Modern Pathology (2009) 22, 373–384

www.modernpathology.org

Classification using hierarchical clustering of tumor-infiltrating immune cells identifies poor prognostic ovarian cancers with high levels of COX expression

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Local immune status is influenced by the tumor microenvironment. This study aims to characterize the local immune/microenvironment status by examining tumor-infiltrating immune cells, as well as cyclooxygenase (COX) expression in tumor cells, and to analyze the relationship with the prognosis of ovarian cancers. Using immunohistochemical staining of 70 ovarian cancer specimens, the numbers of CD8+, CD57+, and CD1a+ cells infiltrating intraepithelial or stromal spaces were counted (six parameters). Hierarchical clustering was used to analyze the six parameters at one time. Expression of COX-1 and COX-2 in tumor cells was also analyzed by immunohistochemistry. Expression of both COX-1 and COX-2 was negatively correlated with intraepithelial CD8+ cells ($P<0.05$ for both). Hierarchical clustering using the six parameters classified ovarian cancers into three clusters. The overall and progression-free survival of cluster 1 with low CD8+ cell and high CD1a+ cell density was poorer than cluster 2 with high CD8+ cell density ($P<0.05$). The cluster classification did not correlate with clinical features, such as histology, stage, age, and amount of residual tumor. In a multivariate analysis, cluster 1 was an independent poor prognostic factor ($P<0.05$). Expression of both COX-1 and COX-2 was higher in cluster 1 than in cluster 2 ($P<0.05$, respectively). In conclusion, hierarchical clustering of tumor-infiltrating immune cells allows poor prognostic COX-high subgroup of ovarian cancer to be detected. COX may influence the pattern of tumor-infiltrating immune cells and prognosis in ovarian cancer.
Modern Pathology (2009) **22,** 373–384; doi:10.1038/modpathol.2008.187; published online 7 November 2008

Keywords: ovarian; cancer; cyclooxygenase; clustering; CD8; CD57; CD1a

Ovarian cancer is the leading cause of death in gynecological malignancies. The majority of patients with ovarian cancer are identified at advanced stages of the disease and eventually die of disease persistence or recurrence. Therefore, novel therapies are of great necessity in order to treat ovarian cancer.[1] Modification of immune responses against tumor cells is one of the important steps in tumor development. Traditionally, a decrease in MHC class I expression and tumor-associated antigens has been the focus of explaining immune tolerance. However, recent findings indicate that tumor cells alter their surrounding microenvironment to activate immune tolerogenic systems, and this mechanism is regarded as a new therapeutic target for cancer.[2]

The cell-mediated immune response, which involves various types of immune cells, comprises a major portion of the immune responses against tumor cells. Cytotoxic T lymphocytes (CTLs) and natural killer cells (NK) are major effector cells, in acquired immunity and innate immunity, respectively. Dendritic cells (DCs) positively and negatively regulate tumor immunity. CD1a, CD8, and CD57 have been used as markers for DC, CTL, and NK, respectively. The levels of tumor infiltration of these individual cells have been extensively studied in cancers.[3–5] However, the interactions between the multiple kinds of immune cells in the tumor microenvironment are yet to be determined.

The first aim of this research was to clarify the immune status of ovarian cancers using the infiltrating pattern of CTL, NK, and DC. In order to understand the complex immunological status, we

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Received 1 August 2008; revised 24 September 2008; accepted 29 September 2008; published online 7 November 2008

independently examined CD1a+, CD8+, and CD57+ cells, followed by a combined analysis using hierarchical clustering. An analysis of multiple factors is likely to lead to a better understanding of the complex natures of cancers. Hierarchical clustering is a powerful bioinformatics method, which analyzes multiple factors to classify tumors by their similarities. It has been frequently used to analyze DNA microarray data. Recently, this method has been used to classify tumors based on the patterns of expression of multiple proteins.[6–10] In this study, we employed hierarchical clustering using the patterns of tumor-infiltrating CD1a+, CD8+, and CD57+ cells. As far as we know, hierarchical clustering has never been used to analyze the patterns of tumor-infiltrating immune cells. We show that classification of ovarian cancers by the infiltrating pattern of immune cells correlates with survival.

Epidemiological studies show that non-steroidal anti-inflammatory drugs, which target cyclooxygenase (COX), reduce the incidence and mortality of colorectal carcinoma and several other types of cancers.[10–13] Prostaglandin E2 (PGE2), generated by COX in tumor cells, has an oncogenic role in cancers, including anti-apoptotic, angiogenic, and invasive functions, which have been extensively studied for decades.[14] In ovarian cancer, two subtypes of COX, COX-1, and COX-2, are upregulated and generate PGE2.[15,16] Recently, after the first report that COX-2 has an immunosuppressive role, there is accumulating evidence to indicate that COX is important in inducing immune tolerance by modulating the microenvironment surrounding tumor cells. PGE2, secreted from tumor cells, alters the Th1/Th2 balance, suppresses DC function, and increases the number of tumor-infiltrating regulatory T cells.[17,18] However, the relationship between COX and immune status has never been studied in ovarian cancer.

The second aim of this research was to analyze the relationship between COX and the immune system in ovarian cancer. We showed that both COX-1 and COX-2 negatively correlate with the intraepithelial CD8+ count. Furthermore, COX-1 and COX-2 are highly expressed in the poor prognostic tumor cluster generated by a pattern of tumor-infiltrating immune cells.

This study proposes a novel strategy to classify ovarian cancers using the patterns of multiple types of infiltrating immune cells. This approach identifies a subset of ovarian cancers that may be treated by targeting the tumor microenvironment, thereby eliciting an immune reaction.

Materials and methods

Patients and Samples

The samples used in this research are the same as we have previously reported, and their features have already been described.[19] Briefly, ovarian cancer specimens from 70 patients were used under written consent and under the approval of the Ethics Committee of Kyoto University Hospital. All of the patients contributing these samples underwent surgery and follow-up at Kyoto University Hospital. We used specimens only from patients who had not received any treatment before the surgeries. The average age of the patients was 55 years (range, 26–78; s.d., 11 years). Of 70 patients, 27 (39%) were diagnosed as International Federation of Gynecology and Obstetrics stage I, 4 (6%) were diagnosed as stage II, 26 (37%) were diagnosed as stage III, and 13 (19%) were diagnosed as stage IV. Histological subtypes of the tumor comprised 28 (40%) cases of serous, 22 (31%) cases of clear cell, 11 (16%) cases of endometrioid, 2 (3%) cases of mucinous, and 7 (10%) cases of other types. Toward the end of the study, 29 (41%) patients had died of their disease, and 41 (59%) were alive. The mean follow-up period was 5 years (range, 0–11; s.d., 3.0).

Immunohistochemistry

Immunohistochemical staining was performed by the streptavidin–biotin–peroxidase method according to the manufacturer's instructions. Briefly, formalin-fixed, paraffin-embedded specimens were cut into 4-μm-thick sections. The tissue sections were deparaffinized in xylene (5 min × 3) and dehydrated through graded alcohols (99, 99, and 70%) to water. Antigens were retrieved by microwave heating for 20 min in citrate buffer (pH 6.0). To block endogenous peroxidase activity, all of the sections were treated with 100% methanol containing 0.3% H_2O_2 for 10 min. Nonspecific binding of IgG was blocked using normal rabbit serum (Nichirei, Tokyo, Japan). The sections were separately incubated with mouse anti-COX-1 monoclonal Abs (1:50) (Cayman Chemical Co., Ann Arbor, MI, USA; Cat. no. 160110), mouse anti-COX-2 monoclonal Abs (1:100) (Cayman Chemical Co.; Cat. no. 160112), mouse anti-CD57 Abs (1:100), or mouse anti-CD1a Abs (1:100) overnight at 4°C. They were then incubated with biotinylated rabbit-anti-mouse secondary Abs (Nichirei), followed by incubation with a streptavidin–peroxidase complex solution for 15 min. Finally, sections were stained with 3,3′-diaminobenzidine and counterstained with hematoxylin.

Evaluation of Immunohistochemistry

CD8+ cells were stained and counted in our previous study.[19] CD57+ and CD1a+ cells were counted using the same method. Briefly, immune cells in intraepithelial or stromal spaces were counted separately using a microscopic field at × 200 (0.0625 mm^2). Five areas with the most abundant infiltration were selected, and an average

count was calculated. The expression of COX-1 and COX-2 was evaluated according to the intensity of the staining and scored as follows: 0, negative; 1, very weak expression; 2, moderate expression; and 3, strongest expression. Cases with scores of 0 or 1 were defined as the low-expression group, and cases with scores 2 or 3 were part of the high-expression group.

Two independent gynecological pathologists examined the immunohistochemical slides without any prior information regarding the clinical history of the patients. In cases of discrepancies in interpretation, a final consensus was achieved between the two observers using a multi-head microscope.

Hierarchical Clustering and Heat Map

In order to conduct a hierarchical clustering, Cluster 3.0, which was available from a website (http://rana.lbl.gov/EisenSoftware.htm), was used. The numbers of tumor-infiltrating CD1a+ cells, CD8+ cells, and CD57+ cells in intraepithelial or stromal spaces counted by the above method (six parameters) were analyzed. Following the instruction of the software, the six parameters were normalized and a complete-linkage hierarchical clustering was conducted. The dendrogram was visualized using Java TreeView 1.1.0, which was also available from a website (http://jtreeview.sourceforge.net/). Immunohistochemical staining scores for COX-1 and COX-2 were determined by the above method. Heat maps for the COX staining scores were generated by R, a language and environment for statistical computing (http://www.R-project.org). The language, 'image(as.matrix(tmp),col = matlab.colors(100))', was used to draw heat maps with R.

Statistical Analysis

The χ^2-test was used to evaluate differences in COX immunological scores and clinicopathological factors. Spearman's correlation coefficient test was employed to analyze the associations between tumor-infiltrating CD8+, CD57+, CD1a+ cell counts, and COX expression. A univariate analysis for survival was performed with a log-rank test. The differences in tumor-infiltrating immune cells and COX expression between different clusters were analyzed by a Mann–Whitney *U*-Test. A multivariate Cox regression model was used to evaluate the independency of prognostic factors. *P*-values <0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism 4.0b and the statistical package SPSS14.

Results

Infiltration of Cd8+, Cd57+, and Cd1a+ Cells in Ovarian Cancer Tissue Samples

Immunohistochemical staining was conducted for 70 ovarian cancer specimens using CD8, CD57, and CD1a antibodies, which label tumor-infiltrating CD8+, CD57+, and CD1a+ mononuclear cells (Figure 1). As described above, their numbers were counted in intraepithelial or stromal spaces. Table 1 shows the

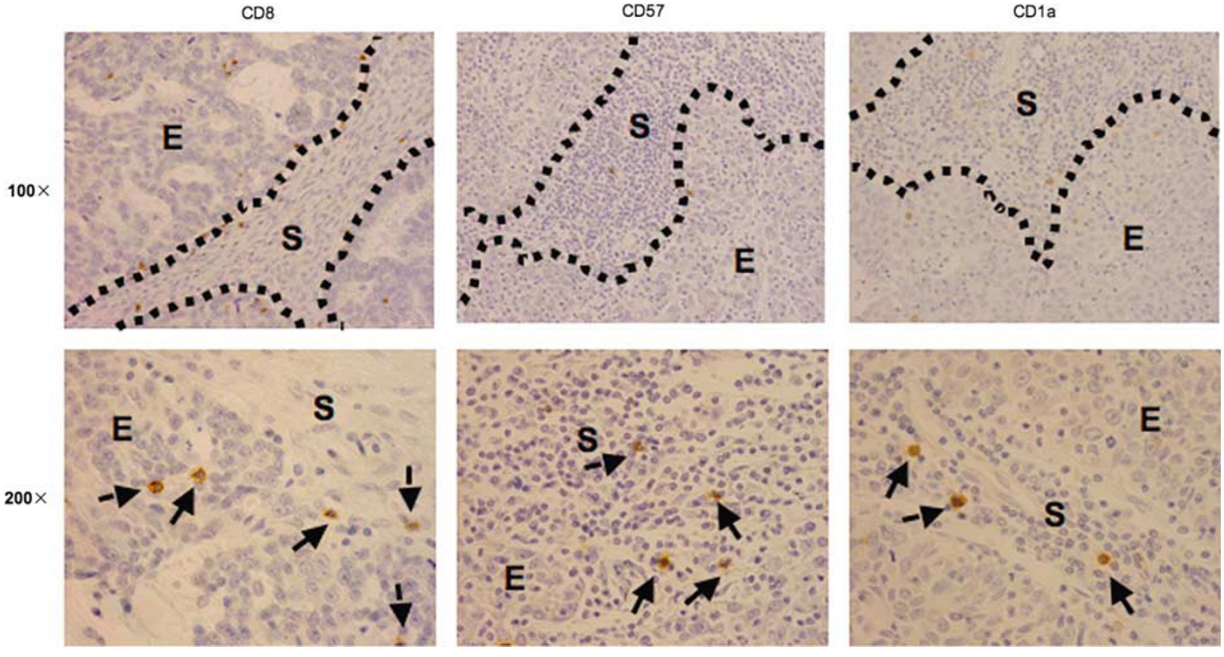

Figure 1 Immunohistochemical staining of human ovarian cancer tissues using anti-CD8, CD57, and CD1a antibodies. They detected tumor-infiltrating mononuclear positive cells; E, intraepithelial space and S, stromal space. Arrows in the photos indicate the positive cells. Original magnification for the above three pictures is ×100, and, for the below three pictures, it is ×200.

Table 1 Mean number and range of the tumor-infiltrating immune cells in different compartments of 70 ovarian cancers

	Intraepithelial space		*Stromal space*	
	Mean cells/ 0.0625 mm²	*Range*	*Mean cells/ 0.0625 mm²*	*Range*
CD8	6.64	0–56.8	7.906	1–60.6
CD57	1.591	0–5	2.329	0–8.2
CD1a	1.39	0–18	1.843	0–15.4

CD, cluster of differentiation.

mean numbers of tumor-infiltrating cells in the 70 ovarian cancers. The numbers of intraepithelial or stromal CD8+ cells (eCD8+ and sCD8+) were higher than intraepithelial or stromal CD57+ and CD1a+ cells (eCD57+, sCD57+, eCD1a+, and sCD1a+).

When the samples were divided into two groups according to the infiltration of immune cells (high 35 samples *vs* low 35 samples), only the eCD8+ levels significantly correlated with survival and high eCD8+ levels correlated with a good prognosis ($P<0.01$, data not shown). Other clinicopathological characteristics, such as histology, age at surgery, tumor status (pT status), lymph node metastasis, distant metastasis, and residual tumor did not significantly correlate with the numbers of tumor-infiltrating immune cells.

Expression of COX-1 and COX-2 in Ovarian Cancer Cells

Expression of COX-1 (Figure 2a) and COX-2 (Figure 2b), as shown by immunostaining, was localized to the cytoplasm of tumor cells. In 70 patients, 22 (31%) showed high expression (score: 2 or 3) of COX-1; whereas, for COX-2, 23 patients (33%) had high expression.

The association between clinicopathological variables and COX-1/COX-2 expression was analyzed using a χ^2-test (Table 2). When patients were divided into COX-1 high/low or COX-2 high/low cases, we did not find any statistically significant deviation for age at surgery, tumor status, lymph node metastasis, distant metastasis, residual tumor, and chemotherapy. However, as for the histological types, higher levels of COX-2 occurred more frequently in serous ovarian carcinoma ($P<0.05$) as compared with other types. Survival was analyzed in relation to COX-1 or COX-2 expression, and we found higher levels of COX-1 or COX-2 tended to correlate with a poor prognosis, but it was not statistically significant (data not shown).

Correlation Between Tumor-Infiltrating Immune Cells and COX Expression

The correlation between COX expression scores and the number of tumor-infiltrating CD8+, CD57+, and CD1a+ cells (eCD8+, sCD8+, eCD57+, sCD57+, eCD1a+, and sCD1a+) was examined (Table 3). We found a positive correlation between COX-1 and COX-2 expression. Both COX-1 and COX-2 expression negatively correlated with the number of eCD8+ cells (Table 3 and Figure 3). When the expression scores of COX-1 and COX-2 were added, these scores (COX sum) showed a stronger negative correlation with the number of eCD8+ cells (Table 3 and Figure 3). Negative correlations between COX sum and number of eCD8+ cells were observed in each histological type independently (serous, $r=-0.222$; clear, $r=-0.190$; endometrioid, $r=-0.517$; data not shown), although they were not statistically significant probably because of the insufficient sample sizes. Neither COX-1 nor COX-2 significantly correlated with the CD57+ or CD1a+ cell count (Table 3).

One of the ligands for programmed cell death 1 (PD-1), an immunoinhibitory receptor belonging to CD28/CTL antigen 4 family is PD-1 ligand 1 (PDL1). Similarly to COX, PDL1 was negatively correlated to the number of eCD8+ cells in our previous report.[19] We also analyzed the correlation between the COX and PDL1 expression scores. However, PDL1 did not show a statistically significant correlation with the level of COX expression (Table 3).

Hierarchical Clustering Classification of Ovarian Cancer Using Tumor-Infiltrating Immune Cells

Hierarchical clustering was used to analyze the 70 ovarian cancers based on the number of tumor-infiltrating immune cells. This analysis generated three main tumor clusters. They were characterized by the following patterns: cluster 1: low eCD8+ low sCD8+, low eCD57+, and high sCD1a+; cluster 2: high eCD8+, high sCD8+, modest eCD57+, and low sCD1a+; cluster 3: low eCD8+, low sCD8+, high eCD57+, low eCD1a, and low sCD1a (Figure 4a and b).

We then analyzed the clinicopathological characteristics among the three clusters. No statistically significant deviation was found for age, histological subtypes, recurrence, tumor state, residual tumor after operation, lymph node metastasis, and distant metastasis (Figure 4a and Table 4). In contrast, there was a statistically significant difference in both the overall and progression-free survival based on the cluster group assignment ($P<0.05$, respectively). In addition, there were significant differences in both overall survival and progression-free survival between clusters 1 and 2 ($P<0.05$, respectively) (Figure 4c).

A multivariate analysis was then conducted using the variables of patient age (>55 *vs* <55 years old), cluster group designation (cluster 1 *vs* the others), tumor stage (stages I and II *vs* stages III and IV), residual tumor (positive *vs* negative), and chemotherapy (with *vs* without paclitaxel). FIGO stage,

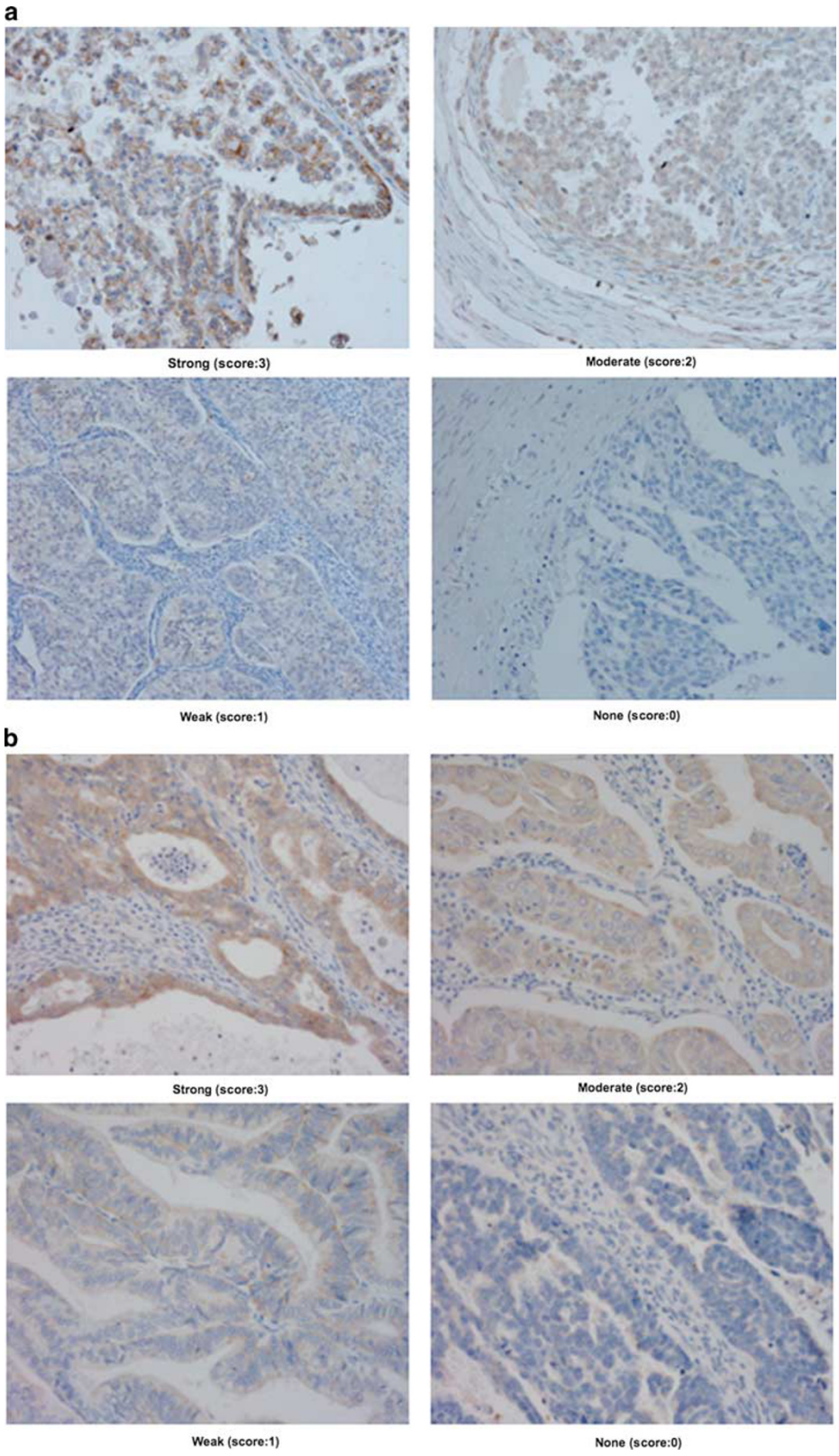

Figure 2 COX-1 (**a**) and COX-2 (**b**) expression in ovarian cancers. Both COX-1 and COX-2 were expressed in the cytoplasm of tumor cells. COX expression was evaluated by staining intensity and scored as follows: negative = 0; weak expression = 1; moderate expression = 2; and strong expression = 3. Representative photos of the staining are shown in the figure.

Table 2 Correlation between clinicopathological characteristics and COX expression analyzed by a χ^2-test

		COX-1 expression			*COX-2 expression*		
Characteristic	n *(%)*	*Negative and weak*	*Moderate and strong*	P-*value*	*None and weak*	*Moderate and strong*	P-*value*
All	70 (100%)	53 (76%)	17 (24%)		48 (69%)	22 (31%)	
Age				0.898			0.585
<55	32 (46%)	24	8		23	9	
⩾55	38 (54%)	29	9		25	13	
Histological type							
Serous	28 (40%)	19	9	0.211	15	13	0.027
Endometrioid	11 (16%)	8	3	1	10	1	0.139
Clear cell	22 (31%)	18	4	0.181	16	6	0.501
Mucinous	2 (3%)	2	0		0	2	
Others	7 (10%)	6	1		7	0	
FIGO stage				0.982			0.747
I	27 (39%)	21	6		19	8	
II	4 (6%)	3	1		2	2	
III	26 (37%)	19	7		17	9	
IV	13 (19%)	10	3		10	3	
Tumor status				0.767			0.894
pT1+pT2	31 (44%)	24	7		21	10	
pT3	39 (56%)	29	10		27	12	
Lymph node metastasis				0.676			0.699
Negative	56 (80%)	43	13		39	17	
Positive	14 (20%)	10	4		9	5	
Distant metastasis				0.32			0.5229
Negative	57 (81%)	41	16		37	20	
Positive	13 (19%)	7	6		10	3	
Residual tumor				0.503			0.736
Optimal	49 (70%)	36	13		33	16	
Suboptimal	21 (30%)	17	4		15	6	
Prognosis (5 years)				0.588			0.131
Survive	41 (59%)	32	9		31	10	
Death	29(41%)	21	8		17	12	
Recurrence				0.886			0.385
No	34 (49%)	26	8		25	9	
Yes	36 (51%)	27	9		23	13	

COX, cyclooxygenase; FIGO, International Federation of Gynecology and Obstetrics.
$^*P<0.05$.

Table 3 Correlation between tumor infiltrating lymphocytes and COX expression analyzed by Spearman's test

Parameter	*eCD8+*	*sCD8+*	*eCD57+*	*sCD57+*	*eCD1a+*	*sCD1a+*	*COX-2*	*PDL1*
COX-1	−0.270 *	−0.117 NS	0.034 NS	−0.015 NS	−0.011 NS	0.134 NS	0.261 *	0.165 NS
COX-2	−0.245 *	−0.152 NS	−0.167 NS	−0.108 NS	−0.023 NS	0.168 NS		0.192 NS
COX sum	−0.317 **	−0.165 NS	−0.073 NS	−0.065 NS	−0.042 NS	0.182 NS		0.145 NS

CD, cluster of differentiation; COX, cyclooxygenase; NS, not significant; PDL1, PD-1 ligand 1.
Coefficients (*r*-values) are listed in the table; $^*P<0.05$ and $^{**}P<0.01$.

existence of residual tumor, and cluster 1 were statistically significant independent predictors of both overall and progression-free survival in ovarian cancer (Table 5).

When the clusters were compared with COX expression, cluster 1 showed higher expression of COX-1 and COX-2 and a higher COX sum than cluster 2 (Figure 4d and e) ($P<0.05$, respectively).

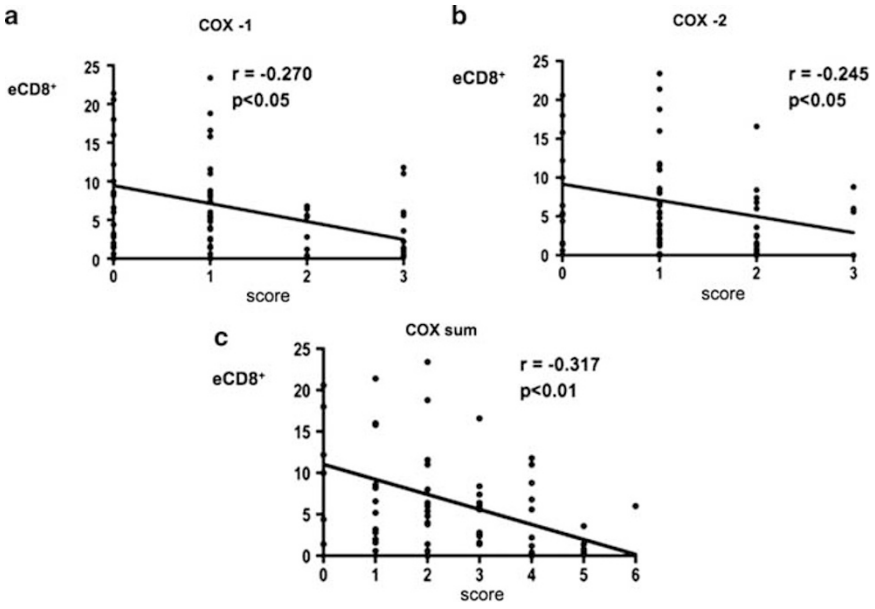

Figure 3 Correlation between COX expression and the number of intraepithelial CD8+ cells (eCD8+). Both COX-1 and COX-2 expression negatively correlated with eCD8+ (**a** and **b**). When the expression scores of COX-1 and COX-2 were added, the score (COX sum) showed a stronger negative correlation with eCD8+ (**c**).

Discussion

Tumor cells develop various strategies to avoid immune-mediated destruction, permitting them to emerge and develop. They alter the microenvironment where the host–tumor interactions occur in order to suppress immune function. The number and the pattern of tumor-infiltrating immune cells are influenced by the tumor microenvironment.[20] CD8+ CTL are regarded as the major effectors of acquired immunity, whereas NK cells exert an innate immune reaction. DCs are powerful antigen-presenting cells that is important in the regulation of immune responses. As we recently reported, intraepithelial infiltration of CD8+ cells positively correlates with good survival in ovarian cancer,[19] although infiltration of CD57+ or CD1a+ cells alone did not significantly correlate with survival in this study (data not shown). Regardless of how they correlate with survival individually, given their important roles in the immune system, the pattern of the infiltration of these cells into the tumor would be expected to contribute to the understanding of the immune status in the tumor microenvironment.

We counted and analyzed the number of intraepithelial and stromal infiltrating cells separately, as differences in the pattern of infiltration between intraepithelial and stromal spaces have been of interest and have been previously studied.[21–23] We analyzed ovarian cancer samples using a total of six parameters. Hierarchical clustering classifies samples by their similarity using raw experimental data. It has been frequently used to analyze DNA microarray data, and, more recently, studies of protein expression using immunohistochemical staining have also taken advantage of this method.[6–9,24,25] As hierarchical clustering uses correlation coefficients to assess similarity, numerical data, such as numbers of tumor-infiltrating immune cells, are suitable for analysis using this method. Therefore, we employed hierarchical clustering to study our data.

Cluster 1 tumors, characterized by high sCD1a+, low eCD8+, low sCD8+, and low eCD57+ had a poor prognosis (Figure 4a and c). As other prognostic factors, such as stage and residual tumors, did not correlate with the cluster classification, cluster 1 was found to be an independent prognostic factor in ovarian cancer (Table 5). Therefore, once a tumor is resected, it is possible to speculate about a patient's prognosis by analyzing the tumor-infiltrating CD1a+, CD8+, and CD57+ cells in the tumor specimen. The correlation between the number of tumor-infiltrating DC and survival has been analyzed in various types of cancers, and conflicting results have been reported.[26–30] DCs is important in the regulation of NK cells and T lymphocytes in both stimulated and tolerant situations.[31] Therefore, the high CD1a+ accompanied with low CD8+ and low eCD57+ could indicate that the DC in cluster 1 are tolerant and may suppress the infiltration of CTL and NK cells. It was interesting to see that sCD1a+ was more prominent than eCD1a+ in this cluster, although the precise mechanism responsible for this difference will be a subject for further investigation.

Cluster 2, characterized by high eCD8+, high sCD8+, and modest eCD57+ had a good prognosis. This correlation is consistent, in part, with

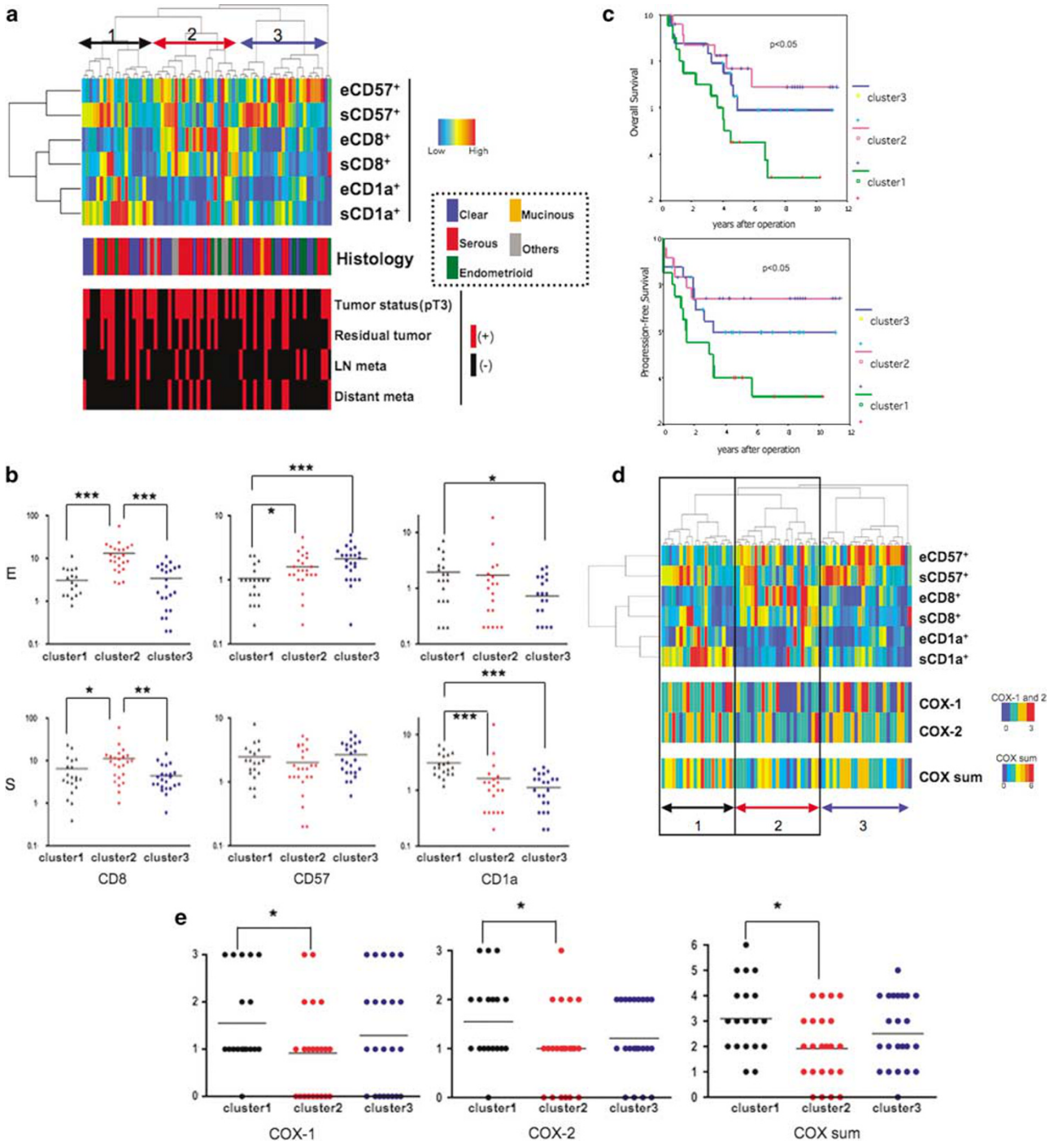

Figure 4 Hierarchical clustering based on the pattern of tumor-infiltrating immune cells. (**a**) All the ovarian cancer samples, except two, were classified into three main tumor clusters; cluster 1, cluster 2, and cluster 3. The intraepithelial and stromal immune cells formed a cluster with their counterparts. The upper heat map represents normalized numbers of infiltrating immune cells. Individual data for the clinicopathological factors are arranged in the same order as the clustering figure. Histological subtypes are shown by the five colors. The other factors are shown in red and black. Tumor status: pT3 or not, residual tumor: residual tumor positive or negative at the initial surgery, LN meta: lymph node metastasis positive or negative, distant meta: distant metastasis positive or negative. These clinicopathological factors did not deviate among the three clusters. (**b**) Raw data for the infiltrating numbers of CD8 + , CD57 + , CD1a + cells in the three tumor clusters; E, intraepithelial spaces and S, stromal spaces; $*P<0.05$, $**P<0.01$, and $***P<0.001$. (**c**) Survival analysis of the three clusters. Kaplan–Meier curves for the clusters are shown with regard to overall (left), and progression-free survival (right). The three tumor clusters were different for both overall and progression-free survival ($P<0.05$, respectively). When individual curves were compared, clusters 1 and 2 were significantly different for both overall and progression-free survival ($P<0.05$, respectively). (**d**) COX expression arranged in the same order as the clustering figure. (**e**) COX-1, COX-2, and COX sum are significantly higher in cluster 1 than cluster 2 by a Mann–Whitney *U*-test; $*P<0.05$.

Table 4 Correlation between the clinicopathological factors and the cluster classification analyzed by a χ^2-test

Characteristic	*Cluster1* *20*	*Cluster2* *24*	*Cluster3* *24*	P-*value*
Age				0.467
<55	8	14	9	
$\geqslant 55$	12	10	15	
Histological type				
Serous	9	9	9	0.847
Endometrioid	2	3	5	0.559
Clear cell	7	6	9	0.623
Mucinous	1	0	1	
Others	1	6	0	
FIGO stage				0.461
I	8	10	9	
II	0	0	4	
III	10	9	6	
IV	2	5	8	
Tumor status				0.573
pT1+pT2	8	10	13	
pT3	12	14	11	
Lymph node metastasis				0.791
Negative	15	21	19	
Positive	5	3	5	
Distant metastasis				0.817
Negative	18	19	19	
Positive	2	5	5	
Residual tumor				0.907
Optimal	13	17	18	
Suboptimal	7	7	6	
Chemotherapy				0.834
No paclitaxel	11	15	12	
With paclitaxel	9	9	12	

FIGO, International Federation of Gynecology and Obstetrics.
None of the factors significantly correlated with the cluster classification.

previous reports that analyzed intraepithelial CD8+ cells.[19,32–36] Cluster 3, characterized by high eCD57+, had a modest prognosis. Analysis using just CD8+ could not discriminate between cluster 1 and cluster 3, which look different in both immune status and prognosis. The number of tumor-infiltrating NK cells has been reported to correlate with good prognosis in colorectal[4] and gastric[5] carcinomas. Our data indicate the significance of intraepithelial infiltration of NK cells as a marker of good prognosis of ovarian cancer, although they might not be as important as CTL.

Tumor cells interact with host cells, including lymphocytes, macrophages, fibroblasts, and endothelial cells.[18] As the many patterns of infiltrating immune cells are thought to reflect the various natures of tumors, we then examined a molecule that alters the microenvironment and is expressed in tumor cells. COX-2, or the 'inducible COX,' generates mainly PGE2, whereas COX-1, or the 'constitutive COX,' generates various types of prostaglandins. COX-2 has been reported to be upregulated in many types of cancers, including ovarian cancer.[37,38] Consistent with a previous report,[37] we found COX-2 expression was higher in serous ovarian cancer, compared with other types of ovarian cancer (Table 2). COX-1 is also known to be upregulated in ovarian cancer, and PGE2, produced by COX-1, contributes to tumor development.[15,39,40] The oncogenic roles of PGE2 have been extensively studied, specifically with regard to angiogenesis, invasiveness, and anti-apoptotic mechanisms.[41,42] However, recent findings indicate that PGE2 is important in modifying the tumor microenvironment to induce immune tolerance.[18,43,44] PGE2 changes the balance of cytokines, suppresses lymphocyte proliferation in response to mitogen stimulation, and inhibits the function of DC.[40,45–47] Several groups, including ours, have shown a negative correlation between COX-2 expression and the number of tumor-infiltrating CD8+ cells in cervical cancer,[46,47] endometrial cancer,[48] hepatocellular carcinoma,[49] and transitional-cell carcinoma of the urinary bladder.[50] Furthermore, in experimental data using mice, COX-2 inhibitors increased the number of tumor-infiltrating CD8+ cells.[17,51–53] In human head and neck cancer, a COX-2 inhibitor administered before operation increased the number of tumor-infiltrating CD8+ cells.[54] In this study, we found a negative correlation between eCD8+ and both COX-1 and COX-2 intensity scores in the 70 ovarian cancer specimens. Given the known function of PGE2 in modulating the tumor microenvironment to suppress an immune response, our data strongly suggest that COX suppresses tumor immunity in ovarian cancer. Furthermore, the result that the sum of the COX-1 and COX-2 intensity scores negatively correlated with eCD8+ infiltration more strongly than either of the COX intensities individually suggests that they may have an additive role in PGE2 production. Finally, the COX expression data was compared with the clustering data. As shown in Figure 4d and e, COX-1 intensity scores, COX-2 intensity scores, and the sum of the two COX intensities were higher in cluster 1 than in cluster 2, whereas cluster 3 showed various intensities for both COX-1 and COX-2. This result indicates that elevated COX expression in a subset of ovarian cancer alters the pattern of tumor-infiltrating CD8+, CD1a+, and CD57+ cells, at least to some extent, and the microenvironment influenced by COX is associated with a poor prognosis of ovarian cancer, although the function of tumor-infiltrating immune cells to attack tumor cells is uncertain.

In conclusion, using hierarchical clustering analysis based on the numbers of tumor-infiltrating CD1a+, CD8+, and CD57+ cells, we found novel classifications of ovarian cancer that predict patient survival. COX expression negatively correlated with the intraepithelial infiltration of CD8+ cells and was high in the poor prognostic subgroup. Given the known function of COX influencing the tumor

Table 5 Multivariate analysis for survival of ovarian cancer

		Overall survival			*Progression-free survival*		
Characters	n	*Multivariate hazard ratio*	*95% CI*	P-value	*Multivariate hazard ratio*	*95% CI*	P-value
Cluster1				0.016			0.029
No	50	1	1		1	1	
Yes	20	2.643	1.201–5.818		2.362	1.0905.121	
Age				0.8			0.743
<55	32	1	1		1	1	
⩾55	38	1.11	0.496–2.485		0.146	0.507–2.589	
Histological type				0.464			0.595
Non-clear cell	48	1	1		1	1	
Clear cell	22	1.41	0.562–3.535				
FIGO stage				0.005			0.004
I+II	31	1	1		1	1	
III+IV	39	5.583	1.684–18.509		5.766	1.744–19.063	
Residual tumor				0.039			0.036
Optimal	49	1	1		1	1	
Suboptimal	21	2.441	1.048–5.687		2.438	1.059–5.610	
Chemotherapy				0.718			0.83
No paclitaxel	39	1	1		1	1	
With paclitaxel	31	1.149	0.541–2.442		0.919	0.425–1.986	

FIGO, International Federation of Gynecology and Obstetrics.
'Cluster 1,' FIGO stage, and the existence of residual tumor at the initial surgery are independent prognostic factors.

microenvironment, COX inhibitors could change the pattern of tumor-infiltrating immune cells in ovarian cancer. In future researches to analyze the effect of COX inhibitors on ovarian cancers, patterns of tumor-infiltrating immune cells should be examined as well as the survival of patients.

Disclosure/conflict of interest

The authors declared no conflict of interest.

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