good prognoses in some other carcinomas [17, 19, 35]. Natural killer group 2, member D (NKG2D) is a well-studied immune receptor expressed on NK cells, $CD8^+$ cytotoxic T cells and $\gamma\delta$-T cells [1, 10, 11, 33]. It is a type II transmembrane-anchored glycoprotein that is expressed as a disulfide-linked homodimer on the cell surface. NKG2D acts as an activating receptor after ligand binding, supporting the cytotoxic activity of NK cells and T cells against tumor cells [1, 4, 10, 20, 22, 25, 26].

Major histocompatibility complex class I-related chains (MIC) A and B and UL-16 binding proteins (ULBPs) are the ligands of NKG2D. They are rarely expressed by normal cells. It has been reported that MICA and MICB are constitutively expressed by intestinal epithelial cells [9, 10], and are broadly expressed in a variety of malignancies [11, 26, 31]. In malignant gliomas, MICA and ULBP2 expressions decreased with increasing WHO grade [7]. MICA expression is reported to be an indicator of good prognoses in colorectal cancer [37]. Tumor cells stably transfected to express MICA and the murine versions of the NKG2D ligands, RAE-1 or H60, at high levels, are rejected by $CD8^+$ T cells and/or NK cells [2, 5, 8, 14], which indicates that tumor cells overexpressing NKG2D ligands become more sensitive to immune cell-mediated cytolysis.

MICA/B and ULBP2 can be cleaved by matrix metalloproteases and released into the bloodstream or tissue culture medium as soluble molecules [12, 30, 31, 36]. These soluble molecules have been correlated with metastasis, advanced clinical stages of disease, and bad prognoses [15, 16, 28, 34], possibly due to their directly blocking NKG2D function or down-regulating NKG2D expression in lymphocytes. Expression of soluble MICA/B and ULBP2 by tumor cells may represent an immune evasion mechanism that leads to the impairment of immunosurveillance by T and/or NK cells [6, 12, 30, 32].

Despite these findings, there have been a few studies on NKG2D and its ligands in ovarian cancer. In the present study, we investigated MICA/B and ULBP2 expressions in ovarian cancers to evaluate their prognostic significance and association with other clinicopathological factors. We evaluated the cell surface expression and the secretion of soluble forms of these ligands in 33 ovarian cancer cell lines and 8 patients in an attempt to investigate the mechanism how NKG2D ligands play a role in ovarian cancer biology.

Materials and methods

Patients and samples

Clinical specimens were obtained from 82 women who underwent primary operations for ovarian cancer at the Department of Obstetrics and Gynecology of Kyoto University Hospital between 1993 and 2003. General patient information is shown in Table 1. The mean age of the ovarian cancer patients was 55.3 ± 11.4 years (26–80 years), and at the end of the study, 32 (39.0%) patients had died of disease and 50 (61.0%) were alive. The mean follow-up period was 68.4 ± 39.8 months (1–154 months). Normal ovarian tissues from six patients who underwent operations for uterine leiomyoma or ovarian cysts were collected as controls. We also studied ten borderline (nine mucinous borderline adenomas and one serous/mucinous borderline adenoma) and six benign ovarian tumors (five mucinous adenomas and one serous adenoma). Also the specimens and corresponding sera of eight ovarian cancer patients were also collected (general information was shown in supplementary Table 1). Clinical data were collected by retrospective review of patient files. Patients provided written informed consent under the approval of the Ethical Committee of Kyoto University Hospital.

Immunohistochemistry

Staining was performed by the streptavidin–biotin–peroxidase method. Formalin-fixed, paraffin-embedded specimens were cut into 4 μm sections. Tissue sections were deparaffinized in xylene (3 × 5 min) and hydrated through graded ethanol (99, 95 and 85%) to water. For MICA/B and ULBP2 antigen retrieval, samples were heated in Tris–EDTA buffer (pH 8.0) at 95°C for 20 min; CD57 staining did not require antigen retrieval. To block endogenous peroxidase activity, sections were treated with 100% methanol containing 0.3% H_2O_2 for 10 min. Nonspecific IgG binding was blocked using normal rabbit serum (Nichirei, Tokyo, Japan). Sections were incubated with mouse anti-MICA/B monoclonal antibodies (Abs) (6D4, Biolegend, San Diego, CA, USA), goat anti-ULBP2 Abs (R & D Systems, Inc. Minneapolis, MN, USA), and mouse anti-CD57 monoclonal Abs (Becton Dickinson, Franklin Lakes, NJ, USA) overnight at 4°C. They were then incubated with biotinylated rabbit-anti-mouse secondary Abs (Nechirei) for MICA/B and CD57 and biotinylated rabbit-anti-goat secondary Abs (Nechirei) for ULBP2, followed by incubation with a streptavidin–perioxidase solution for 30 min. Signals were generated by incubation with 3, 3′-diaminobenzidine. Finally, sections were counterstained with hematoxylin and observed under the microscope. CD8 (C8/144B, Nichirei) staining was performed as described previously [13].

Evaluation of specimens

Two independent gynecological pathologists examined the immunohistochemistry slides without any prior information about the clinical history of the patients.

Table 1 Expression of MICA/B and ULBP2, and tumor infiltration of NK and T cells according to clinico-pathological parameters (χ^2 test)

	Total	MICA/B			ULBP2			NKca			NKstroma			Tca			Tstroma		
		Low	High	*P*	Low	High	*P*	+	−	*P*	+	−	*P*	+	−	*P*	+	−	*P*
Histology	82																		
Serous	36	17	19	0.172	28	8	0.725	23	13	0.472	14	22	0.462	19	17	0.648	20	16	0.229
Clear	25	12	13		18	7		12	13		10	15		10	15		12	13	
Transition	5	4	1		3	2		3	2		3	2		4	1		4	1	
Endometrioid	11	4	7		9	2		8	3		3	8		5	6		3	8	
Undifferentiated	3	3	0		3	0		3	0		1	2		2	1		2	1	
Mucinous	2	0	2		1	1		1	1		2	0		1	1		0	2	
Age	82																		
<55	41	19	22	0.825	31	10	1.000	27	14	0.497	17	24	1.000	22	19	0.659	23	18	0.377
≥55	41	21	20		31	10		23	18		16	25		19	22		18	23	
LN metastasis	81																		
Negative	61	31	30	0.448	46	15	1.000	36	25	0.793	24	37	1.000	30	31	1.000	28	33	0.312
Positive	20	8	12		15	5		13	7		8	12		10	10		12	8	
Stage	82																		
I	29	16	13	0.493	20	9	0.628	15	14	0.551	12	17	0.543	18	11	0.079	13	16	0.300
II	5	1	4		4	1		3	2		1	4		0	5		1	4	
III	33	15	18		25	8		21	12		12	21		16	17		20	13	
IV	15	8	7		13	2		11	4		8	7		7	8		7	8	

NKca, Intra-epithelial infiltration by NK cells; NKstroma, intra-stromal infiltration by NK cells; Tca, intra-epithelial infiltration by T cells; Tstroma, intra-stromal infiltration by T cells

MICA/B and ULBP2 expressions were evaluated according to staining intensity and scored as follows: 0, negative, no staining in cancer cells (same or weaker than the cancer stroma); 1, weak expression, the staining of the cancer cells is a little stronger than that of the cancer stroma in all the area, or much stronger in a limited (less than 20%) area; 2, strong expression, the staining of the cancer cells is much stronger than that of the cancer stroma in whole section. Cases with scores of 0 and 1 were defined as the low-expression group, and cases with scores of 2 were defined as the high-expression group.

CD57 staining was evaluated as follows: tumor-infiltrating $CD57^+$ NK cells were counted in microscopic fields at 200× magnification (0.0625 mm^2) and separated by their localization as intraepithelial (infiltrating into cancer nests) or intra-stromal (infiltrating within the cancer stroma). The ten fields with the most abundant infiltration were selected, and the average count was calculated. The degree of NK cell infiltration was classified into four groups: negative intraepithelial NK infiltration (<10 NK cells found in ten intraepithelial fields); positive intraepithelial NK infiltration (≥10 NK cells found in ten intraepithelial fields); negative intrastromal NK infiltration (<20 NK cells found in ten intrastromal fields); positive intrastromal NK infiltration (≥20 NK cells found in ten intrastromal fields).

CD8 staining was evaluated as previously described; <5 $CD8^+$ cells/field was scored as negative and ≥5 cells/field was scored as positive [13].

Cell culture

In total, 33 ovarian cancer cell lines and 2 normal ovarian surface epithelial cell lines (Supplementary Table 2) were cultured under the conditions recommended for each line. Each line was grown in 10 cm tissue culture dishes with 10 ml medium at 37°C and 5% CO_2. After 24 h of growth, supernatants were collected and frozen at −80°C for ELISA analysis and cells were harvested for flow cytometry.

Flow cytometry

Single-cell suspensions from tumor cell lines were analyzed for ULBP2, MICA/B and HLA-ABC expressions by staining with PE-conjugated mouse monoclonal anti-human ULBP2 (R&D), PE-conjugated mouse anti-human MICA/B (Becton Dickinson), and FITC-conjugated mouse anti-human HLA-A, B, C (Biolegend) Abs followed by analysis in a FACSCalibur flow cytometer (Becton Dickinson). The expression of the NKG2D ligands were represented by intensity of MICs and ULBP2 which were

comparative fluorescence density when compared to isotype control (log).

Elisa

Soluble MICA and MICB in culture supernatants and the sera from eight patients were detected by the human MICA, MICB DuoSet ELISA development kits (R&D) following the manufacturer's instructions. Soluble ULBP2 was detected as described previously [36]. Two non-overlapping-ULBP2-epitope anti-ULBP2 antibodies were used. Plates were coated with 2 μg/ml anti-ULBP2 monoclonal Ab (MAB1298, R&D Systems) in PBS for 1 h at 37°C, then blocked with 300 μl 10% fetal bovine serum (FBS)/PBS at 4°C overnight and washed. Afterwards, ULBP2-Fc (1298-UL, R&D Systems) and the samples were added and plates were incubated for 1 h at 37°C. After incubation, plates were washed and the detection Ab anti-ULBP2 (AF1298, R&D Systems) was added at 0.5 μg/ml in 10% FBS/PBS for 1 h at 37°C. Plates were washed and anti-goat IgG (H + L)-HRP (1:20,000 in 0.05% PBST, ZYMED Laboratories, Carlsbad, CA, USA) was added for 1 h at 37°C. Plates were washed and developed using the Tetramethylbenzidine Peroxidase Substrate System (DY994 and DY999, R&D Systems). Absorbance was measured at 450 nm.

Statistical analyses

χ^2 tests (Fisher's exact test) were used to compare rate differences. The Spearman nonparametric correlation test was used to analyze the relationships between MICA/B and ULBP2 and tumor-infiltrating $CD57^+$ NK and $CD8^+$ T cell counts. Overall and progression-free survival curves were generated by the Kaplan–Meier method and the difference between the survival curves was analyzed by a log rank test. The contribution of variables to survival was tested by multivariate analyses using the Cox proportional hazard model. Variables included MICA/B, ULBP2, tumor-infiltrating $CD57^+$ NK cell count, $CD8^+$ T cell count and other variables such as age, stage, histology, and lymph node involvement. $P < 0.05$ was considered to be statistically significant. All statistics were performed with SPSS 10.0 software (SPSS Inc. USA).

Results

MICA/B and ULBP2 are expressed in most ovarian cancers, but in fewer borderline or benign ovarian tumors

Among 82 ovarian carcinomas, 80 (97.6%) were positive for MICA/B. The number of cases for which the expression level was scored as 0, 1, and 2 was 2/82 (2.4%), 37/82 (45.1%), and 43/82 (52.4%), respectively (Fig. 1a). Among ten borderline tumors, 3/10, 3/10, and 4/10 cases scored as 0, 1, and 2, and among six benign tumors, 2/6, 3/6, and 1/6 cases scored 0, 1, and 2.

ULBP2 was expressed in 82.9% (68/82) of cases, and the expression level was scored as 0, 1, and 2 in 14/82 (17.1%), 48/82 (58.5%), and 20/82 (24.4%) cases, respectively (Fig. 1b). For the ten borderline tumors, 8/10, 2/10, and 0/10 cases were scored as 0, 1, and 2; none of the benign tumors expressed ULBP2. None of the six normal ovarian tissues expressed MICA/B or ULBP2. There was a significant correlation between MICA/B and ULBP2 expressions, $P < 0.05$ (Table 2), but there was no correlation between MICA/B or ULBP2 expressions and patient age, FIGO stage, histological subtype, or lymph node metastasis (Table 1).

ULBP2 expression correlates with poor prognoses in ovarian cancer patients

Analysis using a Kaplan–Meier curve and log rank test revealed that the overall (75 ± 12 months) and progression-free (53 ± 11 months) survival of patients with high-ULBP2 expression was significantly worse than those with low-ULBP2 expression (112 ± 8 months, 107 ± 9 months respectively), $P < 0.05$ (Fig. 2a), whereas there were no differences in overall and progression-free survival between patients with high- and low-MICA/B expression (Fig. 2b).

Tumor infiltration of $CD8^+$ T cells, but not of $CD57^+$ NK cells, has prognostic significance in ovarian cancer

Intra-epithelial infiltration of $CD57^+$ NK cells was observed in 50/82 (61.0%) ovarian carcinoma cases, and intra-stromal infiltration of NK cells was found in 33/82 (40.2%) cases (Fig. 1c). Intra-epithelial infiltration of $CD8^+$ T cells was observed in 41/82 (50.0%) cases, and intra-stromal infiltration of $CD8^+$ T cells was found in 41/82 (50.0%) cases. There was no correlation between tumor infiltration of NK cells and $CD8^+$ T cells. Furthermore, there was no correlation between tumor infiltration of NK cells or $CD8^+$ T cells and patient age, FIGO stage, histological subtype, or lymph node metastasis (Table 1).

Kaplan–Meier curve and log rank test analyses indicated that the overall survival of patients positive only for intra-stromal infiltration of NK cells (but negative for intra-epithelial infiltration) was significantly lower than those positive only for intra-epithelial infiltration of NK cells ($P < 0.05$). In contrast, there was no difference in progression-free survival between the two groups of the patients ($P > 0.05$, Fig. 2c).

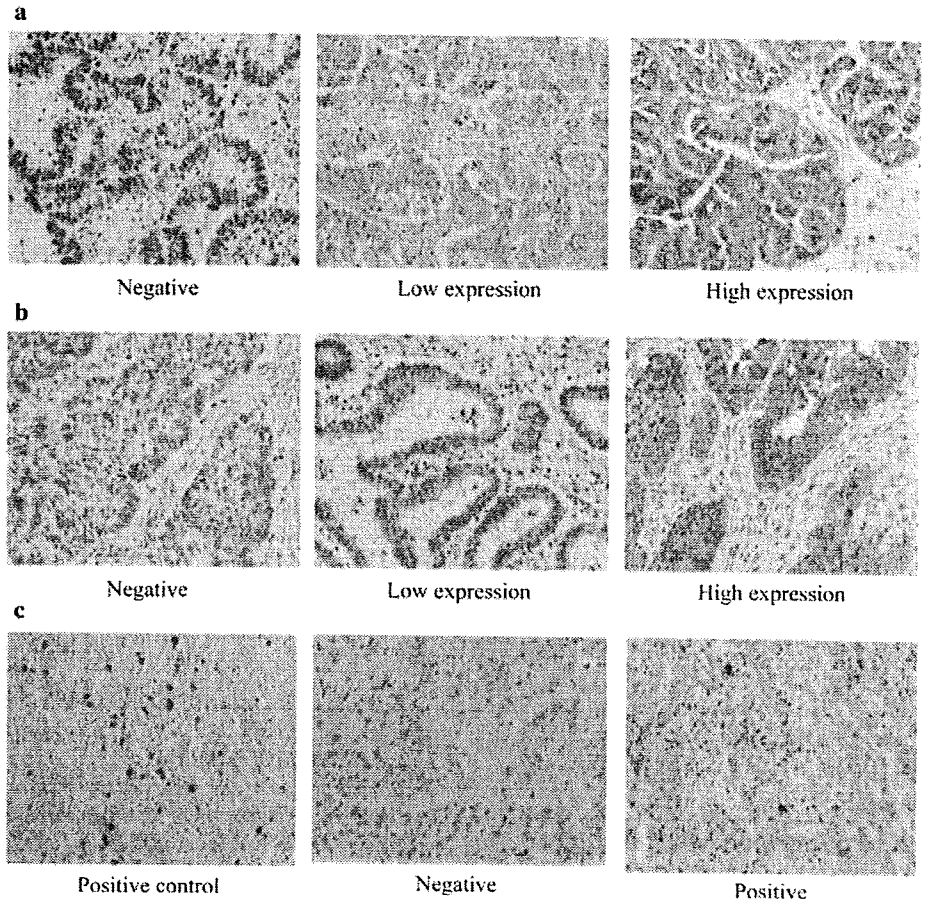

Fig. 1 Immunohistochemical staining of human ovarian cancer tissues using anti-MIC, ULBP2, and CD57 antibodies. **a** Representative staining patterns of MICs. **b** Representative staining patterns of ULBP2. **c** Representative staining patterns of $CD57^+$ NK cells. Spleens were used as positive controls (magnification, ×200)

Table 2 Correlations between NKG2D ligands and NK T cells

	MICs	NKca	NKstroma	Tca	Tstroma
n	82	82	82	82	82
MICs					
*r**	1	−0.236	−0.149	−0.190	−0.102
(*P***)		(0.033)	(0.183)	(0.088)	(0.364)
ULBP2					
*r**	0.384	−0.093	0.055	−0.220	0.014
(*P***)	(<0.001)	(0.406)	(0.626)	(0.047)	(0.898)

NKca, Intra-epithelial infiltration by NK cells; NKstroma, intra-stromal infiltration by NK cells; Tca, intra-epithelial infiltration by T cells; Tstroma, intra-stromal infiltration by T cells

* Spearman's correlation coefficient

** Significance (2-tailed)

The overall and progression-free survival of patients positive for intra-epithelial infiltration of $CD8^+$ T cells was significantly higher than those negative for intra-epithelial infiltration of T cells ($P < 0.05$), in agreement with our previous results [13].

ULBP2 expression correlates with less intra-epithelial infiltration of T cells

High expression of MICA/B in ovarian cancer cases correlated with less intra-epithelial infiltration of NK cells ($P < 0.05$), and high expression of ULBP2 correlated with less intra-epithelial infiltration of T cells ($P < 0.05$). However, there was no correlation between NKG2D ligand expression and intra-stromal infiltration of lymphocytes ($P > 0.05$, Table 2).

ULBP2 expression and intra-epithelial infiltration of T cells are independent prognostic indicators in multivariate analysis

In univariate analyses, FIGO stage, lymph node metastasis, ULBP2 expression, and intra-epithelial infiltration of T cells were prognostic indicators of both overall and progression-free survival (Table 3). High expression levels of ULBP2, FIGO stage, and lymph node metastasis correlated with poor prognoses ($P < 0.05$), whereas intra-epithelial infiltration of T cells correlated with good prognoses for patients ($P < 0.05$).

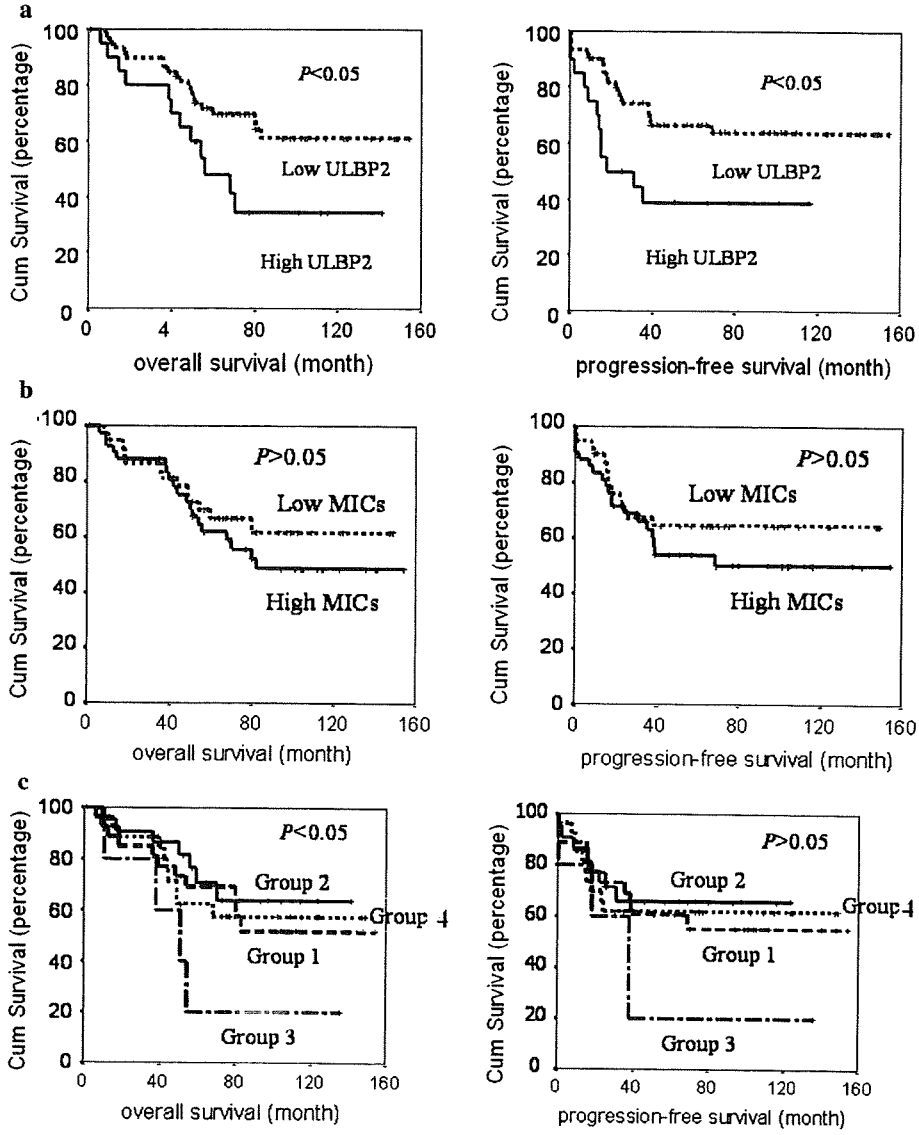

Fig. 2 Overall and progression-free survival analyses of patients with ovarian cancer according to the expression of MICA/B and ULBP2 and tumor infiltration by $CD57^+$ NK cells. **a** Kaplan–Meier curves according to high ($n = 20$) and low ($n = 62$) ULBP2 expressions. **b** Kaplan–Meier curves according to high ($n = 42$) and low ($n = 40$) MIC expressions. **c** Kaplan–Meier curves according to tumor infiltration by $CD57^+$ NK cells. Group 1 ($n = 27$), patients without tumor infiltration by NK cells; Group 2 ($n = 22$), patients only with intra-epithelial infiltration by NK cells; Group 3 ($n = 5$), patients with intra-stromal infiltration NK cells only; Group 4 ($n = 28$), patients with both intra-stromal and intra-epithelial infiltration by NK cells. The difference of overall survival between Group 2 (106 ± 11 months) and 3 (58 ± 19 months) is statistically significant, $P < 0.05$

In multivariate analyses, histology, FIGO stage, and ULBP2 expression were independent prognostic indicators of both overall and progression-free survival, whereas lymph node metastasis, intra-epithelial infiltration of T cells, and intra-stromal infiltration of NK cells were independent prognostic indicators of overall survival (Table 4). High expression of ULBP2 was an indicator of poor prognoses, whereas intra-epithelial infiltration of T cells was an indicator of good prognoses. Intra-stromal infiltration of NK cells was an indicator of poor prognoses only for overall survival.

Most ovarian cancer cell lines express cell surface and soluble forms of MICA/B and ULBP2

Among the 33 examined ovarian cancer cell lines, 32 expressed HLA-class I molecules at high levels and only ovary184 (serous adenocarcinoma) did not express it; 27/33 (81.8%) lines expressed MICA/B, and every line expressed ULBP2 (Fig. 3). None of the two normal ovarian surface epithelial cell lines expressed MICA/B, whereas both of them expressed ULBP2 at low frequencies (13.5 and 15.2% of cells, respectively). There was no correlation between the expression of MICA/B, ULBP2, or HLA ($P > 0.05$).

Soluble MICA was detected in 28/33 (84.8%) ovarian cancer cell lines (the median level is 8.5 pg/ml and the range is 0–238.9); soluble MICB was detected in 21/33 (63.6%) lines (32.5 pg/ml, 0–3095.8); soluble ULBP2 was detected in 18/33 (54.5%) lines (0.2 pg/ml, 0–97.2). No correlation was found between these results. Neither soluble MICA/B nor soluble ULBP2 was detectable in the supernatant of the two normal ovarian cell lines.

Table 3 Univariate analysis of overall and progression-free survival

Variables	Overall survival			Progression-free survival		
	P	Hazard ratio	(95% CI)	*P*	Hazard ratio	(95% CI)
Histology						
Non-clear cell	0.387	1	(0.328, 1.539)	0.360	1	(0.316, 1.519)
Clear cell		0.711			0.693	
Age						
<55	0.311	1	(0.711, 2.918)	0.408	1	(0.659, 2.793)
≥55		1.441			1.356	
Lymph node metastasis						
Negative	<0.001	1	(1.404, 2.858)	0.001	1	(1.299, 2.737)
Positive		2.003			1.886	
Stage						
I and II	<0.001	1	(2.718, 22.348)	<0.001	1	(2.631,
III and IV		7.794			7.595	21.925)
MICs						
Low	0.397	1	(0.669, 2.748)	0.311	1	(0.702, 3.032)
High		1.356			1.459	
ULBP2						
Low	0.036	1	(1.050, 4.420)	0.007	1	(1.308, 5.673)
High		2.154			2.725	
NKca						
Negative	0.383	1	(0.366, 1.470)	0.303	1	(0.335,
Positive		0.734			0.686	1.405)
NKstroma						
Negative	0.367	1	(0.685, 2.780)	0.565	1	(0.600, 2.546)
Positive		1.380			1.236	
Tca						
Negative	0.002	1	(0.139, 0.655)	0.008	1	(0.159, 0.761)
Positive		0.302			0.348	
Tstroma						
Negative	0.614	1	(0.597, 2.398)	0.696	1	(0.563, 2.364)
Positive		1.196			1.154	

NKca, Intra-epithelial infiltration by NK cells; NKstroma, intra-stromal infiltration by NK cells; Tca, intra-epithelial infiltration by T cells; Tstroma, intra-stromal infiltration by T cells

There was no correlation between the soluble levels and the cell surface expression of these NKG2D ligands (Fig. 4).

No correlation was found between the levels of soluble MICA, B and ULBP2 in the sera and their expressions in the cancer tissue of ovarian cancer patients

Soluble MICA was detected in 4/8 (50%) sera of ovarian cancer patients, the median level is 1.0 pg/ml (0, 105.5); soluble MICB was detected in 5/8 (62.5%) sera, median level is 38.3 pg/ml (0, 83.5); soluble ULBP2 was not detectable in the sera of the patients.

Among these eight ovarian carcinomas, the number of cases for which the expression level in cancer tissue was scored as 0, 1, and 2 was 1 case, 5 cases, and 2 cases, respectively. The expression level of ULBP2 was scored as 0, 1, and 2 in 1 case, 6 cases, and 1 case, respectively.

There was no correlation between the soluble levels in sera and the expressions in cancer tissue of these NKG2D ligands (Fig. 5).

Discussion

Recent studies have highlighted the significance of NKG2D function in host-mediated tumor immunity. However, the roles of the NKG2D ligands MICA/B and ULBP2 in the biology of various malignancies remain poorly understood, and the clinicopathological significance of these ligands in ovarian cancer has not yet been reported. Our study is the first to investigate the expression of MICA/B and ULBP2 in a cohort of ovarian cancer cases and a panel of ovarian cancer cell lines with well-defined histological backgrounds. We found that most ovarian cancers express

Table 4 Multivariate analysis of overall and progression-free survival

Factors	*n*	Overall survival		Progression-free survival	
		P	Hazard ratio (95%CI)	*P*	Hazard ratio (95% CI)
Histology[a]	82	0.036	3.064 (1.075, 8.733)	0.033	3.154 (1.094, 9.092)
Age[b]	82	0.819	0.913 (0.420, 1.985)	0.986	0.993 (0.448, 2.200)
Lymph node metastasis	81	0.019	1.722 (1.093, 2.714)	0.059	1.570 (0.982, 2.511)
Stage[c]	82	0.000	10.700 (3.081, 37.161)	0.000	16.029 (4.331, 59.318)
MICs	82	0.513	0.734 (0.290, 1.856)	0.181	0.490 (0.172, 1.394)
ULBP2	82	0.017	3.342 (1.240, 9.005)	0.000	7.578 (2.653, 21.647)
NKca	82	0.274	0.550 (0.188, 1.607)	0.169	0.485 (0.174, 1.358)
NKstroma	82	0.048	2.620 (1.007, 6.818)	0.063	2.525 (0.951, 6.703)
Tca	82	0.026	0.350 (0.138, 0.883)	0.117	0.476 (0.189, 1.203)
Tstroma	82	0.888	1.063 (0.458, 2.468)	0.762	1.144 (0.480, 2.727)

NKca, Intra-epithelial infiltration by NK cells; NKstroma, intra-stromal infiltration by NK cells; Tca, intra-epithelial infiltration by T cells; Tstroma, intra-stromal infiltration by T cells

[a] Patients were divided into non-clear cell carcinoma group and clear cell carcinoma group

[b] Patients were divided into younger women (<55 years) group and elder women (≥55 years) group

[c] Patients were divided into early stage (stages I and II) group and late stage (stages III and IV) group

MICA/B and ULBP2. Borderline and benign tumors expressed them to a lesser extent, but normal ovarian epithelium did not express them. These findings suggest that the expression of NKG2D ligands only occurs after malignant transformation during ovarian cancer development, which is consistent with findings for many other malignancies. Hence, these molecules have the potential to be used as tumor markers for ovarian cancer.

It is well known that the cytotoxic function of NK cells is directly activated by tumor cells, whereas $CD8^+$ T cells require simultaneous pre-sensitization and HLA stimulation. Inducible-surface expression of NKG2D ligands in response to stress or malignant transformation is thought to mark dysfunctional cells for elimination by cytotoxic lymphocytes via NKG2D-mediated mechanisms (the "induced-self" hypothesis) [1, 21, 27]. Indeed, ectopic expression of NKG2D ligands by tumors induces perforin-dependent strong NK and cytotoxic T lymphocyte (CTL) responses in vivo [5, 14]. However, in various human malignancies, including ovarian cancer, it remains unknown whether elevated NKG2D ligand expression in fact leads to enhanced tumor immunity and to better prognoses for patients. Paradoxically, in this study high ULBP2 expression correlated with poor prognoses for patients with ovarian cancer. Expression of another NKG2D ligand, MICA/B, did not correlate with prognoses, suggesting that ULBP2 rather than MICA/B might influence the clinical course of ovarian cancer.

One potential mechanism to explain why high expression of ULBP2 leads to poor prognoses may relate to the shedding of this ligand. Recent studies have shown that both MICA/B and ULBP2 on the cell membrane may be proteolytically cleaved by metalloproteases to produce soluble molecules [12, 14, 30, 36]. These soluble forms systemically down-regulate the surface expression of NKG2D receptors, thereby impairing the anti-tumor reactivity of NK and $CD8^+$ T cells [6, 12]. Taking these results into account, we measured soluble MICA/B and ULBP2 levels in the supernatants of ovarian cancer cell lines and examined whether these levels correlate with the expression levels of NKG2D ligands on the same cells. Our results show that ovarian cancer cells indeed secrete various amounts of soluble NKG2D ligands. However, no correlation was found between the secreted NKG2D ligand levels as measured by ELISA and the expression levels of these ligands on the surface of the cells as detected by flow cytometry. We also collected paired samples of sera and cancer specimen of eight ovarian cancer patients and compared the expression levels of NKG2D ligands. Again, there was no correlation between the soluble molecules levels and cancer tissue expression in vivo (even there was no detectable soluble ULBP2 in sera in accordance with previous report on gastrointestinal malignancies and healthy donors [36]). A recently published report showed that soluble MICA levels in ovarian cancer patients were significantly higher than that in normal women, but no correlation was found between elevated levels of soluble MICA and cancer stage or metastasis [15]. These findings altogether suggest that secreted soluble NKG2D ligands are not responsible for the poor prognosis of the ovarian cancer patient.

To evaluate the influence of NKG2D ligand expression on the immunological microenvironment in ovarian cancer, we investigated the infiltration of both $CD8^+$ T and $CD57^+$ NK cells in the same ovarian cancer tissues. Overexpres-

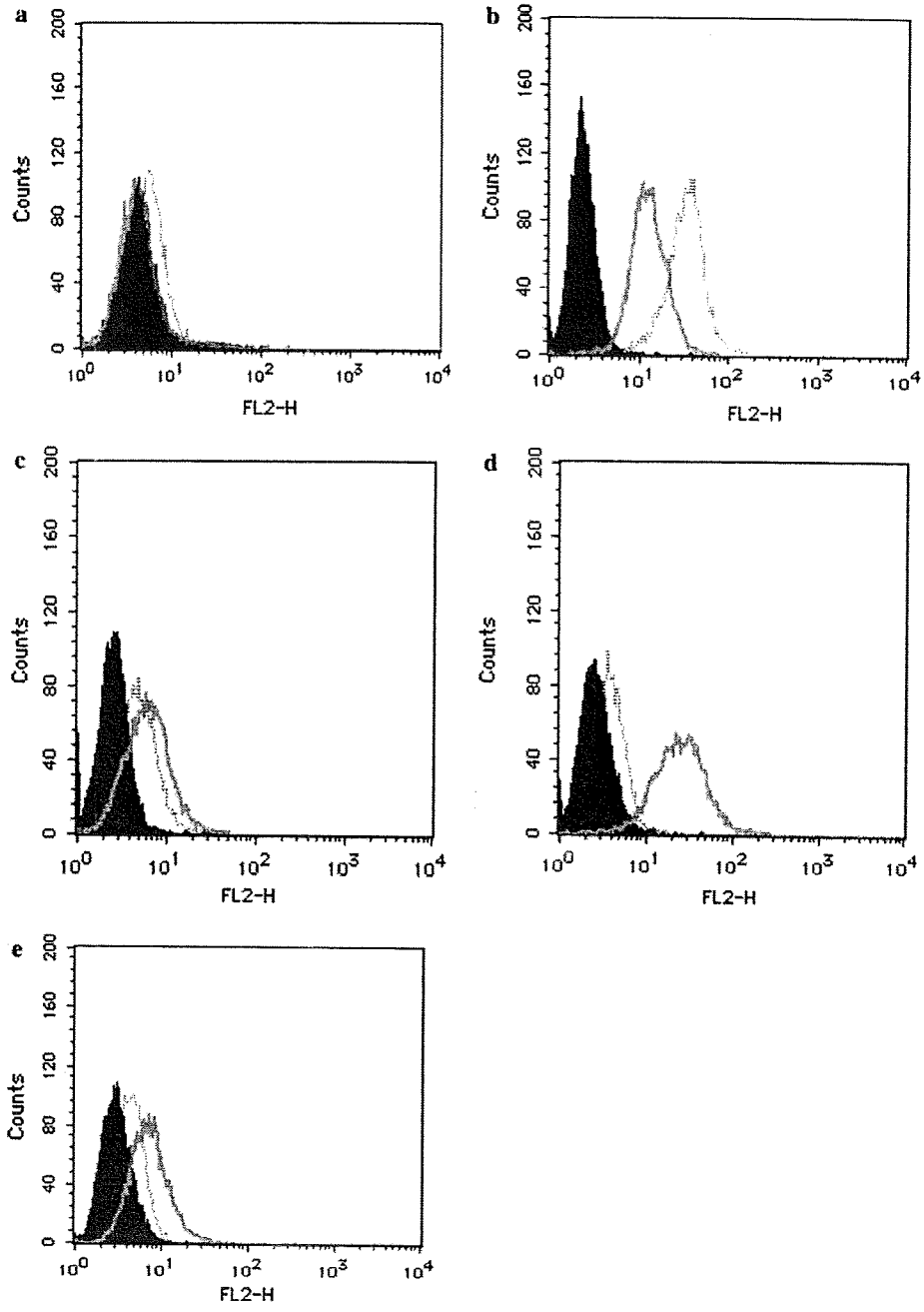

Fig. 3 Representative flow cytometry results for ovarian cell lines. The figure shows the expression of MICs (*solid line*) and ULBP2 (*dot line*), the *filled histograms* represent the isotype control. **a** Normal ovarian cell line OSE6. **b** Endometrioid adenocarcinoma cell line OV2008. **c** Serous adenocarcinoma cell line OVCAR-3. **d** Clear cell carcinoma cell line TOV-21G. **e** Undifferentiated carcinoma cell line A2780

sion of ULBP2 correlated with less tumor infiltration by $CD8^+$ T cells, but not with infiltration of $CD57^+$ NK cells. Furthermore, tumor infiltration of $CD8^+$ T cells correlated with patient prognoses, whereas tumor infiltration of $CD57^+$ NK cells did not correlate with the prognosis of ovarian cancer.

Altogether, these findings suggest that in ovarian cancer, overexpression of ULBP2 rather than MICA/B might hinder the infiltration of cytotoxic T lymphocytes and lead to an unfavorable clinical course by allowing tumor cells to escape from immune surveillance, and this effect may not be induced by the soluble types of the NKG2D ligands. Recently, two mechanisms to explain the link between NKG2D ligand expression and immune dysfunction have been proposed. Chronic engagement of NK cells with NKG2D ligands expressed by tumor cells in vitro and in vivo might not only impair NKG2D function, but also might suppress most of the cytotoxic functions of NK cells [3, 24, 38]. Other reports have shown that MICA/B on target cells can be transferred to NK cells upon conjugation

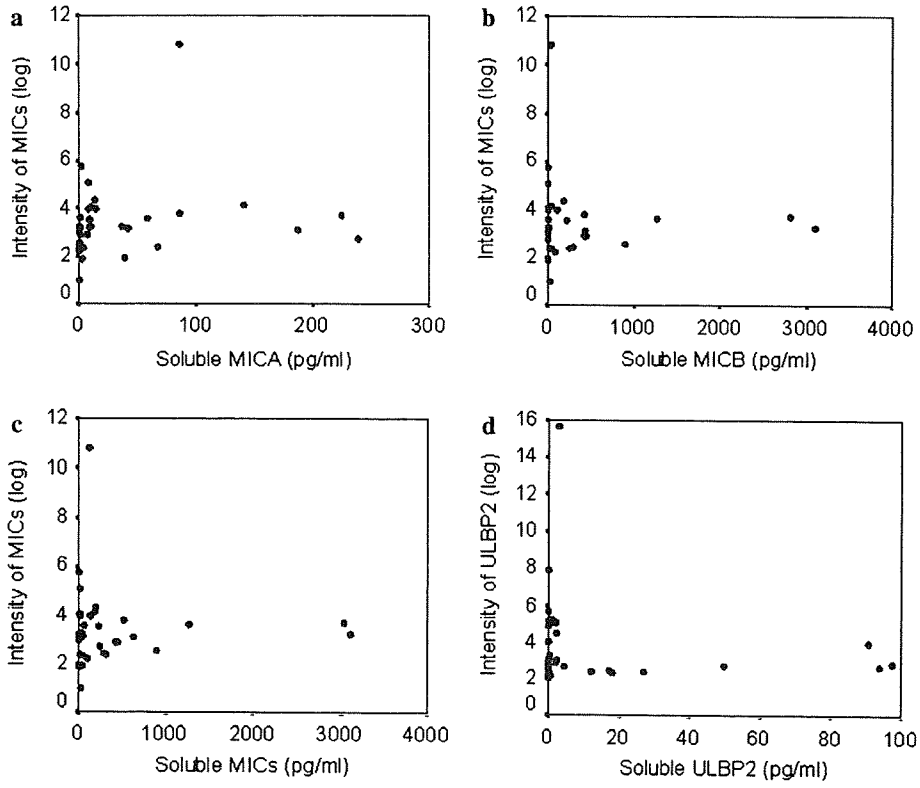

Fig. 4 The relationship between the ligands expression on the cell membrane and the soluble molecules levels in supernatant of ovarian cancer cell lines. *X*-axis represents soluble NKG2D ligands in supernatant which was detected by ELISA. *Y*-axis represents expression of NKG2D ligands detected by flow cytometry, which is shown as comparative fluorescence density compared to isotype control (log). **a** There was no correlation between the soluble MICA and the cell surface expression of MICs. **b** There was no correlation between the soluble MICB and the cell surface expression of MICs. **c** There was no correlation between the soluble MICs (sum of the soluble MICA and MICB) and the cell surface expression of MICs. **d** There was no correlation between the soluble ULBP2 and the cell surface expression of ULBP2

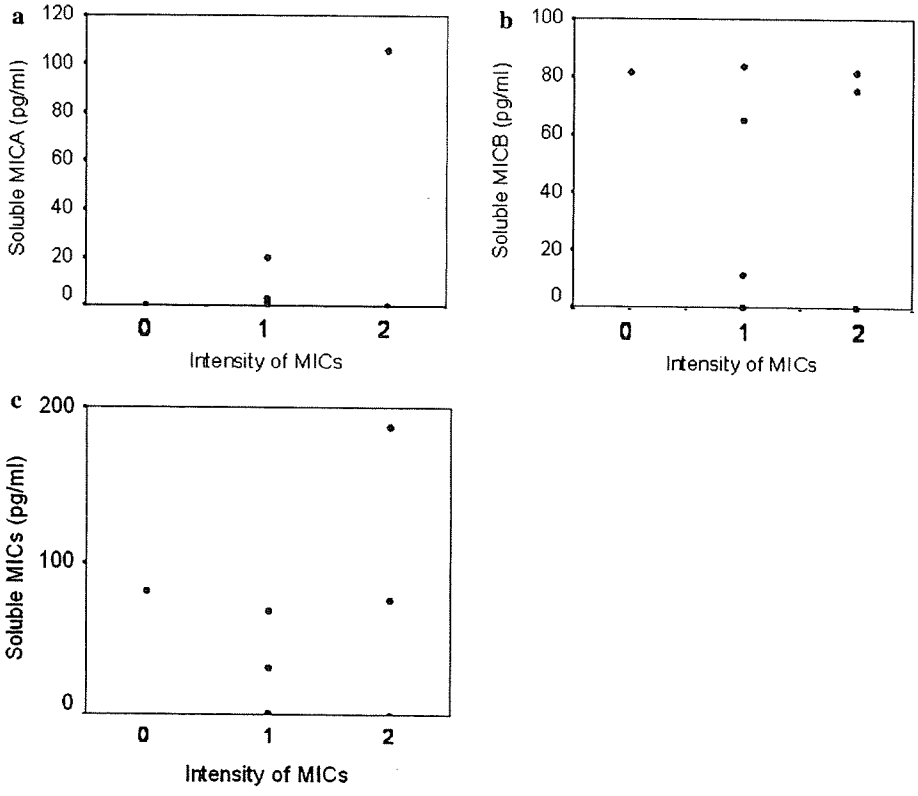

Fig. 5 The relationship between the immunohistochemical expression of MICA/B in the cancer tissue (*x*-axis) and the soluble molecules levels in sera of ovarian cancer patients (*y*-axis). **a** There was no correlation between the soluble MICA and the cancer expression of MICs. **b** There was no correlation between the soluble MICB and the cancer expression of MICs. **c** There was no correlation between the soluble MICs (sum of the soluble MICA and MICB) and the cancer expression of MICs. Intensity of MICs: *0* negative expression, *1* weak expression and *2* strong expression in immunohistochemistry

with NKG2D, and that transferred MICA/B might further down-regulate NKG2D expression in surrounding NK cells or result in cytolysis of the affected NK cells by other NK cells [23, 29]. Similar phenomena have been observed in T cells [18]. Importantly, these effects were mainly caused by cell–cell contact, and the soluble forms of the ligands had little effect [3, 24, 38]. Although direct evidence has not been provided, mechanisms similar to these might explain our finding that elevated ULBP2 expression was associated with less $CD8^+$ T cell infiltration and poor prognoses.

In summary, we show here for the first time that most ovarian cancers express NKG2D ligands, and that strong expression of ULBP2 correlates with poor prognoses for patients, possibly due to the functional inhibition of $CD8^+$ T cells. We have previously reported that intratumoral $CD8^+$ T cells are indicators of poor prognoses in ovarian cancer patients, and the findings described here might partly explain those results. Further investigation is necessary to determine whether the connection between ULBP2 and NKG2D regulates tumor immunity in ovarian cancer and whether it is the exact mechanism underlying this connection.

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