

# Low-dose valproic acid with low-dose gemcitabine augments MHC class I-related chain A/B expression without inducing the release of soluble MHC class I-related chain A/B

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**Abstract.** To improve natural killer group 2 member D (NKG2D)-dependent cytotoxicity, the inhibition of cleavage and release of major histocompatibility complex class I-related chain (MIC) molecules from the tumor surface are required. Valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, is able to induce cell-surface MICA/B on tumor cells. In the present study, the ability of VPA and gemcitabine (GEM) to upregulate MICA/B in pancreatic cancer cells was investigated, resulting in the inhibition of cleavage and release of MIC molecules from the tumor surface. Flow cytometry was used to quantify MICA/B expression in six human pancreatic cancer lines. Functional cytotoxic activity of  $\gamma\delta$ T cells against pancreatic cancer cells treated with VPA and GEM was determined using cytotoxicity assays. At low doses of VPA (0.7 mM) and GEM (0.001  $\mu$ M), which did not induce tumor growth alterations, the agents individually increased cell-surface MICA/B expression in MICA/B-positive cell lines, but not in the MICA/B-negative cell line. Furthermore,

the combination of VPA and GEM synergistically induced cell-surface MICA/B expression. In MICA/B-positive cell lines, the increase in MICA/B expression was dependent on VPA concentration. The combination of low-dose VPA and GEM enhanced the susceptibility of the PANC-1 cell line to  $\gamma\delta$ T cell-mediated tumor cell lysis. It was observed that soluble MIC was released from PANC-1 in the culture supernatant following treatment with GEM. However, the combination of low-dose VPA with low-dose GEM increased MICA/B expression without inducing soluble MIC, resulting in enhanced tumor cell lysis. The results of the present study suggest that the combined administration of low-dose VPA with low-dose GEM has the potential to enhance the therapeutic effects of immunotherapy in pancreatic cancer. Furthermore, it is proposed that the combination acts, in part, by upregulating MICA/B and prevents soluble MIC from being released.

## Introduction

Pancreatic ductal adenocarcinoma (PDAC), one of the most lethal of all types of human cancer, is a common cause of cancer mortality in the USA and Japan (1). PDAC is the fourth most common cause of cancer-associated mortality and its incidence is increasing worldwide (2). PDAC displays local invasion and distant metastasis during early disease stages and this leads to an extremely poor prognosis, with an overall survival rate of <5% (2). As such, there is a requirement for novel and more effective treatment strategies. Research has focused on the development of immunomodulatory approaches (3).

Interactions between the immune system and malignant cells serve an important role in tumorigenesis (4). Tumors have employed multiple mechanisms of immune evasion. One such mechanism, particularly in high-grade cancer, involves cytotoxic T lymphocyte (CTL) evasion through major histocompatibility complex (MHC) class I downregulation (5). Tumor cells express cell-surface MHC class I chain-related gene A/B (MICA/B), frequently induced under cellular stress. MICA/B function as ligands for natural killer group 2 member D (NKG2D), expressed on cytotoxic innate immune cells, specifically  $\gamma\delta$ T cells and

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**Abbreviations:** ADAM, a disintegrin and metalloproteinase; CTL, cytotoxic T lymphocyte; GEM, gemcitabine; HDAC, histone deacetylase; HLA, human leukocyte antigen; MICA/B, major histocompatibility complex class I-related chain A/B; NK, natural killer; NKG2D, natural killer group 2 member D; PBMC, peripheral blood mononuclear cell; PDAC, pancreatic ductal adenocarcinoma; VPA, valproic acid

**Key words:** major histocompatibility complex class I-related chain A/B, low-dose valproic acid, low-dose gemcitabine,  $\gamma\delta$ T cell cytotoxicity, pancreatic cancer, immune evasion

natural killer (NK) cells (6). Unlike conventional  $\alpha\beta$ T cells,  $\gamma\delta$ T cells recognize antigens in an MHC-unrestricted manner, and may provide a novel immunotherapeutic approach against tumor cells (7,8). Therefore, MICA/B expression in PDAC serve as targets for various effector cells expressing NKG2D.

Histone deacetylase (HDAC) inhibitors, which alter histone acetylation, are also promising anticancer agents (9,10). A number of clinical studies are currently investigating HDAC inhibitors, and certain HDAC inhibitors have already been approved by the US Food and Drug Administration for the treatment of cutaneous T cell lymphoma (11). However, certain HDAC inhibitors are of limited therapeutic use owing to toxic side effects at high doses. Valproic acid (VPA) exhibits anti-tumor effects of HDAC inhibitors and has been demonstrated to exert anticancer effects in various cancer models (12,13). The therapeutic range of VPA is between 0.35 and 0.7 mM (14). A number of studies have demonstrated that VPA stimulates the expression of cell-surface MICA/B in a variety of tumors, enhancing the susceptibility of tumor cells to cell-mediated cytotoxicity (7,15-17).

Gemcitabine (GEM), a nucleoside analogue, is commonly administered as an initial chemotherapy drug for the treatment of pancreatic cancer (18). GEM also induces MICA/B expression on the surface of pancreatic cancer cells (19). In our previous study, it was demonstrated that cell-surface MICA/B expression was upregulated following low-dose GEM treatment, at a concentration not affecting cell growth (20).

However, to the best of our knowledge, the combined effects of VPA and GEM have not yet been investigated. In the present study, the effect of VPA with GEM on the expression of MICA/B was investigated in pancreatic cancer cell lines. It was determined that the cytotoxic efficacy of  $\gamma\delta$ T cells directed at tumors is dependent on the ability of VPA and GEM to prevent tumor immune evasion. This is facilitated by pancreatic cell-surface MICA/B expression without cleavage or release of MIC molecules from the tumor surface.

## Materials and methods

**Human pancreatic cancer cell lines.** Human pancreatic cancer cell lines (PANC-1, BxPC-3, HPAF-II, MIA PaCa-2, Capan-1 and AsPC-1) were purchased from the American Type Culture Collection (Manassas, VA, USA). These cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum, 10 mM HEPES, 1 mM sodium pyruvate, 1% non-essential amino acid solution,  $5 \times 10^{-5}$  M 2-mercaptoethanol, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (all from Thermo Fisher Scientific, Inc., Waltham, MA, USA).

**Antibodies and reagents.** VPA was obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). GEM was obtained from Eli Lilly (Indianapolis, IN, USA). Clear Back (human Fc receptor blocking reagent; cat. no. MTG-001), anti-human MICA antibody (dilution, 1:100; cat. no. K0217-3), anti-human MICB antibody (dilution, 1:100; cat. no. K0220-3) and anti-mouse immunoglobulin (Ig)G-fluorescein isothiocyanate (FITC) secondary antibody (dilution, 1:160; cat. no. 238) were obtained from MBL International Co. (Woburn, MA, USA). Phycoerythrin (PE)-conjugated anti-human

MICA/B antibody (dilution, 1:20; cat. no. 320906), mouse IgG2a  $\kappa$  isotype control (dilution, 1:20; cat. no. 400211), FITC-conjugated anti-human leukocyte antigen (HLA)-A, -B and -C antibodies (dilution, 1:50; cat. no. 311403), and mouse IgG2a  $\kappa$  isotype control (dilution, 1:50; cat. no. 400207) were purchased from BioLegend, Inc. (San Diego, CA, USA). FITC-conjugated anti-human V $\gamma$ 9 antibody (dilution, 1:5; cat. no. IM1463) and PE-cyanin 5.1-conjugated anti-human cluster of differentiation (CD) 3 antibody (dilution, 1:10; cat. no. A07749) were acquired from Beckman Coulter, Inc. (Brea, CA, USA). ALyS-203, containing 1,000 IU/ml interleukin-2 (IL-2), was purchased from Cell Science & Technology Institute (Sendai, Japan). Zoledronate (Zometa<sup>®</sup>) was acquired from Novartis International AG (Basel, Switzerland). Calcein-acetoxymethyl ester (AM) was obtained from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Anti-human NKG2D monoclonal antibody (mAb) (catalog No. MAB139) was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). WST-1 was acquired from Roche Diagnostics (Basel, Switzerland).

**Expansion of  $\gamma\delta$ T cells.**  $\gamma\delta$ T cells, obtained from healthy volunteers (n=3) following provision of written informed consent, were expanded from peripheral blood mononuclear cells (PBMCs), as described previously (21). PBMCs were cultured with ALyS-203, containing 1,000 IU/ml IL-2 and 5  $\mu$ M zoledronate. Cell density was maintained at  $(0.5-2) \times 10^6$  cells/ml. Additional PBMCs were cultured with ALyS-203, containing 1,000 IU/ml IL-2, in the absence of zoledronate. On day 12, cells were harvested, and the frequency of  $\gamma\delta$ T cells, identified by subsets CD3<sup>+</sup> and V $\gamma$ 9<sup>+</sup>, were analyzed using flow cytometry. These cells were used as effector cells (E) in the cytotoxicity assay.

**WST-1 assay.** Human pancreatic cancer cell lines were treated with various concentrations of VPA between 0 and 5 mM. After 48 h, the viability of each cell line was analyzed using a WST-1 assay, as described previously (22).

**Immunofluorescence staining and flow cytometry.** Human pancreatic cancer cell lines were cultured in a 10 mm tissue culture dish for 24 h. Each cell line was subsequently treated with VPA alone, or VPA with GEM, for 48 h. Prior to staining with fluorescent antibodies, the Fc receptor was blocked with Clear Back. The expression of MICA/B, and HLA-A, -B and -C on each pancreatic cancer cell line was determined by immunofluorescence staining with PE-conjugated anti-MICA/B antibody and FITC-conjugated HLA-A, -B and -C antibodies. To evaluate MICA/B expression by each pancreatic cancer cell line, cells were first stained with anti-MICA or -MICB antibody and subsequently stained with anti-mouse IgG-FITC secondary antibody. Fluorescence was analyzed using an EPICS XL flow cytometer (Beckman Coulter, Inc., Brea, CA, USA).

**Cytotoxicity assay.**  $\gamma\delta$ T cell cytotoxicity against human pancreatic cancer cell lines were evaluated using a calcein-AM release assay. Pancreatic cancer cell lines were treated with 0.7 mM VPA, 0.001  $\mu$ M GEM or a combination of 0.7 mM VPA and 0.001  $\mu$ M GEM for 48 h. Subsequently, pancreatic

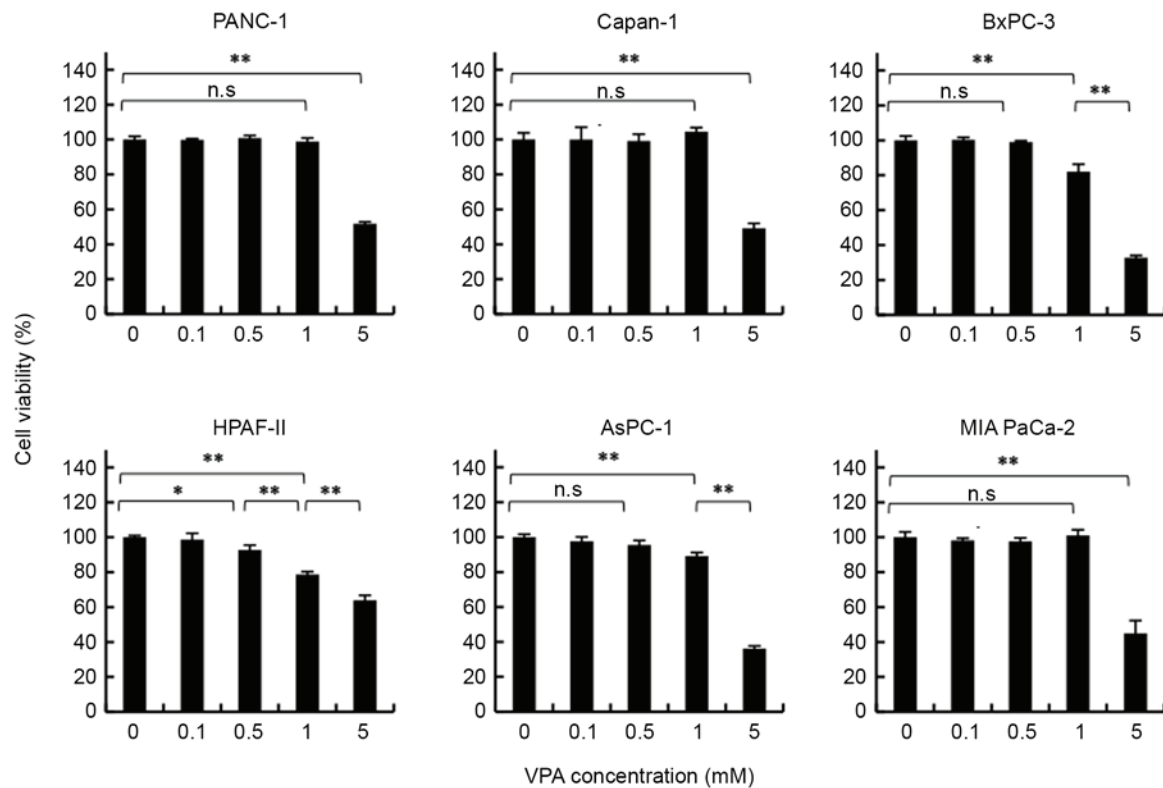


Figure 1. Effects of VPA on cell growth in pancreatic cancer cell lines. Cells were cultured in medium with between 0.1 and 5 mM VPA. At 5 mM VPA, cell viability was decreased in all pancreatic cancer cell lines. \*\*P<0.05; \*P<0.01; n.s, not significant; VPA, valproic acid.

cancer cell lines were labeled with 5  $\mu$ M calcein-AM for 30 min at 37°C. Following washing three times with medium, calcein-AM-labeled cells were used as target cells (T). As effector cells (E),  $\gamma\delta$ T cells were expanded from PBMCs as aforementioned. To evaluate  $\gamma\delta$ T cell cytotoxicity, effector cells were co-cultured with target cells at various E/T ratios (1,5 and 25) at 37°C for 3 h. In blocking experiments, anti-NKG2D mAb was added to  $\gamma\delta$ T cell suspension at 10  $\mu$ g/ml, 30 min prior to co-culturing with target cells. In order to measure the spontaneous release and maximum release of fluorescence intensity from target cells, medium or 6% Triton X-100 was added to target cells. Fluorescence intensity was measured with Terascan VPC (Minerva Tech, Tokyo, Japan) prior to and following culture. The percentage of target cells killed by  $\gamma\delta$ T cells was calculated using Calct-96l software (Minerva Tech, Tokyo, Japan). Triplicate experiments were performed.

**ELISA.** Following treatment of human pancreatic cancer cell lines with 0.7 mM VPA, GEM (0.001, 0.1, 10  $\mu$ M) or a combination of 0.7 mM VPA and GEM (0.001, 0.1, 10  $\mu$ M) for 48 h, in order to detect soluble MICA and MICB in the culture supernatant, sandwich ELISA. An Ab-Match Universal kit (cat. no. 5310), Ab-Match ASSEMBLY Human MICA kit (cat. no. 5330) and Ab-Match Assembly Human MICB kit (cat. no. 5331, all MBL International Co., Woburn, MA, USA) were used according to manufacturers' protocol.

**Statistical analysis.** Differences were analyzed for significance using Student's t-test, Mann-Whitney rank sum test,  $\chi^2$  test or log-rank test, as appropriate. Data management and statistical analysis were performed using SPSS software (version 15;

SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

## Results

**MICA/B, and HLA-A, -B and -C expression by pancreatic cell lines.** MICA/B, and HLA-A, -B, and -C expression by six pancreatic cell lines were analyzed using flow cytometry. As demonstrated in our previous study, the cell-surface MICA/B expression was detected in four pancreatic cell lines (PANC-1, BxPC-3, MIA PaCa-2 and Capan-1), but not in the remaining cell lines (HPAF-II, AsPC-1) (20). The HPAF-II cell line only expressed MICA but not MICB, and AsPC-1 expressed neither MICA nor MICB. Cell-surface expression of HLA-A, -B and -C was detected in all pancreatic cell lines investigated.

**VPA concentration affects pancreatic cancer cell growth.** To determine whether the concentration of VPA had an effect on the viability of each pancreatic cancer cell line, cell lines were analyzed following treatment with various concentrations of VPA for 48 h using a WST-1 assay. As presented in Fig. 1, VPA doses <1 mM did not affect the viability of PANC-1, MIA PaCa-2, Capan-1 and AsPC-1 cells. However, cell viability decreased with 1 mM VPA treatment in BxPC-3 and HPAF-II cell lines. Following treatment with 5 mM VPA, cell viability decreased in all pancreatic cancer cell lines. This result indicated that VPA at a concentration of 5 mM has an antineoplastic effect.

**Effect of MICA/B, and HLA-A, -B and -C expression on pancreatic cancer cell lines following VPA treatment.**

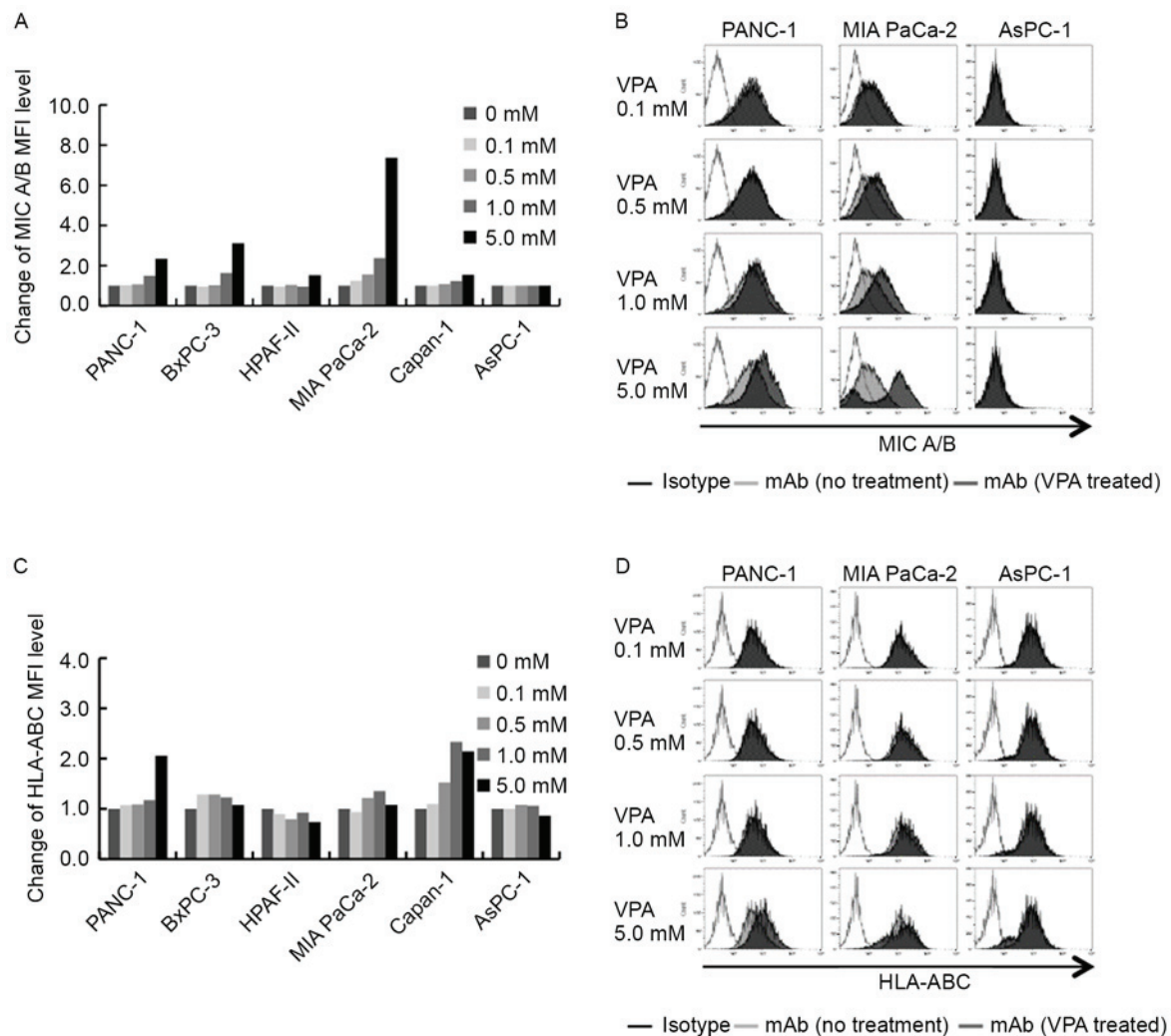


Figure 2. Effects of VPA on the cell-surface expression of MICA/B, and HLA-A, -B and -C. (A) Expression of cell-surface MICA/B was increased in MICA/B-positive cell lines, but not the MICA/B-negative cell line AsPC-1. (B) Representative MICA/B expression profiles of PANC-1, MIA PaCa-2 and AsPC-1 cells in the presence of VPA at between 0.1 and 5 mM. (C) Expression of cell-surface HLA-A, -B and -C was increased in PANC-1 and Capan-1, but not in the other cell lines. (D) Representative HLA-A, -B and -C expression profiles of PANC-1, MIA PaCa-2 and AsPC-1 in the presence of VPA at between 0.1 and 5 mM. VPA, valproic acid; MICA/B, major histocompatibility complex class 1-related chain A/B.

MICA/B expression was quantified on pancreatic cancer cell lines following treatment with various concentrations of VPA. As presented in Fig. 2A, it was determined that VPA treatment resulted in an alteration in MICA/B expression in each pancreatic cancer cell line. MICA/B expression was increased in VPA-treated MICA/B-positive cell lines PANC-1, BxPC-3, HPAF-II, MIA PaCa-2 and Capan-1, but not in the MICA/B-negative cell line AsPC-1. In MICA/B-positive cell lines, the increase in cell-surface MICA/B expression was dependent on VPA concentration. Following 5 mM VPA treatment, cell-surface MICA/B expression increased in PANC-1 and BxPC-3 cells, and markedly in MIA PaCa-2 cells, but not in AsPC-1 cells (Fig. 2B). This result demonstrates that cell-surface MICA/B expression may be increased with high doses of VPA in MICA/B-positive pancreatic cancer cell lines. As presented in Fig. 2C and D, treatment with various concentrations of VPA was able to alter HLA-A, -B and -C expression levels, but not to the level observed with MICA/B. In PANC-1 and Capan-1 cells, HLA-A, -B and -C expression was increased following VPA treatment. However, HLA-A, -B

and -C expression was not observed to be increased in other pancreatic cancer cell lines.

*MICA/B expression following treatment with low-dose VPA and low-dose GEM.* In an effort to increase MICA/B expression effectively on the cell surface, MICA/B expression by pancreatic cancer cell lines was investigated following treatment with low-dose VPA combined with low-dose GEM.

Fig. 3A indicates that the combination of 0.7 mM VPA and 0.001 mM GEM treatment resulted in an alteration in the MICA/B expression level in each pancreatic cancer cell line. VPA at 0.7 mM increased MICA/B expression on the cell surface of the MICA/B-positive pancreatic cancer cell lines PANC-1, BxPC-3, HPAF-II, MIA PaCa-2 and Capan-1. Likewise, MICA/B expression on all MICA/B-positive pancreatic cancer cell lines, except BxPC-3, was increased following low-dose GEM treatment. When treated with a combination of VPA and GEM, MICA/B expression was increased synergistically in pancreatic cancer cell lines to a level greater than that with VPA or GEM treatment, individually. Conversely,

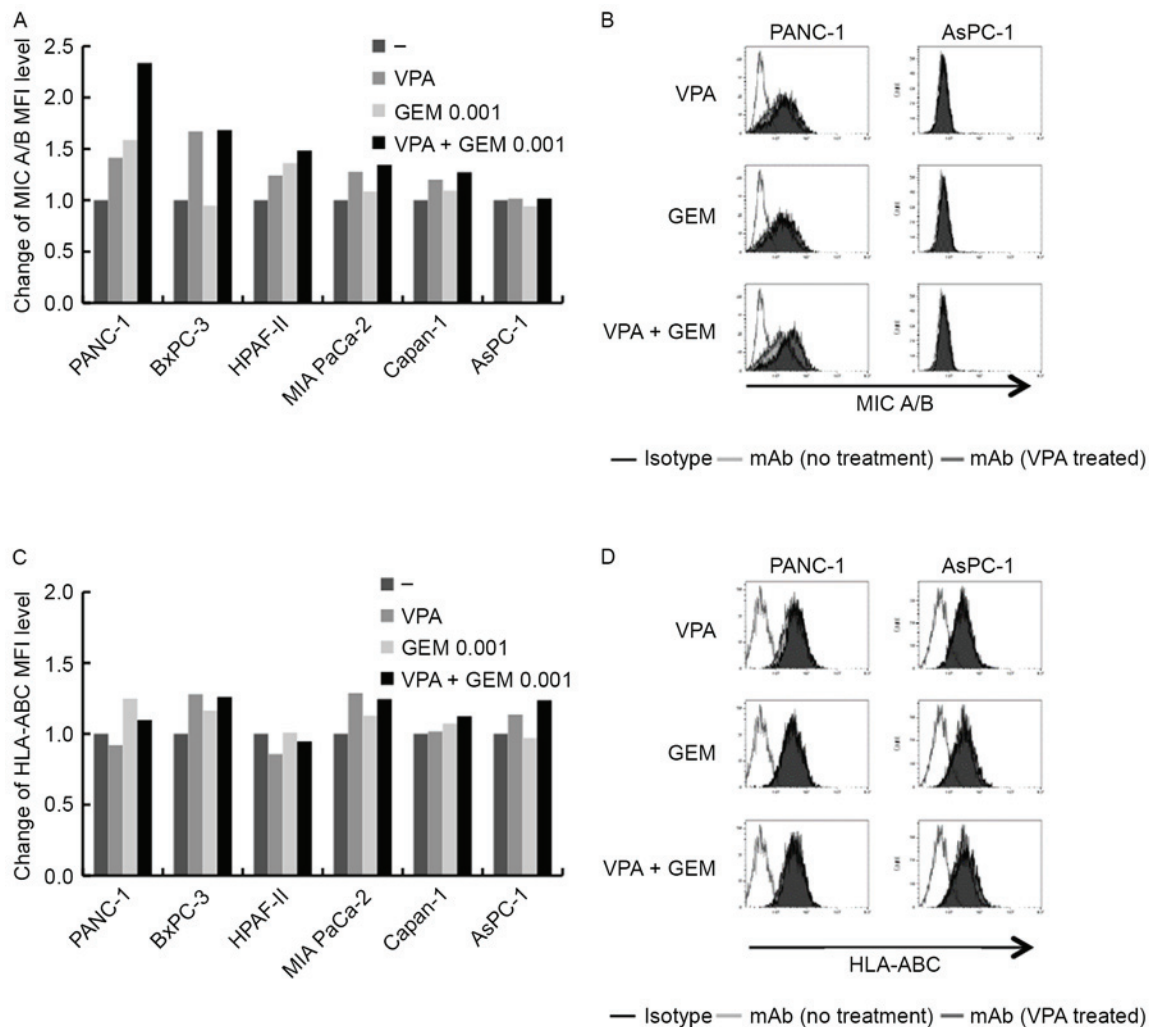


Figure 3. Effects of VPA, GEM and combination of VPA and GEM on the cell-surface expression of MICA/B, and HLA-A, -B and -C. (A) Expression of cell-surface MICA/B with the combination of low-dose VPA and low-dose GEM treatment was increased compared with that following VPA or GEM treatment alone, in MICA/B-positive pancreatic cancer cell lines, but not the MICA/B-negative pancreatic cell line AsPC-1. (B) Representative MICA/B expression profiles for PANC-1 and AsPC-1 cells following treatment with VPA, GEM, and the combination of VPA with GEM. (C) Cell-surface expression of HLA-A, -B and -C was not increased in pancreatic cancer cell lines. (D) Representative HLA-A, -B and -C expression profiles for PANC-1 and AsPC-1 cells in the presence of GEM, VPA, and the combination of VPA with GEM. VPA, valproic acid; GEM, gemcitabine; MICA/B, MICA/B, major histocompatibility complex class 1-related chain A/B.

MICA/B expression was not increased in the MICA/B-negative cell line AsPC-1 using VPA, GEM or a combination of VPA and GEM treatment (Fig. 3B).

As presented in Fig. 3C and D, the administration of low-dose VPA and low-dose GEM resulted in an alteration in the HLA-A, -B and -C expression level in each pancreatic cancer cell line. In all pancreatic cancer cell lines, except HPAF-II, HLA-A, -B and -C expression was slightly increased with a combination of VPA and GEM treatment.

**Cytotoxic activity of  $\gamma\delta$ T cells against pancreatic cancer cell lines treated with low-dose VPA and low-dose GEM combination treatment.** To assess whether NKG2D-dependent cytotoxic activity was enhanced due to increased MICA/B expression on target cells,  $\gamma\delta$ T cell cytotoxicity against pancreatic cancer cell lines treated with 0.7 mM VPA, 0.001  $\mu$ M GEM or a combination of VPA and GEM was evaluated. As presented in Fig. 4, cytotoxic activity of  $\gamma\delta$ T cells against PANC-1 was observed. In addition, this cytotoxic

activity was blocked using the anti-NKG2D antibody.  $\gamma\delta$ T cells killed PANC-1 cells, which was dependent on the interaction of NKG2D with MICA/B. The cytotoxic activity of  $\gamma\delta$ T cells was enhanced with VPA or GEM, when administered individually. However, when target cells were treated with a low-dose combination of VPA and GEM, there was enhancement of  $\gamma\delta$ T cell cytotoxicity against tumor cells. In the MICA/B-negative cell line AsPC-1, the cytotoxic activity of  $\gamma\delta$ T cells was not detected with or without VPA, GEM or a combination of VPA and GEM (Fig. 4).

**Release of soluble MICA/B from pancreatic cancer cell lines treated with GEM or VPA+GEM.** The results of the present study demonstrate that low-dose VPA (0.7 mM) with a cytostatic concentration of GEM (0.001  $\mu$ M) most effectively increased MICA/B expression in PANC-1 cells. To examine the value of the combination of VPA with GEM, the presence of soluble MICA/B in the culture supernatant was determined using ELISA. This would indicate that MICA/B was released

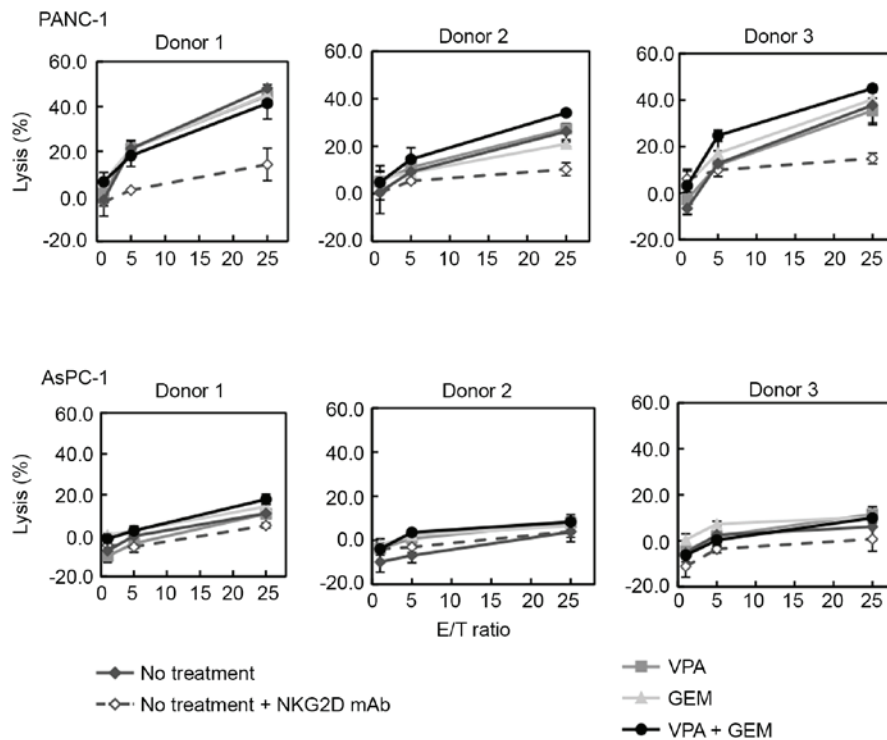


Figure 4. Effect of VPA, GEM and the combination of VPA with GEM on the susceptibility of PANC-1 and AsPC-1 cells to  $\gamma\delta$ T cell-mediated cytotoxicity. The cytotoxicity of  $\gamma\delta$ T cells was enhanced following treatment with VPA and GEM, when administered separately. Furthermore, the cytotoxicity was enhanced additionally following combination treatment of VPA and GEM in PANC-1 cells. However, cytotoxicity was not enhanced with either treatment in AsPC-1 cells. VPA, valproic acid; GEM, gemcitabine.

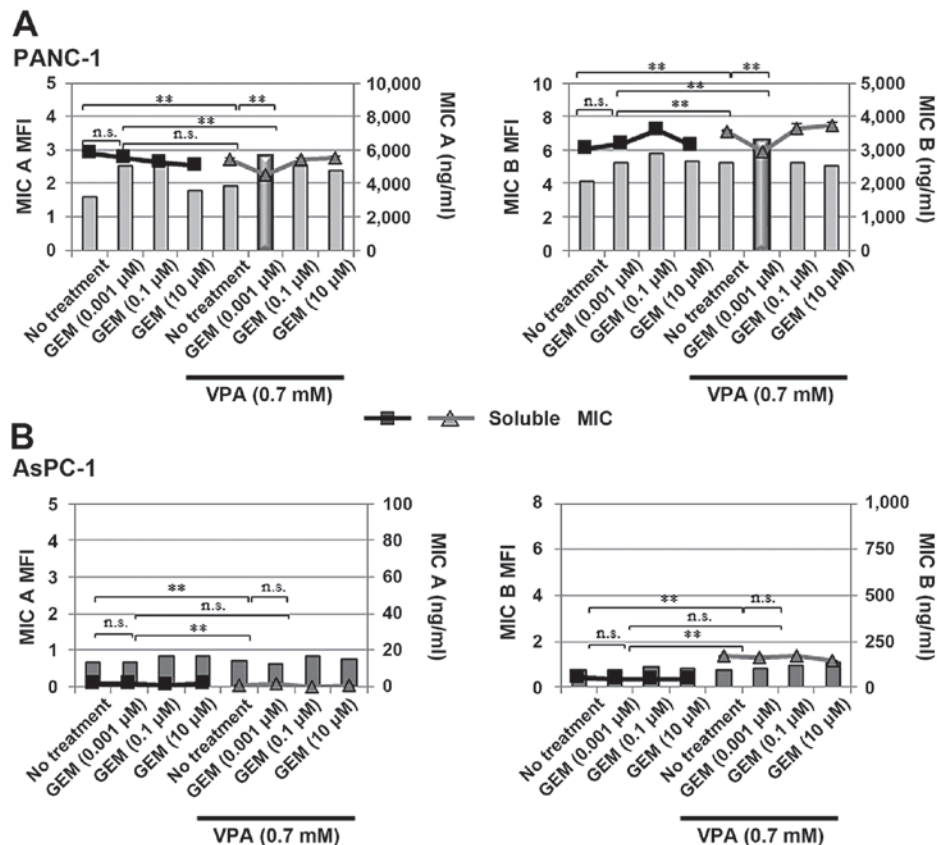


Figure 5. Effect of treatment with GEM or combination of VPA and GEM on the production of soluble MICA/B. (A) In PANC-1 cells, soluble MICA/B remained at high concentrations in the culture supernatant when treated with various doses of GEM, whereas soluble MICA/B decreased following a combination treatment of low-dose VPA with low-dose GEM. (B) In AsPC-1 cells, soluble MICA/B was released slightly into the culture supernatant following treatment with various doses of GEM and the combination treatment of VPA with GEM. \*\* $P < 0.05$ ; n.s., not significant; GEM, gemcitabine; VPA, valproic acid; MICA/B, major histocompatibility complex class 1-related chain A/B.

from pancreatic cancer cell lines treated with GEM, or with a combination of VPA and GEM.

In PANC-1 cells (Fig. 5), a high concentration of soluble MICA/B was detected in the culture supernatant. When PANC-1 cells were treated with various doses of GEM, soluble MICA/B remained at high concentrations in the culture supernatant. Soluble MICA/B decreased when treated with low-dose GEM combined with low-dose VPA.

Soluble MICA/B was released, to a minimal degree, in the culture supernatant of AsPC-1 (Fig. 5). Soluble MICB was detected, despite a lack of cell-surface expression, in this cell line.

## Discussion

In humans, MHC-associated molecules termed MICA/B, are well known as NKG2D ligands. Rarely expressed in healthy cells, MICA/B are frequently expressed in epithelial tumor cells, and in cells under stress. These cells under stress include those undergoing heat shock, viral infection and DNA damage (23,24). The immunoreceptor that recognizes MICA/B, NKG2D, is expressed by  $\gamma\delta$ T and NK cells. Therefore, it may be possible that NKG2D-dependent cytotoxic activity is enhanced with increased MICA/B expression.

VPA has been used clinically to treat migraines and as a mood stabilizer. The therapeutic dose of VPA ranges between 15 and 60 mg/kg/day, and results in a maximum plasma concentrations of 130  $\mu$ g/ml (0.9 mM) (25). VPA levels in epilepsy patients are typically <0.7 mM (26). VPA has been found to act as an HDAC inhibitor (27). Concentrations of VPA for histone acetylation range between 0.25 and 5 mM. In addition, it has been reported that HDAC inhibitors display antineoplastic activity (28,29) and induce NKG2D ligands, such as MICA/B and UL16-binding proteins, on tumor cells (15,30). Furthermore, *in vivo* and *in vitro* studies have demonstrated that VPA enhances NK cell-mediated lysis and upregulates MICA/B expression in pancreatic cancer through the activation of the phosphoinositide 3-kinase/protein kinase B signaling pathway, which has important implications in the pancreatic cancer stroma microenvironment (31,32).

The results of the present study indicate that increased cell-surface MICA/B expression on pancreatic cancer cell lines was dependent on the VPA concentration. Furthermore, when pancreatic cancer cell lines were treated with 5 mM VPA treatment, the viability of those cell lines were decreased and cell-surface MICA/B expression was increased markedly. Therefore, it may be possible that high doses of VPA treatment combined with immunotherapy are able to induce a marked antitumor response. This is due to the enhanced NKG2D-dependent cytotoxicity of immune cells and the antineoplastic effect of VPA. However, because the maximum plasma concentration is 0.9 mM following VPA administration (25), it is necessary to induce the antineoplastic effect of VPA in low doses. Low-dose VPA, <1 mM, was unable to kill pancreatic cancer cell lines with its antineoplastic activity and slightly increased MICA/B expression. Therefore, it was attempted to increase MICA/B expression effectively with low-dose VPA combined with low-dose GEM, as another immunomodulatory reagent.

GEM is a chemotherapeutic agent that has an immunomodulatory effect. It functions by upregulating the cell-surface MICA/B expression for various types of cancer. Results of our previous study indicated that MICA/B expression on the cell surface was increased effectively at low doses of GEM, with no effect on cell viability (20). The results of the present study indicate that the combination of low-dose VPA with low-dose GEM is able to increase cell-surface MICA/B expression on pancreatic cancer cell lines. In addition, NKG2D-dependent cytotoxicity of  $\gamma\delta$ T cells was enhanced with the increase of MICA/B expression by pancreatic cancer cell lines. When PANC-1 was treated with a combination of VPA and GEM, MICA/B expression on the cell surface was increased 2.5-fold in comparison with those untreated in the same cell line.

$\gamma\delta$ T cells are able to recognize and respond to various stress-induced antigens, thereby developing innate immunity (33). They also exhibit potent effector functions, including cytotoxic activity, and the secretion of cytokines/chemokines. The majority of  $\gamma\delta$ T cells in peripheral blood possess the V $\gamma$ 9V $\delta$ 2 T cell receptor. The cytotoxicity of these  $\gamma\delta$ T cells is mediated by perforin-granzyme, CD95/CD95 ligands, tumor necrosis factor (TNF)/TNF receptors and TNF-related apoptosis-inducing ligand (TRAIL)/TRAIL receptor (TRAILR) systems (34). It may be possible that the cytotoxic activity of  $\gamma\delta$ T cells was enhanced through the activation of a number of effector functions. It was reported that HDAC inhibitors were able to increase TRAIL sensitivity in target tumor cells (35). In the present study, it may be possible that cytotoxicity was enhanced by a combination of effector functions, NKG2D-NKG2D ligand interactions and TRAIL/TRAILR sensitivity following the treatment combination of VPA and GEM. In TRAIL-resistant pancreatic cancer cell lines, it was shown that the inhibition of anti-apoptotic B-cell lymphoma extra large (Bcl-XL) protein promoted apoptosis with TRAIL (36). Therefore, the addition of another agent that is able to inhibit Bcl-XL expression may enhance the cytotoxic activity of  $\gamma\delta$ T cells, when TRAIL-resistant tumor cells are treated with VPA and GEM.

A number of clinical studies have emphasized the therapeutic benefits of immunotherapy in combination with chemotherapy (37,38). Therefore, to improve therapeutic benefits, it may be required to combine another chemotherapeutic agent with immunomodulatory agents. One effective approach to improve the therapeutic efficacy of immunotherapy is to prevent immune evasion mechanisms by tumor cells.

In a previous study, it was demonstrated that the release of MIC molecules on the cell surface constitutes an immune evasion mechanism in tumor cells (39). Soluble MICA/B, cleaved on tumor cells, downregulate the cell-surface expression of NKG2D on T cells. This induces the functional impairment of antitumor immune effector cells. As such, proteolytic cleavage may decrease the expression of NKG2D ligands on tumor cell surfaces and contribute to tumor evasion from immunosurveillance. Soluble MIC is perceived as a tumor immune evasion mechanism that may have an effect on the expression of NKG2D, and functional activity of NK cells and T cell subsets (40). MICA/B are cleaved by a disintegrin and metalloproteinase (ADAM) 10 and ADAM17, respectively (40). In the tumor microenvironment, soluble MICA induces the internalization and lysosomal degradation

of the NKG2D receptor in CD8<sup>+</sup> T and NK cells (41). It may be possible that the inhibition of these proteases enhances NKG2D-dependent cytotoxicity. This is due to an increase in MICA/B expression on target cell surfaces, and decrease in soluble MICA/B induction through the prevention of MICA/B cleavage. In our previous study, there was a problem with cleavage and release of MIC molecules from the tumor surface, which resulted in tumors evading immunosurveillance (20). In the present study, soluble MICA/B expression decreased with the combination of low-dose VPA and low-dose GEM, despite increased expression in PANC-1 cells. On the surface of osteosarcoma cells, VPA has been reported to be associated with the downregulation of ADAM10, which cleaves MICA/B (42). In addition, VPA downregulates the activity of matrix metalloproteinases which produce soluble MICA/B (43,44).

In order to prevent the tumor cell immune evasion mechanism and implement effective therapeutic efficacy in immunotherapy, it may be essential to combine a number of immunomodulatory agents and enhance effector functions on immune cells.

The results of the present study indicate that the combined administration of low-dose VPA and low-dose GEM is valuable in enhancing the therapeutic efficacy of immunotherapy by upregulating MICA/B without inducing soluble MIC from being released in pancreatic cancer.

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