		
HLA expression level (dull or negative)	3.86 (1.26–11.85)	0.02	5.36 (1.68–17.11)	0.005
Adjuvant chemotherapy (no)	0.82 (0.30–2.22)	0.70		

chemotherapy, the 5-year DFS rates of those with HLA class I positive expression and those with dull/negative expression were compared. The 5-year DFS rates in the HLA class I positive group and in the dull/negative group were 84% and 68%, respectively (Figure 3). The 5-year DFS in patients with HLA dull/negative expression was lower than that of those with HLA positive expression, although there was no significant difference ($P = 0.10$). On the other hand, no patient with HLA class I positive expression without chemotherapy relapsed, whereas 29% of those with HLA dull/negative expression relapsed. For those without adjuvant chemotherapy, there was a significant difference in 5-year DFS between patients with HLA class I positive expression and dull/negative expression ($P = 0.03$) (Figure 4).

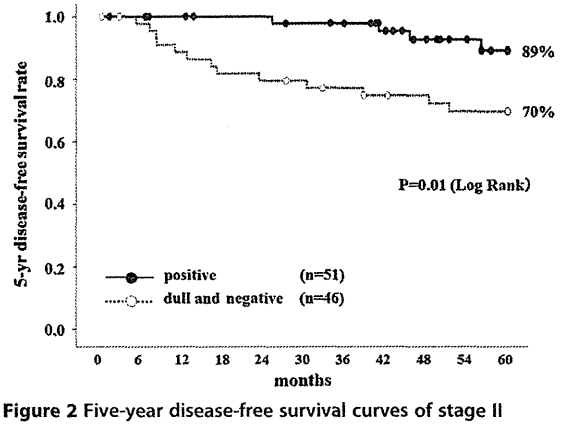

Figure 2 Five-year disease-free survival curves of stage II colorectal cancer patients. The 5-year DFS rates in the HLA class I positive group (*black circle*) and in the dull and negative group (*white circle*) were 89% and 70%, respectively. Patients with HLA class I positive expression had a significantly higher DFS rate than that of those with HLA class I dull and negative expression ($P = 0.01$).

Discussion

Prognostic factors are useful for determination of the therapeutic strategy and follow-up examination after curative operation in cancer treatment. There are various reports of clinical and pathological prognostic factors. However, there are few immunological prognostic factors. The immunological state of the host can influence the prognosis for cancer patients as well as the features of the cancer.

HLA class I molecules have a central role in the anticancer immune system, especially as cancer antigen-presenting molecules for CTLs [13]. CTLs can recognize antigenic peptides presented on the cell surface by HLA

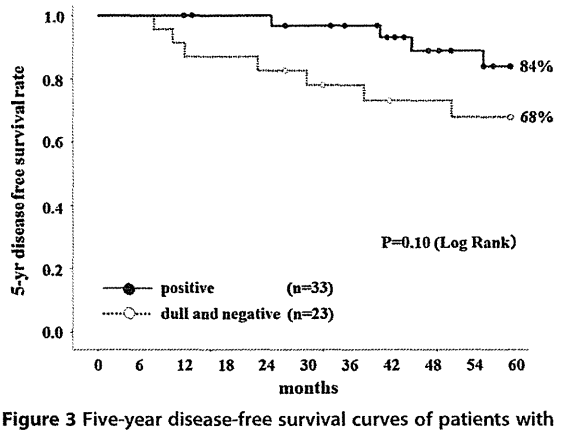

Figure 3 Five-year disease-free survival curves of patients with adjuvant chemotherapy. The 5-year DFS rates of patients with HLA class I positive expression (*black circle*) and with dull and negative expression (*white circle*) were compared. The 5-year DFS in patients with HLA dull and negative expression was decreased more than that of those with HLA positive expression, although there was no significant difference ($P = 0.10$).

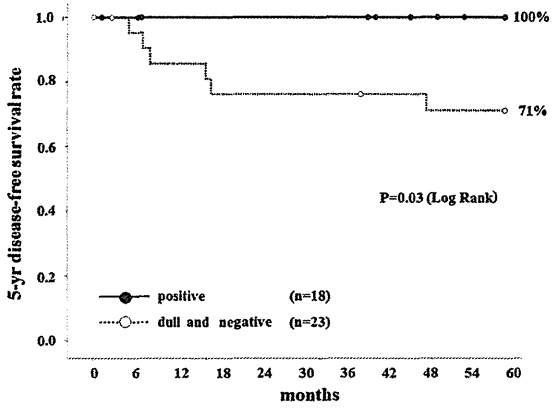

Figure 4 Five-year disease-free survival curves of patients without adjuvant chemotherapy. No patient with HLA class I positive expression (*black circle*) relapsed. Twenty-nine percent of patients with HLA dull and negative expression (*white circle*) relapsed. For patients without adjuvant chemotherapy, there was a significant difference in the 5-year DFS between patients with HLA class I positive expression and those with dull and negative expression ($P = 0.03$).

class I molecules and kill target cells such as cancer cells. However, cancer cells can escape from the immune system by downregulation of HLA class I molecules, secretion of immunosuppressive cytokines, and infiltration of immunosuppressive cells [9-13]. One mechanism of recurrence after curative operation might be immune escape by micrometastatic cancer cells. Therefore, we focused on HLA class I molecules, key molecules in the immune system, to investigate the possibility of new immunological prognostic factors. This investigation was enabled through the use of the novel monoclonal pan-HLA class I antibody EMR8-5 [13], which is suitable for the immunostaining of surgically resected, formalin-fixed tissue specimens stored for a long time.

In this study, we investigated the HLA class I expression level and the prognoses of stage II colorectal cancer patients who underwent curative operation. In patients with stage II cancer, there was a significant difference in 5-year DFS between HLA class I positive patients and dull/negative patients ($P = 0.01$). Patients with HLA class I positive expression had a higher 5-year overall survival (OS) rate than those with HLA class I dull/negative expression, although there was no significant difference ($P = 0.29$) (data not shown). In addition, univariate and multivariate analyses revealed that the HLA class I expression level might be a significant independent prognostic factor. These data suggested that the HLA class I expression level might be a useful prognostic factor, particularly as a predictive factor for relapse, in stage II colorectal cancer. The reason why there was no significant difference in OS for stage II colorectal cancer patients is speculated to be that the beneficial treatments after recurrence might have more influence on OS than the immunological state in the living body such as the HLA class I expression level.

We have also reported that the HLA class I expression level might be a prognostic factor for other cancers such as osteosarcoma, clear cell renal cell carcinoma, and bladder cancer [15-19]. Tsukahara et al. reported that patients with osteosarcoma highly expressing HLA class I had significantly better OS and DFS than those with HLA class I-negative osteosarcoma [15]. Thus, there might be a difference in the impact of the HLA class I expression level on OS or DFS depending on the cancer. Although most reports, including our study, suggested that downregulation of HLA class I expression level was associated with a poor prognosis, Madjd Z et al. reported that total loss of HLA class I was an independent indicator of good prognosis in breast cancer [20]. They considered that the loss of HLA class I might make the tumors more susceptible to natural killer (NK) killing and result in a better prognostic outcome. It is due to the presence of HLA class I allele-specific killer cell inhibitory receptors (KIRs) on the surface of NK cells. Thus, in the absence of HLA class I expression, this KIRs-mediated inhibitory signaling is lost, resulting in the activation of NK cytolytic effector functions [21]. NK cell-mediated cytotoxicity is regulated by a delicate balance between activating and inhibitory signals. So, the prognostic influence brought by the HLA class I expression level might depend on the various cancer immune circumstances.

Surgery alone has relatively favorable results in colorectal cancer patients with stage II disease; hence, any advantage conferred by adjuvant chemotherapy after the curative operation is likely to be small. However, in real life in Japan, approximately 13% of patients with stage II colorectal cancer are found to have recurrence. The seventh edition of the American Joint Committee on Cancer (AJCC) Staging Manual divides stage II into three groups: stage IIA (T3N0), stage IIB (T4aN0), and stage IIC (T4bN0). There is a report that the prognoses for the stage IIB and IIC subgroups are worse than those of some stage III patients [22]. Therefore, stage II patients could be divided into high- and low-risk populations. We should select high-risk stage II patients and give adjuvant chemotherapy to prevent recurrence by micrometastases only to those patients who can obtain a significant benefit from it. The NCCN Guidelines Version 2 (2014) recommended the following risk factors for recurrence: number of lymph nodes analyzed after surgery (<12), poorly differentiated histology, lymphatic/vascular invasion, bowel obstruction, perineural invasion, localized perforation, and close, indeterminate, or positive margins. The ESMO consensus guideline recommended

the following factors: lymph node sampling <12, poorly differentiated tumor, vascular or lymphatic or perineural invasion, T4 stage, and clinical presentation with intestinal occlusion or perforation [8]. In this study, patients with poorly differentiated tumors or moderate and severe lymphovascular invasion were considered to be high-risk stage II patients and underwent adjuvant chemotherapy. We investigated the 5-year DFS in stage II patients with and without adjuvant chemotherapy, respectively. Patients with HLA class I positive expression had a higher DFS rate than those with HLA class I dull/negative expression under both settings. In addition, for low-risk patients without chemotherapy, all patients with HLA class I positive expression did not relapse, although 29% of those with HLA class I dull/negative expression relapsed. These data might make certain of the prognostic value of HLA class I expression for relapse.

Conclusions

The HLA class I expression level might be a very sensitive prognostic factor in colorectal cancer patients with stage II disease.

Abbreviations

DFS: Disease-free survival; CEA: Carcinoembryonic antigen; CTLs: Cytotoxic T lymphocytes; ESMO: European Society for Medical Oncology; NCCN: National Comprehensive Cancer Network; NK: Natural killer; KIRs: Killer cell inhibitory receptors; AJCC: American Joint Committee on Cancer.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YI and TT reviewed all specimens stained with the antibody EMR8-5. TM and TF managed the database of colorectal cancer patients. NT performed the statistical analysis. MM and TT carried out the immunohistochemical staining. NS and KH participated in the design and coordination of this study and helped to draft the manuscript. All authors read and approved the final manuscript.

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第83回　Metabolomicsの最新情報④

肥満の分子メカニズム—オーバービュー—

札幌医科大学消化器・総合，乳腺・内分泌外科学講座

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はじめに

『肥満』とは，「肥満組織が過剰に蓄積した状態で，BMI 25 kg/m^{2}以上のもの」と定義されている。さらに，肥満と判定（BMI ≧ 25）され，

①肥満に起因あるいは関連し，減量を要する有健康障害者

②健康障害をともないやすいハイリスク肥満者

のいずれかの条件を満たすものを『肥満症』と診断する[1)2)]。現在，日本では男性で約3人に1人，女性で約5人に1人がBMI 25を超えているといわれている[3)]。ここでは肥満と密接に関係のある，摂食調節の分子メカニズムと肥満の脂肪組織におけるグルココルチコイド作用の過剰状態を中心に概説する。

脂肪細胞ホルモン，レプチン

摂食調節の分子メカニズムとして，1994年Friedmanらがレプチンを発見し[4)]，これを機に急速に解明が進んでいる。レプチン遺伝子は，21アミノ酸のシグナルペプチドを含む167アミノ酸のレプチン前駆体をコードしており，脂肪細胞で産生されたレプチン前駆体は血中に分泌され，血中においてはシグナルペプチドが切断された146アミノ酸からなる蛋白質（レプチン）として存在している[4)]。遺伝性肥満のモデルマウスであるob/obマウスでは，レプチン遺伝子の点突然変異により105番目のアミノ酸であるアルギニン残基が終止コドンに置換され，レプチンが産生されないために過食による肥満に至る[4)]。レプチン受容体は5種類のアイソフォームが確認されているが，Ob-Rbのみがレプチンのシグナルを細胞内へ伝達する（図1）[5)6)]。レプチンは脂肪細胞の肥大化にともなって分泌量の増加を認め，血中のレプチン濃度は体脂肪量を鋭敏に反映していることが明らかとなっている。視床下部がレプチンの主たる作用部位で，食欲の制御，交感神経活動の亢進を介した熱産生，褐色脂肪組織や骨格筋における糖利用の促進，脂肪酸燃焼の促進効果を生じる[7)]。しかし肥満状態ではレプチンの作用不全が生じ，血中レプチン濃度の

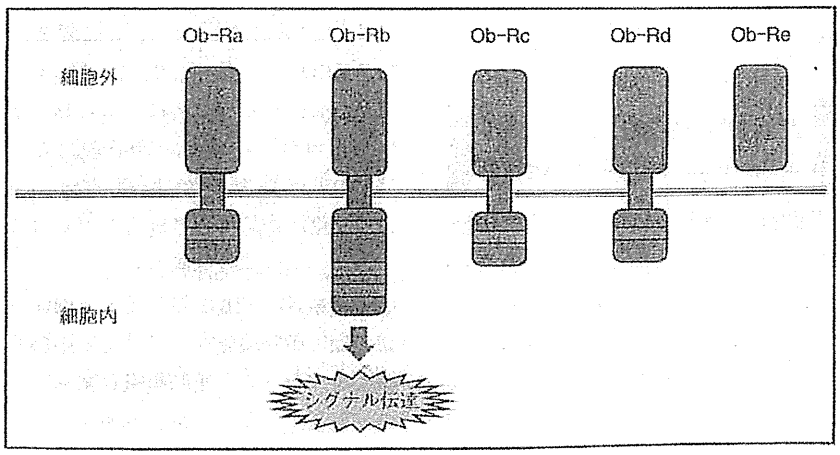

図1　レプチン受容体のアイソフォーム
（文献6より一部改変引用）

Surgery Frontier 21(4)：53-56，2014

上昇にもかかわらず，濃度に見合ったレプチンの効果が発揮されず，レプチン抵抗性を生じている[8]。肥満状態では，可溶型レプチン受容体（Ob-Re）やCRPの血中濃度が上昇し，それらが直接レプチンと結合することによってレプチンのOb-Rbへの結合が阻害される[9][10]。一方で，体重増加にともなって脳脊髄液中ではレプチン濃度が低下し，レプチンの血液脳関門通過障害がレプチン抵抗性を生じる一因とも考えられている[11]。さらにレプチン自体がOb-Rbの発現を抑制することも報告されており，長時間高レプチン濃度状態に曝露されるとOb-Rb発現量が低下すると考えられている[12][13]。また，レプチンによって本来活性化されるSTAT3（signal transducers and activators of transcription 3），PI3K（phosphatidylinositol3）などのシグナル伝達系を傷害する，SOCS（suppressor of cytokine signaling)3，PTP（protein tyrosine phosphatase)1Bの発現の増加も認められ，原因の一端を担っていると考えられる（図2）[14][15]。

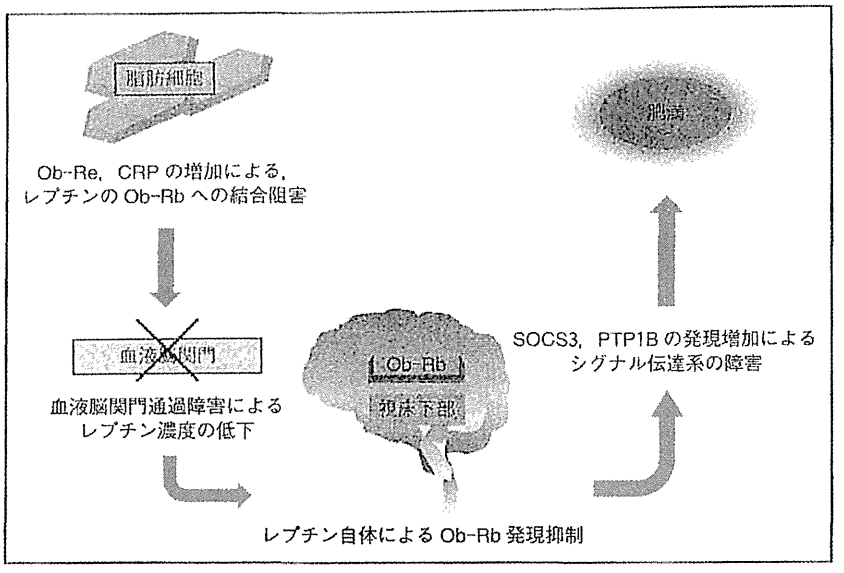

図2　レプチン抵抗性の原因

（文献14，15より引用）

肥満の脂肪組織におけるグルココルチコイド作用の過剰状態

細胞内でグルココルチコイドを活性化する変換酵素である11β-HSD（hydroxysteroid dehydrogenase)1の活性は，肥満脂肪組織内で上昇し，インスリン抵抗性指標を含む種々の代謝パラメーターと強い相関を示すことが報告されている[16]。11β-HSD1は過栄養やストレスによって誘導され，ストレス依存性肥満や肥満脂肪組織の炎症・酸化ストレスの病態に関与している[16]。血中のコルチゾール濃度は視床下部-下垂体-副腎という経路によって制御されているが，個々の細胞におけるコルチゾールの作用強度は細胞内グルココルチコイド活性化酵素，11β-HSD1と不活性化酵素，11β-HSD2のバランスによってコントロールされている[17]。脂肪細胞では11β-HSD2の発現は非常に低く，11β-HSD1が活性化されるとグルココルチコイド作用は抑制されることなく増強を続ける（図3）[18]。元来，内臓脂肪組織の脂肪細胞サイズは皮下脂肪組織に比べ小さく，脂肪組織容積あたりの細胞数も少ない。栄養過多状態では内臓脂肪組織の脂肪細胞が肥大しやすく，機能異常を生じやすい[19]。また，11β-HSD1の発現レベルは皮下脂肪組織よりも内臓脂肪組織で高く，肥満状態ではその差が顕著になる[20]。脂肪細胞で11β-HSD1を過剰発現するトランスジェニックマウスは内臓脂肪蓄積の感受性が高く，インスリン抵抗性，脂質代謝異常，高血圧，脂肪肝をともなう[16]。一方，11β-HSD1ノックアウトマウスではストレスや高脂肪食に対する肝糖新生関連酵素PEPCK（phosphoenolpyruvate carboxykinase）やG6Pase（glucose-6-phosphatase）の誘導を生じないため，糖尿病発症に対して明らかな抵抗性を示し，高脂肪食負荷やob/obマウスとの交配においても内臓脂肪の蓄積は抑制される[21]。また，脂肪組織特異的11β-HSD2トランスジェニックマウス（脂肪組織特異的11β-HSD1ノックア

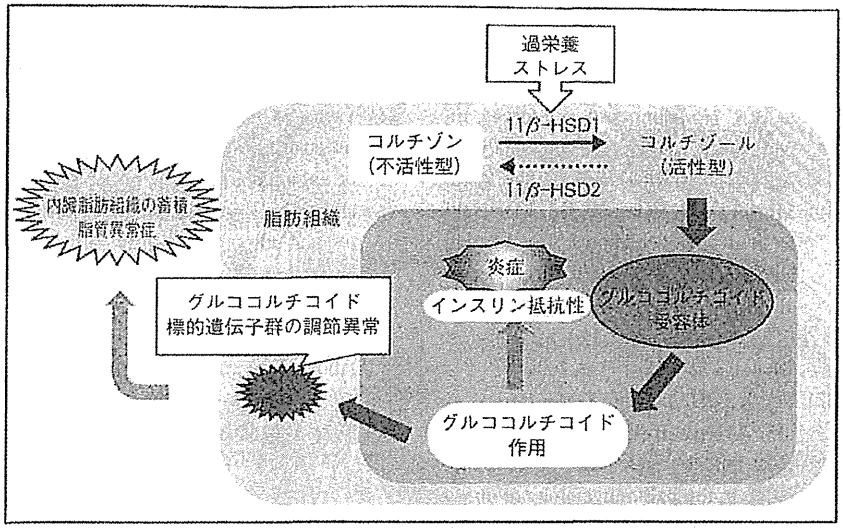

図3　11β-HSD1の活性化によるグルココルチコイド作用の増強

(文献18より引用)

ウトマウス)においても高脂肪食負荷による糖脂質代謝の悪化を認めないことから，脂肪組織で11β-HSD1を抑制することが肥満症治療に有効であることが示唆された[22]。多数例のヒト脂肪組織の解析において，肥満者では脂肪組織での11β-HSD1発現レベルの上昇を認め，ウエスト周囲長，脂質代謝指標やインスリン抵抗性指標と正の相関を示すと報告されている[23][24]。

おわりに

現在，さまざまな肥満に関する研究が進んでいる。今後肥満における分子メカニズムの全容が解明され，増加の一途をたどっている肥満患者への有効な治療法の開発へとつながることを期待する。

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特集によせて

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高等な生物の免疫機構は，自然免疫（innate immunity）と獲得免疫（acquired immunity）で成立している。脊椎動物においては，これら免疫能を相互に連携作用することにより，有効な生体防御機構が構築されている（図1）。

自然免疫はいわゆる抗原提示機能を有する細胞である樹状細胞（dendritic cell：DC），マクロファージが代表的な役割をになっている。これらの細胞は生来から備わった（子孫へと受け継がれてきた）分子を認識する受容体（pattern-recognition receptors：PRRs）を有し，受容体が異物を識別し（パターン認識機能）それを除去することを可能としている。たとえば，病原体を認識した細胞内ではシグナル伝達によって転写因子が活性化され，サイトカインや炎症反応分子を産生し，その後の一連の免疫機構へとつながる。

一方，獲得免疫は生物発生学的に魚類以上の高等動物が有する各個別の免疫能で，子孫へと受け継がれることのない能力である。リンパ球が多様な抗原を認識する能力を備えていることを背景として，その個体の抗原との人為的あるいは非人為的な接触によって識別するもので，外来（？）のペプチド配列を認識するなど高度で緻密な機能を発揮し生体防御にかかわっている。すなわち，分子を認識するにあたってリンパ球はその受容体遺伝子を再編成させることにより，抗原認識のもと，選択的識別を行うという個別化された生体防御能力である。表1に自然免疫と獲得免疫の違いを概括的に紹介した。

自然免疫とパターン認識機構

リガンドともいうべき微生物の有す

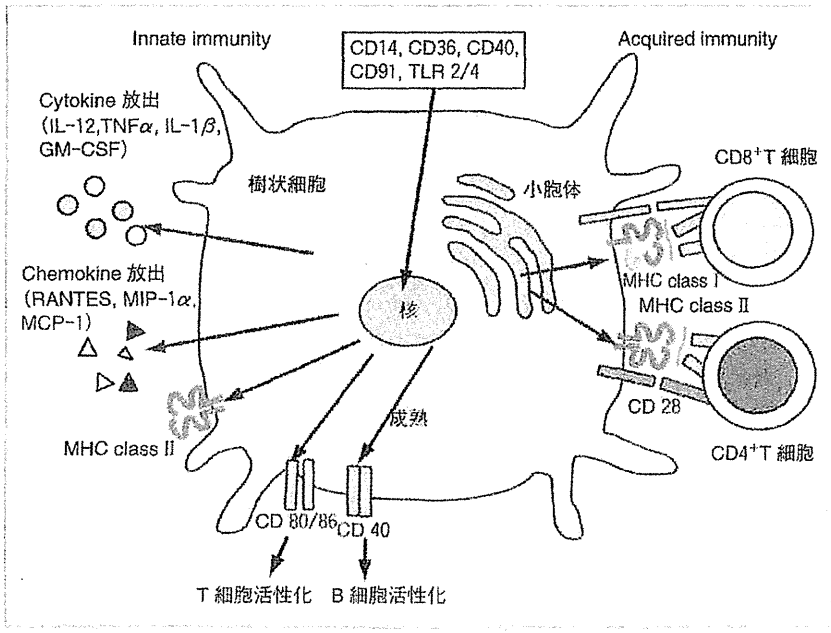

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表1　自然免疫と獲得免疫の比較

比較項目	自然免疫 (innate immunity)	獲得免疫 (adaptive immunity)
担当細胞	抗原提示細胞 (樹状細胞，マクロファージなど)	リンパ球 (T細胞，B細胞)
生物種	昆虫以上の高等進化生物	脊椎動物
反応	早い(数分ないし数時間)	遅い(数日)
受容体形成 発生時期 遺伝子組み換え レパートリー	 生来より 不可 限定	 生後の外的侵入後 可(それ自体が獲得機序) 多様
認識対象	外敵が有する共通な分子 (脂質，核酸，鉱物など)	病原体，腫瘍細胞などの構成成分 (蛋白，ペプチドなど)
個体における記憶	なし	あり

るパターン分子と受容体の解析に関する研究展開はめざましい。

以下に，パターン認識機構の概要と新知見の動向を若干なりとも臨床的視点から紹介し，後述される別稿の論文のための基本的知識として参考にされたい。

1　パターン認識機構のリガンドとパターン認識レセプター

子孫へと遺伝されてきた感染・異物に素早く反応を示すパターン認識機構は，自然免疫の根幹をなす。微生物には特有なパターン分子が存在する。抗原提示細胞のPRRsがそのパターン分子を認識し，外来の微生物に対応する機構の初動を担う。外来のパターン分子をpathogen-associated molecular patterns (PAMPs)と呼ぶ。具体的には，ウイルス由来のRNAパターンやdouble strand (ds) RNAを認識する受容体，細菌由来のmucopeptidesを認識する受容体，ペプチドグリカンのペプチド部分を認識する受容体，あるいは鉱物(尿酸血症，アスベスト，シリカ，アルミニウムなど)を認識する受容体などが次々に明らかにされている。このほか，新しい反応誘発因子として，たとえば微生物パターン分子を認識する代表的受容体として知られるToll-like receptors (TLRs)は，哺乳類ではTLRの分類として1～10が存在するのに対し，マウスではさらにTLR11, 12, 13を有することが知られている(表2)。また，細胞内で菌体成分を認識するnon-TLRsとして，Nodファミリー群，retinoic-acid-inducible gene 1 (RIG-1)，melanoma differentiation-associated gene 5 (MDA5)の存在が知られている。なお，これらについては厳密には受容体の種類が動物種間でその存在数(種類)が異なることも知られるに至っている[1]。一方，病原体により傷害を受けた自己細胞のPRRsの活性分子が遊離すると，それ自体がリガンドとなりうることも知られ，内因性パターン分子(damaged-associated molecular patterns；DAMPs)として注目されてきた。重症感染症や感染合併時の過大侵襲などでは，血中に高頻度で発現してくることが知られ，HMGB1, heat-shock protein, LDLコレステロール，βアミロイドなどが代表的なDAMPsとして知られている[2]。

2　近年注目されてきたパターン認識機構の知見

上記に示したリガンド種別のパターン認識機構の詳細が明白となっていることが注目すべき知見とされている。たとえば，ヒトTLRにおけるリガンドとそのシグナル伝達の相同性が挙げられる。その伝達経路としてMyD88経路やTICAM-1経路が明らかにされるに至っている。その詳細な説明は避けるが，概略を以下に述べたい。TLRはダイマーを形成してリガンド認識とシグナル伝達に預かる。TLR2はTLR1とTLR6がヘテロ複合体を，TLR4はTLR2とMD2が結合して安定化した受容体を形成する。図2にMyD88を“アダプター”とする各種TLRの連関性を示した。最終像としてのTNF，interferon (IFN)-α or βの産生に至るものである。TICAM-1経路はTLR3，TLR4がTICAM-1を“ア

表2 パターン認識機構：受容体とリガンド

受容体	ヒトでの存在	リガンド
TLR1	○	triacyl BLP
TLR2	○	PGN, BLP
TLR3	○	ds RNA
TLR4	○	LPS, Taxol
TLR5	○	flagellin
TLR6	○	diacyl BLP
TLR7	○	ss RNA
TLR8	○	ss RNA
TLR9	○	CpG DNA
TLR10	×（哺乳類）	?
TLR11	×（マウス）	profirin（原虫）
TLR12	×（マウス）	?
TLR13	×（マウス）	?
RIG-1	○	ss RNA, ds RNA
MDA5	○	ds RNA
NOD1	○	G（+）muropeptides
NOD2	○	G（−）muropeptides
NALP3	○	crystals, minerals

BLP：bacterial lipoprotein, PGN：peptideglycan, G：gram

ダプター”とするシグナル伝達で，IRF-3とIFN-βを誘導することが特徴とされている。また，ウイルスRNAを認識する経路として，IRS-1を“アダプター”としてIRF-3活性化を生じる。すなわち，TICAM-1経路に共通する。また，RIP-1を介してNF-$\kappa\beta$活性化経路へ連なる。また，IL-1，IL-18受容体はMyD88経路を活性化し，NF-$\kappa\beta$活性化経路へと連なる。このように紹介した上記経路群については，リガンド種別に研究されてきた歴史はあるが，研究が進むとともに経路間相互に連関性のあることが明らかとなっている。これらのパターン認識機構をどう臨床上へ応用することが可能か，興味深い示唆を得ているがいまだに明確な確証を提案した報告はみられない。

自然免疫から獲得免疫へ

自然免疫から獲得免疫への橋渡しに重要な役割を果たす細胞として，type I IFNを産生するplasmacytoid dendritic cell（pDC）が知られる[3]。このtype I IFNは，CD8Tリンパ球に作用し，キラーT細胞の分化あるいはメモリーT細胞の分化を促すほか，MHC class IあるいはIIの発現上昇など，獲得免疫系への強い影響を有する。このほか，DCの分化の結果，Th1，Th2の分化を促しTh1優位とするなど生体バランスに大きな変化をもたらす（図3）。Th1細胞は，IL-2，IFN-α，TNF-αなどを産生するTh細胞で，細胞障害性T細胞（cytotoxic T cell）やマクロファージを活性化する。その結果，「ウイルス感染細胞」や「細胞内寄生性病原体」の除去や抗腫瘍免疫反応に関与する。Th2細胞は，IL-4，5，6，9，10，13などを産生しB細胞を活性化し，主として細胞外で増殖する微生物の排除に有用となる。このほか，Th17細胞は，IL-17，21，22などを産生し細菌感染，腫瘍免疫に関与している。以上のTh1，Th2，Th17細胞は免疫応答反応として陽に作用し，エフェクターT細胞と総称している。

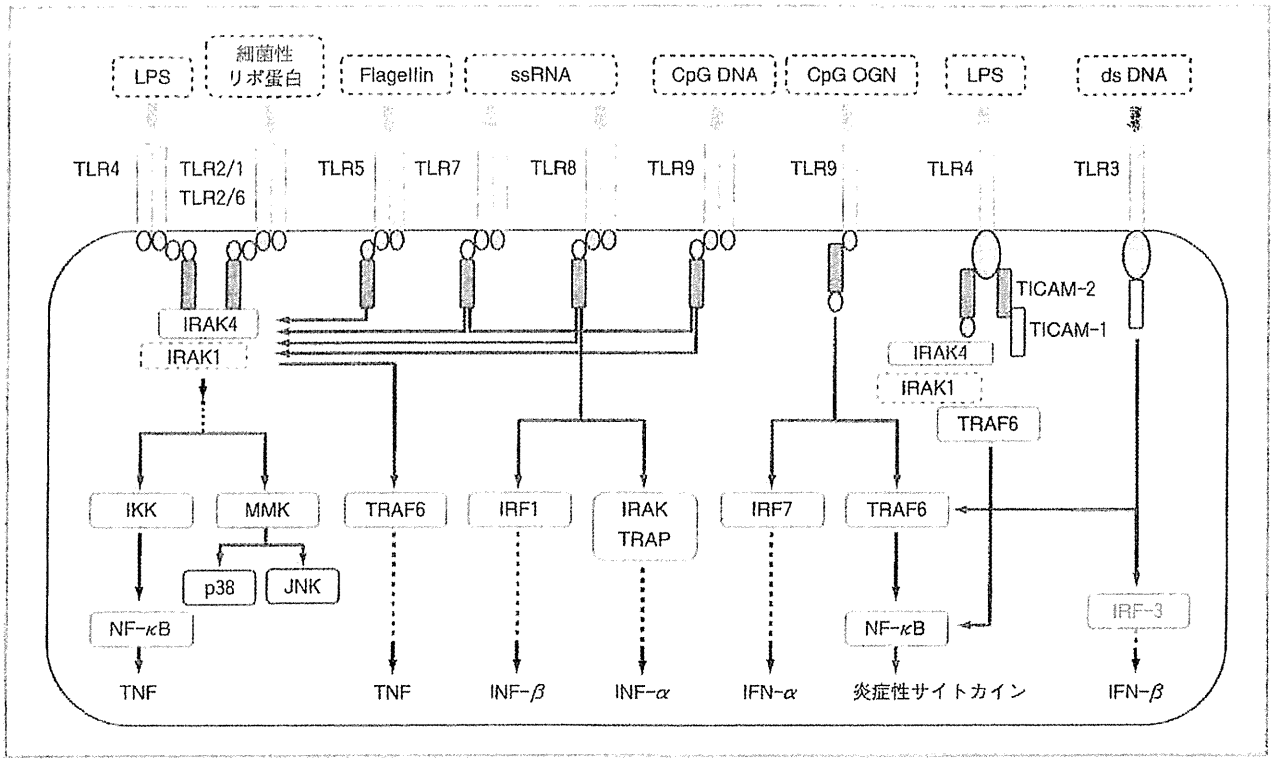

図2　パターン認識機構の主なリガンドと受容体によるシグナル伝達機構

（文献1より改変引用）

一方，負の免疫応答を担うのが抑制性T細胞（regulatory T cell；Treg）が知られている[4]。これらの活性化プロセスにおいてはIFNやサイトカインなどの産生，あるいはMHCクラスⅡ分子（MHC-Ⅱ）を認識するHLA-DRを介してCD4⁺T細胞に抗原を提示し，T細胞の活性化を誘導する。したがって単球・マクロファージのTLRやHLA-DAの発現については，自然免疫から獲得免疫の橋渡しの役割を担っているといえる。

獲得免疫とその機構

獲得免疫は，腫瘍免疫あるいはウイルスに対するワクチン療法に関する研究により急速な分析が成されている[5]。

1　獲得免疫機構の序論

上記のように自然免疫系の応答は獲得免疫系へと情報が伝搬され，細胞傷害性T細胞，エフェクターT細胞あるいはTregなど各機能別のT細胞に活性化を生じる。T細胞は，キラー（$CD8^+$）T細胞をヘルパー（$CD4^+$ T）T細胞の2つのサブセットに大別される。

$CD8^+$ T細胞はT細胞受容体（TCR）を介して，MHCクラスⅠ分子（MHC-I）に結合した抗原ペプチドの複合体を認識し，活性化されて細胞傷害性を発揮する（図4）。この際にMHC-Iに結合する複合ペプチドの由来は，ある種の細胞（たとえば，癌細胞，感染細胞など）内の蛋白質がプロテアソームによる分解を受けて生じる。これが細胞表面上のMHC-I上に移送されて，それを$CD8^+$ T細胞が特異的に認識し攻撃の標的とするものである。

$CD4^+$ T細胞は，抗原ペプチドとMHCクラスⅡ分子（MHC-Ⅱ）の複合