Abstract. UL16 binding protein 1 (ULBP1) expressed on the tumor cell surface binds to the natural killer group 2 member D (NKG2D) receptor presenting on natural killer (NK), cluster of differentiation (CD)8+ T, and γδ T cells. However, the roles of ULBP1 and NKG2D expression and associated immune responses in gastric cancer are unclear. The present study investigated the associations between ULBP1 and NKG2D expression and clinical outcomes in patients with gastric cancer. The levels of ULBP1 and NKG2D expression were examined in human gastric cancer cell lines and gastric cancer tissues from 98 patients who underwent surgery from 2004 to 2008. MKN-74 cells expressed ULBP1 with ULBP2-5, or -6. NKG2D was expressed at a higher level following activation of T cells and NK cells. Among the tissue sections positive for NKG2D expression, 6 patients were positive for CD8 and CD56. In all tissues, NKG2D-expressing cells were typically aCD8+ T cells. Patients with NKG2D expression in tumors exhibited significantly longer overall survival (OS) compared with patients without NKG2D expression in tumors (P=0.0217). The longest OS was observed in patients positive for ULBP1 and NKG2D, whereas the shortest OS was observed in patients negative for ULBP1 and NKG2D. The interaction between ULBP1 and NKG2D may improve OS in patients with gastric cancer, and may have applications in immunotherapy for the induction of adaptive immunity in patients with cancer. Additionally, ULBP1 and NKG2D may be useful as prognostic biomarkers in gastric cancer.

Introduction

Gastric cancer is the fourth most common type of cancer, and the second leading cause of cancer-associated mortality worldwide (1). Therefore, novel therapies for the treatment of gastric cancer are urgently needed.

The expression of natural killer group 2 member D (NKG2D) on natural killer (NK) cells, γδ T cells and CD8+ T cells was first identified in 1991 (2), and its function was subsequently reported in 1999 (3). A total of 6 UL16 binding proteins (ULBP1-6) and MHC class I chain-related proteins A and B (MICA/B) are the two major types of NKG2D ligands (NKG2DLs) for human NKG2D. mRNA export factor, Mult1, and histocompatibility antigen 60 are the 3 major NKG2DLs for murine NKG2D (4). NKG2DLs are induced by cellular or
manner stress. When expressed on CD8+ T cells, NKG2D functions to receive co-stimulatory signals, thereby resulting in CD8+ T-cell activation (5). NKG2DL expression is induced upon stress and malignant transformation (6). In addition, NKG2D expression on mononuclear cells is significantly lower in patients with advanced gastric cancer compared with in patients with early gastric cancer. Additionally, NKG2D expression is significantly higher following surgery compared with prior to surgery in patients with gastric cancer (7). The upregulation of NKG2DLs in cancer cells subjected to stress results in lysis by NK cells (8).

The surface glycoprotein MICA is an NKG2DL broadly expressed in epithelial and hematopoietic tumors, but not in healthy tissue. Subsequent to shedding of MICA in tumors, soluble MICA functions in the immune escape of tumors. Importantly, the level of soluble MICA has been positively correlated with tumor node metastasis (TNM) stage in patients with breast cancer (9). Additionally, soluble MICA has been demonstrated to reduce the expression of NKG2D, impair NK-mediated immune surveillance, which leads to immune escape of breast tumors (9). Interleukin (IL)-12 may also upregulate the expression of NKG2D and increase cytotoxicity of NK cells (10). However, the potential applications of an additional key NKG2DL, ULBP1, as a biomarker or prognostic factor for patients with gastric cancer have not been determined, and the role of the ULBP1/NKG2D interaction in patients with gastric cancer is not yet clear.

The present study investigated whether ULBP1 and NKG2D expression levels were associated with the clinical outcomes of patients with gastric cancer.

Materials and methods

Gastric cancer cell lines and culture conditions. The human gastric cancer MKN-74 cell line, established at the National Cancer Center, was maintained in Dulbecco’s modified Eagle’s medium/F12 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in an atmosphere containing 5% CO2.

Patients. A total of 98 samples were obtained from patients who underwent surgery at the Department of Digestive Surgery and Surgical Oncology, Yamaguchi University Graduate School of Medicine (Yamaguchi, Japan) from January 2004 to December 2008. The patients were diagnosed with gastric cancer with the tumor invaded through the muscularis propria (MP, muscularis propria; SS, subserosa; SE, serosal exposure; SI, serosal invasion).

The clinicopathological characteristics of the patients are summarized in Table I. The study protocol was approved by the Institutional Review Board for Human Use at Yamaguchi Hospital (Yamaguchi, Japan). Written informed consent for the present study was obtained from all patients prior to surgery.

Analysis of ULBP, MICA/MICB expression in cancer cell lines and NKG2D expression in NK/T cells by flow cytometric analysis. Flow cytometric analysis was performed to confirm the expression of NKG2DLs on the MKN-74 gastric cancer cell line and NKG2D on the surface of the NK and T cells. BD FACSFLOW (BD Biosciences, San Jose, CA, USA) was used for buffer of flow cytometric analysis. ULBP1 against MKN-74 was stained using phycoerythrin (PE)-conjugated mouse anti-ULBP1 antibody (dilution, 1:10; cat no. FAB1380P; BD Biosciences), and CD56 against NK and T cells was stained using PE-CF594-conjugated mouse anti-CD56 (dilution, 1:20; cat no. 562289; BD Biosciences). All samples were maintained using PE-CF594-conjugated mouse anti-CD56 (dilution, 1:20; cat no. 562289; BD Biosciences).

The human gastric cancer cell line, MKN-74, mononuclear cells, activated T cells, and NK cells were stained with allophycocyanin (APC)–conjugated mouse anti-ULBP2 (dilution, 1:10; cat no. FAB1298A; R&D Systems, Inc.). CD3 against NK and T cells was stained using v450-conjugated mouse anti-CD3 (dilution, 1:20; cat no. 560351; BD Biosciences), CD8 against NK and T cells was stained using v500-conjugated mouse anti-CD8 (dilution, 1:20; cat no. 557834; BD Biosciences), and CD56 against NK and T cells was stained using APC-Cy7-conjugated mouse anti-CD56 (dilution, 1:10; cat no. 561394; BD Biosciences). CD8 against NK and T cells was stained using APC-Cy7-conjugated mouse anti-CD8 (dilution, 1:20; cat no. 557834; BD Biosciences), CD16 against NK and T cells was stained using v500-conjugated mouse anti-CD16 (dilution, 1:20; cat no. 561394; BD Biosciences), and CD56 against NK and T cells was stained using PE-CF594-conjugated mouse anti-CD56 (dilution, 1:20; cat no. 562289; BD Biosciences). All samples were maintained at 4°C for 30 min.

Table I. Characteristics of the study population.

| Variables | No. of cases |
|-----------|-------------|
| Number of patients | 98 |
| Age, years (range) | 64±14 (23-92) |
| Gender | 65/33 |
| Male/Female | 65/33 |
| Depth of tumor invasion | 21/25/49/3 |
| MP/SS/SE/SI | 21/25/49/3 |
| Histologic grade | 3/28/51/9/7 |
| Papillary adenocarcinoma/tubular adenocarcinoma/poorly differentiated adenocarcinoma/signet-ring cell carcinoma/mucinous adenocarcinoma | 7/42/43 |
| Stroma | 7/42/43 |
| Medullary type/interstitial connective tissue/scirrhous type | 7/42/43 |
| Pattern of tumor infiltration | 4/34/60 |
| a/b/c | 4/34/60 |
| Lymphatic invasion | 7/25/37/29 |
| 0/1/2/3 | 7/25/37/29 |
| Venous invasion | 23/50/21/4 |
| 0/1/2/3 | 23/50/21/4 |
| Regional lymph nodes | 27/20/13/38 |
| N0/N1/N2/N3 | 27/20/13/38 |
| Stage | 11/30/34/23 |
| I/II/III/IV | 11/30/34/23 |

MP, muscularis propria; SS, subserosa; SE, serosal exposure; SI, serosal invasion.
buffer, the cells were resuspended in phosphate-buffered saline (PBS) with 2% FBS at a density of 2x10^6 cells/100 µl. Appropriate isotype control antibodies were stained in the aforementioned manner.

Samples were analyzed with a BD LSRFortessa X-20 instrument (BD Biosciences), and data were analyzed with FlowJo software version x10.0.7r2 (Tree Star, Inc., Ashland, OR, USA). The relative fluorescence intensity (RFI) was calculated as follows: RFI = (FMI of molecule) - (FMI of corresponding isotype control)/FMI of corresponding isotype control, where FMI is the mean fluorescence intensity.

**NK and T cell activation.** Mononuclear cells (LP_241; Cellular Technology, Ltd., Cleveland, OH, USA) were activated to generate NK cells by addition of 5 µl/ml/well of mixed CD2 and CD335 beads with 500 U/ml/well IL-2 (all from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) for 2 h. For T-cell activation, the mononuclear cells were stimulated with 2 µg/ml/well anti-CD3 agonistic antibodies (eBioscience; Thermo Fisher Scientific, Inc.) and 1 µg/ml/well anti-CD28 agonistic antibodies (BioLegend, San Diego, CA, USA) with 100 U/ml/well IL-2 (Miltenyi Biotec GmbH) for 2 h.

**Immunohistochemistry (IHC).** Paraffin-embedded tissue blocks were cut into 5 µm sections, mounted on saline-coated slides, and subsequently dewaxed and rehydrated using xylene and graded alcohol washes. Antigen retrieval was performed by microwaving in Target Retrieval solution (pH 9; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). Endogenous peroxidase was blocked at room temperature (RT) for 15 min with 0.3% hydrogen peroxide (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in PBS containing 0.1% sodium azide. Subsequent to washing twice in PBS, the sections were blocked with Protein Block Serum-Free (Dako; Agilent Technologies, Inc.) at RT for 30 min. The primary antibodies were then added. Anti-ULBP1 antibodies (cat no. HP1007547; 1:100; Sigma-Aldrich; Merck KGaA), anti-NKG2D antibodies (cat no. ab36136; 1:100; Abcam, Cambridge, UK), anti-CD56 antibodies (cat no. B159; 1:50), and anti-CD8 antibodies (cat no. SK1; 1:50) (both from BD Biosciences) were used for IHC according to the manufacturers' protocol. Briefly, following incubation with primary antibodies at 4°C for 15 h, the slides were washed twice in PBS, incubated with horseradish peroxidase-conjugated rabbit/mouse antibodies with original concentration at RT for 30 min (K406311-2; EnVision Plus mouse/HRP system; Dako; Agilent Technologies, Inc.), incubated with Dako-Chromogen solution, and washed in de-ionized water. Following background staining with Mayer's hematoxylin, the sections were dehydrated through ascending alcohols to xylene and mounted. To confirm the specificity of antibodies, negative control slides were incubated with mouse IgG1 monoclonal antibodies (ab81032; 1:100; Abcam). IHC results were evaluated by imaging under low-power magnification (x100) in eight fields of view to identify regions containing positive immunoreactivity. Immunostaining was additionally evaluated at a high-power (x400) magnification (ECLIPSE E200 and Nikon 1 V2 Micro imaging system; Nikon, Tokyo, Japan). Microscopic analysis of ULBP1 and NKG2D expression was performed by three blinded independent observers.

**Scoring of ULBP1 expression in cancer cells.** Evaluation of ULBP1 staining in the cancer cells was performed semi-quantitatively, as described previously by Loos et al (12) (Evaluation of ULBP1 staining was based on the area and intensity of the staining. Scoring system for area of staining is as follows: Score 1, <33% of cancer cells; score 2, 33-66% of cancer cells; score 3, >66% of cancer cells. Scoring for staining intensity is as follows: Score 1, absent/weak staining; score 2, moderately intense staining and score 3, strong staining. Each section was given a final grade derived from the combination of the area and intensity scores. The final score was calculated by adding scores of intensity and area. Sections with a final score of ≥3 were classified as exhibiting low ULBP1 expression (ULBP1 low), whereas sections with a final score of <3 were classified as exhibiting high ULBP1 expression (ULBP1 high).

**Scoring of NKG2D expression on mononuclear cells around gastric tumors.** Absolute numbers of NKG2D-positive cells in 1 µm invasive tumor core biopsies were counted manually using an eyepiece reticle by three blinded independent individuals. The number of NKG2D-positive cells around the tumors was counted using a computerized image analysis system composed of an Olympus DP70 CCD camera on an Olympus AX70 light microscope (Olympus, Tokyo, Japan). Under magnification, x400, there were ≥8 independent and intact computerized microscopic fields for the duplicates of each patient sample. A total of four independent microscopic fields (magnification, x400), representing the densest lymphocytic infiltrates, were selected for each patient sample to ensure representativeness and homogeneity. The scores for four fields were averaged to calculate the final number for one computerized microscopic field at magnification, x400 (0.0768 mm^2/field). The evaluation of NKG2D-positive cells was performed by three independent blinded observers. Discrepancies in enumeration, within a range of 5%, were re-evaluated and a consensus decision was made. The ratio of NKG2D-positive cells/tumor-infiltrating lymphocytes (TILs) was calculated for each specimen. The median value was selected as the cut-off for defining TIL subgroups (median, 0.093). High and low ratios of NKG2D were termed NKG2D high and NKG2D low, respectively.

**Statistical analysis.** Differences were analyzed using Fisher's exact tests. Actuarial OS rates and recurrence-free survival rates were calculated by the Kaplan-Meier method and analyzed by the log-rank test. Univariate analyses were performed using Fisher's exact tests. Multivariate analyses were performed to determine factors affecting overall survival (OS) based on the Cox proportional hazards regression model. A secondary analysis was performed to assess the associations among the expression of NKG2DLs/NKG2D and clinicopathological characteristics by multivariate analysis based on the proportional hazards regression model. The activation of T cells and NK cells was analyzed by Mann-Whitney tests. For comparisons of individual variables, paired-sample t-tests were performed. Two-tailed P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed with EZR version 1.35 (Saitama Medical Center, Jichi Medical University), a graphical user interface for R (The R Foundation for Statistical Computing, v.2.13.0), and more specifically a modified version of R commander.
Expression of NKG2DLs in cancer cell lines. In the flow cytometric analysis, cell surface expression of ULBP1 and ULBP2/5/6 in MKN-74 cells was constitutively high (Fig. 1A). By contrast, cell surface expression of MICA and MICB was not detected in MKN-74 cells (data not shown).

Expression of NKG2D in mononuclear cells. The cell surface expression of NKG2D on CD3+ T cells was significantly increased following T-cell activation (MFI, 621) compared with the expression (MFI, 243) prior to T-cell activation [P=0.0286, n=4, standard deviation (SD) = 378; Fig. 1B]. Similarly, cell surface expression of NKG2D on CD3+CD8+ T cells was significantly higher following T-cell activation (MFI, 1234) compared with expression (MFI, 515) prior to T-cell activation (P=0.0286, n=4, SD=719; Fig. 1C). Cell surface expression of NKG2D on CD16+CD56+ NK cells was significantly higher following NK cell activation (MFI, 974) compared with expression (MFI, 276) prior to NK cell activation (P=0.0283, n=4, SD=698; Fig. 1D).

**IHC staining of ULBP1.** ULBP1 expression was observed in the cell membrane and cytoplasm. Fig. 2A and B show representative images of ULBP1-high staining in gastric cancer (magnification, x100 and x400, respectively). Fig. 2C and D show isotype control staining in gastric cancer (magnification, x100 and x400, respectively). Of the 98 samples examined in the analysis, 70 were positive for ULBP1 expression.

**IHC staining of NKG2D.** NKG2D was expressed on the membrane of mononuclear cells around the gastric tumors. Fig. 2E and F reveal high NKG2D expression in TILs with (magnification, x100 and x400, respectively). Fig. 2G and H demonstrate isotype control staining in TILs.
Figure 2. Representative histopathological images showing immunohistochemical staining of ULBP1, NKG2D, CD8 and CD56 in gastric cancer tissues. (A) High ULBP1 expression in gastric cancer tissues. ULBP1 expression is revealed in the cell membrane and cytoplasm (brown staining). (B) High ULBP1 expression in gastric cancer tissues. Magnification, x400; scale bar, 100 µm. (C) Isotype control for staining of ULBP1. Magnification, x100; scale bar, 200 µm. (D) Isotype control for staining of ULBP1. Magnification, x400; scale bar, 100 µm. (E) High NKG2D expression in tumor-infiltrating lymphocytes. Magnification, x100; scale bar, 200 µm. (F) High NKG2D expression in tumor-infiltrating lymphocytes. Magnification, x400; scale bar, 100 µm. (G) Isotype control for staining of tumor-infiltrating lymphocytes. Magnification, x100; scale bar, 200 µm. (H) Isotype control for staining of tumor-infiltrating lymphocytes. Magnification, x400; scale bar, 100 µm. (I) Staining of CD8-positive cells in gastric cancer. Magnification, x100; scale bar, 200 µm. (J) Staining of CD8-positive cells in gastric cancer. Magnification, x400; scale bar, 100 µm. (K) Staining of CD56-positive cells. Magnification, x100; scale bar, 200 µm. (L) Staining of CD56-positive cells. Magnification, x400; scale bar, 100 µm. Immunohistochemical detection of CD8-positive or CD56-positive mononuclear cells around gastric tumors in I-L used the same sections as those in E-H. ULBP, UL16 binding protein; CD, cluster of differentiation; NKG2D, natural killer group 2 member D.

IHC staining of NKG2D⁺, CD8⁺, and CD56⁺. Among the NKG2D-expressing tissue sections, a total of 6 samples were stained for CD8 and CD56 and were used as representative sections. Fig. 2I-L displays CD8 or CD56 expression in the same samples as those analyzed in Fig. 2E-H. In all tissues, the majority of NKG2D-expressing cells were CD8⁺ T cells.

Expression of ULBP1 and NKG2D and clinicopathological findings. Univariate analysis indicated that NKG2D expression in mononuclear cells was associated with lymphatic invasion (P=0.0028) and postoperative recurrence (P=0.0048; Table II). No significant associations were observed between ULBP1 expression and pathological findings or postoperative recurrence (Table II).

Survival curves following surgery were compared between high and low ULBP1 and NKG2D expression groups. A total of 70 patients were positive for ULBP1, whereas 28 patients were negative for ULBP1 (Table II). By contrast, a total of 19 and 79 patients were positive and negative for NKG2D, respectively (Table II). There were no significant differences in OS observed according to ULBP1 expression (P=0.334; Fig. 3A). However, patients with NKG2D expression were indicated
to have significantly longer OS compared with patients without NKG2D expression (P=0.0217; Fig. 3B). Combined expression of NKG2D and ULBP1 was also analyzed and are as follows: ULBP1-high/NKG2D-high (n=12), ULBP1-high/NKG2D-low (n=58), ULBP1-low/NKG2D-high (n=7), and ULBP1-low/NKG2D-low (n=21). With regards to OS, the findings indicated that the ULBP1-high/NKG2D-high group had the best prognosis, and the ULBP1-low/NKG2D-low group had the poorest prognosis (P=0.0096; Table III).

### Discussion

The present study examined the association between prognosis and expression of NKG2D and ULBP1. The results indicated that detecting a combination of ULBP1 and NKG2D expression might be an effective strategy for prediction of OS in patients with gastric cancer, and therefore this may provide important insights into the roles of these two proteins in gastric cancer. A number of solid tumors constitutively express NKG2DLs and are receptive to NKG2D-dependent immunosurveillance (6). However, soluble NKG2DLs are present II. Univariate analysis of overall survival of patients with gastric cancer following gastrectomy.

| Parameters                                                                 | ULBP1                        | NKG2D                        |
|---------------------------------------------------------------------------|------------------------------|------------------------------|
|                                                                           | Cases, n (n=70)              | Cases, n (n=19)              |
| Depth of tumor invasion (T)                                              |                              |                              |
| T2 (muscularis propria or subserosa)                                    | 46, 37, 9                   | 12, 34                      |
| T3 (serosal exposure), T4 (invasion)                                     | 52, 33, 19                  | 7, 45                       |
| Histological classification of gastric tumors                            |                              |                              |
| Papillary adenocarcinoma/tubular adenocarcinoma                          | 30, 21, 9                   | 5, 26                       |
| Poorly differentiated adenocarcinoma/signet-ring cell carcinoma/ mucinous adenocarcinoma | 68, 49, 19                  | 14, 53                      |
| Cancer stromal volume                                                    |                              |                              |
| Medullary type/interstitial connective tissue                            | 49, 36, 13                  | 9, 40                       |
| Scirrhus type                                                             | 48, 29, 14                  | 9, 39                       |
| Tumor infiltrative pattern into the surrounding tissues                  |                              |                              |
| a, b                                                                     | 38, 28, 10                  | 7, 31                       |
| c                                                                        | 60, 42, 18                  | 12, 48                      |
| Lymphatic invasion                                                        |                              |                              |
| 0                                                                        | 7, 6, 1                     | 5, 2                        |
| 1, 2, 3                                                                  | 91, 64, 27                 | 14, 77                      |
| Venous invasion                                                           |                              |                              |
| 0                                                                        | 23, 16, 7                   | 8, 15                       |
| 1, 2, 3                                                                  | 75, 54, 21                  | 11, 64                      |
| Lymph node metastasis                                                    |                              |                              |
| 0                                                                        | 27, 21, 6                   | 6, 21                       |
| 1, 2, 3                                                                  | 71, 49, 22                  | 13, 58                      |
| Stage grouping                                                           |                              |                              |
| I, II                                                                    | 41, 32, 9                   | 10, 31                      |
| III, IV                                                                  | 57, 38, 19                  | 9, 48                       |
| Stage grouping                                                           |                              |                              |
| I, II, III                                                               | 75, 57, 18                  | 16, 59                      |
| IV                                                                       | 23, 13, 10                  | 3, 20                       |
| Postoperative recurrence                                                 |                              |                              |
| Presence                                                                 | 33, 25, 8                   | 2, 31                       |
| Absence                                                                  | 45, 32, 12                  | 15, 30                      |

ULBP1, UL16 binding protein 1; NKG2D, natural killer group 2 member D.
NKG2DLs are often expressed in breast cancer tissues (13). In particular, MIC-A/B and ULBP2 expression is associated with an improved prognosis in patients with cancer (13) and immunological function of NKG2DLs have not been demonstrated (13). However, other studies have suggested that soluble MICA is involved in tumor immune escape (9). In the present study, ULBP1 expression in gastric cancer may have acted as a positive ligand by binding to NKG2D, thereby prognoses are improved when these proteins were co-expressed. Additionally, it was suggested in Osaki et al (14) that decreased NKG2D expression may be responsible for immune evasion by tumors in gastric cancer, which is consistent with the results of the present study in which patients with low NKG2D expression exhibited poorer survival.

The present study revealed that the interaction of NKG2DLs and NKG2D was involved in cancer and T-cell activation in tumors. This interaction is also thought to be important for NK activation in cancer (3). Therefore, the NKG2D/ULBP1 interaction may be involved in the association between cancer and T cells. ULBP1 and NKG2D, and the ULBP1/NKG2D interaction, may be molecular targets for immunotherapy. In patients with advanced non-small-cell lung cancer, melanoma and renal-cell cancer, the inhibition of programmed death ligand 1 (PD-L1) exhibited a robust response and blocked disease progression (15). Anti-PD-1 antibodies produced objective responses in ~20-25% of patients with non-small-cell lung cancer, melanoma or renal-cell cancer (16). Therefore, one approach for blocking the ULBP1/NKG2D interaction is to use specific antibodies against ULBP1 and NKG2D, which may target T cells at sites of solid tumors, and therefore accelerates the activation of T cells. Additional studies are required to determine the effects of the ULBP1/NKG2D interaction on the adaptive immune system.

Therefore, the interaction between ULBP1 and NKG2D in gastric cancer may be associated with OS. In addition, the results of the present study suggested that ULBP1 and NKG2D may be applied in immunotherapy to induce acquired immunity in patients with cancer and for the prediction of prognosis following surgical resection in patients with gastric cancer. The detailed interactions between NKG2DL and NKG2D require additional investigation. The development of NKG2DL-targeted immunotherapy may have important future clinical applications.

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