Granulin–Epithelin Precursor Renders Hepatocellular Carcinoma Cells Resistant to Natural Killer Cytotoxicity

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Introduction

The immune system has the ability to identify and eliminate tumor cells before they invade or progress during carcinogenesis. Natural killer (NK) cells are a major component of the innate immune system and represent the first line of defense against tumors (1). Antitumor cytotoxicity of NK cells is mediated by direct lysis or induction of IFNγ (2). NK cytotoxicity is impaired in patients with hepatocellular carcinoma (HCC; ref. 3), and the reduced activity is associated with HCC progression (4). NK cytotoxicity is regulated through integrated signaling from their cell-surface receptors that interact with ligands expressed on target cells (2). NK group 2 member D (NGK2D) is a stimulatory receptor expressed on the surface of all NK cells, and its recognition is crucial for tumor immunosurveillance (5). MHC class I chain–related molecule A (MICA), ligand of human NKG2D receptor, is frequently expressed on tumors, but not on normal tissues (5). Engagement of MICA and NGK2D strongly activates NK cells, enhancing their cytotoxicity and cytokine production (5). Thus, the NGK2D-MICA pathway is an important mechanism by which the host immune system recognizes and eliminates tumor cells. CD94/NGK2A is an inhibitory receptor controlling NK activity following interactions with non-classic class I human leukocyte antigen-E (HLA-E) on target cells. HLA-E is ubiquitously expressed; its overexpression in tumors is likely a mechanism evolved by tumor cells to protect against NK cytotoxicity through inhibition via interactions with CD94/NGK2A receptor (2).

Granulin–epithelin precursor (GEP) is a pluripotential growth factor regulating fetal development, tissue repair, and tumorigenesis in various cancers (6). Our group has characterized GEP overexpression in more than 70% of human HCCs, and its...
expression was associated with aggressive HCC features (7). Functional studies demonstrated its role in regulating HCC cell proliferation, invasion, tumorigenicity, and chemoresistance (7–9). Moreover, GEP blockade with antibody could inhibit the growth of established HCC in a mouse model (10). Recently, we showed that GEP was a hepatic oncofetal protein defining a cancer stem cell (CSC) population in HCC (11). CSCs are "roots" of cancers responsible for tumor initiation and progression; they are endowed with stem cell properties, including self-renewal, differentiation, and chemoresistance. Conventional therapies can eliminate the tumor bulk, but the stem cell properties of CSCs enable them to survive and repopulate the tumor, resulting in disease relapse (12). Thus, eradication of CSCs may be the key for curing aggressive malignancies such as HCCs.

It has been suggested that immunoevasion is restricted to CSCs, enabling them to survive in the host (13–15). Recently, ABCB5⁺ malignant melanoma—initiating cells were found to modulate antitumor immunity by preferentially inhibiting IL2-dependent T-cell activation and promoting induction of regulatory T cells (14, 16). Moreover, CD200⁺ CSCs were found to suppress antitumor immunity by downregulating the expression of Th1 cytokines and costimulatory molecules, in ovarian cancer, melanoma, and leukemia (17, 18). However, the role of CSCs in immunoevasion has not been defined in HCC.

In this study, we aimed to investigate the role of GEP in HCC tumor immunoevasion. We showed that GEP rendered HCC cells resistant to NK cytotoxicity, which is an important antitumor response. GEP blockade by monoclonal antibody (mAb) A23 significantly sensitized HCC cells to antitumor cytotoxicity. Targeting GEP by A23 therefore represents a novel therapeutic tool to suppress tumor immunoevasion and eradicate the aggressive GEP-expressing CSCs in HCC. Finally, serum GEP and MICA levels in patients with HCC could provide prognostic information and be used to stratify patients for targeted therapy.

Materials and Methods

Clinical specimens

The study protocol was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (HKU/HA HKW IRB; Hong Kong, China). Patients, diagnosed with primary HCC and confirmed by pathologic examinations, underwent curative partial hepatectomy for HCC at Queen Mary Hospital (Hong Kong, China), and were recruited with written informed consent to the study. Tumor and adjacent non-tumor tissue pair from patients with HCC (80 pairs) were collected and examined for the expression of GEP, MICA, and HLA-E by real-time quantitative reverse-transcription PCR (qRT-PCR; \( n = 80 \)), immunohistochemistry (\( n = 10 \)), and flow cytometry (\( n = 3 \)). Sera of patients with HCC (\( n = 80 \)) and healthy donors (\( n = 30 \)) were collected for measurement of soluble MICA (sMICA) and GEP (sGEP) levels. Blood samples were collected from patients at least 1 hour before tumor resection.

Cell culture and assays

Human HCC cell lines, Hep3B and HepG2, were purchased from the American Type Culture Collection and authenticated using short tandem repeat (STR) profiles by the company. The cells were expanded immediately, cryopreserved in multiple aliquots, and used within 6 months of cultured as described previously (7). A stable cell line for GEP overexpression was performed by transfecting GEP full-length cDNA into HepG2, a cell line with low endogenous GEP level; GEP suppression was performed by transfecting GEP shRNA (sh) into Hep3B, a cell line with high endogenous GEP level. Vector control and shRNA negative control were included as controls for transfection in HepG2 and Hep3B, respectively. All transfectants were maintained in 10% advanced minimum essential media (AMEM) with 0.4 mg/mL of G418. GEP blockade in Hep3B was performed by incubating the cells with or without 50 μg/mL anti-GEP mAb A23 (Versitech Ltd.; ref. 10) or mouse IgG isotype control antibody (Sigma-Aldrich) for 24 hours.

Preparation of human effector cells

Human peripheral blood mononuclear cells (PBMC), NK-depleted PBMCs, and NK cells were freshly isolated from the buffy coats of healthy donors (\( n = 28 \)). Detailed procedures were described in Supplementary Methods and Supplementary Fig. S1.

Cell cytotoxicity assay

Cell cytotoxicity was determined by dual-color flow cytometry using the HCC cell lines Hep3B or HepG2 as target cells, whereas PBMCs, NK cells, or NK cell–depleted PBMCs as effector cells (19).

Immunofluorescence staining and flow cytometric analysis

For HCC cell-surface expression of ligands for NK cell immunoreceptors, cells were stained with allophtococyanin (APC)-conjugated mouse anti-human MICA, HLA-A/B/C, HLA-G, R-phycoerythrin (PE)–conjugated mouse anti-human HLA-E and ULBP1 antibodies or equal amount of corresponding isotype control (BD Biosciences). For coexpression with GEP, cells were stained with the above surface markers, followed by permeabilization with ice-cold 0.1% saponin and then incubated with FITC-conjugated mouse anti-human GEP antibody (Versitech Ltd.; ref. 10) or equal amount of FITC-conjugated mouse IgG isotype antibody (Sigma). For NKGD2 surface expression on NK cells, cells were stained with APC-conjugated mouse anti-NKG2D antibody or an equal amount of corresponding isotype control (BD Biosciences). Results were expressed as a percentage of cells positive for the above markers and/or mean fluorescence intensity (MFI) of the markers, after subtracting the nonspecific background signal (isotype controls).

Immunohistochemical staining

Immunohistochemistry was performed using the Dako Envision Plus System (Dako) following the manufacturer’s instruction with modifications. Briefly, antigen retrieval was performed by microwave with sections immersed in citrate
buffer. Followed by endogenous peroxidase blocking, tissues were stained with the mouse anti-human GEP mAb A23 (Versitech Ltd.; ref. 10), goat anti-human MICA, rabbit anti-human matrix metalloproteinase (MMP)2, rabbit anti-human MMP9 (R&D Systems), and mouse anti-human HLA-E (Abcam). The signal was detected by horseradish peroxidase–conjugated secondary antibody, and color was developed with diaminobenzidine as the chromogen. The tissue sections were then counterstained with hematoxylin.

**Zymography**

MMP2 and MMP9 activity was determined using SDS–PAGE zymography. Briefly, cell supernatants were electrophoresed on a gel containing gelatin and stained with Coomassie Brilliant Blue. MMP2 and MMP9 activity appeared as clear bands on a blue background.

**Orthotopic mouse model**

The establishment of an orthotopic mouse model and drug treatments were performed as described previously (20). Paraffin tissue blocks were retrieved for immunohistochemistry investigation.

**Enzyme-linked immunosorbent assay to determine GEP and MICA levels**

sMICA levels in culture supernatants and human serum samples were determined by a DuoSet MICA ELISA kit (R&D Systems). GEP levels in human serum samples were detected by a human progranulin ELISA kit (Adipogen Inc.).

**Real-time qRT-PCR**

Real-time qRT-PCR was performed as described previously (7). Quantification was performed with the ABI Prism 7700 sequence detection system (Applied Biosystems). The MICA and HLA-E data presented were new data, whereas part of the GEP data have been extracted from a previous cohort (8), and the current study has increased sample size to parallel datasets on MICA and HLA-E. The relative amount of GEP, MICA, and HLA-E, which had been normalized with control 18s for RNA amount variation and calibrator for plate-to-plate variation, was presented as the relative fold change.

**Statistical analysis**

All analyses were performed using the statistical software GraphPad Prism Version 3.00 for Windows (GraphPad Software) or SPSS version 16.0 for Windows (SPSS Inc.). A P value of <0.05 was considered statistically significant.

Details have been described in Supplementary Materials and Methods.

**Results**

**GEP expression of HCC cells modulates antitumor cytotoxicity of immune cells**

To demonstrate the role of GEP in antitumor cytotoxicity, we established stable transfectant of GEP suppression using shRNA (sh) in Hep3B, a HCC cell line with high endogenous GEP level, and stable transfectant of GEP overexpression using GEP full-length cDNA in HepG2, a HCC cell line with low endogenous GEP level. shRNA negative control and vector control were included as controls for transfection in Hep3B and HepG2, respectively. Results from qPCR and flow cytometry showed that GEP mRNA and protein levels were significantly downregulated in Hep3B and upregulated in HepG2 cells (Fig. 1A). We evaluated the cytotoxicity of human PBMCs against HCC cells and their GEP transfectants (n = 4). The cytotoxicity of human PBMCs against GEP-suppressed Hep3B cells was significantly higher than that of control Hep3B cells. On the contrary, the cytotoxicity of PBMCs against HepG2 cells was significantly reduced when GEP was overexpressed. The results indicated that GEP expression was crucial for rendering HCC cells resistant to antitumor cytotoxicity of PBMCs (Fig. 1B).

We then evaluated whether NK cells were responsible for the GEP-modulated immunoevasion. CD56+ NK cells were depleted from human PBMCs by magnetic sorting (n = 4). Upon NK cell depletion, the increased cytotoxicity against GEP-suppressed Hep3B cells was abolished, whereas the reduction of cytotoxicity against GEP overexpressed HepG2 was restored (Fig. 1C). To further confirm the role of NK cells, HCC cells were cocultured with NK cells at different effector:target cell (E:T) ratios (n = 4). NK cytotoxicity against GEP-suppressed Hep3B cells was significantly higher than that against control cells, whereas a significant reduction in NK cytotoxicity was demonstrated in GEP overexpressed HepG2 cells when compared with control cells. Cytotoxicity against HCC cells increased as the number of NK cells increased (Fig. 1D). The data therefore indicated that the GEP-regulated antitumor cytotoxicity was dependent on NK cells.

**GEP differentially regulates the expression of MICA and HLA-E in HCC cells**

NK activity is regulated through integrated signaling from a panel of stimulatory and inhibitory receptors that interact with their ligands expressed on tumor cells (2). To elucidate the mechanism for GEP-regulated NK cytotoxicity, we determined the effect of GEP on HCC cell-surface expression of ligands for both stimulatory and inhibitory receptors of NK cells. MICA, ULBP1, and HLA-A/B/C are ligands for stimulatory immunoreceptors NKG2D and KIR2DL1/3DL1; HLA-E and HLA-G are ligands for inhibiting immunoreceptor NKG2A and KIR2DL4, respectively. We measured the surface expression of MICA, ULBP1, HLA-A/B/C, HLA-E, and HLA-G on Hep3B and HepG2 cells, and their GEP transfectants by flow cytometry.

Upon GEP suppression, the expression of surface MICA significantly increased (Fig. 2A), whereas surface HLA-E expression significantly decreased compared with Hep3B parental cells (Fig. 2B). When GEP was overexpressed in HepG2, the expression of surface MICA significantly decreased (Fig. 2A), whereas surface HLA-E level significantly increased compared with parental cells (Fig. 2B). However, no significant or conclusive change could be observed for ULBP1, HLA-A/B/C, and HLA-G expression upon GEP modulation in both cell lines (Fig. 2C–E). To further study the
The effect of GEP on MICA expression, we measured sMICA in the culture supernatants of HCC cells and their GEP transfecteds. GEP suppression in Hep3B decreased sMICA level in the supernatant, whereas GEP overexpression in HepG2 increased sMICA release significantly (Fig. 2F), suggesting that GEP regulated surface MICA levels at least partially by controlling its shedding from HCC cells.

Because sMICA was shown to suppress NKG2D expression on NK cells (5), we examined the effect of GEP modulation on the expression of NKG2D on NK cells (Fig. 2F). Upon coculture with GEP-suppressed Hep3B cells, surface NKG2D levels on NK cells significantly increased when compared with those cocultured with Hep3B control cells. When NK cells were cocultured with HepG2 cells with GEP overexpression, their surface NKG2D levels significantly decreased when compared with those cocultured with HepG2 control cells (Fig. 2G).

### MMPs were involved the GEP-induced MICA shedding from HCC cells

MMP9 was shown to mediate MICA shedding in various human cancers (21, 22). Therefore, an MMP inhibitor was used to investigate the role of MMP2 and MMP9 on GEP-induced MICA shedding. By gelatin zymography, MMP2 and MMP9 activity was lower in GEP-suppressed Hep3B cells (Fig. 3A). The MMP inhibitor (10 mmol/L), but not vehicle control DMSO (1 μL), prominently reduced the activity of MMP2 and MMP9 in Hep3B cells (Fig. 3A). Upon inhibition of MMP2 and MMP9 activity, GEP-induced sMICA release from HCC cells was significantly reduced in Hep3B (Fig. 3B), suggesting that MMP2 and MMP9 might, at least partially, contribute to the shedding of MICA regulated by GEP.
To validate the relationship of GEP with MMP2 and MMP9 in clinical settings, 10 HCC tumor tissues with different expression levels of GEP were examined for their expression of MMP2 and MMP9 proteins by immunohistochemical staining. Strong signals of MMP2 and MMP9 were detected in tumor tissues with high GEP expression, and weak MMP2 and MMP9 signals were observed in HCCs with low GEP level (Fig. 3C), suggesting that expression levels of GEP, MMP2, and MMP9 were positively correlated in human HCC specimens. To further investigate the association between MMP levels with GEP and MICA levels, we have categorized the 10 HCC cases into two groups according to MMP levels, high MMP (high levels of MMP2 and MMP9; \( n = 7 \)) and low MMP (low levels of MMP2 and MMP9; \( n = 3 \)) expression groups. Serum GEP and MICA levels in the corresponding patients were measured and compared. Serum levels of both GEP (Fig. 3D) and MICA (Fig. 3E) were elevated in the high MMP group when compared with the low MMP group. However, statistical significance was not reached, which could be due to small sample size.

**GEP blockade by anti-GEP antibody differentially regulates MICA and HLA-E expression of HCC cells**

To further validate the regulatory role of GEP on the expression of MICA and HLA-E, GEP blockade by the anti-GEP mAb A23 was performed in Hep3B cells. A23, but not mouse IgG, significantly suppressed GEP expression in Hep3B cells (Fig. 4A), demonstrating that the suppression was specific. A23 significantly increased surface MICA expression, whereas it decreased sMICA release and surface HLA-E expression on
Hep3B, compared with those resulting from control and iso-
type control treatment (Fig. 4A).

To confirm the regulatory role of the anti-GEP antibody A23
in vivo, the orthotopic xenografts of Hep3B cells in nude mice
(20) were examined. The expression of GEP, MICA, and HLA-E
proteins in the tumors was detected by immunohistochemical
staining. The anti-GEP antibody A23 markedly reduced the
protein expression of GEP, but increased MICA expression
compared with control treatment (Fig. 4B). However, HLA-E
protein could not be detected in the xenograft tumors (data not
shown), which was probably due to the low constitutive
expression level of HLA-E protein in Hep3B cells.

Figure 3. MMPs were involved in GEP-induced MICA shedding from HCC cells. A, gelatin zymogram gels showing inhibition of MMP2 and MMP9 in Hep3B
cells. Cells were treated with or without MMP inhibitor (10 mmol/L) or vehicle control DMSO (0.2% v/v) for 24 hours. B, MMP inhibitor significantly abrogated
the GEP-induced sMICA released from Hep3B cells. C, immunohistochemical staining showed similar protein expression patterns of GEP with MMP2 and MMP9 in HCCs (n = 10). Representative photos showing high and low expression levels. D and E, the 10 HCC cases were categorized into two groups,
high (strong and intermediate levels of MMP2 and MMP9; n = 7) and low (low levels of MMP2 and MMP9; n = 3) expression groups. Serum GEP and
MICA levels in the corresponding patients were measured by ELISA and compared. nc, shRNA negative control; P, parental cells; sh, GEP shRNA. *P < 0.05;
**P < 0.01; #P < 0.05, compared between groups as denoted by horizontal bars.

GEP blockade by anti-GEP antibody A23 sensitizes HCC
cells to NK cytotoxicity via MICA

To elucidate the functional role of A23, HCC cells were
treated with A23 or isotype control (mouse IgG1) at various
concentrations for 24 hours before assessing the effect of A23
on antitumor cytotoxicity against HCC cells. A23 and isotype
control alone did not exert any cytotoxic effect on HCC cells at
all concentrations used. Upon coculture, the cytotoxicity of NK
cells against HCC cells treated with 50 and 100 µg/mL A23 was
significantly higher than those against control cells and isotype
control–treated cells (n = 4; Fig. 5A). Thus, 50 µg/mL was
chosen for A23 treatment in subsequent experiments.

To examine the role of MICA in A23-induced NK cytotoxicity
against HCC cells, blocking antibody of MICA was used. HCC
cells were treated with A23 for 24 hours, and then incubated
with anti-MICA neutralizing antibody or isotype control
(mouse IgG2a) and cocultured with NK cells (n = 4). The
addition of anti-MICA blocking antibody, but not mouse
IgG2a isotype control, markedly reduced the A23-induced
cytotoxicity against Hep3B and HepG2 cells, suggesting that
the A23-induced cytotoxicity against HCC cells was dependent
on MICA (Fig. 5B).

To further validate the involvement of the MICA-NKG2D
signal in the A23-induced NK cytotoxicity against HCC cells, a
blocking antibody of NKG2D was used. NK cells (n = 4) were
treated with anti-NKG2D blocking antibody or isotype control
(mouse IgG2a) for 4 hours before coculture with HCC cells. The
addition of anti-NKG2D blocking antibody, but not mouse
IgG2a isotype control, significantly decreased the A23-induced
cytotoxicity against HCC cells, indicating the involvement of
NKG2D in the A23-induced effect (Fig. 5C).

Expression patterns of GEP, MICA, and HLA-E in HCC
clinical specimens

GEP and MICA transcript levels were found to be significantly elevated in HCC when compared with adjacent nontumor liver tissues and normal livers from healthy individuals (n = 80; both P < 0.001). The HLA-E transcript
level was also elevated in HCC when compared with non-

tumor liver tissues and normal livers, though statistical
significance was not reached (n = 80; P = 0.089; Fig. 6A).
By immunohistochemical staining, it was revealed that total protein expression levels of GEP, MICA, and HLA-E were stronger in HCC than in their adjacent nontumor liver tissues \( n=10 \); Fig. 6B). Serum GEP and MICA levels were found to be significantly higher in patients with HCC \( n=80 \) than those in healthy individuals \( n=30; P=0.010 \) and \( P<0.001 \), respectively; Fig. 6C).

We have shown that GEP conferred resistance to NK cytotoxicity by differentially regulating surface MICA and HLA-E expression in HCC cell lines (Figs. 1, 2, 4, and 5). To confirm the immunoevasion ability of GEP-expressing cells in clinical settings, cell-surface MICA and HLA-E were coexpressed with GEP in HCC clinical specimens by immunofluorescence staining and flow cytometry. The Expression of MICA and GEP on the cell surface was mutually exclusive \( n=3 \); Fig. 6D). However, surface HLA-E were coexpressed with GEP in HCC clinical specimens \( n=3 \); Fig. 6D). This result recapitulated that in HCC cell lines in which GEP downregulated MICA and upregulated HLA-E on the cell surface, rendering them less susceptible to NK cytotoxicity. Note that in primary HCC fresh tissues, GEP- cells contributed to less than 5% of the whole tumor bulk, which was comparable with the other hepatic CSC markers such as CD133 (25) and CD90 (26). Such a small proportion was actually in accordance with the definition of CSCs, that only a minority of cells in the tumor bulk was able to self-renew and regenerate into tumors (12).

We demonstrated earlier that GEP could promote the release of sMICA from HCC cells and suppress NKG2D expression on NK cells (Fig. 2). We investigated the relationship of serum GEP and MICA with NKG2D expression on NK cells from patients with HCC. No significant correlation was shown between NKG2D and serum GEP or serum MICA \( n=9 \); Supplementary Fig. S2). The insignificant correlation could be due to small sample size.

**Association of serum GEP and MICA levels with clinicopathologic parameters**

To investigate the potential of GEP and MICA as predictive or prognosis markers for HCC, we determined the association of serum GEP and MICA levels with clinicopathologic parameters in patients with HCC \( n=80 \). We observed that serum GEP levels significantly correlated with tumor size (Supplementary Table S1; Spearman \( \rho \) correlation coefficient \( =0.569; P<0.001 \)). A previous study has reported serum MICA levels as a potential prognostic marker for HCC (27). Here, we showed that patients with high levels of serum GEP or MICA trended toward poor recurrence-free survival, though statistical significance had not been reached with single molecule (Supplementary Fig. S3). The Kaplan–Meier plot was used to examine patient outcome in association with serum GEP and MICA levels. Patients were segregated into the low expression group (low in both serum GEP and MICA) and the high expression group (either one or both high in serum GEP and MICA), with the Youden index maximized to determine the optimal cutoff value. There were 25 patients in the low expression group (median recurrence-free survival, 51.2 months) and 55 patients in the high expression group (median recurrence-free survival, 12.8 months). Patients with high serum GEP and/or MICA levels were found to have poor recurrence-free survival in early and advanced tumor stages, patients with high serum GEP and/or MICA levels were also found to have poor recurrence-free survival (Fig. 6E).
To examine the prediction power for recurrence-free survival, Cox regression analysis was used to compare the serum GEP and MICA levels and tumor stage. By univariate Cox regression analysis, high expression level of serum GEP and/or MICA [hazard ratio (HR), 1.9; 95% confidence interval (CI), 1.0–3.5; \( P = 0.045 \)] and advanced tumor stage (HR, 2.3; 95% CI, 1.5–3.5; \( P < 0.001 \)) were significantly associated with poor recurrence-free survival. By multivariate Cox regression analysis, only high expression level of sGEP and/or sMICA (HR, 1.9; 95% CI, 1.0–3.5; \( P = 0.045 \)), but not advanced tumor stage (\( P = 0.126 \)), was found to be independent prognostic factors for recurrence-free survival (Supplementary Table S2). The above findings showed that high sGEP and/or sMICA levels influenced the prognosis of patients with HCC undergoing curative partial hepatectomy.

Discussion

As a hallmark of tumor progression and recurrence, tumors have developed diverse mechanisms to evade the immune system (13). Tumor cells have evolved to downregulate their surface MICA by proteolytic shedding, therefore reducing their susceptibility to NKG2D-mediated NK cytotoxicity (5). In this study, we showed that GEP downregulated MICA levels on HCC cell surface, therefore rendering GEP-expressing cells less susceptible to NK cytotoxicity. Engineered GEP overexpression promoted the production of sMICA from HCC cells. sMICA released from tumor cell surface not only blocked the NKG2D-binding site for other NKG2D ligands, but it also suppressed NKG2D expression on NK cells (5). Production of sMICA therefore represents a mechanism for tumor immunoevasion (28, 29). Here, we also showed that upon coculture with HCC cells overexpressing GEP, surface NK2D on NK cells...
Expression patterns of GEP, MICA, and HLA-E in HCC clinical specimens. A, GEP, MICA, and HLA-E mRNA levels were upregulated in HCC ($n = 80$) compared with the paralleled adjacent nontumor liver tissues ($n = 80$) and normal livers from healthy individuals ($n = 30$; $P = 0.000$, $0.000$, and $0.089$, respectively). B, immunohistochemical staining showed stronger expression of GEP, MICA, and HLA-E proteins in HCC than in the paralleled adjacent nontumor liver tissues. C, serum GEP (sGEP) and MICA (sMICA) levels were significantly higher in patients with HCC ($n = 80$) than those of healthy individuals ($n = 30$; $P = 0.010$ and $P < 0.001$, respectively). D, liver tissues were digested into disaggregated cells, and assessed for the expression of surface MICA, HLA-E, and GEP. Expression levels of surface MICA and GEP were mutually exclusive (left). Surface HLA-E coexpressed with GEP (right). Table summarizes the mean percentage of positive cells ± SD from three HCC clinical specimens, respectively. E, Kaplan–Meier recurrence-free survival plot according to serum GEP (sGEP) and MICA (sMICA) levels. Patients ($n = 80$) were segregated into the low expression group (low in both sGEP and sMICA) and the high expression group (either one or both high in sGEP and sMICA), with the Youden index maximized to determine the optimal cutoff value. There were 25 patients in the low expression group (median recurrence-free survival, 51.2 months) and 55 patients in the high expression group (median recurrence-free survival, 12.8 months). Patients with high expression levels of sGEP and/or sMICA (sGEP/MICA high) were found to have poor recurrence-free survival (log-rank test, $P = 0.042$). When the patients were segregated into early and advanced tumor stages, patients with high sGEP and/or sMICA (sGEP/MICA high) were also shown to have poor recurrence-free survival (log-rank test, $P = 0.032$).
significantly decreased (Fig. 2G). Hence, GEP might confer immunoevasion ability to HCC cells by promoting the shedding of surface MICA, which blocks NKG2D binding and perturbs NK cytotoxicity.

sMICA levels were frequently elevated in patients with advanced HCC (3, 27, 30–32), and such elevation was associated with downregulated NKG2D expression and impaired NK activity in HCC (3). sMICA was reported as predictive and prognostic marker in hepatitis B virus (HBV)-induced HCC and advanced HCC (27, 30). To investigate the potential of sGEP and sMICA as predictive or prognosis markers for HCC, we determined the association of sGEP and sMICA levels with clinicopathologic parameters in patients with HCC (n = 80). We found that sGEP levels significantly correlated with tumor size; patients with high sGEP and/or sMICA have poor recurrence-free survival in early and advanced tumor stages. Further analysis by Cox regression confirmed that high sGEP and/or sMICA levels were independent prognostic factors for recurrence-free survival. Therefore, sGEP and/or sMICA levels can be used as potential prognostic markers for patients with HCC.

Proteolytic cleavage and/or exosome secretion has been reported for shedding of MICA (29, 33). MMP9 and MMP14 have been shown to mediate MICA shedding in various human cancers (21, 22). MMP9 is produced in a latent form, pro-MMP9, which requires activation to achieve catalytic activity. GEP was reported in an ovarian cancer cell line model to regulate activation of MMP2 (23), which, in turn, activates pro-MMP9 (24). Therefore, it is possible that GEP promotes shedding of MICA from HCC cells by regulating MMP2 activation. Here, we showed by gelatin zymography that GEP regulated the activity of MMP2 and MMP9. Inhibition of MMP activity could significantly reduce GEP-induced MICA shedding from HCC cells. Further characterization in human HCC specimens demonstrated the association between the expression of GEP, MICA, MMP2, and MMP9. These findings suggested that GEP promoted the shedding of MICA from HCC cells at least partially through regulating MMP2 and MMP9 activity.

In addition to MICA, members of the unique long 16-binding protein (ULBP) family are also important ligands for NKG2D in humans (34). ULBP1 was reported to be expressed abundantly in HCC, and the loss of ULBP1 expression was significantly associated with an early recurrence of HCC after hepatic resection (35). Here, we studied the relationship between GEP and ULBP1 expression in HCC cell lines. Although GEP suppression significantly induced the surface expression of ULBP1, GEP overexpression did not affect ULBP1 expression. It is possible that the lack of correlation results from a small sample size and more HCC cell lines should be included for further study to delineate their relationship.

Upregulation of HLA-E was suggested to represent a mechanism of tumor escape from NK cytotoxicity in ovarian cancer and melanoma (36, 37). HLA-E mRNA was reported to be upregulated in HCC tissues when compared with their adjacent nontumor counterparts (38). Furthermore, HLA-E polymorphism was associated with risk of hepatitis B or HCC (39). However, the role of HLA-E in HCC pathogenesis and the cell populations overexpressing HLA-E has not been elucidated in HCC. We showed that GEP expression upregulated surface expression of HLA-E on HCC cells. Therefore, HLA-E might play a role in HCC pathogenesis by promoting the ability of GEP-expressing cells to evade the immune system.

In summary, we have demonstrated that GEP rendered HCC cells resistant to NK cell immunosurveillance through the MICA–NKG2D pathway. GEP blockade by mAb A23 could significantly sensitize HCC cells to antitumor cytotoxicity. Targeting GEP by A23, therefore, represents a novel therapeutic tool to suppress immunoevasion and eradicate the aggressive GEP-expressing CSCs in HCC. Because A23 can enhance NK sensitivity of HCC cells, an additional antitumor effect against HCC cells will be expected if hepatic NK cells are efficiently activated during A23 treatment. The combination therapy of anti-GEP targeted therapy and immunotherapy targeting activation of NK cells might improve the antitumor effect against unresectable HCC. Because we have shown that GEP expression induces shedding of MICA from HCC cells, patients with a higher sMICA level in their serum are likely to be more responsive to the anti-GEP mAb treatment. This will provide a basis for designing a strategy to define criteria to select patients who are potentially responsive to A23 treatment. This study will shed light on future preclinical studies to establish effective therapies targeting the highly aggressive CSCs and may bring new hope for improving treatment for patients with HCC.

Disclosure of Potential Conflicts of Interest

The sponsors had no role in study design as well as in data collection, analysis, and interpretation.

Authors’ Contributions

Conception and design: P.F.Y. Cheung, S.T. Cheung
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