Decreased NKG2D Expression on CD8⁺ T Cell Is Involved in Immune Evasion in Patients with Gastric Cancer

Tomohiro Osaki, Hiroaki Saito, Toshiaki Yoshikawa, Sachiko Matsumoto, Shigeru Tatebe, Shunichi Tsujitani, and Masahide Ikeguchi

Abstract
Purpose: Some studies suggest that the immunoreceptor NKG2D expression on CD8⁺ T cells is down-regulated and this reduction may be involved in immune evasion in cancer patients. The present study was designed to investigate NKG2D expression on CD8⁺ T lymphocytes and its relationship to immune evasion in gastric cancer patients.

Experimental Design: NKG2D expression on both circulating and tumor-infiltrating CD8⁺ T cells was evaluated by multicolor flow cytometry. Soluble MHC class I chain-related gene A (MICA) in the sera was quantitated by ELISA. Transwell experiments were carried out to determine the effect of cancer cells on NKG2D expression.

Results: NKG2D expression on circulating CD8⁺ T cells was down-regulated and significantly correlated with IFN-γ production in gastric cancer patients (r = 0.68; P = 0.007). NKG2D expression was closely related to undifferentiated cancer (P = 0.021) as was the depth of invasion (P = 0.012). There was no difference in soluble MICA between gastric cancer patients and normal controls. NKG2D expression on CD8⁺ T cells was remarkably reduced in the tissue of gastric cancer compared with peripheral blood (P = 0.046). Complete removal of tumor by surgery restored NKG2D expression on CD8⁺ T cells (P = 0.0049). Transwell experiments showed that this down-regulation was induced by direct contact between cancer cells and CD8⁺ T cells and that soluble factors did not affect the NKG2D expression. This phenomenon was blocked by the addition of anti-MICA antibodies.

Conclusions: Decreased NKG2D expression may be one of the key mechanisms responsible for immune evasion by tumors in gastric cancer.

Gastric cancer is one of the most common cancers in Asia, and its mortality still ranks second among all cancer deaths worldwide (1). Despite the expression of tumor rejection antigens, such as MAGE1, MAGE2, and MAGE3 (2), and the presence of tumor-specific cytotoxic T cells (3), the immune system fails to exhibit immune responses against gastric carcinoma. However, the mechanisms by which gastric cancers overcome antitumor immunologic responses are poorly understood.

NKG2D is a type II C-lectin-like protein encoded by a gene located next to the NKG2A, NKG2C, and NKG2E genes within the natural killer (NK) gene complex on human chromosome 12p12-p13 and mouse chromosome 6 (4). NKG2D is an activating cell surface receptor expressed by NK cells, γ-δ T cells, some cytolytic CD8⁺ α-β T cells, and NKT cells and a minor subset of CD4⁺ α-β T cells (5 – 8). Retinoic acid early inducible-1, H60, human MHC class I chain-related gene A (MICA), and MICB are ligands for NKG2D (8 – 11). It has been reported that up-regulation of the inducible gene products MICA (human) and retinoic acid early inducible-1 (mouse) may promote tumor surveillance and autoimmunity by engaging the activating receptor NKG2D on NK cells and T cells. In fact, NKG2D-mediated responses of NK cells and memory T cells to experimentally engineered retinoic acid early inducible-1 expression reduce the tumorigenicity of mouse thymoma and melanoma inocula (12, 13). Thus, sustained NKG2D ligand expression seems to promote immune activation against cancer.

In cancer patients with MICA⁺ or MICB⁺ tumors, on the other hand, tumor-infiltrating and systemic NK cells and CD8⁺ T cells often express little NKG2D and are functionally compromised. This state has been attributed to trans-acting effects of soluble MICA (sMICA) and MICB cleaved from solid tumors and leukemia by a tumor-associated metalloproteinase (14 – 16). Engagement of sMICA then promotes NKG2D internalization and degradation. This mechanism might explain the selection of tumor cells that sustain MICA and/or MICB expression and, rather than suggesting chronic immune activation, ‘predicts’ that sustained NKG2D ligand expression in vivo would promote chronic immune suppression.
Accumulating evidences indicate that advanced tumor-associated expression of NKG2D ligands may signify host tumor susceptibility instead of resistance (14, 17). However, it still remains unclear whether sustained NKG2D ligand expression in vivo promotes chronic immune activation or chronic immune suppression in patients with gastric cancer. This is an important point to clarify from both a biological and a clinical perspective. In the current study, therefore, we determined NKG2D expression on CD8+ T cells obtained from gastric cancer patients. We also analyzed the correlation between NKG2D expression on CD8+ T cells and various clinicopathologic factors. CD8+ T cells from patients with poorly differentiated gastric cancer expressed significantly lower NKG2D than those with differentiated.

### Materials and Methods

**Patients and normal donors.** Thirty-seven patients (22 males and 15 females), treated at Tottori University Hospital (Yonago, Japan) and pathologically diagnosed with gastric cancer, were enrolled in this study. None of the patients received radiotherapy, chemotherapy, or other medical interventions before surgery. Informed consent for blood donations was obtained for all individuals. Patient characteristics are shown in Table 1. Healthy controls (n = 22; 13 males and 9 females) were age matched (60.6 ± 13.9 years for the controls versus 62.8 ± 13.8 years for the patients), and each experiment was done in parallel for the patients and the healthy controls. The clinicopathologic findings were determined according to the Japanese classification of gastric carcinoma (18).

**Preparation of peripheral blood mononuclear cells.** Peripheral blood (30 mL) was drawn from each of the controls or from the patients before surgery and centrifuged by a Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient. Informed consent was obtained from all subjects before blood donation.

### Table 1. NKG2D expression on CD8+ T cells and clinicopathologic characteristics in gastric cancer patients

<table>
<thead>
<tr>
<th>Histopathology</th>
<th>NKG2D</th>
<th>P</th>
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<tbody>
<tr>
<td>Differentiated</td>
<td>19</td>
<td>70.7 ± 12.0</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>18</td>
<td>59.2 ± 16.7</td>
</tr>
<tr>
<td>Depth of invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (early)</td>
<td>19</td>
<td>71.2 ± 11.6</td>
</tr>
<tr>
<td>T2/T3/T4 (advanced)</td>
<td>18</td>
<td>58.5 ± 16.6</td>
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<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>22</td>
<td>69.0 ± 12.8</td>
</tr>
<tr>
<td>Present</td>
<td>15</td>
<td>59.4 ± 17.4</td>
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<tr>
<td>Lymphatic invasion</td>
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<tr>
<td>Absent</td>
<td>15</td>
<td>70.4 ± 13.0</td>
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<tr>
<td>Present</td>
<td>22</td>
<td>61.6 ± 16.1</td>
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<tr>
<td>Vascular invasion</td>
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<td></td>
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<tr>
<td>Absent</td>
<td>20</td>
<td>67.6 ± 14.1</td>
</tr>
<tr>
<td>Present</td>
<td>17</td>
<td>62.2 ± 16.7</td>
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<tr>
<td>Stage</td>
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</tr>
<tr>
<td>I/II/III</td>
<td>31</td>
<td>69.5 ± 12.0</td>
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<tr>
<td>IV</td>
<td>6</td>
<td>42.4 ± 10.8</td>
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</tbody>
</table>

*Differentiated, papillary or tubular adenocarcinoma; undifferentiated, poorly differentiated or mucinous adenocarcinoma or signet-ring cell carcinoma.

**Flow cytometry analysis.** Fluorescence-activated cell sorting was done on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ), and the following antibodies were used to classify the cells: anti-CD-3-PE-Cy5, anti-CD4-PE, anti-CD8-PE, purified anti-human NKG2D monoclonal antibody (mAb), purified anti-human MICA and MICB mAbs, and FITC-labeled goat anti-mouse Ig (BD Pharmingen, Franklin Lakes, NJ). Intracellular cytokine staining. Peripheral blood mononuclear cells (PBMC) were cultured for 6 h in the presence of phorbol 12-myristate 13-acetate (50 ng/mL; Sigma-Aldrich, St. Louis, MO) and ionomycin (1 µg/mL; Sigma-Aldrich). At 2 h of stimulation, GolgiStop (Becton Dickinson) was added to the culture. At 6 h of stimulation, cells were harvested and first stained with CD3-PE-Cy5 and CD8-PE mAbs, fixed and permeabilized with BD Cytofix/Cytoperm solution (Becton Dickinson), and then stained with anti-IFN-γ FITC (BD Pharmingen).

**Measurement of sMICA in sera.** sMICA in human sera was measured by ELISA using human MICA ELISA kit (Immunetics Biotechnologies, Tubingen, Germany).

**Medium.** A complete culture medium consisting of RPMI 1640 (Life Technologies, Grand Island, NY), 1% l-glutamine (Invitrogen, Carlsbad, CA), 1% penicillin/streptomycin (Invitrogen), 2.5% HEPES solution buffer (Life Technologies), 1% sodium pyruvate (Sigma-Aldrich), 1% essential amino acids (Sigma-Aldrich), and 10% heat-inactivated FCS (Thermo Trace Ltd., Melbourne, Victoria, Australia) was used. For the T-cell cultures, the FCS was replaced by 10% human serum AB (Gemini Bio-Products, Woodlands, CA).

**Cell lines.** The gastric cancer cell lines TUMK1 and MKN45 were purchased from the RIKEN Cell Bank (Tsukuba, Japan).

**Transwell experiments.** Transwell experiments were done in six-well plates. PBMCs (5 x 10^6) and cancer cells (3 x 10^5) were either cocultured directly or placed in Transwell chambers (Millicell, 0.4 um; Millipore, Billerica, MA). After 48 h of coculture, PBMCs were harvested for the analysis of NKG2D expression. For the mAb blocking experiment, cancer cells were treated with the anti-MICA antibody (R&D Systems, Minneapolis, MN) for 1 h before coculture with cancer cells, and the mAbs were washed throughout the culture period. Irrelevant mAbs (R&D Systems) were used as isotype controls.

**Statistical analysis.** To determine statistical differences between the two groups, either paired or unpaired t tests were used. Correlation between NKG2D expression and IFN-γ production was analyzed using the Spearman rank correlation coefficient. The accepted level of significance was P < 0.05. StatView software (Abacus Concepts Inc., Berkeley, CA) was used for all statistical analyses.

### Results

**NKG2D expression on circulating CD8+ T lymphocytes in patients with gastric cancer.** We determined NKG2D expression on circulating CD8+ T lymphocytes in both normal controls and gastric cancer patients. NKG2D expression of CD8+ T lymphocytes in gastric cancer patients (65.1 ± 15.4%; n = 37) was significantly lower than those in normal controls (77.0 ± 12.4%; P = 0.009; Fig. 1). Next, we have done intracellular IFN-γ staining to show that reduction in NKG2D on CD8+ T cells is functionally significant. Figure 2 shows that NKG2D expression was significantly correlated with IFN-γ in CD8+ T cells (r = 0.68; P = 0.007).

Table 1 shows the correlation between NKG2D expression on CD8+ T cells and various clinicopathologic factors. CD8+ T cells from patients with poorly differentiated gastric cancer expressed significantly lower NKG2D than those with differentiated.
gastric cancer (59.2 ± 16.7 versus 70.7 ± 12.0; P = 0.021). NKG2D expression in advanced gastric cancer (58.5 ± 16.6%) was significantly lower than that in early gastric cancer (71.2 ± 11.6%; P = 0.012). Moreover, significantly lower NKG2D expression was observed in stage IV in comparison with stage I/II/III (69.5 ± 12.0 versus 42.4 ± 10.8; P < 0.0001). These results indicate that reduced NKG2D expression on CD8+ T cells is closely correlated with disease progression.

Concentration of sMICA in patient’s sera. Groh et al. (14) showed that tumor shedding of MIC systemically down-regulated NKG2D expression and impairs the responses of CD8+ T cells. Therefore, we next wanted to determine the concentration of sMICA in sera from our patient group. The concentration of sMICA in gastric cancer patients and normal controls was 87.7 ± 22.8 pg/mL and 79.8 ± 12.9 pg/mL, respectively, and there was no significant difference between the two groups (P = 0.71; Fig. 3). In fact, patient’s sera had minimal effect on NKG2D expression in CD8+ T cell (data not shown). On the other hand, NKG2D expression on CD8+ T cells obtained from patients recovered after 48 h in culture with cRPMI containing 10% AB serum (Fig. 4). These results indicate the possibility that tumor cells, but not soluble factors, are responsible for down-regulation of NKG2D expression on CD8+ T cells observed in gastric cancer patients.

Recovery of NKG2D expression on circulating CD8+ T cells after complete removal of tumor by surgery. Because we observed a recovery of NKG2D expression on CD8+ T cells after 48 h in culture with cRPMI containing 10% AB serum, we wondered if the same phenomenon could be observed in vivo after complete removal of tumor. NKG2D expression on CD8+ T cells before and after surgery was 68.8 ± 12.4% and 84.0 ± 7.9% (n = 10), respectively, and the differences were statistically significant (P = 0.0049; Fig. 5).

NKG2D expression on CD8+ T lymphocytes in gastric cancer tissue. We then determined NKG2D expression on CD8+ T cells obtained from gastric cancer tissue. NKG2D expression on CD8+ T cells in the tissue of gastric cancer (51.8 ± 27.4%) was significantly lower than that of circulating CD8+ T cells (70.4 ± 12.4%; P = 0.046; Fig. 6).

MICA expressed on tumor cells induces down-regulation of NKG2D expression on CD8+ T cells. Finally, we carried out Transwell experiments using gastric cancer cell lines to show the effect of cancer cells on NKG2D expression. We used two gastric cancer cell lines: TMK1 expressing strong MICA and no NKG2D and MKN45 expressing moderate MICA and no NKG2D. Figure 7A shows that coculture of TMK1 and PBMCs resulted in a remarkable reduction in NKG2D expression on CD8+ T cells. This phenomenon was not observed when TMK1 was placed in Transwell chambers to inhibit direct contact between cancer cells and CD8+ T cells, indicating that soluble factors did not affect NKG2D expression on CD8+ T cells. Reduction in NKG2D expression was closely correlated with MICA expression on tumor cells because TMK1 expressing
strong MICA induced more reduction in NKG2D than MKN45 expressing moderate MICA did (Fig. 7B). Moreover, reduction in NKG2D expression was blocked when anti-MICA antibody was added to culture, indicating that MICA expressed on tumor cells induced NKG2D expression on CD8⁺ T cells (Fig. 7C).

**Discussion**

CD8⁺ T cells are thought to play an important role in the control of tumors as a result of their cytotoxic activity and by releasing soluble factors. It has been reported that the function of CD8⁺ T cells is impaired in cancer patients, which is related to immune evasion by cancer. Takahashi et al. (19) showed that alteration in the signal-transducing molecules associated with T-cell receptor was responsible for the impaired T-cell response in patients with gastric cancer. However, the detailed mechanisms responsible for impaired T-cell function remain unclear.

In the current study, we showed that NKG2D expression of circulating CD8⁺ T cells in gastric cancer patients was significantly lower than that in normal controls. Down-regulated NKG2D expression is closely related to low responsiveness of CD8⁺ T cells against cancer. In fact, NKG2D expression was significantly correlated with IFN-γ production in CD8⁺ T cells in the current study. Moreover, Groh et al. (14) reported that NKG2D low MART-1-specific CD8⁺ T cells isolated from tumor-infiltrating lymphocytes from a MIC-positive melanoma showed no or little induction of IFN-γ after stimulation with MART-1 peptide, whereas a substantial proportion of identically treated NKG2D high MART-1-specific T cells from a MIC-negative melanoma produced a strong IFN-γ response. Therefore, the down-regulated NKG2D expression of circulating CD8⁺ T cells that we observed in gastric cancer patients might be one of the key mechanisms by which gastric cancer impaired CD8⁺ T-cell function.

To further determine the detailed mechanisms behind this reduced NKG2D expression on CD8⁺ T cells, we then examined the concentration of sMICA in patient’s sera. MICA is the ligand for NKG2D and absent from most cells and tissues but can be induced by viral and bacterial infections and is frequently expressed in epithelial tumors. Elevated levels of sMICA were found in sera of patients with various malignancies by shedding of surface MICA (14, 15, 20). A small study revealed a correlation between sMICA serum levels and disease progression in 23 patients with prostate cancer (17). Groh et al. (14) reported that binding of sMICA to NKG2D induced endocytosis and degradation of NKG2D, which induced down-regulation of NKG2D expression and in turn severe impairment of the responsiveness of tumor antigen-specific CD8⁺ T cells. The same phenomenon was also reported in NK cells (17). In fact, our preliminary data showed that recombinant sMICA could down-regulate NKG2D expression on CD8⁺ T cells (data not shown). However, there was no difference in sMICA concentration between gastric cancer patients and normal controls, indicating that sMICA was not responsible for down-regulation of NKG2D in gastric cancer patients. It was also reported that transforming growth factor-β down-regulated NKG2D expression on NK cells (21). This is not likely, however, because down-regulation of NKG2D expression of CD8⁺ T cells was not observed after 48 h of culture with patient’s serum, indicating that soluble factors included in serum were not responsible for NKG2D down-regulation on CD8⁺ T cells in gastric cancer.
On the other hand, decreased NKG2D expression on CD8+ T cells from gastric cancer patients was restored after a 48-h incubation in medium. Furthermore, recovery of NKG2D expression on CD8+ T cells was observed after complete removal of tumor by surgery. One recent article showed that NKG2D expression of NK cells was decreased by direct chronic exposure to NKG2D ligand-expressing tumor cells (22). Furthermore, Oppenheim et al. reported that constitutive retinoic acid early inducible-1 transgene expression in normal epithelium elicited local and systemic NKG2D down-regulation, generalized but reversible defects in NK cell–mediated cytotoxicity, and mild CD8+ T-cell defects. The extent of NKG2D down-regulation correlated well with the incidence and progression of cutaneous carcinogenesis, emphasizing the use of NKG2D as a marker of tumor resistance. These results indicate that NKG2D engagement is a natural mediator of immunosurveillance, which can be compromised by locally sustained ligand expression (23). Therefore, we hypothesized that direct contact between CD8+ T cell and tumor cells might be responsible for the decreased NKG2D expression observed in gastric cancer patients. In fact, NKG2D expression on CD8+ T cells in tissue of gastric cancer was significantly lower than that on circulating CD8+ T cells. Transwell experiments showed that direct contact of CD8+ T cells and cancer cells induced a reduction in NKG2D expression on CD8+ T cells. This phenomenon was closely related to the degree of MICA expression on tumor cells and blocked by the addition of anti-MICA antibodies, indicating that the MICA expressed on tumor cells was responsible for the reduction in NKG2D expression on CD8+ T cells. On the other hand, soluble factors secreted by tumor cells do not affect NKG2D expression. Collectively, our results indicate that NKG2D down-regulation observed in gastric cancer patients is dependent on prolonged encounters with MICA in a cellular context and is reversible.

Coudert et al. (22) showed that dysfunction of NK cell–mediated target cell lysis induced by chronic engagement with tumor cell–bound NKG2D ligand was due to the striking reduction of the NKG2D signaling adaptor molecules DNAX-activating protein of 10 kDa and killer cell activating receptor-associated protein/DNAX-activating protein of 12 kDa. It

**Fig. 6.** NKG2D expression on CD8+ T cells in tissue of gastric cancer was significantly lower than that on circulating CD8+ T cells (P = 0.046).

**Fig. 7.** NKG2D down-regulation on CD8+ T cells is partially mediated by MICA expressed on tumor cells. A, 5 × 10⁶ PBMCs and 3 × 10⁶ cancer cells were either cocultured directly or placed in Transwell chambers. After 48 h of coculture, NKG2D expression on CD8+ T cells was remarkably down-regulated when PBMCs were cocultured directly with TMK1. However, down-regulation of NKG2D expression was minimal when PBMCs were cocultured separately with TMK1 using Transwell membranes. B, TMK1 strongly expressing MICA down-regulated NKG2D expression on CD8+ T cell more than MKN45 moderately expressing MICA did. C, NKG2D down-regulation is partially blocked by the addition of anti-MICA antibodies.
remains unclear if the same phenomenon can be observed in CD8+ T cells in gastric cancer patients. Further investigation of the molecular function of DNAX-activating protein of 10 kDa and killer cell activating receptor-associated protein/DNAX-activating protein of 12 kDa is urgently required.

Recently, Groh et al. (24) observed MIC-driven proliferation of NKG2D+CD4+ T cells that produced the cytokine Fas ligand as a result of NKG2D costimulation. Soluble Fas ligand secreted by NKG2D+CD4+ T cells inhibited the proliferation of NKG2D-CD4+ T cells, indicating that the NKG2D+CD4+ T cells they observed had properties of immunosuppressive T cells. Furthermore, some articles showed decreased NKG2D expression on NK cells that were involved in innate immunity and play important role in tumor immunity (16, 17). Therefore, the MIC-NKG2D system might more exclusively contribute to immunosuppression by tumors than we first expected.

In conclusion, our data indicate that decreased NKG2D expression on CD8+ T cells was correlated with progression of disease and might be induced by chronic exposure to MICA-expressing tumor cells. Decreased NKG2D expression might be one of the key mechanisms responsible for immune evasion by tumor in gastric cancer. The down-regulation of MICA expression using antibodies or small interfering RNA may be a promising strategy for treatment of gastric cancer. Further investigation of molecular function of MICA and NKG2D is urgently required.

References
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