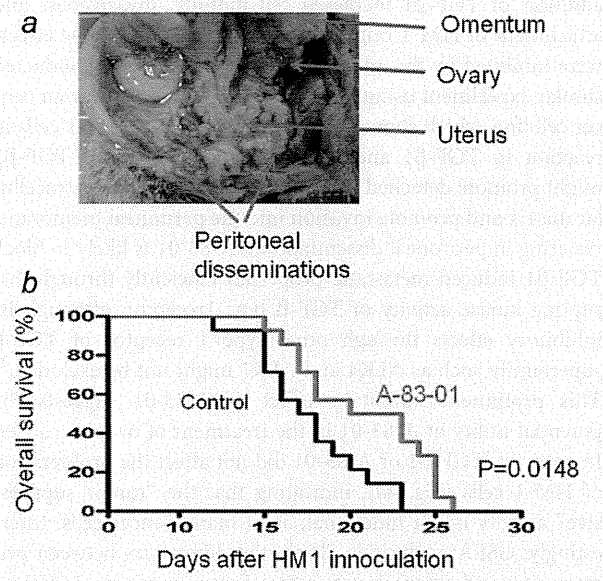


**Figure 5.** Identification of genes regulated by TGF- $\beta$ 1 in HM-1 cells. Microarray analysis of HM-1 cells for the four treatment groups; control ( $n = 3$ ), TGF- $\beta$ 1 ( $n = 3$ ), TGF- $\beta$ 1 + A-83-01 ( $n = 3$ ) and A-83-01 only ( $n = 1$ ). Average-linkage hierarchical analysis was performed using the 241 differentially expressed probes (FDR  $q < 0.25$ ) between the control and TGF- $\beta$ 1-treated cells. Upregulated genes were used for GSEA analysis to examine relevance with the clinical cancer dataset GSE2109.

activated signaling pathways in cancer.<sup>10–12,17,18</sup> In our study, we found that the TGF- $\beta$  pathway activity was upregulated in the omental metastases compared to primary sites (Fig. 1). GSEA also demonstrated enrichment of gene sets relevant to the TGF- $\beta$  signaling pathway. Based on the results from the computational screening, we further examined expression of pSMAD2, a representative molecule indicating activated TGF- $\beta$  signaling.<sup>19</sup> Immunohistochemical analysis revealed significant upregulation of pSMAD2 expression in the omental metastases (Fig. 2b). These results demonstrated that the TGF- $\beta$  signaling pathway is activated in ovarian cancer metastases. Furthermore, pSMAD2 expression was stronger in the invasive front of cancer nests (Fig. 2d). Localized TGF- $\beta$  signaling within cancer tissue may be attributed to the inherent heterogeneity of the tumor. Here, we found higher expression of pSMAD2 in invasive fronts and small cancer nests, and this would result in higher expression overall in metastases because invasive fronts tended to be more abundant in omental metastasis than in primary sites (data not shown). Nonetheless, activation of the TGF- $\beta$  signaling pathway suggests a role in the metastatic process of ovarian cancer.

Expression of *TGFBR2*, a core component of the TGF- $\beta$  signaling pathway, showed a significant positive correlation with TGF- $\beta$  signature probability scores (Fig. 2a), and *TGFBR2* was upregulated in metastases similar to pSMAD2 (Figs. 2c, 2e). Thus, transcriptional regulation of *TGFBR2* could play a crucial role in the regulation of TGF- $\beta$  signaling. Although TGF- $\beta$  signaling leads to cell cycle arrest and/or apoptosis of normal epithelial cells, this “tumor-suppressive



**Figure 6.** Mouse model of intraperitoneal dissemination using HM-1 cells and treatment with A8301. (a) Intraoperative findings after 10 days of HM-1 cells inoculation. Disseminated nodules can be seen on the peritoneum and abdominal organs, especially omentum, mesentery, uterus and ovary. (b) Survival curves of the A-83-01 treated group and the control group ( $n = 14$  each).

function” of TGF- $\beta$  signaling is inhibited through genetic or epigenetic changes in pathway components in virtually all types of cancers.<sup>20</sup> Lynch et al.<sup>21</sup> reported that in ovarian cancer, expression of *TGFBR2* is lost by mutations in the coding regions of the *TGFBR2* gene. However, there have been several reports that challenge this observation. Another group showed that the frequency of *TGFBR2* mutation is quite rare in ovarian cancer.<sup>22</sup> In most cases of ovarian cancer, addition of TGF- $\beta$  to primary cancer cells leads to activation of internal signals.<sup>23,24</sup> In our study, given the specific expression of pSMAD2 (Fig. 2d) in tumor cells, loss of internal signaling by receptor mutations is unlikely. Epigenetic regulation, however, could be one possible mechanism that alters transcriptional regulation of *TGFBR2*. We recently found that promoter DNA methylation of the *TGFBR2* gene is often found in ovarian cancer and its status negatively correlates with *TGFBR2* expression (submitted for publication). However, further studies are necessary to clarify the precise mechanisms of activation of the TGF- $\beta$  signaling pathway in omental metastases.

Next, to reveal the functional role of the TGF- $\beta$  signaling pathway in ovarian cancer, we conducted *in vitro* assays using the HM-1 mouse ovarian cancer cell line.<sup>6</sup> In our preliminary experiments, HM-1 cells expressed *Muc16* (data not shown), an ortholog of human *MUC16*, which encodes CA125, a specific biomarker of human epithelial ovarian cancer. The addition of TGF- $\beta$ 1 increased expression of pSmad3 in HM-1 cells (Fig. 3), indicating that the signaling pathway is not disrupted at the receptor level. In *in vitro* experiments,

addition of TGF- $\beta$ 1 increased cell motility, invasiveness and attachment of HM-1 cells (Figs. 4*b*, *c* and *d*), and these effects were inhibited by the addition of A-83-01. We also conducted similar experiment using SK-OV-3, human serous ovarian cancer cell line, which showed the same tendency as HM-1 cells in reaction to TGF- $\beta$ 1 and A-83-01. These effects of TGF- $\beta$ 1 might promote detached cancer cells to attach to the extracellular matrix and promote invasion into the peritoneal membrane, resulting in peritoneal dissemination. A-83-01 is likely to block TGF- $\beta$ 1-induced metastatic properties efficiently through disrupting kinase activity of TGF- $\beta$  type 1 receptor, although its inhibitory effects through other type 1 receptor of TGF- $\beta$  superfamily such as ALK4 and ALK7 might not be excluded.<sup>25</sup> This prominent inhibitory effect of A-83-01 suggests the potential utility of A-83-01 in the treatment of ovarian cancer. In contrast, TGF- $\beta$ 1 or A-83-01 did not affect the proliferation of HM-1 cells (Fig. 4*a*), indicating that the “tumor suppressive” activity is not functional, as in many cancer cells. Interestingly, GSEA analysis to clarify the differences between primary sites and omental metastases of ovarian cancers indicated that gene sets relevant to cell motility, adhesion and invasion were enriched among the omental metastases, suggesting an *in vivo* role of the TGF- $\beta$  pathway in ovarian cancer.

We next examined transcriptional changes caused by TGF- $\beta$ 1 and/or A-83-01 in HM-1 cells and found that A-83-01 reversed the effects of TGF- $\beta$ 1 (Fig. 3). From GSEA analysis, genes upregulated by TGF- $\beta$ 1 in HM-1 cells were highly expressed in omental metastases of ovarian cancer. These results indicated that the TGF- $\beta$  signaling pathway in HM-1 cells bears relevance to omental metastasis in human ovarian cancer. Genes upregulated by TGF- $\beta$ 1 in HM-1 cells included several “metastasis promoter genes”, such as acylglycerol kinase (*AGK*), granulins (*GRN*), heparanase (*HPSE*), oncostatin M (*OSM*), *slingshot homolog2* (*SSH2*) and TGF- $\beta$ -induced (*TGFBI*) (Supporting Information Table 1). *AGK* increases formation and secretion of lipoprotein(a) LPA, resulting in increased migration of prostate cancer cells.<sup>26</sup> *HPSE* increases metastatic potential by remodeling extracellular matrix in many types of cancers.<sup>27</sup> *GRN* increases the motility and invasion of ovarian cancer cells<sup>28</sup> and in hepatocellular carcinoma.<sup>29</sup> *OSM* increases the invasive capacity of breast cancer cells.<sup>30</sup> *SSH2*-mediated dephosphorylation of cofilin/ADF (actin depolymerizing factor) increases the migration and invasion of hepatoma cells.<sup>31</sup> *TGFBI* promotes metastasis of colon cancer cells by

accelerating cell extravasation.<sup>32</sup> Although further investigation is necessary, increased metastatic properties resulting from TGF- $\beta$  signaling in ovarian cancer may be attributed to upregulation of multiple metastasis-promoting genes.

Finally, we analyzed the *in vivo* therapeutic effects of A-83-01. As A-83-01 has never been used for *in vivo* experiments, we first examined side effects of A-83-01 in mice; no significant side effects were observed as the previous reports with the other TGF- $\beta$  inhibitors.<sup>14,15</sup> It is known that considerable amounts of TGF- $\beta$ 1 exist in the ascitic fluid of ovarian cancer patients.<sup>33</sup> Similarly, the concentrations of TGF- $\beta$ 1 were high in the ascitic fluid associated with peritoneal dissemination of HM-1. Therefore, this mouse model is appropriate to examine the *in vivo* therapeutic effects of A-83-01. Accumulation of ascitic fluid in A-83-01-treated mice tended to be slower than the control group (Supporting Information Fig. 1). In addition, A-83-01 significantly improved survival (Fig. 6*b*). Recently, inhibition of the TGF- $\beta$  pathway has been shown to be a promising treatment modality against cancers. AP12009, an antisense oligonucleotide against TGF- $\beta$ 2, was found to be very effective against recurrent glioma in a phase I/II clinical trial,<sup>5</sup> and a phase III clinical trial is now underway. Efficacy of TGFBR kinase inhibitors, such as A-77, Ki26894 and LY2109761 was demonstrated in preclinical studies of gastric cancer, breast cancer and pancreatic cancer.<sup>14,15,34</sup> Although there may be a risk of carcinogenesis<sup>20</sup> with these drugs, this has never been reported in clinical and preclinical studies for TGF- $\beta$  inhibitors. Therefore, A-83-01 may be a useful drug in the treatment of advanced ovarian cancer.

In summary, starting from gene expression profiling analysis, we found that the TGF- $\beta$  signaling pathway is activated in omental metastases of ovarian cancers. We then demonstrated the *in vitro* and *in vivo* efficacy of A-83-01, a TGFBR1 kinase inhibitor. This report shows for the first time that inhibition of the TGF- $\beta$  signaling pathway by a small molecule is a promising strategy in the treatment of ovarian cancer.

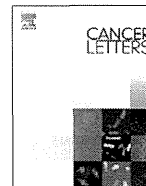
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## Mini-review

## Ovarian clear cell carcinoma as a stress-responsive cancer: Influence of the microenvironment on the carcinogenesis and cancer phenotype

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## ABSTRACT

Although it is well known that ovarian endometriosis occasionally gives rise to ovarian cancers with specific histology such as endometrioid and clear cell carcinomas, its etiology is not fully understood. We have shown that a stressful microenvironment within the endometriotic cyst may lead to cancer development by inducing unique gene expressions, which potentially serves as a molecular marker for treatment modality. In this review, by referring to other articles in this field, we explore how the carcinogenic microenvironment affects the phenotype and gene expression of a cancer, and how we can develop new treatment based on this concept.

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### 1. Introduction

Endometriosis is a benign gynecological disease that affects 5–10% of women [1]. It is clinically well known that ovarian cancer arises from ovarian endometriosis with a relatively high frequency. A prospective randomized trial in Japan, in which over 8000 asymptomatic postmenopausal women were followed for up to 17 years, demonstrated that 0.72% of women with endometriosis develop ovarian cancer [2]. This rate is higher than the incidence of cancer in other benign ovarian tumors such as serous or mucinous cystadenomas [2], suggesting the presence of discrete risk factors for oncogenesis in ovarian endometriosis. In addition, unlike typical ovarian cancers, the cancers arising from endometriosis more commonly comprise clear cell and endometrioid subtypes [3,4]. These findings strongly suggest that the carcinogenic process in ovarian endometriosis is unique and different from that of ordinary

ovarian cancers. With this in mind, we sought to clarify the mechanism by which endometriosis gives rise to a discrete phenotype of cancer with a high frequency, and we hypothesized that the unique microenvironment in the endometriotic cyst plays a pivotal role in cancer development.

Pathological evidences as well as recent molecular analyses have been clarifying the precancerous property of endometriosis. Pathologically, co-existence of endometriosis and ovarian cancer is frequently observed, and they sometimes accompany “atypical endometriosis”, a putative precursor lesion [5]. Common genetic events such as LOH and genetic mutation have been demonstrated in carcinoma and adjacent endometriosis [5]. Overexpression of HNF-1 $\beta$ , a putative hallmark of clear cell carcinoma, is also frequently expressed in benign and atypical endometriosis [6]. Moreover, mutation of the ARID1A gene, which is recently identified in approximately a half of clear cell carcinoma and one thirds of endometrioid carcinoma, is also found in a part of atypical endometriosis adjacent to the carcinoma [7]. These data collectively suggest that endometriosis really transform into carcinoma, occasionally

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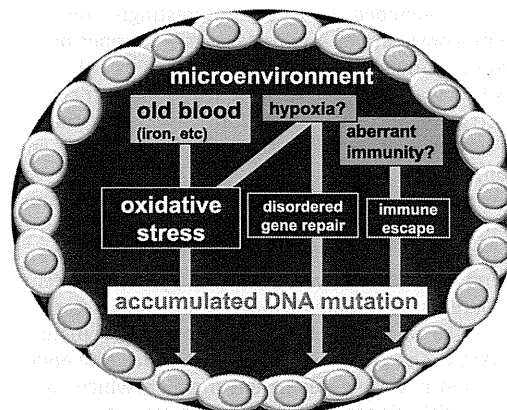
passing through an intermediate lesion, namely, atypical endometriosis. However, little is known about the mechanisms that drive benign epithelium into malignant transformation within the endometriotic cyst.

## 2. High iron concentration and its potential influence in the development of ovarian cancer in endometriosis

Endometriosis is characterized by repeated bleeding into the cyst cavity during the menstrual cycle. As a result, the content of an endometriotic cyst consists of highly concentrated old blood, and the endometriotic epithelial cells within the cyst are consistently exposed to an unusual microenvironment that is rarely encountered in the body. Recently it has been recognized that carcinogenesis and tumor progression are significantly influenced by the microenvironment in which the tumor arises [8,9]. We therefore sought to identify elements specifically included in the cystic fluid of endometriosis. As expected, the concentration of iron was markedly high in endometriotic cysts [10], in accordance with previous reports [11,12]. In our study, the free iron concentration was also markedly high in endometriotic cysts compared with other benign ovarian cysts. Free iron is associated with cancer development through induction of persistent oxidative stress in several organs such as liver and lung [13,14]. There is also a ferric nitrilotriacetate-induced renal carcinogenesis model that shows the contribution of catalytic iron to cancer development [15]. Therefore, persistent exposure to highly concentrated free iron may lead to ovarian cancer, possibly via production of oxidative stress.

To examine this possibility further, we evaluated the extent of oxidative stress within the endometriotic cyst compared to other benign ovarian tumors. Lactose dehydrogenase (LDH), a marker of tissue damage; potential antioxidant (PAI), an antioxidant marker; lipid peroxidase (LPO), a marker of oxidative stress; and 8-hydroxy-2-deoxyguanosine, a marker of DNA damage; were all significantly elevated in endometriotic cysts compared with other ovarian cysts [10]. Obviously, the epithelial cells within the endometriotic cyst are exposed to extensive oxidative stress (reactive oxygen species, ROS), and as a result, they are subjected to more cellular and DNA damage than other benign ovarian tumors (Fig. 1). Actually, our *in vitro* experiments revealed that the fluid in chocolate cysts is more mutagenic than that of other cysts [10]. Hence, epithelial cells within the endometriotic cyst are subject to “microenvironment-induced mutagenesis” [15]. The link between the stressful microenvironment and cancer development is supported by several other reports. (1) Chromosomal aberrations are present at a higher frequency in ovarian endometriotic cysts compared to extra-gonadal endometriosis where the epithelium is less exposed to old blood elements [16]. (2) The occurrence of ovarian cancer increases with the duration of endometriosis [17].

It is, of course, an oversimplification to ascribe the cause of cancer development in endometriosis solely to the accumulated iron. The characteristic microenvironment within the endometriotic cyst may include other factors such as



**Fig. 1.** Influence of the microenvironment within the endometriotic cyst on carcinogenesis. Fluid in the endometriotic cyst contains various special elements including free iron and constitutes a unique stressful microenvironment, which thereby leads to the cancer development.

hypoxia, poor nutrition or altered immune status (Fig. 1). Hypoxia, for instance, also increases ROS leading to increased DNA damage and less efficient DNA repair [18,19]. To investigate these aspects further, more comprehensive analyses using genome-wide analysis was needed.

## 3. Influence of microenvironment on the gene expression and phenotype of cancer arising in endometriosis

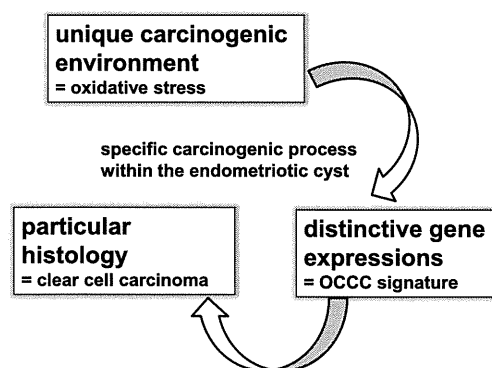
There is likely to be an association between endometrioid adenocarcinoma and endometriosis because of the similarity to the relationship between endometrioid adenocarcinoma and the uterine endometrium. Several studies suggest the etiological role of estrogen in both cases [20]. In contrast, no clear explanation has been found for the observation that clear cell carcinoma frequently occurs in endometriosis.

We then hypothesized that the microenvironment within the endometriosis, which appears to be associated with the development of ovarian cancer, also affects the phenotype of the cancer arising there. To verify this possibility, we first identified the gene signature that distinguishes clear cell carcinoma from other types of ovarian cancer using a microarray dataset of ovarian cancers [21]. The signature consisted of 437 genes and was designated as the ovarian clear cell carcinoma (OCCC) signature. It could efficiently distinguish clear cell carcinomas in multiple independent databases, indicating that the OCCC signature is indeed specific for clear cell carcinoma. Next, we analyzed what kinds of genes were included in this signature. A categorical analysis, Allez, demonstrated that genes belonging to three categories, stress response, sugar metabolism and coagulation, are frequently involved in this signature. Especially stress-related genes occupy a significant part, suggesting that they play a central role in the manifestation of the phenotypical features of clear cell carcinoma. Then, a computer-based pathway analysis was employed to elucidate the functional relationships among

the genes included in OCCC. Interestingly, one signal network consisting of a relatively large number of genes, including many stress-related genes such as HIF1- $\alpha$ , IL-6, and SOD2, was supposed to be activated in clear cell carcinoma. This result suggests that at least one of the major functions evoked by OCCC signature genes is the stress-response.

Our next question was whether the microenvironment within the endometriotic cyst is in any way associated with the expression of the OCCC signature. To address this question, we treated ovarian surface epithelial cells with the contents of endometriotic cysts and assessed the time-course of the alteration of the OCCC signature by microarray. The OCCC signature was induced by endometriotic fluid in a time dependent manner, which suggests that the OCCC signature is primarily a response of epithelial cells to the microenvironment within the endometriotic cyst [21]. If this is the case, the expression of the OCCC signature in the case of clear cell carcinoma should be based on some other mechanism than merely gene induction because they are constitutively expressed in clear cell carcinoma without any specific stress in the culture medium. We speculated that epigenetic regulation may have something to do with the constitutive expression of the OCCC signature and examined the methylation status in ovarian cancer cells. In two genes we examined, VCAN and HNF-1 $\beta$ , methylation in the regulatory region of the gene is more frequent in clear cell carcinoma than in serous carcinomas, suggesting that, at least in part, OCCC signature expression is caused by epigenetic alteration [21]. Thus, it is estimated that the OCCC signature should first be induced by the stressful environment in the endometriotic cyst and then fixed in the course of development of the clear cell carcinoma (Fig. 2).

Taken together, the identification and analysis of the OCCC signature gave us the idea that the specific microenvironment induces unique gene expressions and leads to a cancer of a special phenotype (Fig. 3). Recently, the effect of the local microenvironment such as ROS or inflammation was implicated in cancer development [22]. However, a link between the microenvironment and the occurrence

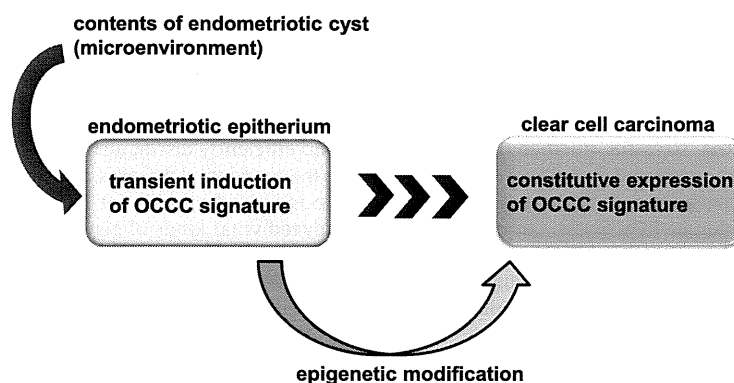


**Fig. 3.** A specific carcinogenic process within the endometriotic cyst. A stressful microenvironment not only induces carcinogenesis but also affects the phenotype and character of the cancer derived under its influence.

of a specific cancer phenotype is a new insight, and further evidence should be collected to verify this concept.

#### 4. Possible application of the OCCC signature in the treatment of disease

Is it possible to apply the OCCC signature to the treatment of ovarian clear cell carcinoma? To answer this question, we evaluated the malignant tumors in the whole body to ascertain whether there are cancers with elevated expression of the OCCC signature [23]. Among various cancers, only renal cell carcinomas (RCC) express significantly higher levels of the OCCC signature. In hierarchical clustering using the OCCC signature, ovarian clear cell carcinoma was indistinguishable from RCC, indicating the similarity in gene expressions between these cancers. RCC is generally chemoresistant, and until recently, IFN- $\alpha$  and IL-2 were the only therapeutic agents clinically used for RCC, and they had only modest response rates [24]. However, the recently introduced molecular target drugs sorafenib and sunitinib provide significant therapeutic efficacy, drastically changing the modality of this tumor [25,26]. Given



**Fig. 2.** Transient induction of the OCCC signature and subsequent fixation of the expression by epigenetic alteration. The expression of the OCCC signature is transiently induced in the presence of endometriotic fluid and subsequently becomes constitutive partly by epigenetic modifications.

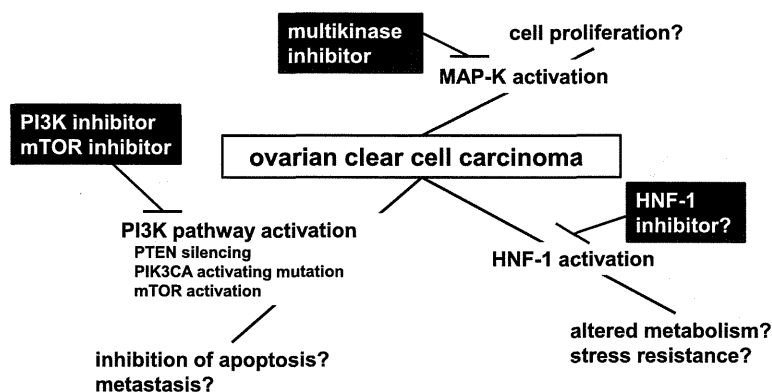


Fig. 4. Multimodality molecular targeting therapy for ovarian clear cell carcinoma. Clear cell carcinoma, a chemoresistant cancer, should be treated by multimodal targeting therapy based on the biological nature of the tumor.

the results indicating the similarity between OCCC and RCC, we sought to further investigate the efficacy of sorafenib and sunitinib against OCCC. A mouse xenograft model using the human OCCC cell line, RMG-2, which is known to be chemoresistant, clearly indicated that sorafenib is effective in the suppression of RMG-2.

We then evaluated the mechanism by which sorafenib exerts its antitumor effect. Sorafenib is known to inhibit multiple kinase activities, including those activated by epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR) and vascular endothelial growth factor receptor (VEGFR) [27,28]. In our analyses of various oncogenic pathways among ovarian cancers, OCCC is characterized by the activation of MAPK, while oncogenic signals related to cell proliferation, such as E2F3 and Myc, are not activated compared with other ovarian cancers such as serous adenocarcinoma. *In vitro* experiments revealed that sorafenib can inhibit the MAPK activity of RMG-2, in accordance with other reports [29,30], suggesting that this might be the primary consequence of the antitumor effect of sorafenib in our study. These data suggest that comprehensive assessment of specific gene expressions in one type of cancer provides a reasonable background for the selection of a therapeutic reagent that targets an activated intracellular signal, especially in the case of a slowly proliferating, chemoresistant tumor such as OCCC.

##### 5. Further directions, especially future treatment approaches for clear cell carcinoma (Fig. 4)

Most of the clear cell carcinomas are resistant to current chemotherapy, and the treatment of this tumor remains an important clinical challenge in the treatment of ovarian cancer. As mentioned above, our comprehensive gene expression analyses revealed that the molecular targeting drugs effective for RCC are potentially useful in the treatment of OCCC, one of which is sorafenib. There are several other reagents that are expected to be effective in RCC, and such drugs are thought to be candidates for therapeutic reagents against OCCC. For instance, mTOR inhibitors (temsirolimus, everolimus) as well as VEGF signaling blockers

(bevacizumab) are shown to be promising therapeutic reagents in RCC [31–33] (Fig. 4). Both are induced by HIF-1 $\alpha$  activation, a fundamental feature in RCC harboring VHL aberrations [34].

Another possible target molecule in OCCC is HNF-1 $\beta$ . This molecule is known to be a specific marker for clear cell carcinoma [35]. In our analyses, HNF-1 $\beta$  is included in the OCCC signature and in the signal network estimated by pathway analysis [21]. Moreover, gene expression of this molecule is regulated by DNA methylation in cancer cells [21]. The DNA binding motifs of HNF-1 $\beta$  were significantly enriched in OCCC [23]. These data collectively suggest that HNF-1 $\beta$  plays a pivotal role in the biological behavior of OCCC. However, the precise function of this protein is not fully understood. To determine whether this molecule could be a therapeutic target, we are now evaluating the effect of suppressing the expression of this gene in HNF-1 $\beta$ -overexpressing OCCC cells.

Obviously, in a chemoresistant malignancy like OCCC, the future treatment modality consists mainly of combinations of the various molecular targeting drugs mentioned above. In this respect, the most important strategy in successfully applying those reagents in each case of OCCC is to reliably identify the molecular marker that can predict the efficacy of those reagents. The OCCC signature, which reflects the fundamental character of OCCC and represents the 'OCCC-likeness', might serve as such a marker. However, to estimate more precisely which drug is most effective in each case, it will be necessary to develop individual signatures to reflect signal pathways corresponding to each drug.

##### Conflict of interest

All the authors in this study do not have any financial and personal relationships with other people or organizations that could inappropriately influence (bias) our work.

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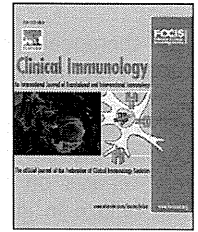




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# The comprehensive assessment of local immune status of ovarian cancer by the clustering of multiple immune factors

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## KEYWORDS

Ovarian cancer;  
Tumor immunity;  
Tumor immune escape

**Abstract** The aim of this study was to evaluate the local immune status of human ovarian cancers by the comprehensive analysis of tumor-infiltrating immune cells and immunosuppressive factors, and to elucidate the local immunity in clinical course. The numbers of CD1 $\alpha$ +, CD4+, CD8+, CD57+, forkhead box P3+ and programmed cell death-1+ cells were counted, and the intensity of immunosuppressive factors, such as programmed cell death-1 ligand (PD-L)1, PD-L2, cyclooxygenase (COX)-1, COX-2 and transforming growth factor  $\beta$ 1, were evaluated in 70 ovarian cancer specimens stained by immunohistochemistry. Then hierarchical clustering of these parameters showed the four clusters into ovarian cancer cases. Cluster 1, which had significantly better prognosis than the others, was characterized by high infiltration of CD4+ and CD8+ cells. In conclusion the comprehensive analysis of local immune status led to subdivide ovarian cancers into groups with better or worse prognoses and may guide precise understanding of the local immunity.

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## 1. Introduction

Ovarian cancer is the most lethal gynecologic cancer in the world with >200,000 patients diagnosed every year and

over a half of them dying annually. These deaths are partly due to the fact that more than half of the patients with ovarian cancer are diagnosed at advanced tumor stages (stage III or IV). Although platinum or taxane-based chemotherapies are effective in the treatment of the majority of ovarian cancer cases, most of the patients suffer from recurrence and eventually develop chemo-resistance. Considering the high mortality rate of ovarian cancer due to the absence of curative treatment in the advanced stage or at recurrence,

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new therapeutic modalities other than chemotherapy and surgery are urgently needed [1–3].

Tumor immune therapy has long been considered as an alternative modality in the treatment of solid tumors including ovarian cancer. Nevertheless, there have been few reports on clinically successful immune therapies. The failure in immune therapies in such clinical trials is partly ascribed to the phenomenon designated as “tumor immune escape”. It is increasingly understood that the dynamic interaction between tumor cells and immune cells in the local microenvironment plays a pivotal role in cancer development and progression [4]. In the case of advanced cancers, tumor cells establish an immunosuppressive environment regionally and make it difficult to induce immune activation to eliminate cancer cells. In this situation, adoptive immunotherapy, such as a tumor vaccine, is not sufficient to eradicate tumors [5,6].

The differences in the phenotypes or populations of tumor-infiltrating immune cells, such as CD4<sup>+</sup> (helper) T cells, CD8<sup>+</sup> (cytotoxic) T cells, CD57<sup>+</sup> (NK) cells and CD11c<sup>+</sup> (dendritic) cells, have been shown to be associated with different clinical outcomes of solid tumors including colorectal cancer [7], breast cancer [8], gastric cancer [9,10], lung cancer [11,12], hepatic cancer [13,14], melanoma [15], kidney cancer [16] and uterine cervical cancer [17]. In ovarian cancer, several recent studies have shown an association between tumor-infiltrating immune cells and clinical outcomes [18,19]. We also reported that CD8<sup>+</sup> T cell infiltration [20] and NK cell infiltration [21] are associated with a favorable prognosis in the ovarian cancer patient. On the other hand, regulatory T cells, most specific marker of which is FOXP3, in the tumor site play a suppressive role in the local tumor immunity, leading to tumor progression [22,23].

With respect to the tumor, there are also a wide variety of mechanisms that enable tumor cells to evade an immune attack. These mechanisms include a loss of MHC [24], the upregulation of immunosuppressive factors, such as transforming growth factor  $\beta$  (TGF $\beta$ ) [25], IL-10, indoleamine 2,3-dioxygenase (IDO) [26] and cyclooxygenases (COX-1 and COX-2) [27] or upregulating negative regulatory signals, such as programmed cell death-1 (PD-1) ligands (PD-L1, PD-L2) and cytotoxic T lymphocyte antigen-4 (CTLA-4) [28–31]. We reported that PD-L1 expression in ovarian cancer is inversely correlated with tumor-infiltrating CD8<sup>+</sup> T cells and is associated with a poor prognosis of the patient [20]. The expression of the immune suppressive factors COX and UL-16 binding protein 2 is also inversely associated with CD8<sup>+</sup> T cell infiltration and the prognosis of the patient with ovarian cancer [21,32]. Thus, there are a variety of reports that suggest that a certain immunosuppressive factor influences the local tumor immunity. However, there are few comprehensive analyses that integrate various immune factors and evaluate the immune status as a whole.

Therefore, in this study, we attempted to explore the status of local immunity in ovarian cancers by integrating various immune parameters presented by the immunohistochemical analysis of clinical specimens. For this purpose, we employed bioinformatics analyses, such as hierarchical clustering, that allows the comprehensive assessment of multiple factors and enables us to determine the relationships among them.

## 2. Materials and Methods

### 2.1. Patients and Samples

Formalin-fixed, paraffin-embedded specimens were obtained from 70 patients who underwent primary surgery for epithelial ovarian cancer at the Kyoto University Hospital. After surgery, all patients received platinum- and paclitaxel-based chemotherapy. The average age of the patients was 55 years old (range, 26–78; standard deviation [SD], 11). At the end of the study, 29 (41%) patients had died from their disease, and 41 (59%) patients were alive. The mean follow-up period was 5 years (range, 0–11; SD, 3.0). All 70 tissue specimens were collected under the approval of the Ethics Committee of the Kyoto University Hospital.

### 2.2. Immunohistochemistry

The primary antibodies and antigen retrieval methods are listed in Supplementary Table 1. Briefly, formalin-fixed, paraffin-embedded specimens were cut into 4  $\mu$ m-thick sections. The tissue sections were deparaffinized in xylene and dehydrated. For antigen retrieval for TGF $\beta$ 1 and CD4 staining, the samples were boiled in Tris-EDTA buffer (pH 9.0) in a pressure cooker. For FOXP3 and PD-1 staining, the samples were boiled in citrate buffer (pH 6.0) in a pressure cooker. To block endogenous peroxidase activity, all of the sections were treated with 100% methanol containing H<sub>2</sub>O<sub>2</sub>. Nonspecific binding of IgG was blocked using normal rabbit serum (Nichirei, Tokyo, Japan). The sections were incubated with a mouse anti-TGF $\beta$ 1 monoclonal antibody (Ab) (clone TB21), anti-CD4 monoclonal Ab (clone 1F6), anti-FOXP3 monoclonal Ab (clone 236A/E7) and PD-1 monoclonal Ab (clone NAT) overnight at 4 °C. Then the sections were incubated with biotinylated rabbit anti-mouse secondary Abs (Nichirei), followed by an incubation with a streptavidin–peroxidase complex solution. Signals were generated by incubation with 3, 3'-diaminobenzidine. Finally, the sections were counterstained with hematoxylin and observed under a microscope.

### 2.3. Evaluation of the specimens

Immune cells in the intraepithelial space were counted using a microscopic field at 200 $\times$  magnification (0.0625 mm<sup>2</sup>). Five areas with the most abundant infiltration of immune cells were selected, and an average count was calculated. The result was interpreted as negative when fewer than five cells per 0.0625 mm<sup>2</sup> were observed and as positive when more than or equal to five cells were observed. The expression of TGF $\beta$ 1 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 of 2 or 3 were defined as the high-expression group. Two independent gynecological pathologists examined the immunohistochemical slides without any prior information regarding the clinical history of the patients.

## 2.4. Hierarchical clustering and statistics

Hierarchical clustering analysis of our immunohistochemical data was performed using the software Cluster 3.0 that was originally designed for manipulating cDNA microarray data [33]. Following the instructions of the software, the eleven parameters (six tumor-infiltrating immune cells and five immune suppressive factors) were normalized, and a complete-linkage hierarchical clustering was conducted. The dendrogram and heat map were graphically viewed using Java TreeView [33]. Cluster and Treeview software are freely available programs that can be accessed at <http://jtreeview.sourceforge.net/>.

## 2.5. Statistical analysis

Fisher's exact test and the  $\chi^2$  test were used to analyze the associations between each cluster and various clinicopathological factors. Spearman's correlation coefficient test was employed to analyze the associations among 11 immunological factors. Univariate analysis for overall survival was performed and evaluated with the log rank test, and Kaplan–Meier curves were generated. A multivariate Cox proportional-hazard model was used to evaluate the independency of Cluster 1 as a prognostic factor. Two-sided p values of  $<0.05$  were considered to be significant.

## 3. Results

### 3.1. Expression of immune-suppressive factors in ovarian cancer specimens and patient prognosis

Immunohistochemical expression of TGF $\beta$ 1, PD-L1, PD-L2, COX-1 and COX-2 was evaluated in 70 ovarian cancer tissues (Fig. 1). High expression (score 2 or 3) of TGF $\beta$ 1 was observed in 22 cases (31.4%) and low expression (scored 0 or 1) was observed in 48 cases (68.6%). There was no correlation between the expression of these factors and clinicopathological characteristics such as age, histological type, FIGO stage, TNM classification, and residual tumor state (Supplementary Table 2) [20,32].

The log rank test showed that the 5-year survival rate of patients with high expression of TGF $\beta$ 1, COX-1 or COX-2 was not significantly different from the patients with low expression (Supplementary Fig. 1). Only the high expression of PD-L1 was an independent worse prognostic factor, whereas PD-L2 expression was not related to patient prognosis [20].

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### 3.2. Tumor-infiltrating immune cell count and prognosis

The number of tumor-infiltrating CD1 $\alpha$ + (dendritic cells), CD4+ (helper T cells), CD8+ (killer T cells), CD57+ (NK cells), FOXP3+ (regulatory T cells) and PD-1+ immune cells was evaluated using the same 70 ovarian cancer specimens (Fig. 1). The average numbers of these cells were shown in Supplementary Table 3, respectively. There was positive correlation between tumor-infiltrating FOXP3+ cells and several clinicopathological factors such as age, histology, tumor status and residual tumor, while there was no correlation between the number of CD4+, CD8+, CD57+ or PD-1+ cells and clinicopathological characteristics (Supplementary Tables 4 and 5).

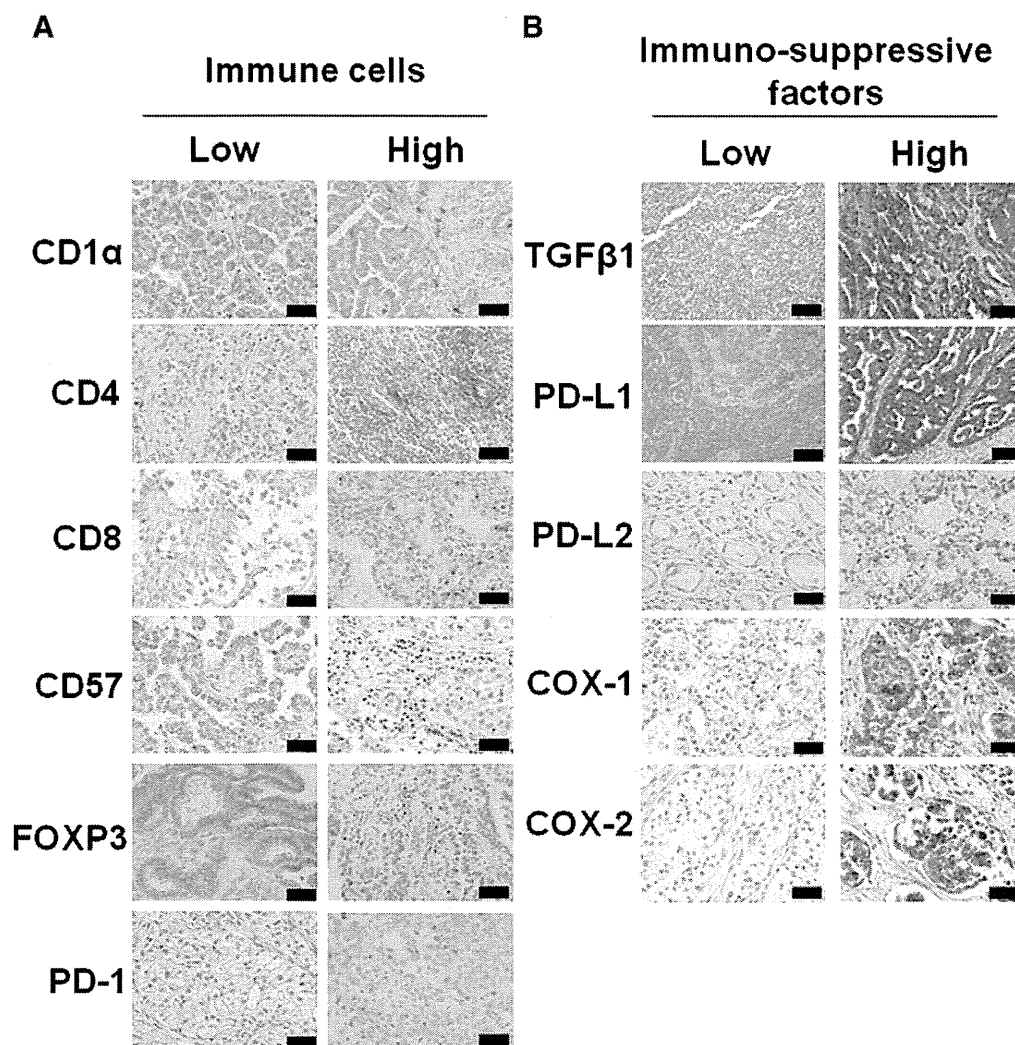
A significant correlation was found between parameters below; CD4+ cell infiltration vs. CD8+ cell infiltration, COX-1 and COX-2 expression, CD4+ cell vs. PD-1+ cell infiltration, CD8+ cell vs. PD-1+ cell infiltration, CD57+ cell vs. PD-1+ cell infiltration, FOXP3 cell infiltration vs. PD-L2 expression and FOXP3 vs. CD4+ cell infiltration (Supplementary Table 6), although a negative correlation between COX-1 vs. COX-2 expression, CD8+ cell infiltration vs. PD-L1 expression, CD8+ cell infiltration vs. COX-1 expression and CD8+ cell infiltration vs. COX-2 expression [20,32].

The log rank test showed that the overall survival rate of patients with high levels of CD1 $\alpha$ +, CD4+, CD57+, FOXP3+ or PD-1+ immune cells was not significantly different from patients with low infiltration (Supplementary Fig. 1), whereas a high infiltration of CD8+ cells was the only beneficial prognostic factor ( $p < 0.001$ ) [20]. Combination of any two factors such as CD8+ and PD-L1 low did not serve as a superior prognostic factor compared with single factor. Besides we found a higher ratio of CD8/FOXP3 in Cluster 1 than that in Cluster 2–4, although there was no statistic significance (mean  $\pm$  SD, Cluster 1,  $3.4 \pm 2.4$  vs. Cluster 2–4,  $1.9 \pm 1.9$ ).

The log rank test showed that the overall survival rate of patients with high levels of CD1 $\alpha$ +, CD4+, CD57+, FOXP3+ or PD-1+ immune cells was not significantly different from patients with low infiltration (Supplementary Fig. 1), whereas a high infiltration of CD8+ cells was the only beneficial prognostic factor ( $p < 0.001$ ) [20]. Combination of any two factors such as CD8+ and PD-L1 low did not serve as a superior prognostic factor compared with single factor. Besides we found a higher ratio of CD8/FOXP3 in Cluster 1 than that in Cluster 2–4, although there was no statistic significance (mean  $\pm$  SD, Cluster 1,  $3.4 \pm 2.4$  vs. Cluster 2–4,  $1.9 \pm 1.9$ ).

### 3.3. The correlation among eleven immunological factors

The correlation among eleven immunological factors (the expression of PD-L1, PD-L2, COX-1, COX-2 and TGF $\beta$ 1 and the number of tumor-infiltrating immune cells expressing CD1 $\alpha$ +, CD4+, CD8+, CD57+, FOXP3+ and PD-1+) was examined (Supplementary Table 6). The expression of PD-L1 or COX expression was negatively correlated with the number of CD8+ cells in the tumor site, respectively [20,32]. In this study, we found that the number of CD4+ cells was



**Figure 1** Immunohistochemical staining of human ovarian cancer tissue. (A) Representative staining patterns of ovarian cancers with low expression or with high expression of immunosuppressive factors, such as TGF $\beta$ 1, PD-L1, PD-L2, COX-1, and COX-2, are shown. (B) Representative staining patterns with low or high infiltrating immune cells, such as CD1 $\alpha$ +, CD4+, CD8+, CD57+, FOXP3+ or PD-1+ cells, in the tumor site are shown. Original magnification; (A and B)  $\times$ 200. White bar, 200  $\mu$ m.

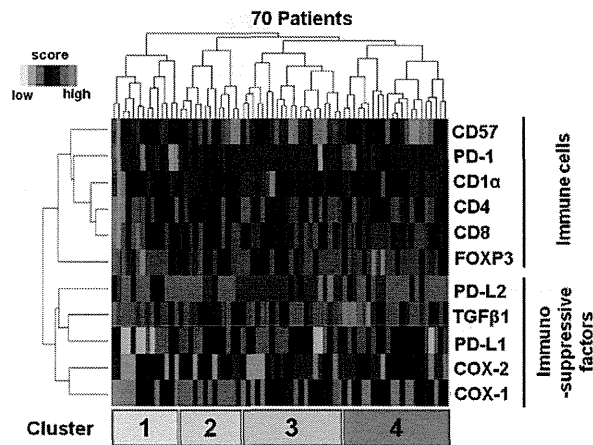
positively correlated with the number of CD8+ cells (correlation coefficient ( $R=0.240$ ;  $p=0.045$ ) and FOXP3+ cells ( $R=0.410$ ;  $p<0.001$ )). In addition, the number of PD-1+ cells showed a positive correlation with the number of CD4+ cells ( $R=0.302$ ;  $p=0.011$ ), CD8+ cells ( $R=0.366$ ;  $p=0.002$ ) and CD57+ cells ( $R=0.365$ ;  $p=0.002$ ). The number of FOXP3+ cells was negatively correlated with PD-L2 expression ( $R=-0.262$ ;  $p=0.028$ ).

### 3.4. Evaluation of the local immune status by hierarchical clustering of immune factors in ovarian cancer

Hierarchical clustering analysis of the expression levels of five immune suppressive factors and the cell counts of the six tumor-infiltrating immune cells were used to divide the 70

ovarian cancers into 2 major clusters and subdivided one of the major clusters into three clusters, which were designated as Cluster 1 and Clusters 2, 3 and 4, respectively (Fig. 2). When Cluster 1 was compared to the other clusters (Clusters 2–4), it was characterized as having significantly higher immune cell infiltration, such as CD4+ cells ( $p=0.004$ ), CD8+ cells ( $p<0.0001$ ) and PD-1+ cells ( $p=0.0037$ ), and as having lower expression of immunosuppressive factors such as TGF $\beta$ 1, PD-L1, PD-L2, COX-1 and COX-2 (Figs. 3A–C, F and 4).

The characteristics of the other three clusters were relatively common in terms of low immune cell infiltration and partially high expression of immune suppressive factors with the following patterns: Cluster 2, high COX-1 expression ( $p<0.0001$ ) and high CD57+ cell (NK cell) infiltration ( $p=0.0042$ ); Cluster 3, high PD-L2 ( $p=0.0002$ ), low FOXP3+ cells ( $p=0.0288$ ) and low PD-1+ cell infiltration ( $p=0.011$ ); and Cluster 4, low CD4+, low CD8+, low CD1 $\alpha$ +, low CD57+,



**Figure 2** Graphic representation of the immune status of 70 ovarian cancer tissues. Patterns of immune status were classified into four clusters by hierarchical clustering based on six phenotype of immune cells, such as CD1α+, CD4+, CD8+, CD57+, FOXP3+ or PD-1+ cells, in the tumor site and five immunosuppressive factors, such as TGFβ1, PD-L1, PD-L2, COX-1 and COX-2. Separated clusters are indicated by dendrograms. The color bar indicates that red is the high score (expression or infiltration), while green is the low score.

low PD-1, high PD-L1, high TGFβ1, and high COX-2 (Figs. 3 and 4).

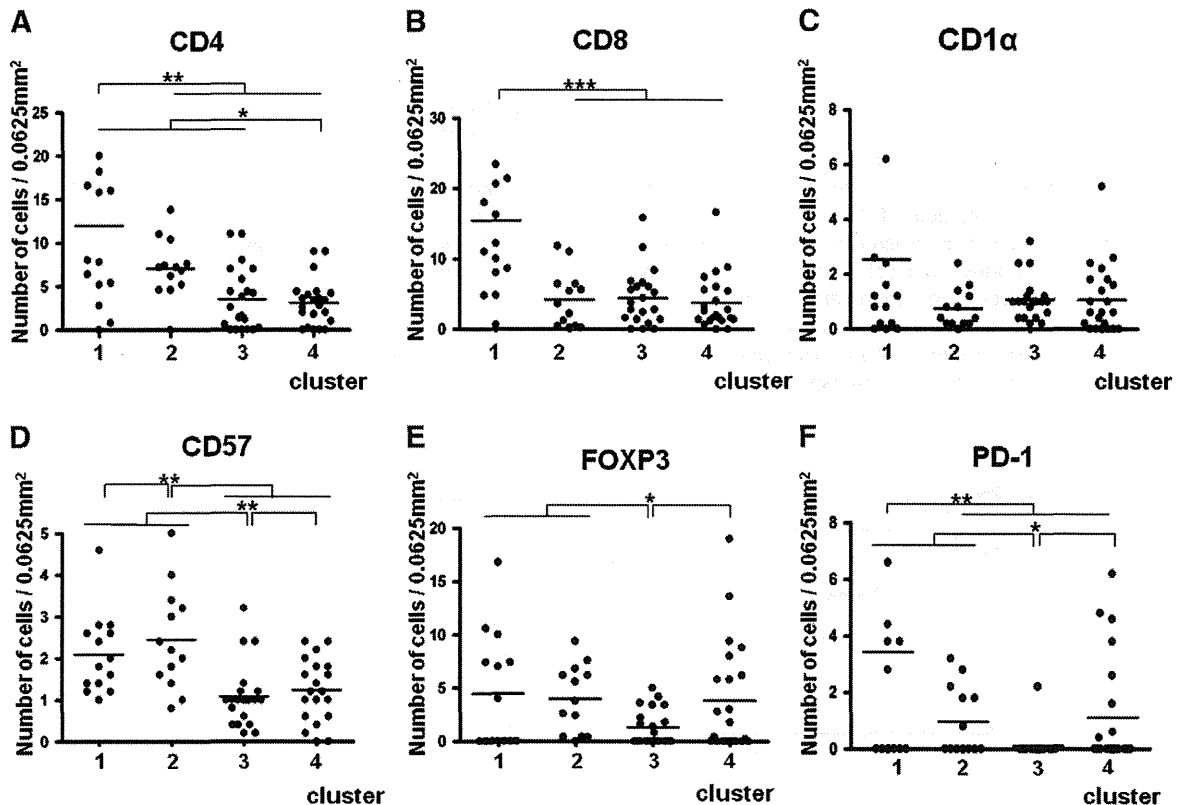
**3.5. Univariate analysis and correlation between four clusters and clinicopathological factors**

The Kaplan–Meier curve and log rank test showed that the overall survival rate of patients in Cluster 1 was significantly better than those in the other clusters (5-year survival rate in Cluster 1 vs. Clusters 2–4, 84.6% vs. 55.2%;  $p=0.041$ ) (Fig. 5 and Table 1). The progression-free survival rate of patients in Cluster 1 was not significantly, but was relatively, better than other clusters (5-year survival rate of Cluster 1 vs. Clusters 2–4, 78.6% vs. 44.4%;  $p=0.061$ ).

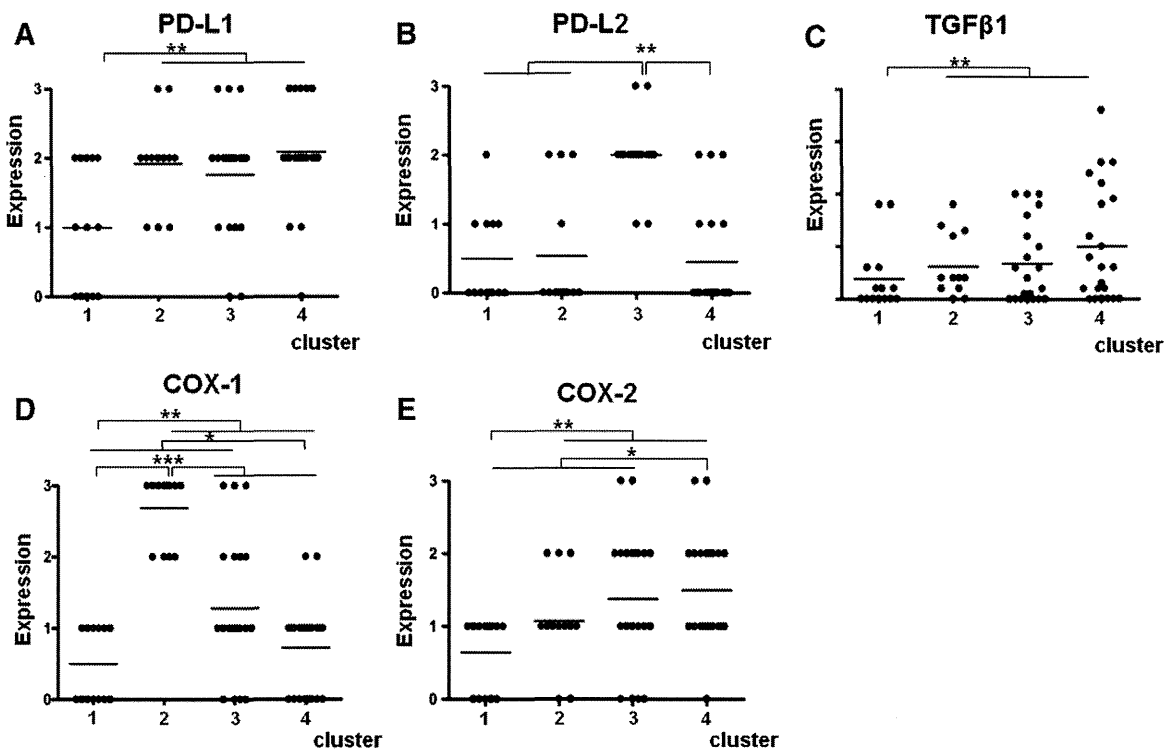
There was no statistical correlation between the four clusters and the clinicopathological factors such as primary tumor status, lymph node metastasis, distant metastasis, residual tumor status, the age of the patient, histology, and adjuvant chemotherapy (Table 2).

**3.6. Multivariate analysis**

Multivariate analysis showed that Cluster 1 was an independent favorable prognostic factor for overall survival (RR, 4.93) (Table 1). Other factors contributing to overall poor survival were tumor status (RR, 5.36), lymph node metastasis (RR, 2.78), and residual tumor status (RR, 5.86) (Table 1).



**Figure 3** The patterns of immune cell infiltration into tumor sites in each cluster. The dot plots represent the number of immune cells in the four clusters; (A) CD4; (B) CD8; (C) CD1α; (D) CD57; (E) FOXP3; and (F) PD-1 (\* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.0001$ ).

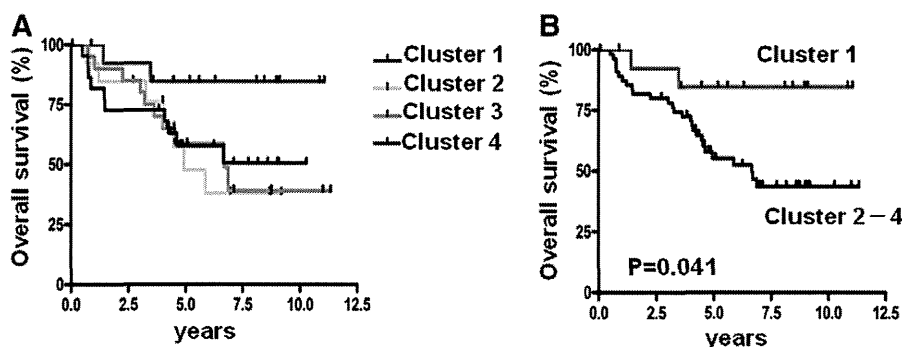


**Figure 4** The patterns of immunosuppressive factor expression in each cluster. The dot plots represent the expression levels of immunosuppressive factors in the four clusters; (A) PD-L1; (B) PD-L2; (C) TGFβ1; (D) COX-1; and (E) COX-2 (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.0001$ ).

**4. Discussion**

Recent studies have shown that local tumor immunity is closely associated with clinical course of cancer patient, and several immunological factors, including CD8 T cell count shown in our previous study, serve as prognostic indicator. However, these analyses were mainly done using single factor or combination of several factors, and there are few papers which tried to clarify the immunological background by analyzing multiple immune factors simultaneously. The application of hierarchical clustering allowed us to manage the complex data

sets of immunohistochemical staining with multiple antibodies [34,35] and to identify new groups of patients with similar local immunological patterns that may be caused by similar consequences. Ovarian cancers were divided into two groups, Cluster 1 and Clusters 2–4, by hierarchical clustering analysis according to the local immunological state. The patients in Cluster 1 had a significantly better prognosis than those in other clusters ( $p = 0.041$ , Fig. 5B). In this group, immune cells, including CD4+ cells, CD8+ cells and PD-1+ cells, were highly infiltrated into tumor sites compared to the other clusters ( $p = 0.004$ ,  $p < 0.001$  and  $p = 0.0037$ , respectively), while the expression of TGFβ1, PD-L1, PD-L2, COX-1 and COX-2



**Figure 5** Overall survival analyses of patients with ovarian cancer according to the four clusters. (A) Kaplan–Meier curves according to Cluster 1 and the other clusters. (B) Kaplan–Meier curves according to Cluster 1 and the combination of other clusters.

**Table 1** Univariate and multivariate analysis demonstrating the independent risk factors, including Cluster 1, on overall survival of patients with ovarian cancer (n=70).

	n	Overall survival			
		Univariate hazard ratio <sup>a</sup>	p	Multivariate hazard ratio <sup>a</sup>	p
Cluster			0.041		0.035
Cluster 1	38	1		1	
Clusters 2–4	32	3.98 (1.04–5.90)		4.93 (1.11–21.76)	
Tumor status			<0.001		0.013
pT1+pT2	31	1		1	
pT3	39	7.90 (2.73–22.83)		5.36 (1.42–20.20)	
LN metastasis			0.003		0.041
pN0	56	1		1	
pN1	14	3.24 (1.50–7.00)		2.78 (1.04–7.38)	
Distant metastasis			0.047		0.122
pM0	57	1		1	
pM1	13	2.28 (1.01–5.16)		2.44 (0.79–7.58)	
Residual tumor			<0.001		0.001
Optimal	49	1		1	
Suboptimal	21	4.54 (2.17–9.50)		5.86 (1.98–17.34)	
Histology			0.477		0.103
Serous type	33	1.33 (0.59–3.02)		3.27 (1.20–8.93)	
Non-serous type	37	1		1	
Chemotherapy			0.122		0.936
Paclitaxel	31	1		1	
No paclitaxel	39	1.79 (0.86–3.77)		1.03 (0.48–2.23)	
Age			0.486		0.562
<55	32	1		1	
≥55	38	1.31(0.62–2.77)		1.268 (0.57–2.83)	

<sup>a</sup> The numbers in parenthesis represent the 95% confidence interval (C.I.).

were significantly low. In this group, PD-1+ cells may represent T cells in the late active phase [36], though its significance is to be clarified. Thus, Cluster 1 was characterized by high immune cell infiltration and low expression of all immunosuppressive factors studied (Figs. 3 and 4), suggesting that host-tumor immunity in the tumor microenvironment is still maintained in this group, which may lead to the significantly better prognosis. Besides a ratio of CD8/FOXP3ratio in Cluster 1 was higher than that in Cluster 2–4, although there was no statistic significance, which is a similar tendency to the previously published report [19].

Clusters 2–4 were characterized by a low level of immune cell infiltration and high expression of immunosuppressive factors and had significantly worse prognoses than Cluster 1. Cluster 2 was characterized by a significantly high expression of COX-1, whereas Cluster 3 and 4 had significantly high expressions of COX-2 ( $p=0.0053$  and  $p=0.0048$ , respectively). The immunoregulatory function of COX-2-induced prostaglandin E2 (PGE<sub>2</sub>) is known to be important in inducing immune tolerance in the tumor microenvironment [37]. Secreted from tumor cells, PGE<sub>2</sub> alters the Th1/Th2 balance, suppresses lymphocyte proliferation, and regulates the function of antigen presenting cells [37,38]. There is a report that expression of COX-2 is an independent prognostic factor in human ovarian carcinoma [39]. Hence, high expression of COX-2 in Cluster 3 and Cluster 4 may contribute to poorer prognosis associated with low CD8+ cell infiltration (Figs. 3–5 and Supplementary Table 6). Similarly, COX-1 expression was inversely correlated with CD8+ cell infiltration in

Cluster 2, which may partly explain the poor prognosis of Cluster 2.

Cluster 3 was characterized by high PD-L2 expression and low PD-1+ cell infiltration and had a worse prognosis. We previously reported that the patient with high expression of PD-L2 had a tendency for poor prognosis, although the difference was not statistically significant. In this respect, high expression of PD-L2 may partly explain the poor prognosis of this group, possibly by negatively influencing the infiltration of CD8+, CD4+ and PD-1+ cells. Cluster 4 was characterized by high expression of PD-L1, TGFβ1 and COX2 and low CD8+ cell infiltration. Previous studies on PD-L1 expression in malignant tumors, such as in kidney, bladder, breast, gastric, pancreatic and ovarian cancer, have shown that PD-L1 has a negative impact on the survival of the patient [29]. In addition, PD-L1 expression was inversely correlated with intraepithelial infiltrating CD8+ T cells, suggesting that PD-L1 inhibits the intratumoral infiltration of CD8+ T cells. TGFβ signaling has been implicated in tumor progression, metastasis and immunosuppression in the advanced tumor phase [25]. These results suggest that PD-1 ligand and/or COX expression are associated with an unfavorable clinical outcome of the patient by influencing the local immune environment.

Recently, three phases of cancer immunoediting, namely, "elimination", "equilibrium" and "escape" [4,6,40], have been proposed. In the first "elimination" phase, innate and adaptive immune cells recognize and eliminate tumor cells by immunosurveillance, protecting the host against cancer. In the second "equilibrium" phase, ongoing tumor growth

**Table 2** Correlations between the four clusters and clinicopathological characteristics in ovarian cancer (*n*=70).

	n	Cluster 1	Cluster 2	Cluster 3	Cluster 4	p
Age						0.922
<55	32	6	8	10	10	
≥55	38	8	5	11	12	
Stage						0.972
I	27	6	3	9	9	
II	4	1	0	1	2	
III	26	5	5	8	8	
IV	13	2	5	3	3	
Histology						0.672
Serous	33	7	9	8	9	
Clear cell	22	5	2	9	6	
Endometrioid	11	1	1	4	5	
Mucinous	2	0	0	0	2	
Others	2	1	1	0	0	
Tumor status						0.889
pT1+pT2	31	6	4	10	11	
pT3	39	8	9	11	11	
LN metastasis						0.566
Positive	14	2	5	4	3	
Negative	56	12	8	17	19	
Distant metastasis						0.499
Positive	13	2	5	3	3	
Negative	57	12	8	18	19	
Residual tumor						0.773
Optimal	49	10	7	16	16	
Suboptimal	21	4	6	5	6	
Chemotherapy						0.965
No paclitaxel	39	9	7	12	11	
Paclitaxel	31	5	6	9	11	

and immune surveillance enter into a dynamic balance with one another, yielding in a protracted period. In the last "escape" phase, the tumor avoids immune-mediated destruction and develops into a clinically apparent neoplasm [4]. This hypothesis is mainly applied to the process of cancer development in which immunosurveillance is gradually impaired. However, in clinical situations, cancer patients sometimes experience an asymptomatic period coexisting with known cancer lesions, or even a spontaneous regression, without any medical interventions, suggesting that the balance between tumor growth and host immunity significantly influences the clinical course of cancer patients. Nevertheless, there have been few studies that intended to comprehensively analyze the local immune status of each case, which would thereby establish the means to predict the clinical outcome. In this study, Cluster 1 may represent a phenotype of the "equilibrium" phase, where immune cells infiltrate into the tumor site to eliminate the tumor. Clusters 2, 3 and 4 may be in "escape" phase in which the local immune environment has already fallen into an immunosuppressive status. For an effective immune therapy, an understanding of the immune status in each case is particularly important. This study provides a model to analyze the complicated immune reaction in a local tumor site.

This study may also provide a future direction for order-made immunotherapy in each ovarian cancer patient. Currently, therapeutic modalities to target specific immunosuppressive factors are being developed. Blocking antibodies

against PD-1 (MDX-1106) have been developed and are in Phase I clinical trials for advanced refractory malignancies [41]. Phase II and III clinical trials using selective COX2 inhibitors, celecoxib and rofecoxib, in combination with a chemotherapeutic have shown a clinical benefit [42]. Clinical trials focusing on the inhibition of the TGF $\beta$  signaling pathway by a monoclonal antibody or a small molecule inhibitor of the TGF $\beta$  receptor I kinase are being performed. To select the most efficient single or combined immune targeting therapies, precise assessments with multiple immune parameters in each case is essential.

In conclusion, hierarchical clustering of tumor-infiltrating immune cells and immunosuppressive factors was used to identify a subgroup of ovarian cancer patients with a better prognosis. This study also suggested that immunosuppressive factors might influence the pattern of tumor-infiltrating immune cells. The approach to comprehensively analyze multiple immune factors shown here may lead to a precise understanding of the local immune status and provide a tool for the application of immune therapies to treat ovarian cancer patients.

Supplementary materials related to this article can be found online at doi:10.1016/j.clim.2011.08.013.

### Conflict of interest statement

The authors declare no conflict of interest.



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## 特集

## 婦人科がんの Molecular Biology

## 11. cancer stem cell

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## 要旨

cancer stem cell という概念は、癌細胞中に自己複製能・多分化能をもち、腫瘍の形成・維持に必須の細胞があることを想定したものである。cancer stem cell は再発・抗がん剤耐性を克服し、癌を完治するために重要なターゲットであると考えられている。しかしながら婦人科領域を含めた固形癌では、その定義・生物学的性格・意義ともにまだ十分に確立されておらず、臨床応用のためにはさらに基礎的な検討が必要である。

**Key Words** cancer stem cell, 卵巣癌, 子宮体癌

新しい概念が想像される時に言葉が独り歩きすることはよくある。cancer stem cell (CSC) という言葉はこの数年で腫瘍の分野では知らない者はいないくらいに有名になったが、改めて“その本質は？その臨床的意義は？”と聞かれると、なかなか明確には答えにくい。本稿では、改めて CSC という概念とその意味について考察してみたい。

癌を顕微鏡で見ると、その多くは形態の異なる癌細胞で構成されており、核異型や分化の程度も場所や細胞によってかなり異なっているように見える。この癌の形態的多様性は遺伝子の不安定性や微小環境によって引き起こされるとも考えられるが、癌細胞自体が体内で持続的に分化しており、それをある一時点で観察するために多様性があるように見えるとも考えられる。このような癌細胞の分化モデルは仮説と

しては古くからあるが、この10年間にその分化のおおもとになるべき幹細胞、すなわち CSC が実際に同定され、その性質が調べられるようになったことで、一躍脚光を浴びることになった、と同時に、様々な誤解や混乱も引き起こしているのが現状である。

## CSC の定義とその検証法

現在、一般的に CSC の定義として受け入れられているのは、①自己複製能がある、②様々な phenotype の腫瘍細胞から構成された腫瘍そのものを再生することができる、③腫瘍細胞のなかでごくわずかな割合しか存在しない、④造腫瘍能をもたない細胞にも分化する多分化能をもつ、⑤特異的な分化マーカーを発現する、といった特徴を満たすものである。このなかでも

特に①と②は必須のものとしてされている。

そこで、CSCを同定したことを示すためには、その細胞が①、②をともに満たすことを証明する必要があるが、このための gold standard とされている方法が、NOD/SCID マウス等での継代移植である。すなわち、マウスで3~4代以上、継代できれば①、②を満たしているとみなされている。しかしながら、この方法自体にも移植する場所での間質細胞の影響といった本質的な問題とともに、アッセイに時間がかかり過ぎるといった実際的な問題があり、もう少し high-throughput なアッセイ法の開発が望まれる。このため *in vitro* でのアッセイ法である、sphere assay, serial colony-forming unit assay などが用いられている。Zhang らは、漿液性卵巣癌から足場非依存性増殖を手掛かりに CSC を分離し、これが sphere を形成、幹細胞のマーカーを発現し、かつ造腫瘍能を有することを示した<sup>1)</sup>。しかし、たとえば乳癌の CSC は sphere assay で sphere を形成しないなど、これらの性質だけで CSC とするには、問題が多い。

一方、CSC を分離同定する方法として、Hoechst dye の排出によって規定された SP (side population) 分画という概念もよく用いられる。ABC トランスポーターを介して色素を細胞外に排出する能力が亢進している幹細胞の性質を利用したこの方法により、多くの癌腫から CSC が *in vitro* で同定された。Hirschmann-Jax らは 2004 年に卵巣癌細胞株である SK-OV-3, PA-1 中の SP 細胞を同定し、これに多剤耐性遺伝子である p-glycoprotein が発現していることを示した<sup>2)</sup>。Szotek らは、ヒト卵巣癌細胞株 MOVCAR 等から SP 細胞を分離し、これらが non-SP 細胞に比べて、より少数で早期に癌を形成することを示した<sup>3)</sup>。また、Moserle らは卵巣癌患者から樹立した細胞から SP 細胞を分離し、これが non-SP に比べて高

い増殖能、低いアポトーシスを示し、かつ造腫瘍能が高いことを報告した<sup>4)</sup>。一方、子宮内膜癌においても、Kato らはヒトやラットの内膜癌細胞から SP 細胞を分離同定し、これらが自己複製能・造腫瘍能を有していること、さらには、癌の間質細胞にも分化しうる可能性を示した<sup>5)</sup>。このように、SP 細胞は CSC の性格をもつものが多く、その同定に有用ではあるが組織によっては SP 分画に CSC がほとんど含まれていないこともあり、また、CSC 以外の細胞がここに含まれることもあるので、やはり分離した細胞に機能解析を行って、CSC であることを確認する必要がある。

CSC に特有のマーカー、特に細胞表面マーカーを見つけることができれば、flow cytometry 等で簡便に分離・解析できるので、この探索は CSC 研究で現在最も盛んに研究されているテーマの一つである。CD133, CD44, Scd1, Thy1 等が CSC のマーカーとして報告されている。様々な癌腫で最も広く用いられているのは CD133 であり、Ferrandina らは卵巣癌組織では良性卵巣腫瘍等と比べて CD133 陽性細胞の割合が高いことを報告した<sup>6)</sup>。Baba は卵巣癌細胞株を用いて、CD133 陽性、陰性細胞を分離し、陰性細胞からは陰性細胞のみが増殖するが、陽性細胞からは陽性細胞・陰性細胞の両者が産生されること、後者はヌードマウスでより高い造腫瘍能を示し、かつシスプラチン抵抗性であることを示した<sup>7)</sup>。子宮内膜癌においても、Rutella らは CD133 陽性細胞が造腫瘍能や化学療法抵抗性といった CSC の性格を示すことを報告した<sup>8)</sup>。一方、Bapat らは卵巣癌の腹水から分離した CD44 陽性細胞が、また Deng らは ALDH1 陽性の卵巣癌細胞が、それぞれ CSC の性格をもっていることを報告した<sup>9)10)</sup>。このような特異的な細胞表面マーカーの探索は現在も精力的に続けられているが、ある癌では CSC のマーカーとして使えても、別の癌で