Clinical significance of tumor expression of major histocompatibility complex class I-related chains A and B (MICA/B) in gastric cancer patients

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Abstract. Gastric cancer (GC) is the third most common cause of cancer death worldwide. Natural killer cells play an important role in the immune defense against transformed cells. They express the activating receptor NKG2D, whose ligands belong to the MIC and ULBP/RAET family. Although it is well established that these ligands are generally expressed in tumors, the association between their expression in the tumor and gastric mucosa and clinical parameters and prognosis of GC remains to be addressed. In the present study, MICA and MICB expression was analyzed, by flow cytometry, in 23 and 20 pairs of gastric tumor and adjacent non-neoplasic gastric mucosa, respectively. Additionally, ligands expression in 13 tumors and 7 gastric mucosa samples from GC patients were evaluated by immunohistochemistry. The mRNA levels of MICA in 9 pairs of tumor and mucosa were determined by quantitative PCR. Data were associated with the clinicopathological characteristics and the patient outcome. MICA expression was observed in 57% of tumors (13/23) and 44% of mucosal samples (10/23), while MICB was detected in 50% of tumors (10/20) and 45% of mucosal tissues (9/20). At the protein level, ligand expression was significantly higher in the tumor than in the gastric mucosa. MICA mRNA levels were also increased in the tumor as compared to the mucosa. However, clinicopathological analysis indicated that, in patients with tumors >5 cm, the expression of MICA and MICB in the tumor did not differ from that of the mucosa, and tumors >5 cm showed significantly higher MICA and MICB expression than tumors ≤5 cm. Patients presenting tumors >5 cm that expressed MICA and MICB had substantially shorter survival than those with large tumors that did not express these ligands. Our results suggest that locally sustained expression of MICA and MICB in the tumor may contribute to the malignant progression of GC and that expression of these ligands predicts an unfavorable prognosis in GC patients presenting large tumors.

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Introduction

Gastric cancer (GC) is the third most common cause of cancer-related death worldwide (1), with approximately one million new cases diagnosed each year (2). Even though the incidence of GC has declined in most endemic countries, and despite considerable therapeutic improvements in surgical techniques, innovations in clinical diagnosis and the development of new chemotherapy regimens, it still remains a global challenge, since the prognosis for patients with GC is generally poor, particularly in advanced stages of the disease (3).

Several lines of evidence indicate that immune cells in the tumor microenvironment have an important role in regulating tumor progression, which may determine the clinical parameters and prognosis of GC (4). Therefore, a better comprehension of the immune mechanisms that govern tumor

surveillance and tumor evasion strategies is essential to investigate new therapeutic tools to treat this disease.

Among the innate immune effectors that participate in the early control of transformed cells are the natural killer cells (5), which directly lyse tumor cells without prior sensitization (6). The recognition of target cells by NK cells is mediated by activating receptors that detect self-molecules induced in conditions of cellular stress (7). This is the case for NKG2D, a type II C-type lectin-like transmembrane activating receptor expressed on NK cells, some cytolytic $CD8^+$ αβ T cells, γδ T cells and NKT cells (8,9). Its functional outcome is the release of granules containing perforin and granzyme, which consequently triggers cell-mediated cytotoxicity (10). The target cell ligands recognized by the NKG2D receptor are the MHC class I chain-related molecules A and B (MICA and MICB) and UL16-binding proteins (ULBP) 1-6 (11), which are cell surface glycoproteins expressed at low levels in most tissues, but upregulated under cellular transformation (12). Since NK cells can efficiently recognize and kill tumors bearing NKG2D ligands (NKG2DLs) (13), the presence of these molecules on the cell surface potentially serves as 'danger signals' to alert the innate immune system to the existence of transformed cells, thus contributing to their elimination (14).

Nevertheless, several types of tumors have evolved mechanisms to evade immune surveillance mediated by cytolytic cells. Among these strategies, the shedding of NKG2DLs from the surface of tumor cells results in the release of soluble MICA, which dampens NK cell and $CD8^+$ T lymphocyte cytotoxicity due to downregulation of NKG2D receptor and leads to impairment of NKG2D-dependent cell activation (15-17). In addition, the expression of low levels of NKG2DLs on the cell surface as a result of intracellular deposits of immature forms of these ligands also prevents NK cell-mediated lysis and favors tumor development (18).

On the contrary, tumors that secrete MICA still express significant amounts of this NKG2DL on their cell surface (19,20). Several reports have demonstrated that sustained surface tumor expression of NKG2DLs can also elicit NKG2D receptor downregulation (21). Therefore, chronic exposure of NK cells to tumor-associated cell surface or soluble NKG2DLs may lead to disease progression and poor prognosis in cancer as a result of the impairment of NKG2D receptor-dependent activation, which contributes to tumor escape (19,22,23).

In GC patients, systemic and local immune defects have been demonstrated, which correlate with disease progression and prognosis. For instance, tumor-infiltrating and systemic NK cells, cytotoxic $CD8^+$ T cells and γδ T cells in GC patients express low levels of NKG2D receptor, resulting in compromised cytotoxic activity and contributing to disease severity (24-26). Serum levels of soluble MICA and MICB in patients with GC are increased compared to healthy donors (27). In addition, MICA and MICB mRNA levels in the gastric tumor tissue are higher compared to patient gastric mucosa (28). Although MICA and MICB have been detected in gastric adenocarcinoma tissue (29) and gastric mucosa of healthy donors (30), both, tumor and gastric mucosa-associated MICA and MICB expression in GC and its clinical significance still remain to be established.

In the present study, we sought to determine tissue expression of MICA and MICB in GC patients and examine the clinical relevance of these NKG2DLs in this disease. The expression of MICA and MICB in the primary tumor and the adjacent non-tumor gastric mucosa from GC patients who received radical surgery was evaluated by immunohistochemistry (IHC) and flow cytometry. MICA mRNA levels were determined by real-time quantitative PCR (RT-qPCR). Additionally, we evaluated the relationship between MICA and MICB expression in the tumor and clinicopathological features of the disease, including tumor size, differentiation, depth of invasion, status of lymph node metastasis and TNM staging, as well as their prognostic value to post-resection survival of GC patients.

Table I. Clinicopathological characteristics of gastric cancer patients.

Variables	N (%)
Gender	
Male	22 (76)
Female	7 (24)
Age at surgery (years)	
Mean (range)	64 (41-90)
Tumor size (cm)	
≤5	10 (34.5)
>5	19 (65.5)
Tumor differentiation	
High/medium	12 (41.4)
Low/none	17 (58.6)
Invasion status[a]	
T1, T2	7 (24.1)
T3, T4	22 (75.9)
Lymph node metastasis (N)	
No (N0)	8 (27.6)
Yes (N1,2,3)	21 (72.4)
TNM stage[b]	
I, II	12 (41.4)
III, IV	17 (58.6)

[a]Invasion status corresponds to tumor invasion of lamina propria, muscularis mucosae or submucosa (T1), muscularis propria (T2), subserosal connective tissue (T3) and serosa (visceral peritoneum) or adjacent structures (T4). [b]Stage according to the TNM Classification for Gastric Cancer (AJCC) (31).

Materials and methods

Patients and samples. During 2010 and 2012, a total of 29 patients (7 female, 22 male) aged 64±12 years (range, 41-90 years) treated at the Department of Gastrointestinal Surgery, Hospital del Salvador (Santiago, Chile), and pathologically diagnosed with gastric adenocarcinoma, were enrolled in this study. None of the patients received chemotherapy, radiotherapy, or other medical interventions for GC treatment before surgery. Primary gastric tumor and their matched adjacent non-malignant gastric mucosa samples were collected immediately

after surgical resection of the stomach. Patient characteristics and clinicopathological features of tumors (Table I) were determined according to the disease staging system of the American Joint Committee on Cancer (AJCC) (7th Edition) (31). Tumor size was given as the maximum tumor diameter measured on the freshly resected stomach. Histopathological diagnosis was carried out by the team of pathologists from Hospital del Salvador (Santiago, Chile). The patient survival was assessed for 36 months after potentially curative surgery or until death due to tumor-specific disease. Written informed consent for tissue donation was obtained from all the patients. This study was approved by the Committee on Human Ethics Investigation of the Faculty of Medicine, University of Chile, and the Committee on Scientific Ethics of the Metropolitan Health Service of the Chilean Government.

Isolation of single cells from dissociated gastric tissues. Fresh tumor and their matched mucosal tissue samples from 23 GC patients were transported to the laboratory in Hank's balanced salt solution (HBSS) medium (Gibco, Invitrogen, Waltham, MA, USA) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (GE Healthcare Hyclone, Inc., South Logan, UT, USA). Tissues were cut into small pieces using sterile scalpel blades and minced in RPMI-1640 medium (GE Healthcare-HyClone Laboratories) supplemented with penicillin/streptomycin and 3% fetal bovine serum (FBS) with the help of syringe needles and plungers. To obtain single-cell suspensions, the resultant dissociated tissues were passed through 70-μm cell strainers (BD Biosciences, San Jose, CA, USA) to remove tissue fragments. Cells were then centrifuged at 2,000 rpm for 10 min at 4˚C, and the pellet was incubated with erythrocyte lysis buffer for 10 min at room temperature (RT). Cells were washed with RPMI-supplemented medium, centrifuged and counted. Cell viability was assessed by an exclusion method using Trypan blue staining (Merck KGaA, Darmstadt, Germany). Cell suspensions were then used for flow cytometric analysis.

Flow cytometric analysis. Cells derived from the primary tumors and their matched non-tumor gastric mucosa were resuspended with 1% paraformaldehyde (PFA) in 1% FBS/phosphate-buffered saline (PBS) and incubated on ice for 30 min. Cells were then transferred to V-bottomed 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA) and centrifuged at 2,000 rpm for 3 min at 4˚C. At least $2x10^5$ cells were stained with purified mouse anti-human MICA or MICB monoclonal antibodies (both from R&D Systems, Inc., Minneapolis, MN, USA) (1:25 dilution in 1% FBS/PBS). Purified mouse IgG2b (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used as the isotype control to exclude non-specific fluorescence. Cells were then incubated overnight at 4˚C. After three washes with 1% FBS/PBS, a secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (KPL, Inc., Gaithersburg, MD, USA) was added (1:50 dilution in 1% FBS/PBS), and cells were incubated for 30 min at 4˚C. Further washing steps were carried out, and cells were finally fixed in 2% PFA before flow cytometric analysis. An average of 20,000 events was collected per sample. MICA and MICB staining was detected as median fluorescence intensity (MFI). Flow cytometry was performed using a FACSCalibur flow cytometer, acquired with the CellQuest program (both from BD Biosciences), and analyzed using FlowJo vX.0.7 software (Tree Star, Inc., Ashland, OR, USA).

IHC. Formalin-fixed, paraffin-embedded tumor samples from 13 GC patients enrolled in this study were obtained from the Department of Pathology from Hospital del Salvador (Santiago, Chile). In 7 paired cases, non-neoplastic gastric mucosa was available for comparative analysis. Only specimens fixed and included in optimal conditions for quantitative immunohistochemical studies were selected. Serial sections (4 μm thick) of tissue samples were plated on silanized glass slides, deparaffinized and rehydrated. Antigen retrieval was performed by heating the tissue sections in a steam bath at 90-95˚C for 30 min in 10 mM citrate buffer (pH 6.0). The slides were rinsed in cool running water and immersed in 3% hydrogen peroxide for 10 min at RT to block the endogenous peroxidase activity. To reduce non-specific binding, slides were incubated with 2% bovine serum albumin (BSA) in PBS for 10 min at RT. The sections were then incubated with mouse anti-human MICA/B polyclonal antibody generated as previously described (32,33) (1:50 dilution in 2% BSA/PBS) for 1 h at 37˚C in a humid chamber. The specificity of the reaction was tested by omission of the primary antibody. Slides were further incubated with a secondary antibody, a biotinylated goat anti-mouse IgG (Sigma-Aldrich, St. Louis, MO, USA) (1:200 dilution in 2% BSA/PBS), for 30 min at RT. Next, sections were incubated with streptavidin-horseradish peroxidase (HRP)-conjugate (Dako/Agilent Technologies, Glostrup, Denmark) in 2% BSA/PBS for 20 min at RT. As a peroxidase substrate, liquid 3,3-diaminobenzidine tetrahydrochloride (DAB Chromogen; Dako/Agilent Technologies) was used. Each step was followed by extensive slide washes with PBS (pH 7.4). The sections were then counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene, coverslipped and evaluated in a Leica DM2500 microscope. Photographs were digitally processed using a Leica Application Suite V3.6.0 (Leica Microsystems, Wetzlar, German). Peroxidase staining intensity was assessed in 10 randomly-selected microscopic fields per tissue section, and the immune-reactive areas were quantified in pixels/μm^2 using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Coloring cytoplasm and membrane of cells present in the tumor and mucosal tissues was considered as positive reaction for MICA/B.

Quantitative real-time PCR (qRT-PCR). qRT-PCR was performed to determine the mRNA level of MICA. After stomach surgical resection, the fresh tissue samples were immediately immersed in RNAlater RNA stabilization reagent (Qiagen, KJ Venlo, The Netherlands) and stored at -20˚C until RNA extraction. Total RNA was extracted from 9 pairs of matched gastric tumor and adjacent non-tumor gastric mucosa of GC patients using E.Z.N.A® Total RNA kit I (Omega Bio-Tek, Inc., Norcross, GA, USA), according to the manufacturer's instructions. After treatment with DNase I (Thermo Fisher Scientific), total RNA concentration and purity were assessed using the Synergy HT Multi-detection microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). cDNA

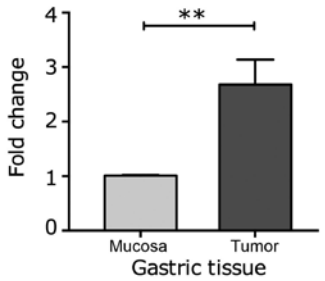

Figure 1. MICA mRNA levels are increased in the primary tumor tissue of patients with gastric adenocarcinoma. The levels of MICA mRNA in 9 pairs of tumor tissue and non-neoplasic gastric mucosa of GC patients were assessed by qRT-PCR. The relative fold differences were calculated using the $2^{-\Delta\Delta CT}$ method. Results show the fold change in the expression of MICA in the tumor tissue relative to the mean values of gastric mucosal samples. Bars represent the mean values ± standard error of the mean (SEM). The Wilcoxon paired test was used for statistical analysis. **P<0.01.

synthesis was carried out using the Affinity Script Multi-Temp RT & RT-PCR kit (Agilent Technologies, Inc., Santa Clara, CA, USA), using 2 μg of total RNA as the template. The resulting cDNA was amplified by qRT-PCR using the Stratagene Mx3000P QPCR System (Agilent Technologies), which measures the binding of SYBR® Green (Brilliant III Ultra-Fast SYBR-Green QPCR Master Mix; Agilent Technologies) to the double stranded DNA. The housekeeping gene HPRT was used as an internal control. Gene-specific primers were designed using the Amplifx 1.7 software (University of Marseille, Marseille, France). Primers for MICA were 5'-GAGACTTGACAGGG AACGGAAA-3' (sense) and 5'-GAAGACAACAGCACC AGGAG-3' (antisense). Primers for HPRT were CAAGCT TGCTGGTGAAAAGGAC (sense) and GTCAAGGGCATA TCCTACAACAAA (antisense). PCR reactions were performed in triplicate in a final volume of 20 μl as follows: 95˚C for 5 min, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 20 sec. Regression curves were calculated for each sample, and the threshold cycles were obtained using the instrument's software (MxPro QPCR software; Agilent Technologies) and normalized to the mean values of the internal control gene HPRT. For relative quantification of MICA in the tumor and mucosa, the fold change levels, calculated using the $2^{-\Delta\Delta CT}$ method, were determined relative to the mean values of each gastric mucosa sample.

Statistical analysis. Data were expressed as mean ± standard error of the mean (SEM) for each group. Normal distribution was tested by Kolmogorov-Smirnov test. Normally distributed data were analyzed using Student's t-tests, while non-parametric data were evaluated using Wilcoxon or Mann-Whitney U tests to compare results between groups. For survival analysis, groups of patients were distinguished according to positive or negative MICA and MICB expression on tumor tissue (observed by flow cytometry). Survival rates, which were defined as the period from surgery until GC-related death or survival for 36 months after surgery, were analyzed by the Kaplan-Meier method, and the log-rank test was performed to assess survival differences. Data were evaluated with GraphPad Prism v6.01 software (GraphPad Software, Inc., La Jolla, CA, USA). All statistical tests were two-tailed, and P-values <0.05 were considered statistically significant.

Results

MICA and MICB are expressed on gastric tumor and mucosal tissues of GC patients. NKG2DLs are frequently expressed by tumor cells, and their presence on the surface of target cells may determine the NK cell-mediated immune response against the tumor (12). The levels of MICA mRNA in 9 pairs of gastric tumor and adjacent non-tumor gastric mucosa from GC patients were estimated by qRT-PCR. The MICA levels were significantly increased in the tumor tissue as compared with the gastric mucosa (P=0.039) (Fig. 1), in accordance with results previously described by others using Multiplex RT-PCR (28).

Nevertheless, since the levels of mRNA do not usually predict its protein abundance (34), we decided to perform immunohistochemical staining of MICA/B in paraffin-embedded gastric tumor and mucosal tissue sections of histopathologically confirmed gastric adenocarcinoma. Representative photomicrographs are shown in Fig. 2A. The analysis of 13 gastric tumors and 7 non-neoplasic gastric mucosa from GC patients showed that MICA/B immunoreactivity in the tumor tissue was significantly higher than in the gastric mucosa (P=0.0009) (Fig. 2B).

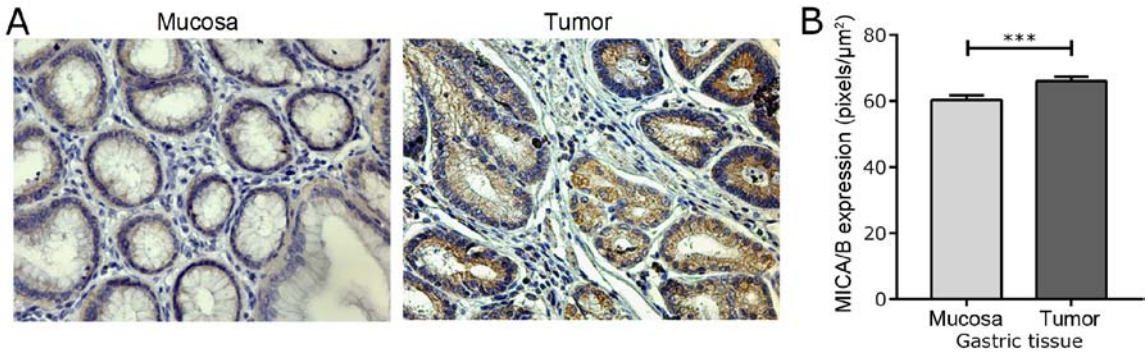

Figure 2. MICA/B expression is higher in the tumor tissue than in the gastric mucosa of GC patients. (A) Representative IHC for MICA/B in the gastric tumor and non-neoplasic gastric mucosal tissue sections of a GC patient. Magnification, x400. (B) Quantification, in pixels/μm^2, of IHC staining of 13 tumors and 7 non-neoplasic gastric mucosal tissues of GC patients. Mean values ± SEM are shown. Statistical analyses were performed using the Mann-Whitney U test. ***P<0.001.

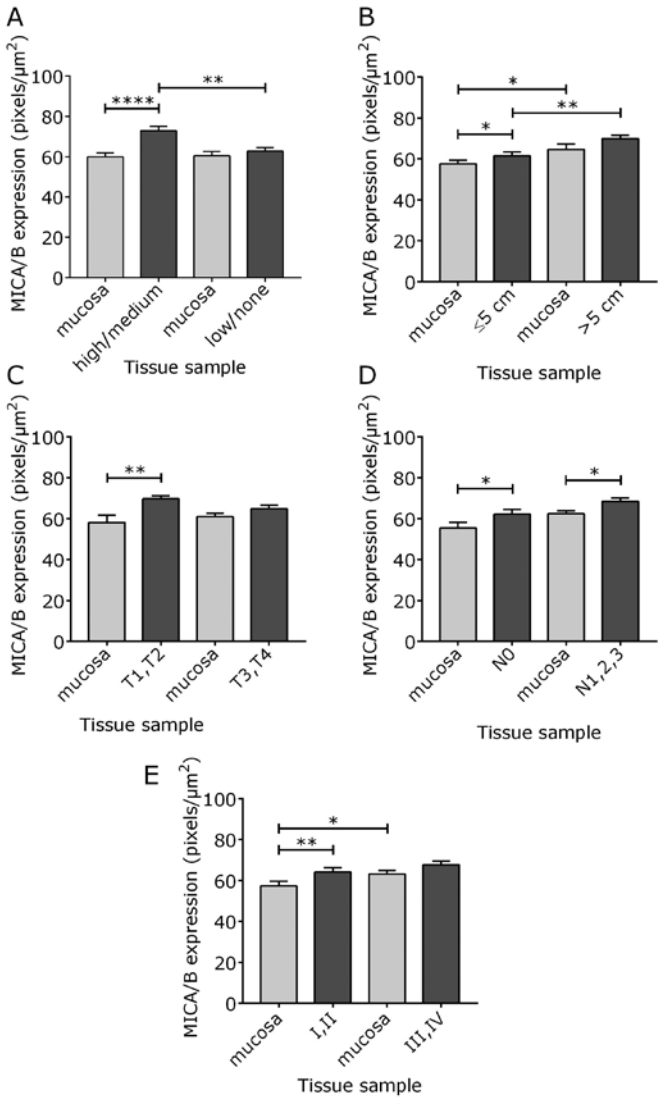

Figure 3. MICA/B expression in gastric adenocarcinoma associates with clinicopathological parameters of the disease. Quantification, in pixels/μm^2, of IHC staining of 13 tumors and 7 non-neoplasic gastric mucosal samples of GC patients. Tumor tissue and mucosa were classified according to disease parameters: (A) tumor differentiation (high/medium and low/none), (B) tumor size (≤5 and >5 cm), (C) invasion status (T1,T2 and T3,T4), (D) lymph node metastasis (N0 and N1,2,3), and (E) TNM stage (I,II and III,IV). Mean values ± SEM are shown. The Mann-Whitney U test or unpaired t-test was used for statistical analysis. *P<0.05; **P<0.01; ****P<0.0001.

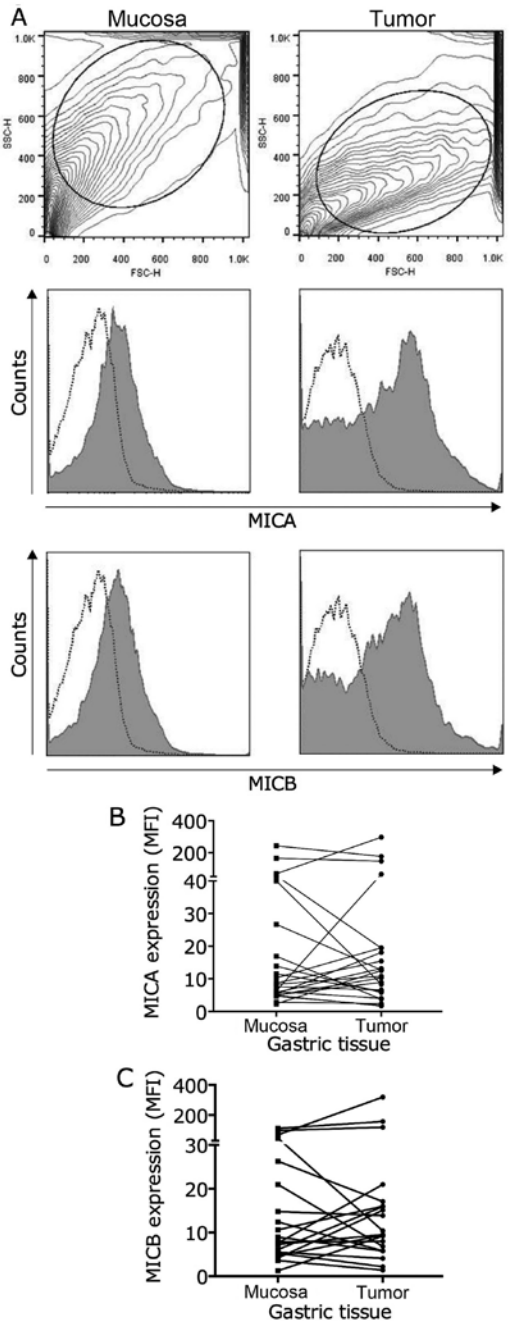

Figure 4. MICA and MICB are expressed in the tumor and non-neoplasic gastric mucosal tissues of GC patients. (A) Representative contour plots of the forward (FSC-H) and side scatter (SSC-H) properties of cells derived from the primary tumor and adjacent gastric mucosal tissues of one patient with gastric adenocarcinoma. Expression of NKG2DLs was assessed on the selected gates and depicted in the gray-filled histograms. Dotted line histograms: isotype control. (B) MICA and (C) MICB expression in the tumor and gastric mucosal cells from 23 and 20 GC patients, respectively, is depicted as median fluorescence intensity (MFI) values for each patient. The Wilcoxon paired test was used for statistical analysis.

MICA/B expression associates with clinicopathological characteristics of gastric adenocarcinoma. We further asked whether our immunohistochemical analysis of MICA/B expression in human GC presented an association with clinicopathological parameters of the disease. MICA/B expression in the tumor was found to be significantly increased when compared to the gastric mucosa in patients with well differentiated tumors (P<0.0001) (Fig. 3A), in tumors of small size (≤5 cm) (P=0.0359) (Fig. 3B), tumors with lower invasion status (T1 and T2) (P=0.0015) (Fig. 3C), tumors with no lymphatic invasion (N0) (P=0.0269) (Fig. 3D), and tumors at TNM stages I and II (P=0.0056) (Fig. 3E). However, MICA/B immunoreactivity in the tumor did not differ significantly from that of the corresponding gastric mucosa in patients diagnosed with poorly or non-differentiated tumors (Fig. 3A), with tumors >5 cm (Fig. 3B), tumors with deeper invasion status (T3 and T4) (Fig. 3C), and tumors at TNM stages III and IV (Fig. 3E), although patients presenting with lymph node metastasis (N1, N2 and N3) showed higher MICA/B expression in the tumor than in the gastric mucosa (P=0.0115) (Fig. 3D). Notably, while well differentiated tumors displayed higher MICA/B expression than non-differentiated tumors (P=0.0049)

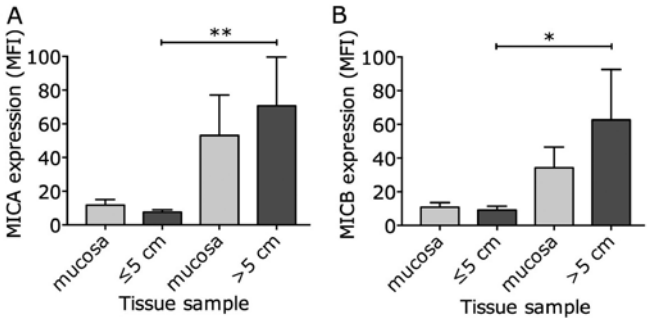

Figure 5. MICA and MICB expression is increased in gastric tumors of large size. (A) MICA and (B) MICB expression was assessed in the tumor and gastric mucosal cells from 23 and 20 GC patients, respectively, by flow cytometry. Data were obtained in MFI values and classified according to tumor size (≤5 and >5 cm). Mean values ± SEM are shown. The Mann-Whitney U test or unpaired t-test was used for statistical analysis. *P<0.05; **P<0.01.

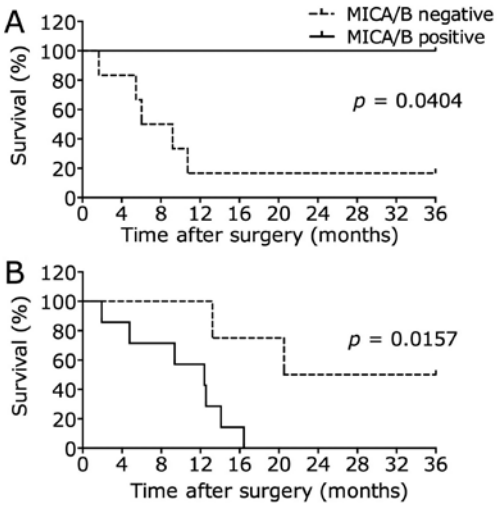

Figure 6. Kaplan Meier curves for overall GC patient survival according to positive and negative expression of both, MICA and MICB, as assessed by flow cytometry, in tumors ≤5 and >5 cm. The P-values were calculated by the log-rank test. (A) The survival rate of GC patients with tumors ≤5 cm that expressed MICA and MICB (n=3) was significantly higher than that of tumors ≤5 cm that did not express these ligands (n=6) (P=0.0404). (B) Patients bearing tumors >5 cm that expressed MICA and MICB (n=7) showed significantly shorter survival than patients with tumors >5 cm that did not express these NKG2DLs (n=4) (P=0.0157).

(Fig. 3A), tumors ≤5 cm showed significantly lower MICA/B immunoreactivity than tumors >5 cm (P=0.0024) (Fig. 3B). Accordingly, the non-neoplasic gastric mucosa of tumors ≤5 cm presented decreased MICA/B expression compared to the gastric mucosa of tumors >5 cm (P=0.0205) (Fig. 3B), which was also observed in tumors at stages I and II of the disease (P=0.0477) (Fig. 3E).

Next, we performed flow cytometry to evaluate MICA expression on cells derived from 23 pairs of gastric tumor and mucosa of GC patients. MICB expression was also analyzed on 20 pairs of tumor and mucosal tissues. Contour plots of freshly isolated gastric tumor and mucosal cells of a representative patient are shown in Fig. 4A. Expression intensity of MICA and MICB, shown in the corresponding histograms, was assessed on the selected contour plot gates and compared to the isotype control (Fig. 4A). The median fluorescence intensity (MFI) values of MICA and MICB present in the tumor and mucosa, for all patients analyzed, are shown in Fig. 4B and C, respectively. MFI values >10 were established as positive ligand expression. MICA expression was observed in 57% of tumors (13 out of 23 samples) (Fig. 4B), while MICB was detected in 50% of tumors (10 out of 20 samples) (Fig. 4C). Although the presence of MICA and MICB was observed in 10 (44%) and 9 (45%) non-neoplasic gastric mucosal samples, respectively, paired tissue analysis showed no significant difference between tumor and gastric mucosa expression of either ligand (Fig. 4B and C).

We then evaluated whether gastric tumor and mucosa expression of MICA and MICB, observed by flow cytometry, associates with clinicopathological features of the disease. We found that, as observed by IHC (Fig. 3B), tumors ≤5 cm presented lower expression of MICA (P=0.0045) and MICB (P=0.0461) than tumors >5 cm (Fig. 5A and B). Although MICA and MICB expression in the gastric mucosa of tumors >5 cm did not differ significantly from that of the mucosa of tumors ≤5 cm, no difference in the expression of either ligand was observed between the tumor and gastric mucosa when tumors were ≤5 or >5 cm (Fig. 5).

GC patients presenting large tumors that express MICA and MICB show lower overall survival rate. It has been reported that tumor size is an independent prognostic indicator in GC patients, and that patients bearing tumors >5 cm present more aggressive clinicopathological characteristics than patients with tumors ≤5 cm (35). The 3-year overall survival rate in our cohort, after gastrectomy, was 30.4%, with a median survival of 12.6 months (range, 2-36 months). We observed no statistically significant difference in the survival rate between patients with tumors ≤5 cm and tumors >5 cm (data not shown). Nevertheless, in patients presenting tumors ≤5 cm, the 3-year survival rate when tumors expressed MICA and MICB was 100%, whereas in patients whose small tumors did not express MICA and MICB, the survival rate was 17% (P=0.0404) (Fig. 6A). On the contrary, all the patients with tumors >5 cm that expressed MICA and MICB succumbed within 16.4 months after gastrectomy, while in patients whose large tumors did not express MICA and MICB, the 3-year survival rate reached 50% (P=0.0157) (Fig. 6B). These results suggest that tumor-associated expression of MICA and MICB may not guarantee a survival benefit for GC patients with tumors >5 cm.

Discussion

NK cells participate in the immune surveillance of several types of tumors, including gastrointestinal malignancies (29,36). Nevertheless, in GC patients, the downregulation of NKG2D receptor on tumor-infiltrating and systemic NK cells, cytotoxic $CD8^{+}$ T cells and γδ T lymphocytes has been associated with impaired function of these cells and also with tumor progression and poor disease prognosis (24-26). Low surface expression of NKG2D receptor has been attributed to the effects of soluble NKG2DLs that are shed from solid tumors by metalloproteinases, compromising the NKG2D-dependent NK cell activation and favoring tumor escape (19,37). Indeed, we have detected

higher levels of soluble MICA and MICB in the serum of GC patients compared with healthy individuals (data not shown), as previously described by others (27). Conversely, persistent cell surface expression of NKG2DLs by tumors and other cells can also elicit NKG2D receptor downregulation, promoting an impairment of NK and $CD8^+$ T cell cytotoxicity (21). Tumor-associated or soluble NKG2DLs have been related with disease progression and poor prognosis in several types of cancer, such as pancreatic and prostate cancer (MICA/B) (15,22), ovarian cancer (ULBP2 and ULBP4) (38), melanoma (ULBP2) (39) and multiple myeloma (MICA) (40). In GC patients, MICA and MICB mRNA levels have been described in the tumor and non-neoplasic gastric mucosal tissue (28). Accordingly, MICA/B has been detected, at the protein level, in the primary tumor of these patients (29). However, to date, the levels of MICA and MICB expression in both gastric tumor and mucosal tissues of GC patients have not been addressed.

In the present study, we evaluated the presence of MICA and MICB in the tumor tissue and gastric mucosa obtained from GC patients who underwent potentially curative surgery to treat the disease. We observed that MICA mRNA levels, estimated by qRT-PCR, were detected in both, gastric tumor and mucosal tissues, and that MICA mRNA levels are higher in the tumor as compared with the gastric mucosa. These results are in agreement with Park *et al* (28), who reported, using Multiplex PCR, that the mRNA levels of MICA and other NKG2DLs (MICB, ULBP-2 and ULBP-3) are significantly increased in the primary tumor of GC patients compared to the adjacent non-neoplasic gastric mucosa.

Here, we also observed that MICA and MICB are expressed in 57 and 50% of tumors, respectively. Conversely, 44 and 45% of patients presented MICA and MICB, respectively, in their non-neoplasic gastric mucosa, as evidenced by flow cytometry. Our IHC data showed that the immunoreactivity for MICA/B in the tumor was increased in relation to the gastric mucosa, although we did not detect such significant differential tumor and mucosal tissue expression of MICA and MICB by flow cytometry. The use of different antibodies and techniques to detect these NKG2DLs in gastric tissues might have contributed to this result. Nevertheless, we can conclude that MICA and MICB are expressed in both, the primary tumor and gastric mucosa of patients with gastric cancer.

The prognostic value of MICA/B expression in gastric adenocarcinoma has been studied. A recent report by Mimura *et al* (29) demonstrated, using a large cohort of GC patients, that 40% of these patients express MICA/B in the tumor, which correlated with clinicopathological parameters that characterized early disease stages and better overall survival. These authors also observed that most patients at advanced stages of GC do not express MICA/B (29). In this study, we related MICA/B expression, by IHC, in both, gastric tumor and mucosal tissues, to clinicopathological characteristics of the disease. MICA/B expression in the tumor was significantly increased compared to the gastric mucosa in patients with well differentiated tumors, in tumors of small size (≤5 cm), tumors with lower invasion status (T1 and T2), tumors with no lymphatic invasion (N0), and tumors at early TNM stages (I and II). In contrast, MICA/B expression in the tumor was similar to the gastric mucosa in patients with poorly or non-differentiated tumors, with tumors >5 cm, tumors with deeper invasion status (T3 and T4), and tumors at advanced TNM stages (III and IV), although GC patients presenting with lymph node metastasis (N1, N2 and N3) showed higher MICA/B expression in the tumor than in the gastric mucosa. Notably, the non-neoplasic gastric mucosa of patients bearing large tumors and tumors at stages III and IV presented higher levels of MICA/B than the gastric mucosa of small tumors and tumors at earlier disease stages, respectively. Altogether, these results suggest that both, the equivalent expression of MICA/B in the tumor and non-neoplasic gastric mucosa and the increased expression of MICA/B in the gastric mucosa of GC patients presenting advanced features of the disease reflects a persistent cell-associated expression of these NKG2DLs. Whether patient tumor and gastric mucosal expression of MICA/B favors NKG2D receptor downregulation in cytotoxic lymphocytes at advanced stages of GC needs further investigation.

In the present study, we also observed that, by IHC, well differentiated tumors presented higher levels of MICA/B than non-differentiated tumors. These results are in agreement with Mimura *et al* (29), who demonstrated that MICA/B-positive tumors were more prevalent in patients presenting well-differentiated tumors. It has been well established that the prognosis of patients with GC is determined by tumor extension and lymph node involvement (41,42). Although tumor differentiation may influence patient survival (43), its value as an independent prognostic indicator in GC is still controversial (44). On the contrary, several reports have identified gastric tumor size as an independent prognostic factor in gastric cancer. For instance, Zu *et al* (35), in a study using a large cohort of patients and adopting a 5-cm cut-off value for tumor size, clearly demonstrated that GC patients bearing tumors >5 cm presented more aggressive clinicopathological characteristics than patients with tumors ≤5 cm, which was also correlated to worse patient survival, regardless of tumor stage and lymph node metastasis, supporting that tumor size may be considered an independent prognostic indicator in patients with gastric cancer.

By IHC, we demonstrated that tumors ≤5 cm had significantly lower MICA/B expression than tumors >5 cm, which was confirmed by flow cytometry. Even though the overall survival rate between GC patients with tumors ≤5 cm and tumors >5 cm did not differ in our cohort, the 3-year survival percentage of patients whose tumors were ≤5 cm and that presented both, MICA and MICB, was significantly higher than in patients with small tumors that did not express these NKG2DLs. In contrast, GC patients with tumors >5 cm that expressed MICA and MICB had significant lower survival rates than patients presenting large tumors that did not express either of the ligands. Therefore, these results indicate that tumor-associated expression of MICA and MICB may be protective for patients bearing tumors of small size, while MICA and MICB expression in large tumors may contribute to tumor progression.

The association between MICA/B expression and tumor stage has been previously studied for other types of cancer. In colorectal and pancreatic cancers, MICA was detected as an independent marker of favorable prognosis for stages I and II, but not later stages of the disease (15,45). These data, along with those obtained by Mimura *et al* (29) for GC, suggest a protective role for NKG2DL expression at early, but

not advanced stages of tumors. Accordingly, here we provide evidence that MICA/B expression in small gastric tumors, which has been associated with better patient survival (35), may contribute to a better prognosis of the disease, but that persistent expression of MICA/B in large tumors may not be beneficial for GC patients.

It is intriguing that the high expression of a molecule involved in the alertness of the immune response may conversely be associated with a tumor immune evasion strategy. It is well established that cell transformation results in increased NKG2DL expression in relation to their basal levels, thus converting these cells in a specific target for NK cell-mediated cytotoxicity, which may determine their elimination (8,9). However, high MICA and MICB expression in the primary tumor tissue may provide a selective advantage for tumor cells that sustain MICA and MICB expression and, instead of immune activation, this prevalent expression would promote immune suppression, since persistent NKG2DL expression may desensitize NK cells and render them dysfunctional, which is a determinant of tumor aggressiveness (21,46,47). However, it is worth mentioning that other factors, such as soluble NKG2DLs (19) and TGF-β released from tumors (48), may more strongly impair NK cell and other lymphocytes cytotoxicity, compromising their function and favoring malignant progression.

It is becoming clear that the delineation of alternative pathways for antagonizing the deleterious effects of NKG2DL overexpression in cancer patients remains an important issue for further investigation. Defining the contributory role of persistent NKG2D receptor ligands on tumor progression could help the development of novel treatment strategies or improve the efficacy of standard therapy for GC. Our findings suggest that MICA/B expression within large tumors can serve as a prognostic indicator for GC and a potential target for immunotherapy.

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