



# Clinical Impact of Natural Killer Group 2D Receptor Expression and That of Its Ligand in Ovarian Carcinomas: A Retrospective Study

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**Purpose:** Natural killer (NK) cells are innate immune cells with antitumor activity. NKG2D is the most important activating receptor expressed on the NK cell surface; this receptor binds to the ligands MICA/B and ULBPs to activate NK cells. The current study aimed to evaluate the expression of NKG2D by NK cells, and to evaluate expression of its ligands in ovarian carcinomas; it also examined the clinical relevance of NK receptor/ligand expression by analyzing the relationship between expression, clinicopathological parameters, and prognosis.

**Materials and Methods:** Formalin-fixed paraffin-embedded archival ovarian high-grade serous carcinoma (HGSC, n=79) tissue samples were used for tissue microarray analysis. The expressions of NK cell markers (CD56 and NKG2D) and NKG2D ligands (MICA/B, ULBP1, ULBP3, and ULBP2/5/6) in carcinoma tissues were evaluated by immunohistochemical staining, and the association between these results and clinical prognostic parameters was analyzed statistically.

**Results:** ULBP1 was highly expressed in 51 cases (64.6%), and ULBP2/5/6 was highly expressed in 56 cases (70.9%) of HGSC. High expression of ULBP1 and ULBP2/5/6 was significantly associated with lower recurrence of HGSC, whereas high expression of ULBP3 was significantly associated with higher recurrence. Multivariate Cox regression analysis revealed that high expression of ULBP1 was associated with increased overall survival and a decreased hazard ratio (0.150,  $p=0.044$ ), suggesting that it is an independent predictor of better survival.

**Conclusion:** High expression of ULBP1 predicts a better prognosis for HGSC, suggesting that ULBP1 expression could be a novel prognostic indicator in this subset of carcinomas.

**Key Words:** NK cell, ovarian carcinoma, NKG2D, NKG2D ligands, ULBP1

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## INTRODUCTION

Natural killer (NK) cells are key cells that undertake innate immune surveillance against cancer. NK cells recognize target cells via an antigen-independent, receptor-ligand interaction, and then kill them by releasing cytotoxic granules such as perforin and granzyme, or by activating death ligands (e.g., tumor necrosis factor-related apoptosis-inducing ligand or Fas ligand).<sup>1</sup> The activation of NK cells is regulated by the balance between activating and inhibitory receptor signaling. Normal

cells are protected from NK cell attack due to the expression of self-defining major histocompatibility complex (MHC) class I molecules, which bind to inhibitory receptors on NK cells. Tumor cells may lose the expression of MHC class I molecules to evade T cell-mediated cytotoxicity, which facilitates killing by NK cells. Additionally, NK cells are activated by malignant cells that show upregulated expression of ligands that bind to activating NK receptors.

The natural killer group 2 member D (NKG2D) receptor is a major activating receptor on NK cells; it is expressed abundantly by cytotoxic NK cells and CD8<sup>+</sup> T cells, and is also detectable on CD4<sup>+</sup> T cells,  $\gamma\delta$  T cells, NKT cells, and regulatory T cells (Tregs).<sup>2,3</sup> Activating ligands for NKG2D, such as MICA/B and ULBP1-6, are rarely expressed by normal healthy cells; however, they are induced upon malignant transformation of cancer cells, whereupon they activate NK cells by binding to the NKG2D receptor.<sup>2,4,5</sup> Activated NK cells can then kill tumor cells. Therefore, increased expression of NK cell activating receptors and their ligands may suppress tumor growth and development. On the other hand, some clinical studies have shown that several human cancers, including breast and pancreatic cancers,<sup>6,7</sup> evade immune surveillance by NK cells, leading to a poor prognosis. However, the clinical relevance of NK cell surveillance and the roles of NKG2D and its ligand in the context of gynecological malignancies remain unclear.

Ovarian high-grade serous carcinoma (HGSC) is the most lethal gynecologic malignancy, with an estimated 13940 deaths expected in the United States in 2019. Despite optimal treatment with surgery and adjuvant chemotherapy, recurrence rates are approaching 70–80%. Therefore, understanding NK cell biology and function in these tumors may lead to the development of promising new immunotherapeutic approaches.<sup>8</sup>

Here, we investigated the expression of NKG2D by immune cells and the expression of NKG2D ligands by tumor cells. We also examined the relationship between receptor/ligand expression and clinicopathological prognostic parameters to ascertain the clinical relevance of NK cell activation in HGSC.

## MATERIALS AND METHODS

### Patients and tissue samples

Formalin-fixed paraffin-embedded archival tissues from

HGSC samples (n=79) were examined. The samples were obtained from patients who underwent oophorectomy and were subsequently diagnosed with HGSC at CHA Bundang Medical Center, School of Medicine, CHA University. Histological subtype and clinical stage were evaluated according to the WHO classification system and a tumor-node-metastasis staging system. The samples were divided into chemosensitive and chemoresistant groups according to the responsiveness to firstline chemotherapy (taxol/platinum-based combination therapy) within 6 months after surgery. The following clinical and pathological data were reviewed: patient's age, surgical procedure, tumor size, lymph node metastases, distant metastasis, recurrence, chemoresistance, and overall survival (OS). The study was approved by the Ethics Committee of CHA Bundang Medical Center (IRB No. 2020-04-048-003).

### Generation of a tissue microarray and immunohistochemical staining

For immunohistochemical (IHC) analysis, all 79 cases of ovarian cancer were used to construct a tissue microarray (TMA). In each case, two tissue cores with a diameter of 3 mm were punched out from the donor tissue block and arranged into recipient paraffin blocks using a manual microarray device (UNITMA; Quick-RAYTM UNITech Science, Seoul, Korea). IHC staining was performed to detect the NKG2D ligands MICA/B, ULBP1, ULBP3, and ULBP2/5/6; the NK cell marker CD56; and the NK activating receptor NKG2D (Table 1). The sections of TMA were stained on a Ventana Benchmark automated staining platform (Ventana Medical Systems, Inc., Tucson, AZ, USA) using a VENTANA OptiView diaminobenzidine tetrahydrochloride IHC Detection Kit (P/N 760–700) and its staining protocol. The formalin-fixed paraffin-embedded TMA tissues were cut, dried, deparaffinized, rehydrated, and heated following the protocol. The antigen-retrieval time, retrieval buffer, and incubation time of each antibody are shown in Table 1. IHC staining was interpreted by two independent pathologists, blinded to the clinical data and patient outcomes. For CD56 and NKG2D staining, the number of positive inflammatory cells in each TMA core was counted. For NKG2D ligands, the intensity and percentage of immune-stained tumor cells were evaluated. The cut-off values were determined by analysis with statistical significance, as described below. MICA/B and ULBP1 staining was interpreted as low (<50% positive tumor cells) or high ( $\geq$ 50%

**Table 1.** Primary Antibodies, Dilution Factors, and Incubation Times

Antibody	Company	Number	Dilution	Retrieval time	Retrieval buffer	Antibody incubation
NKG2D	Abcam	ab203353	1:200	30 min	CC2	1 hr
MICA/B	Abcam	ab224702	1:100	1 hr	CC1	2 hr
ULBP1	Novus	NBP1-80856	1:100	2 hr	CC1	Overnight
ULBP3	Novus	NBP2-31866	1:100	2 hr	CC1	2 hr
ULBP2/5/6	R&D Systems	AF1289	1:200	1 hr	CC2	1 hr
CD56	Roche	123C3	-(RTU)	30 min	CC1	30 min

RTU, ready to use.

positive tumor cells). ULBP3 expression was categorized as high when the staining was 10% or more positive tumor cells. For ULBP2/5/6, staining was interpreted using an immunoreactive score (IRS). The staining intensity was categorized as follows: 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). The percentage of positive cells was scored as follows: 0 (negative), 1 (10% or less), 2 (11–50%), 3 (51–75%), or 4 (>75%). The final IRS was calculated by multiplying these two scores. An IRS <6 was classified as low expression, and an IRS ≥6 was classified as high expression.

### Statistical analysis

Statistical analysis was performed using SPSS 24.0 software (IBM Corp., Armonk, NY, USA). The association between the IHC expression of each protein and clinicopathological parameters was evaluated using the chi-square test and Fisher’s exact test. OS curves were generated using the Kaplan-Meier method. Multivariate survival analysis was conducted using the Cox proportional hazards model.  $p < 0.05$  was considered statistically significant.

## RESULTS

### Clinicopathological characteristics of HGSCs

The clinicopathological characteristics of patients with HGSCs are summarized in Table 2. The mean age of the 79 patients was 54.59±11.03 years. Most patients (81.0%) were stage III and IV. Metastasis was shown in approximately half of the patients, lymph nodes in 51.8%, and distant metastasis in 48.1%. Nineteen (24.0%) cases were chemoresistant, and disease recurrence was observed in 54.4% of cases. All but five of the patients with chemoresistance showed recurrence.

### Infiltration of CD56+ or NKG2D+ NK cells and its clinical impact in HGSCs

Tumor tissue cores from 6 (7.6%) of the 79 HGSCs showed infiltration by CD56-positive NK cells (Fig. 1A). Among these, two cases had two, three cases had three, and one had six CD56-positive NK cells per TMA core. All CD56-positive cells were in the intratumoral area. None of the patients with tumor-infiltrating CD56-positive NK cells experienced recurrence during follow-up ( $p < 0.01$ ) (Table 3).

NKG2D-positive cells were observed in 5 (6.3%) of 79 HGSCs: two positive cells per TMA core in four cases and four positive cells in one case (Fig. 1B). These cases, including NKG2D-positive cells, were not matched with the cases showing CD56-positive cells. As with CD56, NKG2D-positive cells were usually present in the intratumoral area, and not in the stromal (peritumoral) area. There was no association between the presence of NKG2D-positive cells and the clinicopathological parameters of HGSCs (Table 3).

**Table 2.** Clinical Characteristics of Patients with Ovary Serous Carcinoma (n=79)

Age (yr)	
Mean	54.59±11.03
<55	43 (54.4)
≥55	36 (45.5)
Lymph node metastasis	
No	38 (48.1)
Yes	41 (51.8)
Stage	
I	6 (7.5)
II	9 (11.3)
III	50 (63.3)
IV	14 (17.7)
Distant metastasis	
No	41 (51.8)
Yes	38 (48.1)
Chemoresistance	
No	60 (75.9)
Yes	19 (24.0)
Recurrence	
No	36 (45.5)
Yes	43 (54.4)

Data are presented as n (%).

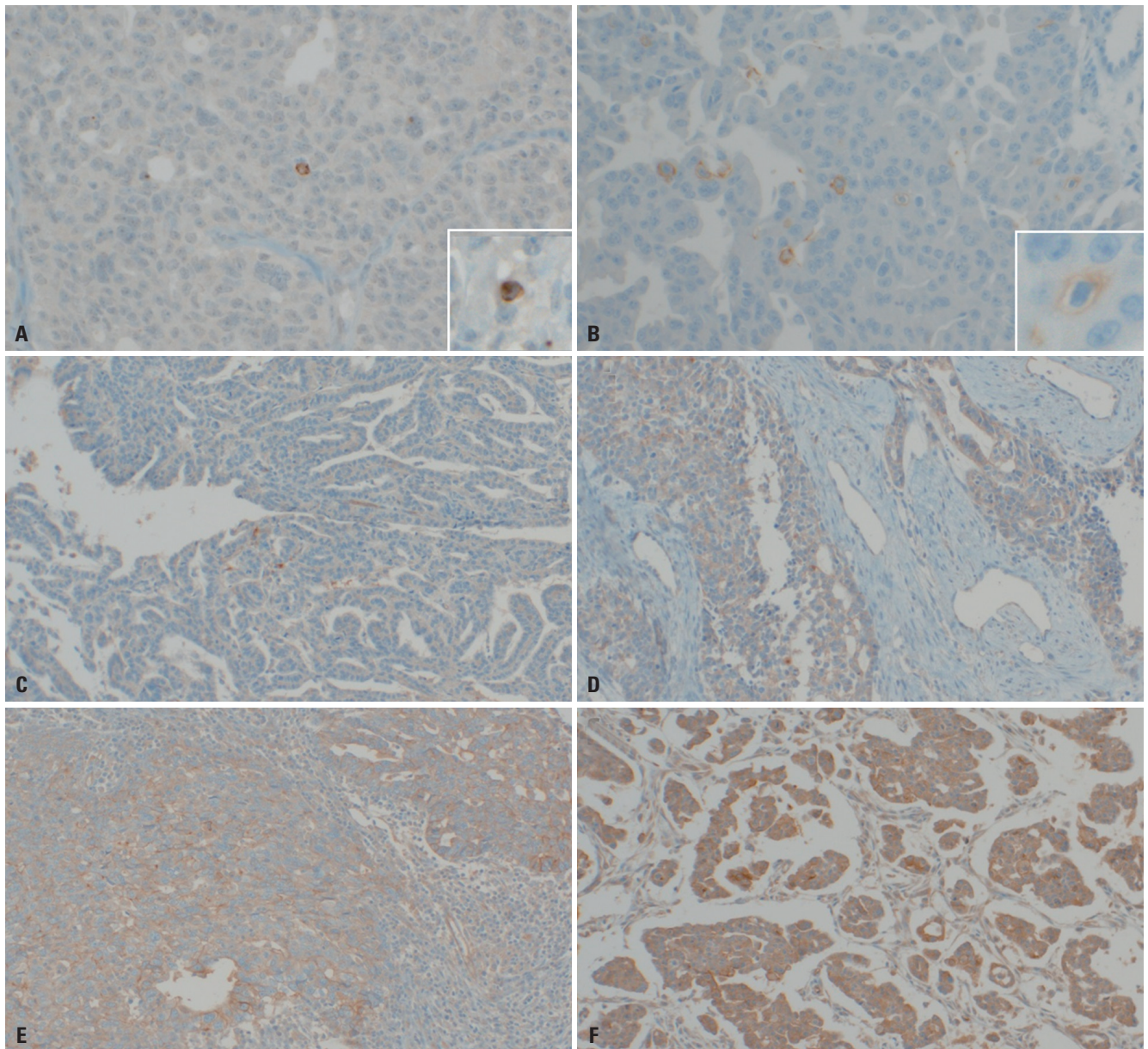
### Expression of NKG2D ligands and their clinicopathological correlation in HGSCs

To assess the clinical impact of NKG2D ligand expression in HGSCs, we performed IHC analysis of the NKG2D ligands MICA/B, ULBP1, ULBP3, and ULBP 2/5/6 in all 79 HGSC cases. Representative IHC images showing the expression of individual NKG2D ligands are shown in Figs. 1 and 2. No ligands were expressed in normal control tissue (e.g., fallopian tube epithelium and stromal cells).

MICA/B was expressed mainly on the membrane of tumor cells (Fig. 1C-F). It was expressed in 51 (64.5%) of 79 HGSCs; 28 cases (35.4%) showed high expression in which more than 50% of tumor cells were positively stained. However, the expression level was not associated with any clinicopathological parameters (Table 3).

ULBP1 was expressed in 51 cases (64.6%), all of which had staining in more than 50% of the tumor cells in the cytoplasm and cell membrane (Fig. 2A, B). Therefore, all ULBP1-positive cases belonged to the high-expression group. The ULBP1-high-expression group was significantly associated with no recurrence ( $p=0.025$ ) (Table 3). Among 28 ULBP1-negative cases, 20 experienced recurrence (71.4%), whereas 23 (45.1%) of the 51 ULBP1-high-expression group experienced recurrence.

ULBP3 was expressed in the cytoplasm of tumor cells (Fig. 2C-F). The ULBP3-high-expression group (≥10% of tumor cells stained) comprised 47 cases (59.5%). Unexpectedly, however, high expression of ULBP3 was positively associated with re-



**Fig. 1.** Immunohistochemical staining of NKG2D, CD56, and MICA/B in ovarian serous carcinoma. (A and B) A representative image of ovarian serous carcinoma showing NKG2D- (A) and CD56 (B)-positive cells ( $\times 200$ ). NKG2D- and CD56-positive cells at high magnification (small boxes). These cells were present mainly in the intratumoral area. (C-F) MICA/B staining of ovarian serous carcinoma. Intensity score: 0 (C), 1+ (D), 2+ (E), and 3+ (F) ( $\times 100$ ). MICA/B is expressed mainly on the membrane of tumor cells. When 50% or more tumor cells were positively stained and the intensity was 1+ or more, it was considered as high expression.

currence ( $p=0.042$ ) (Table 3). The recurrence rate in the ULBP3-high-expression group was 63.8% (30/47), whereas that in the ULBP3-low-expression group was 40.6% (13/32).

For ULBP2/5/6, both membrane and cytoplasmic staining were observed (Fig. 2G-J). Among the various NKG2D ligands, ULBP2/5/6 was expressed in most HGSCs, even in part (71 cases, 88.6%). Therefore, we interpreted the IHC staining of this protein as a semiquantitative IRS score. Fifty-six (70.9%) of 79 HGSCs had an IRS of  $\geq 6$ , which was considered high. This group of HGSCs was significantly associated with better prognosis (i.e., no recurrence). Among the 23 cases in the low IRS

group of ULBP2/5/6, recurrence occurred in 18 cases (78.3%). In the high IRS group, 25 (44.6%) out of 56 cases experienced recurrence ( $p=0.006$ ) (Table 3).

### Survival analysis

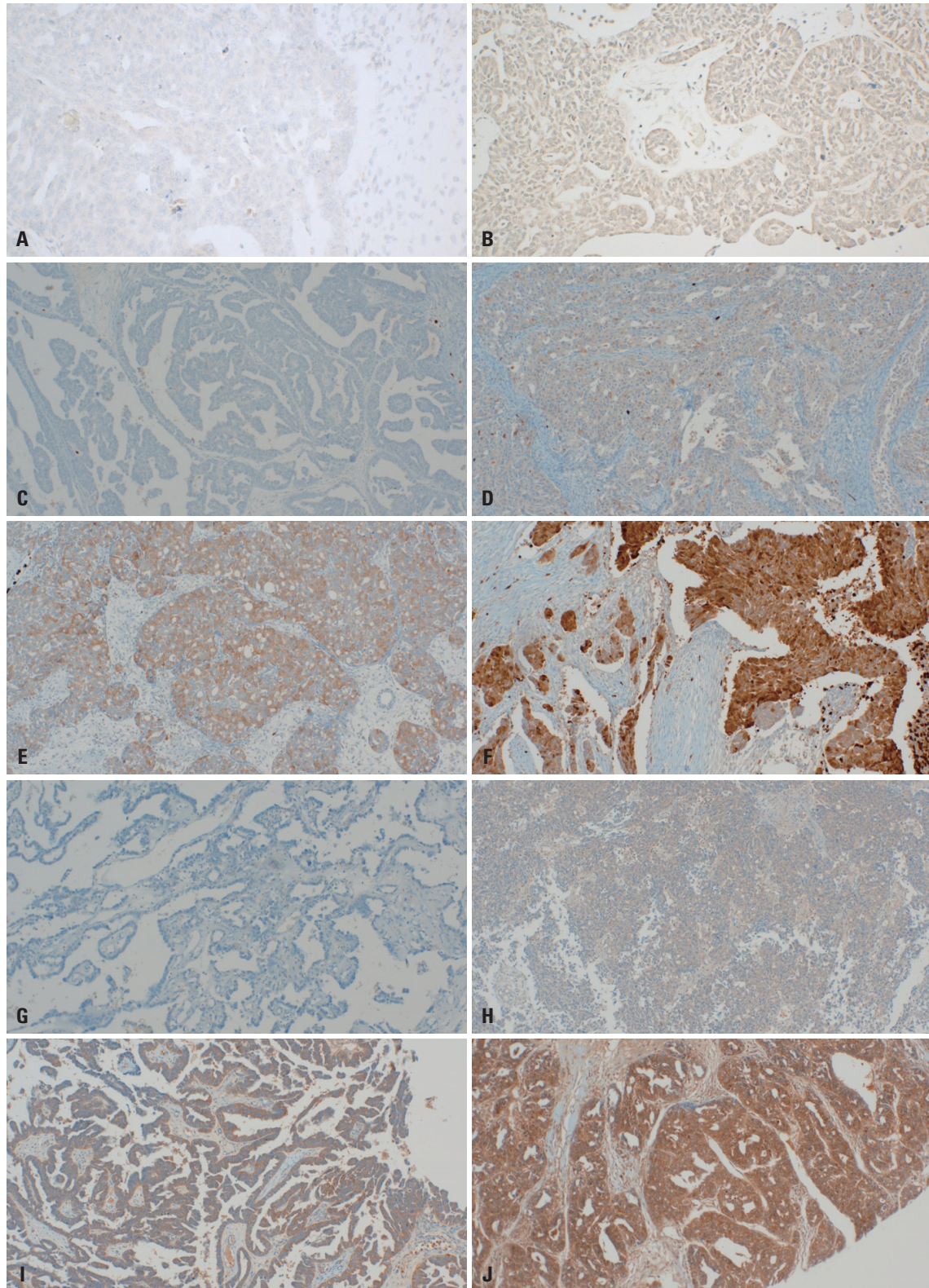
Survival analysis of the 79 patients with HGSC was based on the expression of NK cell markers (CD56 and NKG2D) and NKG2D ligands in tumor cells (Fig. 3). The median follow-up period was 31 months (range, 1–112 months). During the follow-up period, 22 patients (27.8%) died of disease. Twenty-two (30%) out of 73 cases without CD56-positive NK cells died,

**Table 3.** Relationship between Expressions of CD56, NGK2D, and Its Ligands and Clinicopathological Parameters of Ovarian Serous Carcinoma

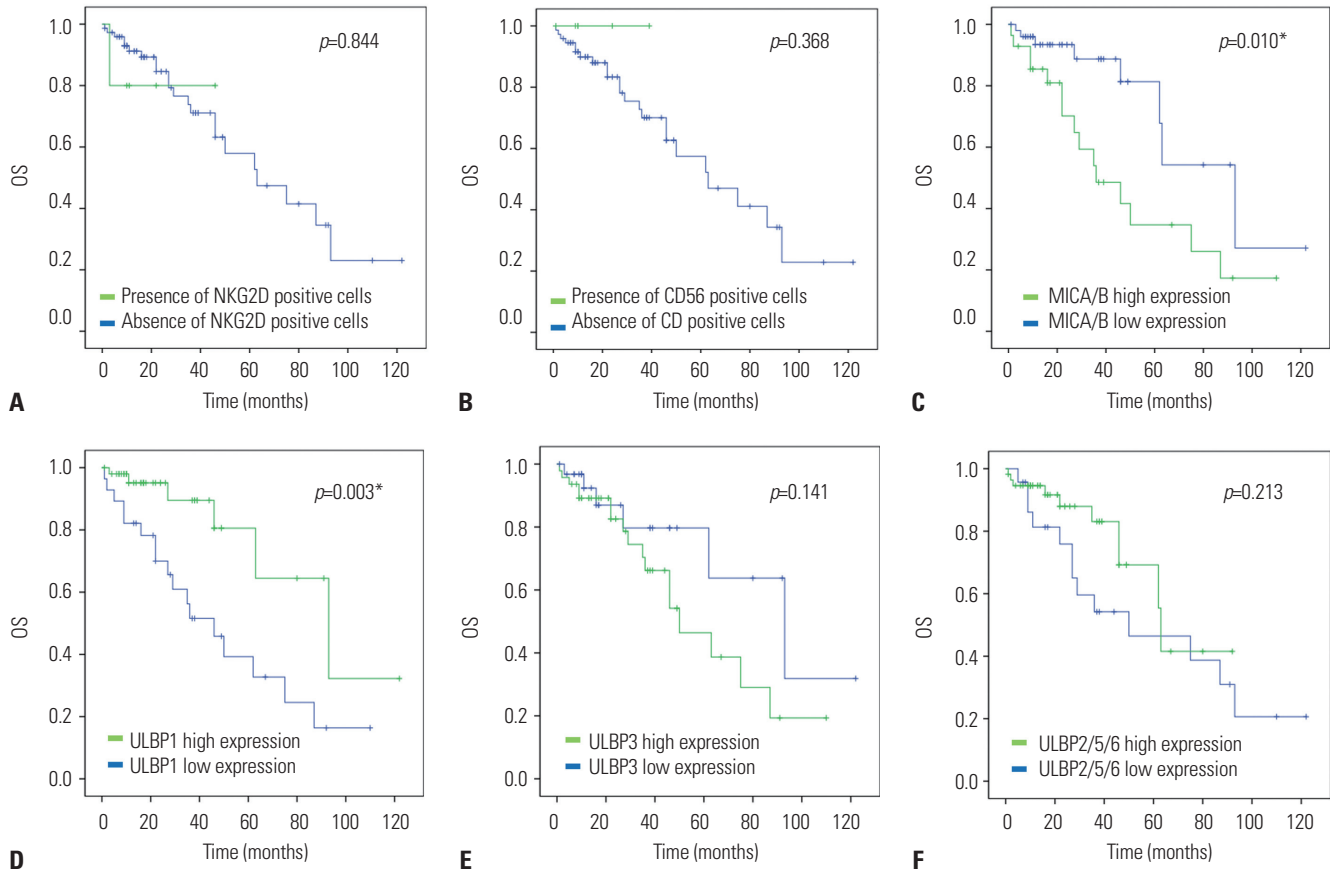
	CD56 (+) cells <sup>†</sup>	p value	NGK2D (+) cells <sup>†</sup>	p value	MICA/B-high expression <sup>‡</sup>	p value	ULBP1-high expression <sup>‡</sup>	p value	ULBP3-high expression <sup>‡</sup>	p value	ULBP2/5/6-high expression <sup>§</sup>	p value
Age (yr)		0.280		0.796		0.290		0.558		0.035		0.796
<55	2/43 (4.7)		3/43 (6.9)		13/43 (30.2)		29/43 (67.4)		21/43 (48.8)		31/43 (72.1)	
≥55	4/36 (11.1)		2/36 (5.6)		15/36 (41.7)		22/36 (61.1)		26/36 (72.2)		25/36 (69.4)	
Stage		0.246		0.305		0.308		0.700		0.650		0.233
I, II	2/13 (15.4)		0/13 (0.0)		3/13 (23.1)		9/13 (69.2)		7/13 (53.8)		11/13 (84.6)	
III, IV	4/66 (6.1)		5/66 (7.6)		25/66 (37.9)		42/66 (63.6)		40/66 (60.6)		45/66 (68.2)	
Lymph node metastasis		0.923		0.190		0.103		0.103		0.232		0.337
No	3/38 (7.9)		4/38 (10.5)		10/38 (26.3)		28/38 (60.5)		20/38 (52.6)		25/38 (65.8)	
Yes	3/41 (7.3)		1/41 (2.4)		18/41 (43.9)		23/41 (56.1)		27/41 (65.9)		31/41 (75.6)	
Distant metastasis		0.923		0.708		0.233		0.471		0.523		0.642
No	3/41 (7.3)		3/41 (7.3)		12/41 (29.3)		28/41 (68.3)		23/41 (56.1)		30/41 (73.2)	
Yes	3/38 (7.9)		2/38 (5.3)		16/38 (42.1)		23/38 (60.5)		24/38 (63.2)		26/38 (68.4)	
Chemoresistance		0.660		0.389		0.486		0.686		0.363		0.786
No	5/60 (8.3)		3/60 (5.0)		20/60 (33.3)		38/60 (63.3)		34/60 (56.7)		43/60 (71.7)	
Yes	1/19 (5.3)		2/19 (10.5)		8/19 (50.0)		13/19 (68.4)		13/19 (68.4)		13/19 (68.4)	
Recurrence		0.007**		0.796		0.406		0.025*		0.042*		0.006*
No	6/36 (16.7)		2/36 (5.6)		11/36 (30.6)		28/36 (77.8)		17/36 (47.2)		31/36 (86.1)	
Yes	0/43 (0.0)		3/43 (7.0)		17/43 (39.5)		23/43 (53.5)		30/43 (70.0)		25/43 (58.1)	

Data are presented as n (%).

<sup>†</sup>Cases in which NGK2D-positive cells were present, <sup>‡</sup>A case was considered "high-expression" when positive immunostain ≥10% of tumor cells for ULBP3, and of ≥50% of tumor cells for MICA/B and ULBP1, <sup>§</sup>The immunoreactive scores, calculated by multiplying the intensity score (0–3) by the positivity score (0–4) was ≥6 for ULBP2/5/6 high-expression (\* p<0.05, Pearson's  $\chi^2$  test, \*\* p<0.05, Fisher's exact test).



**Fig. 2** Immunohistochemical staining of ULBP1, ULBP3, and ULBP 2/5/6 in ovarian serous carcinoma. (A and B) Negative (A) and high expressions (B) of ULBP1 in ovarian serous carcinoma ( $\times 200$ ). ULBP1 was stained in the cytoplasm and membrane of tumor cells. (C-F) ULBP3 immunostaining in ovarian serous carcinoma according to the intensity score: 0 (C), 1+ (D), 2+ (E), and 3+ (F) ( $\times 100$ ). It is expressed in the tumor cell cytoplasm. When 10% or more of tumor cells were stained, high expression of ULBP3 was considered. (G-J) ULBP2/5/6 immunostaining in ovarian serous carcinoma. Intensity scores: 0 (G), 1+ (H), 2+ (I), and 3+ (J) ( $\times 100$ ). ULBP2/5/6 was evaluated as the immunoreactive score (IRS), multiplying intensity-scoring and percentage (0, negative; 1, 1–10%; 2, 11–50%; 3, 51–75%; and 4, >75%). IRS  $\geq 6$  is considered high expression of ULBP2/5/6.



**Fig. 3.** Overall survival (OS) of patients with ovarian serous carcinoma according to the expressions of NKG2D, CD56, MICA/B, ULBP1, ULBP3, and ULBP2/5/6. (A) Kaplan-Meier survival curves of OS according to the absence (n=74) or presence of (n=5) NKG2D-positive cells. (B) Kaplan-Meier survival curves of OS according to the absence (n=73) or presence of (n=6) CD56-positive cells. (C) Kaplan-Meier survival curves of OS according to low (n=51) and high (n=28) expression of MICA/B. Low expression of MICA/B was significantly associated with better OS ( $p=0.010$ ). (D) Kaplan-Meier survival curves of OS according to low (n=28) or high (n=51) expression of ULBP1. ULBP1 high expression was significantly associated with better OS ( $p=0.003$ ). (E) Kaplan-Meier survival curves of OS according to low (n=32) or high (n=47) expression of ULBP3. (F) Kaplan-Meier survival curves of OS according to low (n=23) or high (n=56) expression of ULBP2/5/6.

whereas all patients with infiltrating CD56-positive NK cells remained alive without disease. However, Kaplan-Meier analysis did not show a significant difference between these groups.

Multivariate Cox regression analysis was performed to examine the association between clinicopathological parameters and NK cell markers and NKG2D ligands (Table 4). ULBP1 expression was an independent predictor of better survival (hazard ratio=0.150,  $p=0.044$ ), suggesting that ULBP1 is a novel prognostic indicator in HGSC; however, the expression of other NKG2D ligands was not significantly associated with OS. As expected, chemoresistance was associated with poor survival (hazard ratio=8.919,  $p=0.002$ ).

**DISCUSSION**

NK cells play a key role in innate immune responses against tumors;<sup>9,10</sup> they also regulate tumor growth.<sup>11</sup> In this context, NK cells act as cytotoxic effector cells that play a significant role in

**Table 4.** Multivariate Cox Analysis of Overall Survival of Patients with Ovarian Serous Carcinoma

	Hazard ratio (95% CI)	p value
Age <sup>†</sup>	4.424 (1.533–12.766)	0.006*
Lymph node involvement	1.556 (0.451–5.363)	0.484
Stage <sup>‡</sup>	2.306 (0.256–20.761)	0.456
Metastasis	1.726 (0.521–5.718)	0.372
Chemoresistance	8.919 (2.209–36.012)	0.002*
NKG2D	2.243 (0.176–28.516)	0.533
MICA/B	0.636 (0.108–3.753)	0.617
ULBP1	0.150 (0.024–0.949)	0.044*
ULBP3	0.908 (0.292–2.819)	0.867
ULBP2/5/6	0.708 (0.247–2.027)	0.520
CD56	0.000 (0.000, NA)	0.988

CI, confidence interval; NA, not applicable.

<sup>†</sup>Patients were divided into younger (<55 years) and older (≥55 years) age groups, <sup>‡</sup>Patients were divided into early stage (stages I and II) and late stage (stages III and IV) groups (\* $p<0.05$ ).

eliminating transformed cells.<sup>12</sup> NK cell activity against tumor cells is regulated by the balance between inhibitory and stimulatory signals. NKG2D is an activating receptor expressed by NK cells, CD8<sup>+</sup> T cells, some NKT cells, and a rare population of CD4<sup>+</sup>CD28<sup>-</sup> T cells<sup>2</sup>; as such, it is a key regulator of both innate and adaptive immune responses. NKG2D delivers an activating signal via the adaptor protein DAP10.<sup>13</sup> MICA/B<sup>2,14</sup> and ULBPs are representative and well-known NKG2D ligands, and many previous studies have reported that the expression of NKG2D ligand is associated with the destruction of transformed cells by immune cells.<sup>15,16</sup>

Contemporary studies have revealed the importance of NKG2D function in host-mediated tumor immunity.<sup>17,18</sup> Many studies have demonstrated that in various human tumor microenvironments, NKG2D receptor expression is significantly lower on immune cells such as NK cells and CD8<sup>+</sup> T cells, thereby allowing tumor cells to escape immune surveillance. This has been shown for cervical cancer and pancreatic cancers.<sup>19,20</sup> Changes in NKG2D expression by immune cells might be regulated by a variety of factors in the tumor microenvironment, including changes in cellular activity factors or soluble factors secreted by tumor cells, cancer-associated fibroblasts, tumor-associated macrophages, and Treg cells.<sup>21,22</sup> However, the roles of NKG2D ligands such as MICA/B, ULBP1, ULBP3, and ULBP 2/5/6 in numerous malignancies are unclear. Therefore, we investigated the role of NK cells and NKG2D ligands such as MICA/B, ULBP1, ULBP3, and ULBP 2/5/6 in HGSC, which is the most lethal carcinoma among gynecological malignancies.

We identified NKG2D-positive cells in only five cases of HGSC; these cases were not matched with the six cases showing CD56-positive cells. The NK cell population consisted of CD56<sup>high</sup> cells (less than 10% in peripheral blood) and CD56<sup>low</sup> cells (approximately 90% in peripheral blood). Therefore, NKG2D-positive cells were not CD56<sup>high</sup> NK cells; rather, they could be CD56<sup>low</sup> NK cells or NKG2D-positive T lymphocytes. We expected a longer OS when more NKG2D-positive cells were shown; however, these cells were not associated with a better prognosis. Additionally, the results suggested that NKG2D expression by immune cells, such as NK or T cells, falls markedly in the tumor microenvironment of HGSCs, as mentioned in several previous reports.<sup>23</sup> Surface expression of NKG2D ligands may activate antitumor responses in the early stages of a tumor immune response; however, sustained NKG2D ligand expression and the shedding of soluble ligands inhibit NKG2D-dependent NK cell activity in the later stages.<sup>24</sup> The shed soluble NKG2D ligands may combine with NKG2D, leading to endocytosis of NKG2D, thereby disrupting the tumor immune surveillance function of NKG2D.<sup>25</sup> In addition, NKG2D expression can be altered by soluble factors secreted by tumor cells.<sup>24,26</sup> For example, TGF $\beta$  induces downregulation of NKG2D-activating receptors, thereby impairing NK cell-mediated cytotoxicity.<sup>21</sup> NKG2D expression might also be impaired by macrophage migration inhibitory factor, which is

another soluble factor present in ascites of ovarian cancers.<sup>22</sup>

ULBPs, human ligands for NKG2D, are expressed in several carcinomas, including ovarian cancers. Previous studies have reported that the expression of ULBP3 or ULBP4 correlates with better prognosis in patients with lymphoma or ovarian cancer,<sup>27,28</sup> and that ULBP recruits NK cells and T cells.<sup>29</sup> In our study, we found that higher ULBP1 expression is significantly associated with low recurrence rates and increased OS as well as with a decreased hazard ratio in multivariate Cox regression analysis, suggesting that ULBP1 is a good independent prognostic indicator for HGSC.

In contrast, higher ULBP3 expression was associated with high recurrence rates, and higher MICA/B expression was more common in the recurrence group (39.5%) than in the nonrecurrence group (30.5%), with no statistical significance; higher MICA/B expression was also associated with poor OS. Similar to our study, Li, et al.<sup>30</sup> reported that high expression of MICA/B was associated with a poor prognosis in ovarian cancer. Another study suggested that soluble MICA/B released by tumor cells downregulates the expression of NKG2D, which results in an adverse clinical outcome. In the same context, strong expression of ULBP2 correlates with a poor prognosis in patients with ovarian or pancreatic cancer.<sup>30,31</sup> However, in our study, ULBP2/5/6 expression was associated with low recurrence of HGSC. The discrepancy between our results and those of the previous report may be due to the fact that they studied a heterogeneous group of ovarian cancers, whereas we studied only HGSCs to eliminate any possible influence of different pathologic types. Another possible reason is that we used an antibody specific for ULBP2/5/6.

We also found that CD56-positive NK cells were scarce in most cases analyzed; however, the presence of CD56-positive NK cells tended to be associated with low recurrence and better survival, although the result was not statistically significant. High levels of tumor-infiltrating NK cells are associated with a good prognosis in several human cancers, including breast cancer,<sup>32</sup> advanced gastric carcinoma,<sup>33</sup> neuroblastoma,<sup>34</sup> and prostate cancer.<sup>35</sup> However, the identification of NK cells in the tumor microenvironment seems ambiguous, as there are two distinct subsets of NK cells (CD56<sup>bright</sup> and CD56<sup>dim</sup>), and they have a short lifespan of approximately 14 days.<sup>36</sup> In addition, this study was limited due to the small size of the TMA tissue as well as the sample size. It would be more helpful if the whole slides of more cases were evaluated for analyzing CD56-positive or NKG2D-positive immune cells. Also, due to these limitations, some prognostic factors, including the stage, were not independent prognostic factors in our regression analysis.

In conclusion, this study demonstrated that most HGSC tissues expressed NKG2D ligands, high expression of ULBP1 was an independent prognostic indicator of OS, and ULBP2/5/6 was associated with a better prognosis. In contrast, high expression of ULBP3 was associated with cancer recurrence in patients with HGSC. Further studies are needed to examine the



levels of soluble NKG2D ligands in patient blood samples to better understand the association between NKG2D and its ligands in HGSC, and to develop new immunotherapies for this lethal gynecological cancer.

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