

# Anticancer Chemotherapy Inhibits MHC Class I–Related Chain A Ectodomain Shedding by Downregulating ADAM10 Expression in Hepatocellular Carcinoma

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## Abstract

MHC class I–related chain A (MICA) is a ligand for the NKG2D-activating immunoreceptor that mediates activation of natural killer (NK) cells. The ectodomain of MICA is shed from tumor cells, which may be an important means of evading antitumor immunity. We previously reported that patients with hepatocellular carcinoma (HCC) display high levels of soluble MICA in circulation, which could be downregulated by chemotherapy. The present study shows that anti-HCC drugs suppress MICA ectodomain shedding by inhibiting expression of a disintegrin and metalloproteinase 10 (ADAM10). Both ADAM10 and CD44, a typical substrate of the ADAM10 protease, were expressed in human HCC tissues and HCC cells but not in normal liver tissues or cultured hepatocytes. Small interfering RNA–mediated knockdown experiments revealed that ADAM10 is a critical sheddase for both MICA and CD44 in HCC cells. Of interest is the finding that epirubicin clearly downregulated ADAM10 expression and MICA shedding in HCC cells; its suppressive effect on MICA shedding was abolished in ADAM10-depleted cells. Epirubicin treatment also enhanced the NKG2D-mediated NK sensitivity of HCC cells. Patients with HCC had significantly higher levels of serum-soluble CD44, which correlated well with serum-soluble MICA levels, thus suggesting a close link between ADAM10 activity and MICA shedding in these patients. Soluble MICA and CD44 levels were downregulated with a significant correlation in patients treated by transarterial chemoembolization using epirubicin. In conclusion, anticancer drugs can modulate expression of ADAM10, which is critically involved in MICA ectodomain shedding. Epirubicin therapy may have a previously unrecognized effect on antitumor immunity in HCC patients. [Cancer Res 2009;69(20):8050–7]

## Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer deaths worldwide. Chronic liver disease caused by hepatitis virus infection and nonalcoholic steatohepatitis leads to a predisposition for HCC, with liver cirrhosis, in particular, being considered a premalignant condition (1, 2). With regard to

treatment, surgical resection or percutaneous techniques such as ethanol injection and radiofrequency ablation are considered to be choices for curable treatment of localized HCC, whereas transcatheter arterial chemoembolization (TACE) is a well-established technique for more advanced HCC (3). The liver contains a large compartment of innate immune cells [natural killer (NK) cells and natural killer T cells] and acquired immune cells (T cells; refs. 4, 5), but the activation of these immune cells after HCC treatments remains unclear. If such treatments can efficiently activate abundant immune cells in the liver, this could lead to the establishment of attractive new strategies for HCC treatment.

MHC class I–related chain A and B (MICA and MICB) are ligands for NKG2D expressed on a variety of immune cells (6). In contrast to classic MHC class I molecules, MICA/B are rarely expressed on normal cells but frequently on tumor cells (7–10). The engagement of MICA/B and NKG2D strongly activates NK cells and costimulates T cells, enhancing their cytolytic activity and cytokine production (11). Thus, the MICA/B–NKG2D pathway is an important mechanism by which the host immune system recognizes and kills transformed cells (12). In addition to those membrane-bound forms, MICA/B molecules are also cleaved proteolytically from tumor cells and appear as soluble forms in sera of patients with malignancy (13–15). Soluble MICA/B in circulation downregulates NKG2D expression and disturbs NKG2D-mediated antitumor immunity (9, 10, 13). We previously reported that soluble MICA could be detected in sera of HCC patients (16) and that TACE treatment reduces the levels of soluble MICA and thereby upregulates the expression of NKG2D (17). Thus, cancer therapy may have a beneficial effect on NKG2D-mediated immune responses.

The release of soluble MICA/B from tumor cells is impaired by metalloproteinase inhibitors, suggesting the involvement of members of the metzincin superfamily, such as ADAM proteins (14, 18). In addition, ERp5, related to protein disulfide isomerase, is required for the MICA shedding as it reduces disulfide bond of the  $\alpha 3$  domain of MICA (19). Although it may not be a direct protease for MICA, it may enable proteolytic cleavage through conformational change. Recently, it was reported that MICA shedding of 293T fibroblast cells and HeLa cervical cancer cells was inhibited by silencing of the ADAM10 and ADAM17 proteases (20). This suggests that ADAM family proteins may be a therapeutic target for enhancing antitumor immunity, but how to therapeutically modulate these proteins is still not clear. Furthermore, it remains to be determined whether ADAMs can regulate MICA shedding in a clinical setting.

In the present study, we showed that ADAM10, but not ADAM17, was critically required for MICA shedding in human HCC cells. Of importance is the discovery that epirubicin, a widely used anti-HCC drug, was capable of downregulating ADAM10 expression and

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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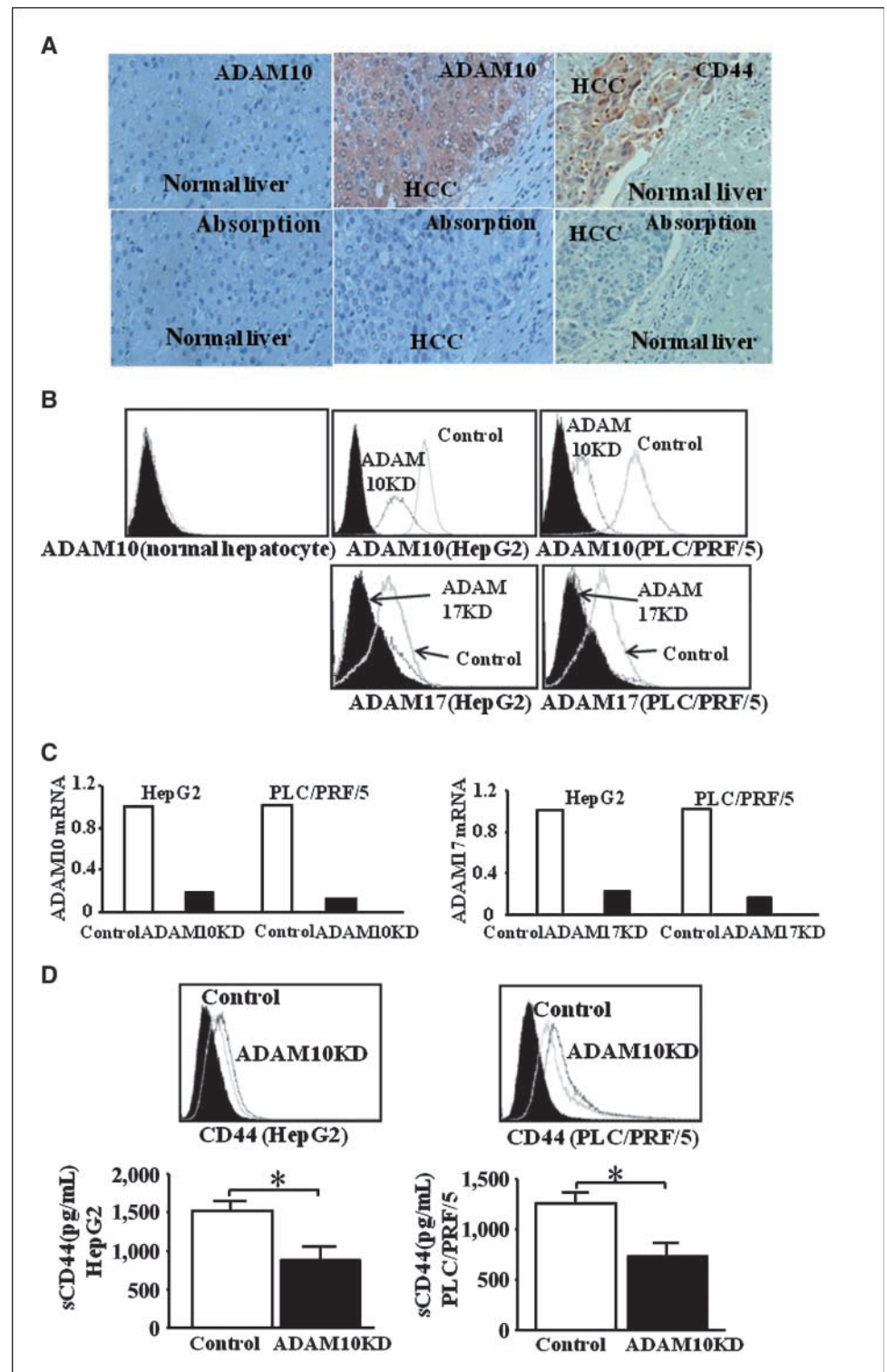
activity in HCC cells; it can thus inhibit MICA shedding and enhance NK sensitivity. ADAM10 was immunohistochemically detected in HCC tissues and a correlation was observed between soluble MICA levels and ADAM10 activity determined by soluble CD44 levels in HCC patients. The present study sheds light on previously unrecognized effects of an anticancer drug on modulating ADAM family proteins and MICA shedding and thus

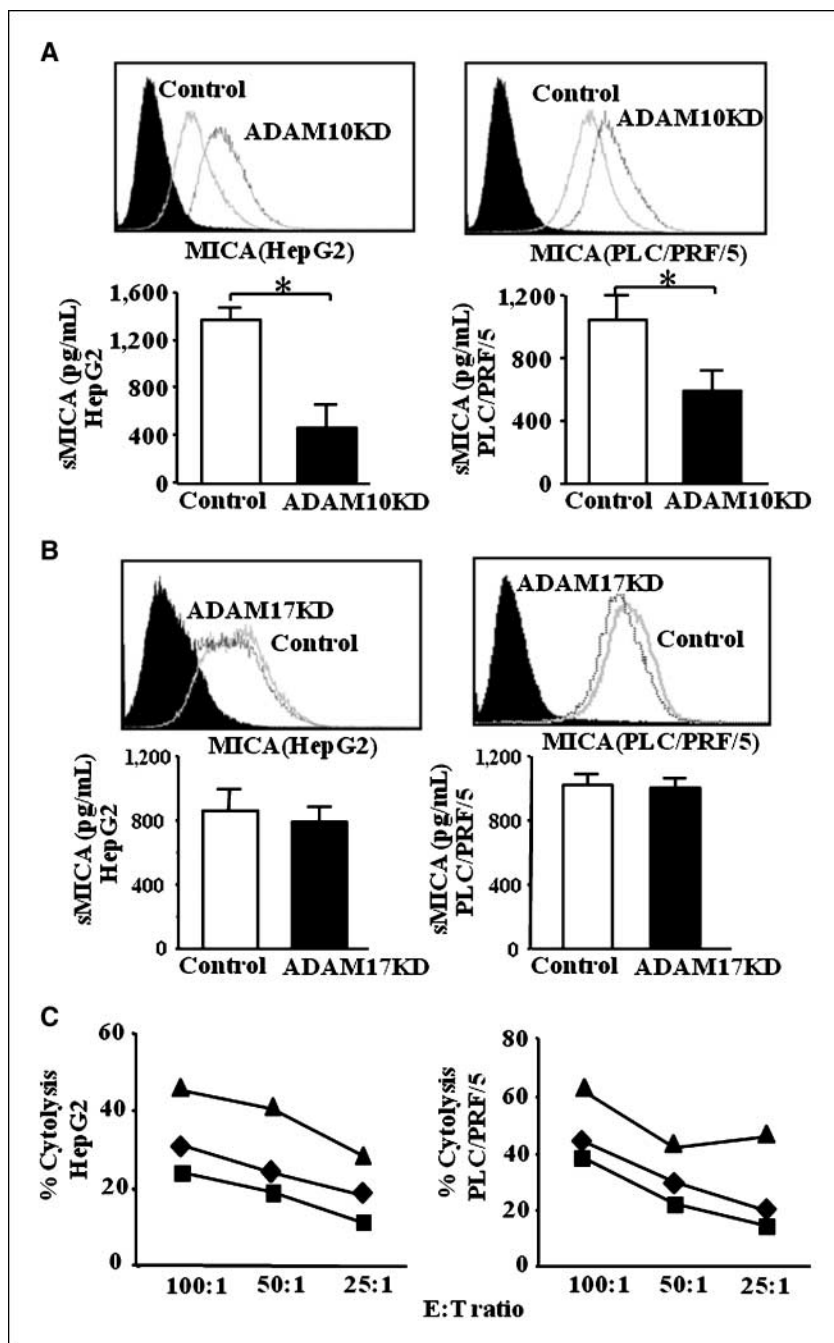
suggests a promising aspect for chemoimmunotherapy against human HCC.

## Materials and Methods

**Liver tissues and immunohistochemistry.** Human HCC tissues ( $n = 8$ ) and normal liver tissues ( $n = 2$ ) obtained at surgical resection were used. Informed consent, under an institutional review board-approved protocol,

**Figure 1.** Expression of ADAM10 and CD44 in human HCC tissues and ADAM10 or ADAM17 knockdown in human HCC cells. **A**, immunohistochemical detection of ADAM10 and CD44 in human HCC tissues ( $n = 8$ ) and normal liver tissues ( $n = 2$ ). Liver sections were stained with the corresponding antibodies (*top panels*). Both primary antibodies were incubated with recombinant CD44 and ADAM10 proteins and then applied to liver sections in parallel as the absorption test (*bottom panels*). Representative images are shown. **B** and **C**, expression of ADAM10 or ADAM17 in human primary hepatocyte and HCC cell lines (*HepG2* and *PLC/PRF/5*). Cells were treated with ADAM10 siRNA, ADAM17 siRNA, or control siRNA, and subjected to analysis of ADAM10 or ADAM17 expression by flow cytometry (**B**) or real-time RT-PCR (**C**). *Histograms*, anti-ADAM10 or anti-ADAM17 staining of ADAM10 or ADAM17 siRNA-treated cells (*ADAM10KD* or *ADAM17KD*, *black dotted line*) and control siRNA-treated cells (*Control*, *gray line*), respectively. *Closed histograms*, control IgG staining. **D**, the expression of membrane-bound CD44 on HCC cells treated with ADAM10 siRNA (*ADAM10KD*, *black line*) or control siRNA (*Control*, *gray line*) was evaluated by flow cytometry (*top panels*). *Closed histograms*, control IgG staining. Soluble CD44 (*sCD44*) production from HCC cells treated with ADAM10 siRNA or control siRNA were evaluated by specific ELISA (*bottom panels*). \*,  $P < 0.05$ .





**Figure 2.** Expression of MICA in ADAM10 or ADAM17 knockdown HCC cells and NK sensitivity in ADAM10 knockdown HCC cells. *A* and *B*, the expression of membrane-bound MICA on HCC cells treated with ADAM10 siRNA (*ADAM10KD*, black line; *A*), ADAM17 siRNA (*ADAM17KD*, black line; *B*), or control siRNA (*Control*, gray line) was evaluated by flow cytometry (top panels). Closed histograms, control IgG staining. Soluble MICA (sMICA) production from HCC cells treated with ADAM10 siRNA (*A*), ADAM17 siRNA (*B*), or control siRNA were evaluated by specific ELISA (bottom panels). \*,  $P < 0.05$ . *C*, HCC cells treated with ADAM10 siRNA or control siRNA were subjected to  $^{51}\text{Cr}$ -release assay against NK cells. Cytolytic activity of NK cells against control HCC cells (■) or ADAM10 knockdown HCC cells without (▲) or with blocking antibody of MICA/B (6D4; ◆). Representative results are shown. Similar results were obtained from three independent experiments.

was obtained from all patients before sample acquisition. Liver sections were subjected to immunohistochemical staining using the ABC procedure (Vector Laboratories, Burlingame, CA). The primary antibodies used were anti-ADAM10 and anti-CD44 (R&D Systems). To confirm the specificity of the staining, primary antibodies were incubated with recombinant CD44 or ADAM10 protein (R&D Systems, Minneapolis, MN) for 3 h and then applied onto liver sections in parallel with staining of the primary antibodies as the absorption test.

**HCC cell lines.** Human HCC cell lines HepG2 and PLC/PRF/5 were purchased from the American Type Culture Collection and were cultured with DMEM supplemented with 10% fetal bovine serum (GIBCO/Life Technologies, Grand Island, NY) in a humidified incubator at 5%  $\text{CO}_2$  and 37°C.

**RNA silencing.** The small interfering RNA (siRNA) method was used to knockdown ADAM10 and ADAM17. Stealth RNAi oligonucleotide targeting ADAM10 or ADAM17 and scrambled oligonucleotides as a

control were purchased from Invitrogen (Carlsbad, CA). Cells were transfected by RNAi Max transfection reagent (Invitrogen) with 50 nmol/L siRNA. At 24 h posttransfection, the cells were analyzed for specific depletion of the mRNAs of ADAM10 and ADAM17 by real-time reverse transcription-PCR (RT-PCR; Applied Biosystems, Foster City, CA). The following siRNAs were used: ADAM10, 5'-AUAUCUGGGCAAUCACAGCUUCUCG-3'; scramble control, 5'-AUACUUGGUCAACGCACUUCGAUGG-3'; ADAM17, 5'-UGAACAAAGCUCUUCAGGUGGUUCUC-3'; scramble control, 5'-UGAAUAGAACUCUCGACUGGUGUC-3'.

**ELISA.** The supernatants of cultured cells were harvested at 24 h after transfection with siRNA as well as sera from HCC patients ( $n = 97$ ) and age-matched healthy volunteers ( $n = 32$ ) were subjected to analysis of soluble MICA and soluble CD44 levels. Informed consent, under an institutional review board–approved protocol, was obtained from all patients before sample acquisition. The levels of soluble MICA and soluble CD44 were

determined by DuoSet MICA eELISA kit (R&D Systems) and soluble CD44 ELISA (Abcam, Cambridge, MA), respectively.

**Flow cytometry.** For the detection of membrane-bound MICA and CD44, cells were incubated with an anti-MICA-specific antibody (2C10, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-CD44 antibody (R&D Systems) and stained with phycoerythrin (PE)-goat anti-mouse immunoglobulin (Beckman Coulter) as a secondary reagent and then subjected to flow cytometric analysis. For the detection of ADAM10 or ADAM17, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences, San Jose, CA) and stained with PE-conjugated anti-ADAM10 or anti-ADAM17 antibody (R&D Systems). Flow cytometric analysis was performed using a FACScan flow cytometer (Becton Dickinson).

**Plasmid construction of pMyc-MICA.** MICA full coding cDNA was isolated from Huh7, human HCC cells, using a conventional RT-PCR method (Supplementary Fig. S1, DDBJ/EMBL/Genbank accession number AB506764) and inserted into the *Hind*III-*Xba*I site of pcDNA3 (Invitrogen). A C-myc tag was placed between the leader peptide and the  $\alpha$ 1 domain of MICA by site-specific mutagenesis using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) referred to as pMyc-MICA. Cells were transfected with pMyc-MICA using a Lipofectamine LTX reagent (Invitrogen). The green fluorescent protein (GFP)-expressing vector (pEGFP-C1, Clontech, Mountain View, CA) was cotransfected to evaluate the transfection efficiency.

**Immunoprecipitation.** Cells or tissues were homogenized in lysis buffer containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50  $\mu$ g/mL aprotinin, 100  $\mu$ g/mL phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, and PBS. To the cell supernatants, 0.5% NP40 and a cocktail of protease inhibitors were added. The protein contents of the samples were determined by BCA protein assay kit (Pierce, Rackford, IL). Immunoprecipitation with anti-c-Myc beads was performed for 1 h at 4°C. Immunocomplexes were eluted by a c-Myc-tagged peptide solution (MBL, Woburn, MA). The samples after immunoprecipitation were treated with 250 mU of N-glycosidase F (Roche, Mannheim, Germany) for 3 h at 37°C.

**Western blotting.** The total cellular protein was electrophoretically separated using SDS-12% polyacrylamide gels and transferred onto polyvinylidene difluoride membrane. The membrane was blocked in TBS-Tween containing 5% skim milk for 1 h and then probed with anti-Myc mouse monoclonal antibody (Cell Signaling Technology, Danvers, MA) at 4°C overnight. Horseradish peroxidase-conjugated anti-rabbit antibody and SuperSignal West Pico System (Pierce) were used for the detection of blots.

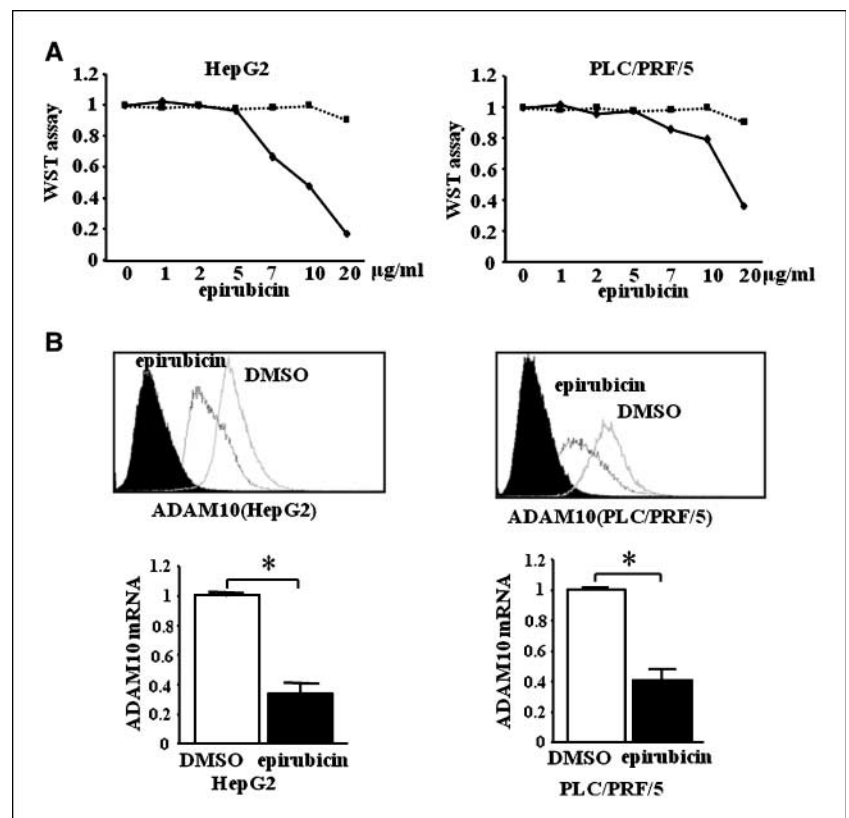
**Real-time RT-PCR.** Total RNA was isolated using RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan) and was reverse transcribed using SuperScript III First-Strand Synthesis System (Invitrogen). The mRNA levels were evaluated using ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Ready-to-use assays (Applied Biosystems) were used for the quantification of ADAM10 (Hs00153853\_m1), ADAM17 (Hs00234221\_m1), MICA (Hs00792195\_m1),  $\beta$ -actin (Hs99999903\_m1), and CD44 (Hs00174139\_m1) mRNAs according to the manufacturer's instructions. The thermal cycling conditions for all genes were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.  $\beta$ -Actin mRNA from each sample was quantified as an endogenous control of internal RNA.

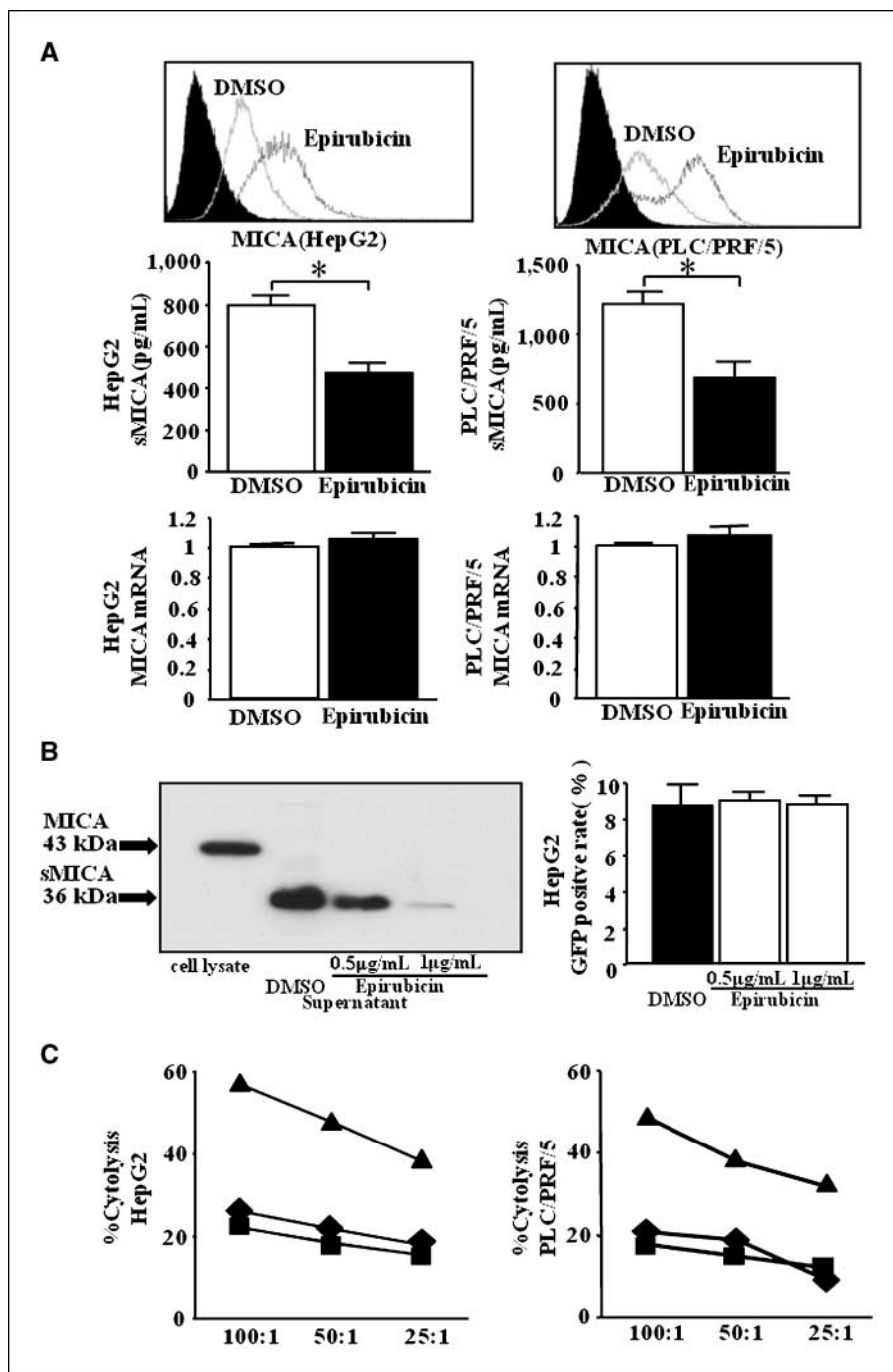
**WST-8 assay.** HepG2 and PLC/PRF/5 cells were treated with different concentrations of epirubicin for 24 h. Cell growth of epirubicin-treated HCC cells was determined by WST-8 assay (Nacalai Tesque, Kyoto, Japan) as previously described (21).

**NK cell analysis.** NK cells were isolated from human peripheral blood mononuclear cells by magnetic cell sorting using CD56 MicroBeads (Miltenyl Biotech, Auburn, CA) as previously described (16). The cytolytic ability of NK cells was assessed by 4-h <sup>51</sup>Cr-releasing assay with or without MICA/B-blocking antibody (6D4; ref. 7), which binds to the  $\alpha$ 1 and  $\alpha$ 2 domains of MICA and MICB. 6D4 was a generous gift from Drs. Veronika Groh and Thomas Spies (Fred Hutchinson Cancer Research Center, Seattle, WA).

**Statistics.** All values were expressed as the mean and SD. The statistical significance of differences between the groups was determined by applying Student's *t* test or two-sample *t* test with Welch correction after each group

**Figure 3.** Expression of ADAM10 in epirubicin-treated HCC cells. **A**, the cytotoxicity of epirubicin to human HCC cells was evaluated by WST-8 assay. Cells were treated with different doses of epirubicin (solid lines) or vehicle (DMSO; dotted lines) for 24 h, and the viability of the cells was evaluated by the WST-8 assay. **B**, ADAM10 expression of epirubicin-treated HCC cells. Cells were treated with a nontoxic dose of 1  $\mu$ g/mL epirubicin (black lines) or vehicle (DMSO, gray lines) for 24 h and their ADAM10 expression was evaluated by flow cytometry (top panels). Closed histograms, control IgG staining. Total RNA was extracted at 24 h of epirubicin treatment and mRNA levels of ADAM10 were evaluated by real-time RT-PCR (bottom panels). \*, *P* < 0.05.





**Figure 4.** Expression and shedding of MICA in epirubicin-treated HCC cells. *A*, HCC cells were treated with a nontoxic dose of 1  $\mu$ g/mL epirubicin (black lines) or vehicle (DMSO, gray lines) for 24 h and their expression of membrane-bound MICA and MICA mRNA was evaluated by flow cytometry (top panels) and real-time RT-PCR (bottom panels), respectively. Closed histograms, control IgG staining in flow cytometry. At the same time, 24-h culture supernatants were subjected to the analysis of soluble MICA (sMICA) levels by ELISA (middle panels). \*,  $P < 0.05$ . *B*, HepG2 cells were transfected with pMyc-MICA and pEGFP-C1, cultured with 0.5 to 1  $\mu$ g/mL epirubicin or vehicle (DMSO) for 24 h. Cell lysates from HepG2 cells and 24-h culture supernatants of epirubicin- or vehicle-treated HepG2 cells were immunoprecipitated with anti-Myc. The resulting immunoprecipitates were eluted, treated with N-glycanase, and subjected to Western blot analysis for MICA (left). Transfection efficacies were equal in all treatment groups as evidenced by similar GFP-positive cell rates (right). *C*, the cytotoxic activity of NK cells against HCC cells. Vehicle-treated cells (■) or epirubicin-treated cells without (▲) or with blocking antibody of MICA/B (6D4; ◆) were subjected to  $^{51}\text{Cr}$ -release assay. Representative results are shown. Similar results were obtained from three independent experiments.

had been tested with equal variance and Fisher's exact probability test. We defined statistical significance as  $P < 0.05$ .

## Results

**ADAM10 and CD44 are overexpressed in human HCC.** ADAM10 was detected in all human HCC tissues tested by immunohistochemistry but not in normal liver tissues (Fig. 1A). Flow cytometric analysis revealed that ADAM10 was strongly expressed in a variety of HCC cell lines, including HepG2, PLC/PRF/5 (depicted in Fig. 1B), and Hep3B (data not shown), but faintly in primary hepatocytes. CD44, a typical substrate of the ADAM10 protease, was also expressed in all human HCC tissues

but not in normal liver tissues (Fig. 1A). The data suggest that overexpression of ADAM10 and CD44 is a characteristic of human HCC like other malignancies (22).

**ADAM10 is involved in MICA shedding of HCC cells but ADAM17 is not.** To examine the involvement of ADAM family proteins in MICA ectodomain shedding, ADAM10 or ADAM17 were knocked down in HCC cells using a siRNA-mediated procedure. ADAM10 expression was clearly suppressed in HepG2 cells and PLC/PRF/5 cells at both mRNA and protein levels (Fig. 1B and C). Both cell lines expressed CD44 on the cellular surface and produced significant levels of soluble CD44 (Fig. 1D), indicating that CD44 is expressed and shed from those cell lines. ADAM10 knockdown (KD)

led to an increase in CD44 expression on HCC cells and a decrease in soluble CD44 levels in culture supernatants (Fig. 1D). Because ADAM10 has been established as being a sheddase for CD44, siRNA-mediated knockdown of ADAM10 suppressed not only the expression but also the activity of ADAM10 in HCC cells. HepG2 and PLC/PRF/5 cells also expressed ADAM17, which was clearly knocked down by a siRNA-mediated procedure (Fig. 1B).

HepG2 cells and PLC/PRF/5 cells expressed membrane-bound MICA and also produced soluble MICA (Fig. 2A). Knockdown of ADAM10 for both cell lines clearly upregulated MICA expression on their cellular surface and downregulated soluble MICA levels in their culture supernatant (Fig. 2A). In contrast, knockdown of ADAM17 did not affect the expression of membrane-bound MICA or the production of soluble MICA (Fig. 2B). We also examined the involvement of ADAM17 in MICA shedding of phorbol 12-myristate 13-acetate (PMA)-stimulated HCC cells because ADAM17 is considered to primarily affect stimulated shedding. The expression of membrane-bound MICA and the soluble MICA production were equal between PMA-stimulated ADAM17KD-HCC cells and control HCC cells (Supplementary Fig. S2). Thus, ADAM10, but not ADAM17, is critically involved in the shedding of MICA in HCC cells.

We next evaluated the cytolytic activity of NK cells against HCC cells. The cytolytic activity of NK cells against ADAM10KD-HepG2 cells was higher than that against control HepG2 cells. This activity was inhibited by blocking of anti-MICA/B antibody, suggesting that the increase of NK sensitivity depended on the increased expression of membrane-bound MICA on ADAM10KD-HepG2 cells, although we could not exclude the possibility of the involvement of MICB in this cytotoxicity (Fig. 2C). Similar results were also obtained with ADAM10KD-PLC/PRF/5 cells.

**Epirubicin suppresses ADAM10 expression in HCC cells.** We examined the biological modification of human HCC cells by adding epirubicin, which is commonly used in anti-HCC chemotherapy. We first examined the cytotoxicity of epirubicin to human HCC cells by WST-8 assay. Adding  $>5 \mu\text{g/mL}$  of epirubicin resulted in a significant

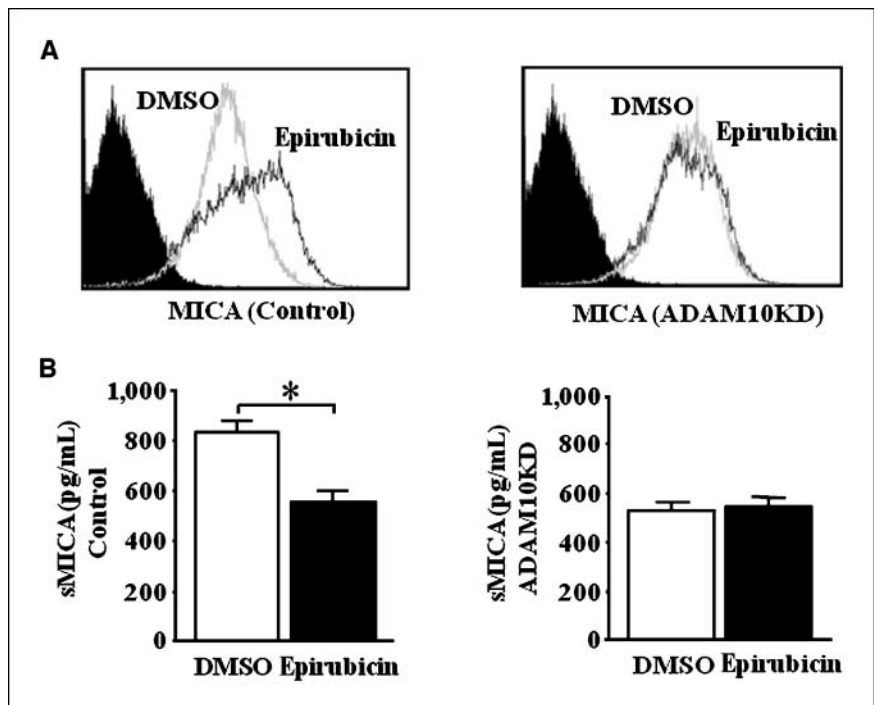
decrease in cell growth of both HepG2 and PLC/PRF/5 cells (Fig. 3B). Based on these findings, we used  $1 \mu\text{g/mL}$  of epirubicin to evaluate the biological effect on human HCC cells without toxicity. Both HepG2 cells and PLC/PRF/5 cells were cultured for 24 h with epirubicin and then subjected to analysis of ADAM10 expression. Epirubicin suppressed ADAM10 expression at the mRNA and protein levels in both cell lines (Fig. 3C). Although the data are not shown, doxorubicin also suppressed ADAM10 expression in HCC cells.

**Epirubicin inhibits MICA ectodomain shedding and enhances susceptibility to NK cells of HCC cells.** The above observations led us to investigate whether epirubicin or doxorubicin treatment would affect MICA ectodomain shedding in HCC cells. Epirubicin treatment led to an increase in membrane-bound MICA expression and a decrease in soluble MICA production in both HepG2 and PLC/PRF/5 cells (Fig. 4A). The mRNA levels of MICA did not change after exposure to epirubicin in both HCC cells (Fig. 4A). Similar data were obtained with doxorubicin-treated cells (data not shown).

To confirm whether the soluble MICA detected by ELISA was actually reflected in the cleaved form, we transfected Myc-tagged MICA into HepG2 cells and collected culture supernatants as well as cellular lysates. Immunoprecipitates from these samples with anti-Myc were subjected to Western blot analysis after treatment with N-glycosidase. MICA in the culture supernatants migrated faster than cellular MICA (Fig. 4B), indicating that the MICA detected by ELISA is actually processed and released from full-length MICA. Epirubicin treatment led to a decrease in soluble MICA protein in HepG2 cells (Fig. 4B).

We next evaluated whether the epirubicin treatment could also modify the NK sensitivity of human HCC cells. Epirubicin-treated HepG2 cells or PLC/PRF/5 cells were more susceptible to NK cells than nontreated HepG2 or PLC/PRF/5 cells (Fig. 4C). The cytolytic activity against epirubicin-treated HCC cells was significantly decreased to the control levels by adding the anti-MICA/B blocking antibody. These results showed that the addition of epirubicin enhanced the NK sensitivity of HCC cell through increased

**Figure 5.** The epirubicin-mediated modification of MICA is ADAM10 dependent. HepG2 cells were transfected with ADAM10 siRNA (*ADAM10KD*) or control siRNA (*Control*) and further cultured with  $1 \mu\text{g/mL}$  of epirubicin (*black lines*) or vehicle (DMSO, *gray line*) for 24 h. The expression of membrane-bound MICA (*MICA*) was evaluated by flow cytometry (A), and the soluble MICA (*sMICA*) production in the culture supernatant was evaluated by specific ELISA (B). Similar results were obtained from two independent experiments. \*,  $P < 0.05$ .



expression of membrane-bound MICA, although the possibility of MICB involvement could not be excluded. The doxorubicin-treated human HCC cells showed similar results to those obtained from epirubicin-treated HCC cells (data not shown).

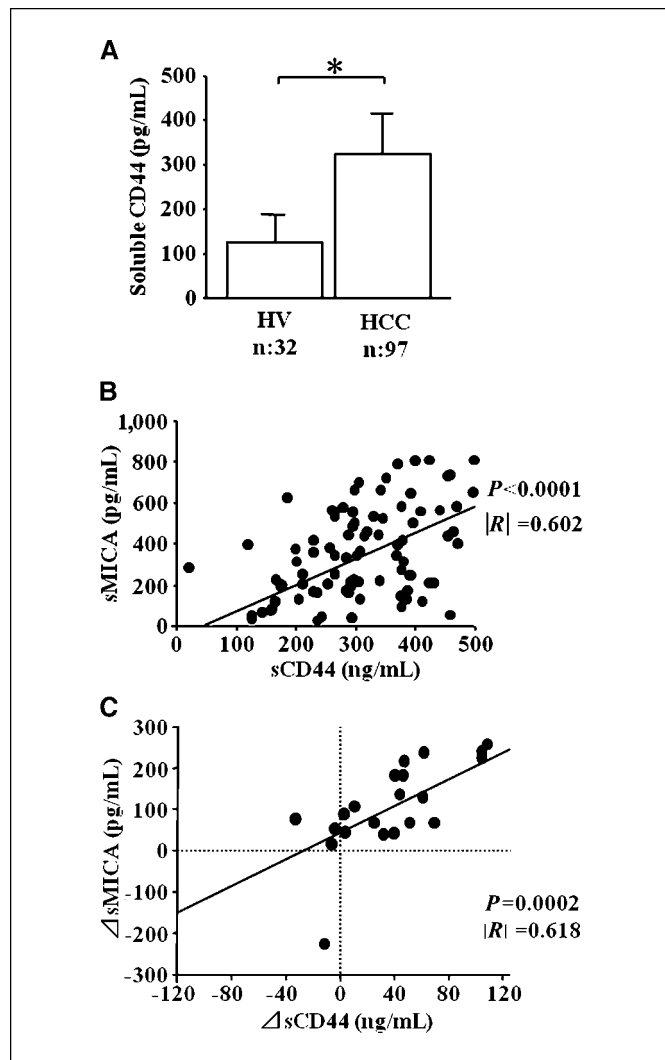
**Epirubicin inhibits MICA ectodomain shedding through suppression of ADAM10.** To examine whether the suppressive effect of epirubicin on MICA shedding occurred through downregulation of ADAM10, HepG2 cells were transfected with ADAM10 siRNA or scramble siRNA as a control and then treated with epirubicin. Consistent with earlier observations, epirubicin upregulated MICA surface expression and downregulated the levels of soluble MICA in control cells (Fig. 5). In contrast, neither upregulation of surface MICA nor downregulation of soluble MICA levels was observed in ADAM10KD-HepG2 cells. These results suggest that the suppressive effect of epirubicin on MICA shedding is mediated by ADAM10 downregulation. We also found similar results with ADAM10KD-PLC/PRF/5 cells (data not shown).

**Soluble CD44 and soluble MICA levels in patients with HCC.** We have shown that ADAM10 is expressed in human HCC tissues. However, it is not clear whether ADAM10 activity in HCC tissues is actually involved in MICA shedding in patients. Because ADAM10 was reported to be the constitutive functional sheddase of CD44 (23), we examined the soluble CD44 levels in HCC patients, which might be produced from tumor cells through ADAM10 activity. As shown in Fig. 6A, the soluble CD44 levels in HCC patients ( $n = 97$ ) were significantly higher than those in age-matched healthy volunteers ( $n = 32$ ). More importantly, soluble MICA levels in HCC patients significantly correlated with soluble CD44 levels (Fig. 6B), suggesting a close link between MICA shedding and ADAM10 activity.

We further examined soluble CD44 levels before and 2 weeks after TACE in HCC patients. Whereas the levels did not change in nontreated HCC patients during the 2-week interval ( $n = 9$ ;  $306.7 \pm 82.5$  ng/mL and  $309.9 \pm 79.9$  ng/mL after 2 weeks), they were significantly decreased in epirubicin-based TACE-treated HCC patients ( $n = 21$ ;  $339.7 \pm 78.1$  ng/mL before TACE and  $308.9 \pm 81.4$  ng/mL after TACE,  $P < 0.003$ ). The changes of soluble CD44 in TACE treatment correlated significantly with those of soluble MICA ( $P = 0.0002$ ; Fig. 6C). These results indicated that ADAM10-mediated CD44 shedding was decreased after TACE in HCC patients, implying that this reduction of ADAM10 activity might be related to the decline in MICA shedding.

## Discussion

MICA shedding is thought to be a principal mechanism by which tumor cells escape from NKG2D-mediated immunosurveillance (13). Thus, inhibition of MICA shedding should be a reasonable strategy for enhancing antitumor immunity. In the present study, we showed that ADAM10 was overexpressed in human HCC tissues and that ADAM10 knockdown resulted in increased expression of membrane-bound MICA, decreased production of soluble MICA, and upregulation of NK sensitivity of human HCC cells. These results point to ADAM10 as a therapeutic target for inhibiting MICA shedding, thereby ameliorating immunity against HCC. Waldhauer and colleagues recently showed that both ADAM10 and ADAM17 proteases are critically involved in the proteolytic release of soluble MICA of human 293T fibroblast cells and HeLa cervix carcinoma cells (20). Interestingly, in the present study, ADAM17 knockdown failed to affect MICA expression in human HepG2 cells or PLC/PRF/5 cells. Thus, ADAM10, not ADAM17, plays an essential role in the shedding of MICA in human HCC cells. Anderegg and colleagues



**Figure 6.** Correlation between soluble CD44 and soluble MICA in human HCC patients. **A** and **B**, soluble CD44 levels and MICA levels in healthy volunteers and HCC patients. Soluble CD44 levels (sCD44) and soluble MICA levels (sMICA) were determined for sera of HCC patients ( $n = 97$ ) and age-matched healthy volunteers (HV;  $n = 32$ ). **A**, comparison of sCD44 levels between groups; **B**, correlation between sCD44 levels and sMICA levels in 97 HCC patients. \*,  $P < 0.05$ . **C**, correlation of sCD44 levels and sMICA levels during TACE therapy. HCC patients ( $n = 21$ ) treated with epirubicin-based TACE therapy were enrolled and examined for sMICA and sCD44 levels before and 2 wk after therapy. Changes in sMICA ( $\Delta$ sMICA = serum level of sMICA before TACE treatment – serum level of sMICA after TACE treatment) and those in sCD44 levels ( $\Delta$ sCD44 = serum level of sCD44 before TACE treatment – serum level of sCD44 after TACE treatment) are plotted.

(23) reported that only ADAM10, not ADAM17, contributed to shedding of CD44 molecules in human melanoma cells although both ADAM10 and ADAM17 proteases were significantly expressed in human melanoma tissues, suggesting that ADAM10 and ADAM17 do not always work in a similar manner. A recent report showed that ADAM10, but not ADAM17, could directly bind to calmodulin (24), which may involve the difference of MICA cleavage between ADAM10 and ADAM17 proteases. Recently, Boutet and colleagues reported that ADAM17 regulates proteolytic shedding of the MICB protein, which is another ligand for the NKG2D receptor on immune cells (25). We previously showed that both soluble MICA and MICB significantly increased in the sera of HCC patients and that therapeutic intervention for HCC leads to reduction of soluble

MICA levels, but not of soluble MICB levels (17), suggesting a more important role of soluble MICA in regulating NKG2D expression after HCC therapy. This led us to focus on the mechanism of MICA shedding in the present study.

Our results revealed that anticancer drugs such as epirubicin and doxorubicin downregulated ADAM10 expression and activity, thereby inhibiting MICA ectodomain shedding. The ADAM family proteins, which are highly expressed in some tumors, play a role in secreting growth factors, such as HB-EGF, and migration of cells. Thus, it is speculated that these proteins could be potential targets for tumor treatment (22). The present study is the first to show that clinically available anticancer drugs have an ability to modulate the expression of ADAM family proteins. They seem to suppress ADAM10 expression at a transcriptional level, but the precise mechanism of this suppression is not yet known.

The MICA ELISA system may not equally detect all soluble MICA (MICA molecules have >60 allelic variants). Our finding that soluble MICA could be detected in all HCC patients suggests that this system was applicable for our cohort of HCC patients. However, special caution should be paid for the use of this ELISA system for widely polymorphic MICA. Because CD44 is well known to be released into circulation from tumors by proteolytic cleavage of ADAM10 (23), the activity of ADAM10 in HCC tissues may be correlated with soluble CD44 levels. If so, our data suggest a close link between ADAM10 activity and the shedding of MICA in HCC. Furthermore, the decline in soluble MICA levels correlated well with the decline in soluble CD44 levels as early as 2 weeks after epirubicin-based TACE therapy. Reducing the tumor volume by such therapy may have led to both decreases but it is also possible that epirubicin suppresses ADAM10 activity, thereby inhibiting the shedding of MICA and CD44. Epirubicin may have a previously unrecognized role in cancer therapy; that is, affecting ADAM10 activity and MICA shedding rather than simply serving as a direct toxic agent for tumor cells.

Our data suggest that anti-HCC chemotherapy could remodel HCC cells, enhancing sensitivity to NK cells by upregulating MICA

expression on the cellular surface. A concomitant decline in soluble MICA levels ameliorates NK cell ability by upregulating its NKG2D expression. We previously showed that activation of local innate antitumor immunity in liver tissues resulted in eliciting tumor-specific acquired immunity (21). If liver innate immunity is efficiently activated after anti-HCC chemotherapy, an additional antitumor effect against HCC cells could be expected. Immune modulators such as  $\alpha$ -galactosylceramide have been shown to efficiently activate liver innate immune cells, including NK cells (21, 26). The combination therapy of anti-HCC chemotherapy and immunotherapy targeting NK cells might improve the antitumor effect of unresectable HCC and the prognosis of HCC patients.

In spite of recent progress in HCC therapies, there remains significant room for improvement, especially with respect to advanced liver cancer. We have shown here that anti-HCC chemotherapy resulted in enhanced NK sensitivity of HCC cells through inhibition of the activity of ADAM10 protease followed by modification of MICA expression. These findings indicate that efficient activation of liver innate immunity after anti-HCC chemotherapy might represent a particularly promising approach to suppress tumor growth and promote regression in liver cancer patients.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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