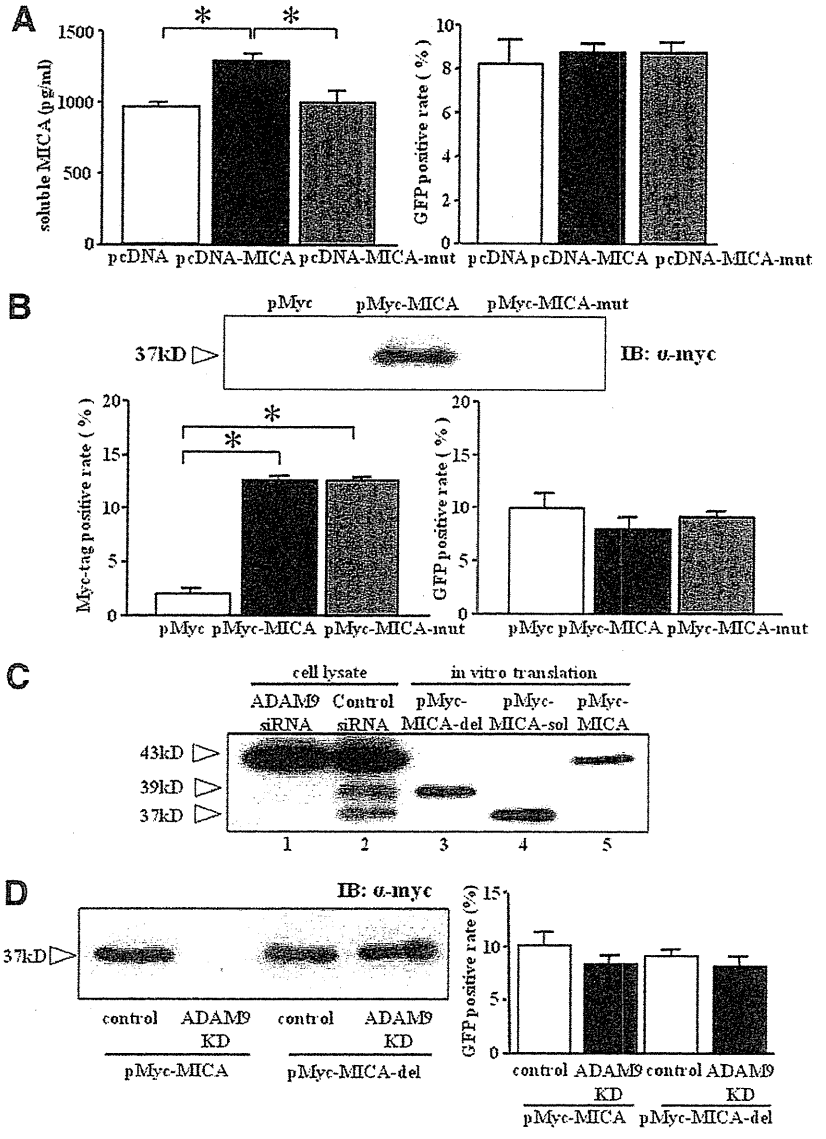

Fig. 3. Truncation of cytosolic domain of MICA by ADAM9 is essential for ectodomain shedding. (A) Blockade of ectodomain shedding of MICA by mutation at the ADAM9 recognition site. HepG2 cells were cotransfected with pcDNA3, pcDNA-MICA, or pcDNA-MICA-mut and pEGFP-C1. After 24 hours incubation, the culture media were collected and assayed for soluble MICA by ELISA. Transfection efficiencies were monitored by measuring GFP-positive cell rates by fluorescent-activated cell sorting (FACS). (B) HepG2 cells were cotransfected with pcDNA3, pMyc-MICA, or pMyc-MICA-mut and pEGFP-C1. After 24 hours incubation, the culture media were immunoprecipitated with anti-myc-tag mAb, deglycosylated with glycanase, and then the expression of myc-tagged MICA was detected by immunoblot (upper panel). Expression of myc-tagged MICA and GFP in the cells was confirmed by FACS (lower panel). (C) Western blotting of cell lysate of HepG2 cells and *in vitro* translation of pMyc-MICA vectors. HepG2 cells were transfected with ADAM9 siRNA (lane 1) or control siRNA transfection (lane 2) followed by transfection of pMyc-MICA. The cell lysates were deglycosylated by tunicamycin as described in Materials and Methods. *In vitro* translation was carried out using pMyc-MICA-del (lane 3), pMyc-MICA-sol (lane 4), and pMyc-MICA (lane 5). Myc-tagged MICA in the samples was detected by immunoblot using anti-myc-tag mAb. (D) No relevance of ADAM9 found to shedding of MICA lacking the cytosolic domain. HepG2 cells were transfected with pMyc-MICA or pMyc-MICA-del after ADAM9 knockdown. The culture media were immunoprecipitated with anti-myc-tag mAb and deglycosylated, then the expression of myc-tagged MICA was detected by immunoblot. Transfection efficiencies were monitored by measuring GFP-positive cell rates by FACS.

raised: (1) ADAM9 activates some protease, which cleaves MICA in the extracellular domain to produce soluble MICA, or (2) 39 kD MICA, which lacks a cytosolic domain, is susceptible to extracellular domain cleavage by some protease. To clarify this, we transfected pMyc-MICA or pMyc-MICA with a stop codon at the ADAM9 cleavage site (pMyc-MICA-del) into control HepG2 or ADAM9KD HepG2 cells. Soluble MICA was detected in the supernatants of pMyc-MICA–transfected control cells, but not of pMyc-MICA–transfected ADAM9KD cells (Fig. 3D). In contrast, pMyc-MICA-del transfection resulted in ectodomain shedding of MICA irrespective of ADAM9 activity. Accordingly, these results suggested that ADAM9 does not directly cleave MICA at the extracellular domain. More importantly, the ADAM9-dependent truncation of cytosolic domain of MICA rendered this molecule susceptible to cleavage to produce soluble MICA.

ADAM9 Is Overexpressed in Human HCC and NK Sensitivity of ADAM9KD HCC Cells. ADAM9 was detected in all human HCC tissues (N = 11) tested by immunohistochemistry, but not in normal liver tissues (Fig. 4A). The data suggest that overexpression of ADAM9 is a characteristic of human HCC, similar to other malignancies.[23] We next evaluated the cytolytic activity of NK cells against HCC cells. The cytolytic activity of NK cells against ADAM9KD-HepG2 or PLC/PRF/5 cells was significantly higher than that against control cells. The cytolytic activities of NK cells against ADAM9KD cells were inhibited by blocking of anti-MICA/B Ab in both HepG2 and PLC/PRF/5 cells, suggesting that the increase of NK sensitivity depended on the

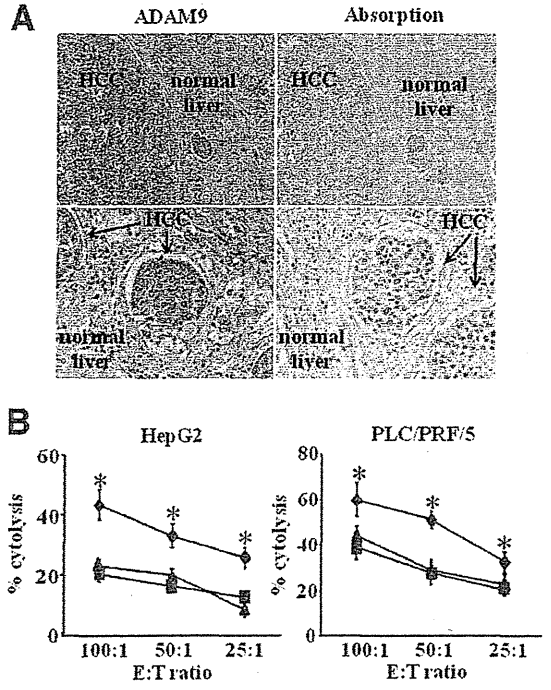

Fig. 4. Expressions of ADAM9 in human HCC tissues and NK sensitivity in ADAM9 KD HCC cells. (A) Immunohistochemical detection of ADAM9 in human HCC tissues (N = 11). Liver sections were stained with the corresponding antibodies (left panels). Primary antibodies were incubated with recombinant ADAM9 protein and then applied to liver sections in parallel as the absorption test (right panels). Representative images are shown. (B) HCC cells (HepG2 and PLC/PRF/5) treated with ADAM9 siRNA or control siRNA were subjected to ^{51}Cr-release assay against NK cells. The cytolytic activity of NK cells against control HCC cells (■) or ADAM9 KD HCC cells without (◆) or with blocking antibody of MICA/B (6D4) (▲). *$P < 0.05$ versus the cytolytic activity of NK cells against control HCC cells at each respective E:T ratio. Representative results are shown. Similar results were obtained from three independent experiments.

increased expression of membrane-bound MICA on ADAM9KD HCC cells (Fig. 4B), although we could not exclude the possible involvement of MICB in this cytotoxicity.

Sorafenib Inhibits MICA Ectodomain Shedding and Enhanced Susceptibility to NK Cells of HCC Cells. The above observations led us to investigate whether sorafenib treatment would affect MICA ectodomain shedding in HCC cells. We first examined the cytotoxicity of sorafenib to human HepG2 cells by WST-8 assay. Adding more than 4 μmol/L of sorafenib resulted in a significant decrease in cell growth of HepG2 cells (Fig. 5A). Based on these findings, we used 1 μmol/L of sorafenib to evaluate the biological effect on HepG2 cells without toxicity. ADAM9 expressions in sorafenib-treated HepG2 cells were decreased in a dose-dependent manner at protein levels (Fig. 5B). The mRNA of ADAM9 was also decreased in sorafenib-treated HepG2 cells (Fig. 5B). We previously reported that anti-HCC chemotherapy including epirubicin and doxorubicin reduced ADAM10 expression resulting in inhibiting the shedding of MICA on human HCC cells.[20] We also examined ADAM10 expression in sorafenib-treated HepG2 cells. The protein and mRNA expressions of ADAM10 did not change between sorafenib-treated HepG2 cell and non-treated HepG2 cells (Supporting Fig. 1).

Sorafenib treatment also led to an increase in membrane-bound MICA expression and a decrease in soluble MICA production in HepG2 cells in a dose-dependent manner (Fig. 6A). Increased membrane-bound MICA expression and a decrease of soluble MICA were observed in

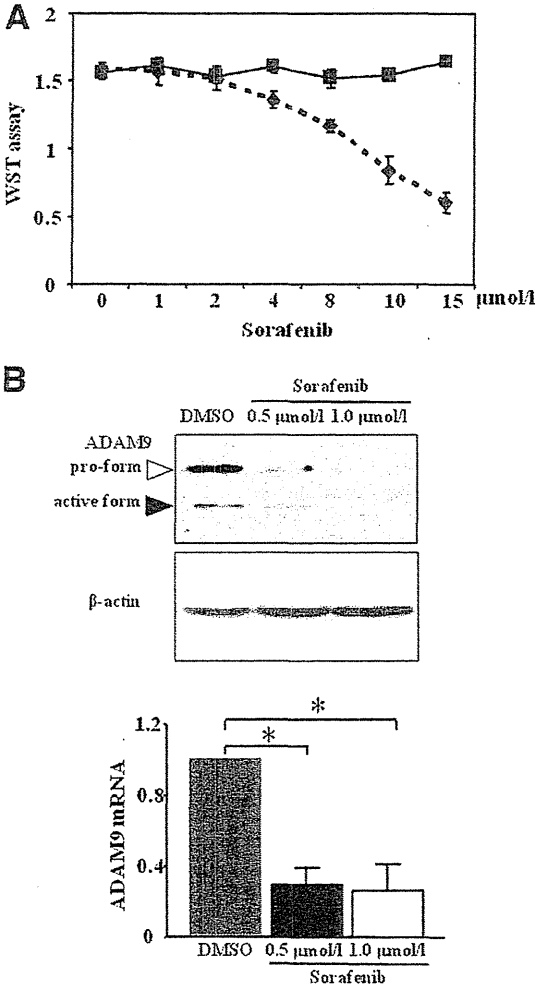

Fig. 5. Expression of ADAM9 in sorafenib-treated HCC cells. (A) The cytotoxicity of sorafenib to HepG2 cells was evaluated by WST-8 assay. Cells were treated with different doses of sorafenib (dotted line) or vehicle (DMSO; solid line) for 24 hours, and the viability of the cells was evaluated by WST-8 assay. (B) HepG2 cells were treated with 0.5 or 1 μmol/L sorafenib or vehicle (DMSO) for 24 hours and their protein and mRNA expressions of ADAM9 by western blotting (upper panel) and real-time RT-PCR (lower panel), respectively. Representative results are shown. Similar results were obtained from three independent experiments. *$P < 0.05$.

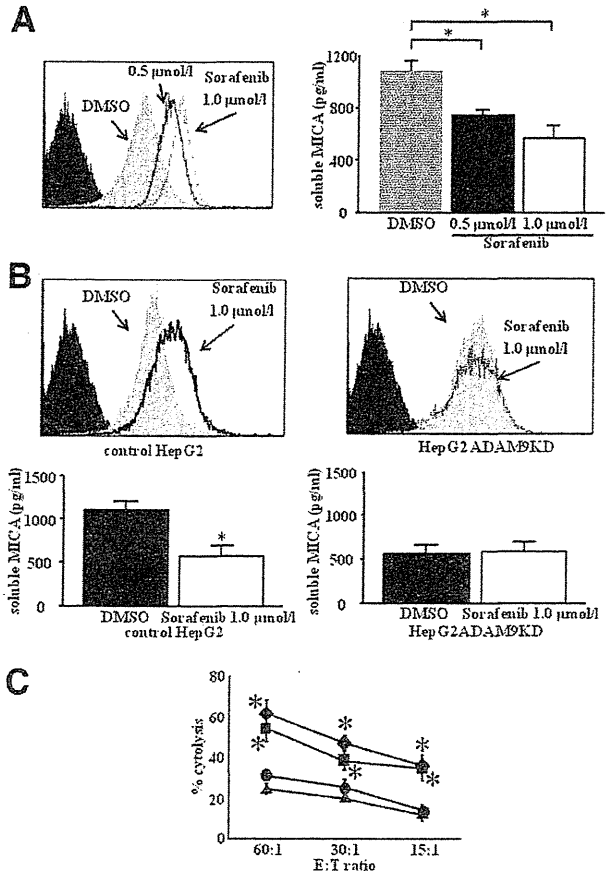

Fig. 6. Expression of MICA in sorafenib-treated HCC cells and NK sensitivity in sorafenib-treated HCC cells. (A) HepG2 cells were treated with 0.5 or 1 μmol/L sorafenib or vehicle (DMSO) for 24 hours and their expressions of membrane-bound MICA and the production of soluble MICA in the culture supernatant were evaluated by flow cytometry and specific ELISA, respectively. Closed histograms indicate control IgG staining in flow cytometry. Similar results were obtained from two independent experiments. $*P < 0.05$. (B) Control HepG2 or ADAM9KD HepG2 cells were treated with 1 μmol/L sorafenib or vehicle (DMSO) for 24 hours, and their expressions of membrane-bound MICA and the production of soluble MICA in the culture supernatant were evaluated by flow cytometry and specific ELISA, respectively. Closed histograms indicate control IgG staining in flow cytometry. Similar results were obtained from two independent experiments. $*P < 0.05$. (C) The cytolytic activity of NK cells against sorafenib-treated HepG2 cells were evaluated by ^{51}Cr-release assay. Vehicle-treated cells (▲), sorafenib-treated cells (0.5 μmol/mL sorafenib [■], 1 μmol/mL sorafenib [◆]), 1 μg/mL sorafenib-treated HepG2 cells with blocking antibody of MICA/B (6D4) (●), respectively. $*P < 0.05$ versus the cytolytic activity of NK cells against vehicle-treated HepG2 cells at each E:T ratio. Representative results are shown. Similar results were obtained from three independent experiments.

sorafenib-treated control HepG2 cells, but not in ADAM9KD-HepG2 cells (Fig. 6B), suggesting that an increase of membrane-bound MICA expression and a decrease of soluble MICA in sorafenib-treated HepG2 cells depended on ADAM9 expression. NK-mediated effector functions are regulated by a balance between inhibitory and stimulatory signals. NK cells can recognize MHC class I molecules on target cells via surface receptors that signals to suppress NK cell function.[24,25] We also examined the human leukocyte antigen (HLA) class I expressions on sorafenib-treated HepG2 cells by flow cytometry. The expression of HLA class I on sorafenib-treated HepG2 cells was similar to that on nontreated HepG2 cells (Supporting Fig. 2), suggesting that sorafenib did not affect the expression of HLA class I molecule.

We next evaluated whether the sorafenib treatment could also modify the NK sensitivity of human HCC cells. The cytolytic activities of NK cells against sorafenib-treated HepG2 cells were significantly higher than those against nontreated HepG2 cells (Fig. 6C). The cytolytic activity against sorafenib-treated HepG2 cells was decreased to the control levels by adding anti-MICA blocking antibody. These results demonstrated that adding sorafenib enhanced the NK sensitivity of HepG2 cells via increased expression of membrane-bound MICA. The sorafenib-treated PLC/PRF/5 HCC cells also showed similar results to those obtained from sorafenib-treated HepG2 cells (data not shown).

Discussion

MICA shedding is thought to be the principal mechanism by which tumor cells escape from NKG2D-mediated immunosurveillance.[13] In this study, we demonstrated that ADAM9 was overexpressed in human HCC tissues and that ADAM9 knockdown resulted in increased expression of membrane-bound MICA, decreased production of soluble MICA, and up-regulation of NK sensitivity of human HCC cells. These results point to ADAM9 as a possible therapeutic target for inhibiting MICA shedding, thereby increasing immunity against HCC.

We identified the ADAM9 cleavage site of MICA *in vitro*, which is located at the intracellular domain of MICA. ADAM9 protease is usually located in the extracellular area, but we revealed that ADAM9 protease is required for the production of not only the 37 kD soluble MICA but also the 39 kD MICA in HCC cells. Based on our present data, it is speculated that ADAM9 protease may enable intracellular cleavage of MICA protein by activating some intracellular protease which can recognize a similar ADAM9-cleavage site of MICA or by direct cleavage of MICA by activating ADAM9 while the intracellular domain of MICA shifts to the extracellular area by a flip-flop mechanism such as that observed with lipids.[26] Further study is needed to clarify the detailed mechanism of the intracellular cleavage. On the other hand, ADAM9 does not directly cleave MICA at the extracellular do-

main, and the ADAM9-dependent truncation of the cytosolic domain of MICA rendered this molecule susceptible to cleavage to produce soluble MICA. These results suggested that 39 kD MICA, which lacks a cytosolic domain, is susceptible to extracellular domain cleavage by some unidentified protease. Interestingly, this unidentified protease is independently activated after ADAM9 activation. This is the first report to show the involvement of ADAM9 in the shedding of MICA in cancer cells, which might offer new insights of the detailed escape mechanism of human HCC cells from the immune-surveillance system.

One of the important findings of the present study is that sorafenib, a new molecular targeted anticancer drug, could remodel HCC cells by down-regulating ADAM9 expressions, thereby inhibiting MICA ectodomain shedding and enhancing sensitivity to NK cells. Liu et al. demonstrated that the antitumor activity of sorafenib in human HCC might be attributed to inhibition of tumor angiogenesis via blocking of VEGF receptor or PDGF receptor and direct effect on HCC cell proliferation/survival through a Raf kinase signaling–dependent and/or Raf kinase signaling–independent mechanism.[27] However, early clinical study revealed that sorafenib treatment did not inhibit the progression of HCC tumor, although sorafenib prolonged the median overall survival of patients with advanced HCC.[21,28] This might be partly because sorafenib may not be distributed to HCC tissues enough to induce apoptosis of HCC cells. The ADAM family proteins, which are highly expressed in some tumors, play a role in secreting growth factors, such as heparin-binding epidermal growth factor, and migration of cells. This study is the first to demonstrate that clinically available molecular targeted anticancer drugs have the ability to modulate the expression of ADAM family proteins and NK sensitivity of tumor cells even if HCC cells were treated with a nontoxic dose of sorafenib. Sorafenib seemed to suppress ADAM9 expression at a transcriptional level, but the precise mechanism of this suppression is not yet known. Because sorafenib enhances NK sensitivity of HCC cells, if liver NK cells are efficiently activated during sorafenib treatment, an additional antitumor effect against HCC cells could be expected. We previously demonstrated that immune modulators such as α-galactosylceramide can efficiently activate liver innate immune cells including NK cells.[29,30] The combination therapy of anti-HCC molecular targeted therapy and immunotherapy targeting activation of NK cells might improve the antitumor effect against unresectable HCC and the prognosis of patients with HCC.

In spite of recent progress and early successes reported for HCC therapies, there remains significant room for improvement, especially with respect to advanced liver cancer. We have shown here that ADAM9 plays essential roles in MICA shedding in human HCC cells and that anti-HCC molecular targeted therapy enhances NK sensitivity of HCC cells via inhibition of the activity of ADAM9 protease followed by modification of MICA expression. These findings indicate that modulation of MICA shedding mediated by ADAM9 might represent a particularly promising approach to suppressing tumor growth and promoting regression in patients with HCC.

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Research Article

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The *let-7* family of microRNAs inhibits Bcl-xL expression and potentiates sorafenib-induced apoptosis in human hepatocellular carcinoma

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Background & Aims: Bcl-xL, an anti-apoptotic member of the Bcl-2 family, is over-expressed in human hepatocellular carcinoma, conferring a survival advantage to tumour cells. The mechanisms underlying its dysregulation have not been clarified. In the present study, we explored the involvement of microRNAs that act as endogenous sequence-specific suppressors of gene expression.

Methods: The expression profiles of microRNAs in Huh7 hepatoma cells and primary human hepatocytes were compared by microarray analysis. The effect of *let-7* on Bcl-xL expression was examined by Western blot and a reporter assay. The involvement of *let-7* microRNAs in human tissues was analysed by western blot and reverse transcription-PCR.

Results: Microarray analysis, followed by *in silico* target prediction, identified *let-7* microRNAs as being downregulated in Huh7 hepatoma cells in comparison with primary human hepatocytes, as well as possessing a putative target site in the *bcl-xl* mRNA. Over-expression of *let-7c* or *let-7g* led to a clear decrease of Bcl-xL expression in Huh7 and HepG2 cell lines. Reporter assays revealed direct post-transcriptional regulation involving *let-7c* or *let-7g* and the 3′-untranslated region of *bcl-xl* mRNA. Human hepatocellular carcinoma tissues with low expression of *let-7c* displayed higher expression of Bcl-xL protein than those with high expression of *let-7c*, suggesting that low *let-7* microRNA expression contributes to Bcl-xL over-expression. Finally, expression of *let-7c* enhanced apoptosis of hepatoma cells upon exposure to sorafenib, which downregulates expression of another anti-apoptotic Bcl-2 protein, Mcl-1.

Conclusions: *let-7* microRNAs negatively regulate Bcl-xL expression in human hepatocellular carcinomas and induce apoptosis in cooperation with an anti-cancer drug targeting Mcl-1.

Cancer

Introduction

MicroRNAs (miRNAs), a novel class of non-coding, small RNAs, repress gene expression by binding to the 3′-untranslated region (3′UTR) of target messenger RNAs (mRNAs) [1]. More than 500 miRNAs have been identified in humans. Each miRNA is capable of modulating the expression of many mRNAs to which it binds by imperfect sequence complementarity, although only a limited number of targeted genes has been identified. Through its activity of gene silencing, miRNA functions in a variety of cellular processes, such as development, organ homeostasis, and cancer development and progression [2]. In the context of cancer development and progression, miRNAs targeting oncogenes function as tumour suppressors, whereas those targeting tumour suppressor genes serve as oncogenes [3]. Accumulating evidence has revealed the aberrant expression of miRNAs in human hepatocellular carcinoma (HCC) [4–6]. *miR-122a* has been reported to be downregulated in HCC, in turn, leading to upregulation of cyclin G1 [7]. On the other hand, recent reports have demonstrated that *miR-21* [8], *miR-221* [9], and *miR-224* [10] are upregulated in HCC, leading to downregulation of PTEN, CDK inhibitors, and API-5, respectively. Furthermore, the miRNA expression signature was reported to be related to the clinical outcome of patients with HCC [11,12]. Thus, miRNAs may play an important role in HCC development and progression by modulating a variety of gene expression and cellular processes.

Apoptosis resistance is an important characteristic of tumour cells, in addition to dysregulated proliferation and aberrant differentiation. Apoptosis is regulated by a fine balance of Bcl-2 family proteins, such as anti-apoptotic Bcl-xL and Mcl-1 and pro-apoptotic Bak and Bax. We previously demonstrated that Bcl-xL

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Abbreviations: miRNA, microRNA; 3′UTR, 3′-untranslated region; mRNA, messenger RNA; HCC, hepatocellular carcinoma; CDK, cyclin-dependent kinase; DMEM, Dulbecco's modified Eagle medium; RT, reverse transcription; PCR, polymerase chain reaction; 7-AAD, 7-amino-actinomycin D; DMSO, dimethyl sulfoxide.

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is over-expressed in one-third of human HCC and confers resistance to hepatoma cells toward a variety of apoptotic insults generated by serum starvation and *p53* activation [13]. Patients with Bcl-xL-overexpressing HCC were shown to have significantly shorter disease-free survival after surgery [14]. Recently, it was proposed that autophagy defect is another mechanism of the malignant phenotype of Bcl-xL-overexpressing HCC through interaction between Bcl-xL and Beclin1 [15]. The underlying mechanisms of Bcl-xL over-expression in HCC are not clearly understood. Several reports show that transcription factors such as NF-κB [16] and STAT3 [17] could upregulate Bcl-xL expression in hepatoma cells. In addition, hepatitis C virus-related proteins, such as core [18] and NS5A [19], could upregulate Bcl-xL at a transcriptional level. However, we noticed that Bcl-xL-overexpressing hepatocarcinoma tissues do not always display upregulation of *bcl-xl* mRNA [13]. This observation led us to examine the possibility that post-transcriptional regulation by miRNAs may be involved in Bcl-xL expression in human HCC.

In the present study, we demonstrate that *let-7* family miRNAs, a prototype of human miRNAs [20], negatively regulate Bcl-xL expression in human HCC. *let-7* miRNAs are downregulated in human hepatoma cells and tissues in association with enhanced expression of Bcl-xL. Over-expression of *let-7* miRNAs in hepatoma cells downregulates Bcl-xL in a *bcl-xl* 3′UTR sequence-specific manner and enhances apoptosis induced by sorafenib, a recently approved anti-cancer drug for HCC [21]. The present study demonstrates for the first time that *let-7* miRNAs directly target Bcl-xL and induce apoptosis in cooperation with an anti-cancer drug targeting Mcl-1 in HCC.

Materials and methods

miRNA target predictions

The algorithms miRanda (http://www.microrna.org/), Pictar (http://pictar.mdc-berlin.de/), and TargetScan (http://www.targetscan.org/) were used to predict miRNAs that could potentially bind to *bcl-xl* mRNA.

Cell lines and tissues

Primary human hepatocytes were obtained from ScienCell Research Laboratories (Carlsbad, CA) and cultured with the provided medium. Human hepatoma cell lines, Huh7 and HepG2, were cultured with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (Sigma, St. Louis, MO). HCCs and adjacent non-tumour counterparts were obtained at the time of surgical resection. Written informed consent was obtained from each patient. All tissues were stored at −80 °C until the time of use.

RNA extractions

Total RNA including the miRNA fraction was isolated from cell lines and tissue samples using the miRNeasy Mini Kit (QIAGEN, Valencia, CA). After extraction, the quality of each RNA sample was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

miRNA microarray analysis

RNA labelling and hybridisation were performed using a human miRNA microarray kit and a miRNA complete labelling and hybridisation kit (Agilent Technologies). After washing with Gene Expression Wash Buffer, the slides were scanned with an Agilent Microarray Scanner and analysed by GeneSpring GX software.

Western blot

Cells or tissues were lysed and Western blotted as previously described [22]. For immunodetection, the following antibodies were used: anti-Bcl-xL polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Mcl-1 polyclonal antibody (Santa Cruz Biotechnology), anti-Bak polyclonal antibody (Millipore, Billerica, MA), anti-Bax polyclonal antibody (Cell Signaling Technology, Danvers, MA). Optical densities of bands in each blot were analysed using ImageJ 1.40 g (NIH, Bethesda, MD).

Real time reverse transcription (RT)-PCR assays for mature miRNAs

To quantify the expression of mature miRNA, we synthesised cDNA from 10 ng of RNA sample using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed with TaqMan MicroRNA Assays (Applied Biosystems) specific for *let-7c* (P/N 4373167) and *let-7g* (P/N 4395393). To normalise the expression levels of miRNAs, we used TaqMan MicroRNA Assays specific for RNU6B (P/N 4373381) as the endogenous control.

Real time RT-PCR assays for bcl-xl mRNA

We reverse-transcribed RNA with High Capacity RNA-to-cDNA Master Mix (Applied Biosystems), and *bcl-xl* mRNA expression was measured using TaqMan Gene Expression Assays (Applied Biosystems, Assay ID: Hs99999146_m1). We also quantified *β-actin* mRNA as an endogenous control (Assay ID: Hs99999903_m1).

Transfections with miRNAs

Huh7 and HepG2 cells were transfected with 50 nM Pre-miR miRNA precursor molecules (Ambion, Austin, TX) of either *let-7c* or *let-7g* using RNAiMAX (Invitrogen, Carlsbad, CA) in six-well plates according to the manufacturer's instructions. Pre-miR negative control (Ambion) was also used as a control.

Luciferase assay

To generate the pMIR-Bcl-xL-3′UTR construct that contains the putative binding site of *bcl-xl* 3′UTR downstream of the firefly luciferase gene, we synthesised oligonucleotides to mimic the target sequence and inserted them into the SpeI–HindIII site of pMIR-REPORT Luciferase vector (Ambion). We also generated a pMIR-Bcl-xL-3′UTR mutant that has a point mutation in the putative binding site, using the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

Each of these constructs was transfected into Huh7 cells together with 50 nM Pre-miR miRNA precursor molecules and pMIR-REPORT β-Gal vector (Ambion), which contains the β-galactosidase gene for normalisation of transfection efficiency. Transfection was performed using Lipofectamine 2000 (Invitrogen). We measured firefly luciferase activity 24 h after transfection using the Luciferase Assay System (Promega, Madison, WI) and normalised it to the β-galactosidase expression level.

In vitro staurosporine or sorafenib treatment

Huh7 cells were transfected with 50 nM Pre-miR miRNA precursor molecules as described above, and 48 h after transfection, the medium was changed to DMEM containing staurosporine (Calbiochem, Gibbstown, NJ) or sorafenib. Sorafenib was kindly provided by Bayer HealthCare Pharmaceuticals Inc. (Wayne, NJ). Cells were additionally cultured and assayed for apoptosis by monitoring the activity of caspase-3/7 using a luminescent substrate assay for caspase-3 and caspase-7 (Caspase-Glo assay, Promega, Madison, WI), or by flow cytometry using the Annexin V-PE Apoptosis Detection Kit I (BD Biosciences, San Jose, CA). We defined apoptotic cells as Annexin V-PE-positive and 7-amino-actinomycin D (7-AAD)-negative cells. Cell viability was determined by the WST assay using cell count reagent SF (Nacalai Tesque, Kyoto, Japan).

Statistical analysis

Data are presented as mean ± SD. Comparisons between two groups were performed by the unpaired *t*-test. Multiple comparisons were performed by ANOVA with the Scheffe post hoc test. $p<0.05$ was considered statistically significant.

Results

let-7 miRNAs were downregulated in hepatoma cells with upregulated expression of Bcl-xL

As observed in human HCC tissues, Bcl-xL was over-expressed, according to Western blot analysis, in Huh7 and HepG2 human hepatoma cell lines compared to normal hepatocytes (Fig. 1A). Previous research established that 30 and 32 kDa species are original and post-translationally modified Bcl-xL, respectively [23]. Mcl-1 was also over-expressed in human hepatoma cells, but the levels of expression of Bak and Bax did not differ between hepatoma cells and normal hepatocytes. We reasoned that miRNA regulating Bcl-xL expression would be downregulated in those hepatoma cell lines. To search for the candidate miRNA, microarray analysis was performed. More specifically, miRNA expression in Huh7 cells and normal hepatocytes was compared. When levels of expression less than 50% were considered significant, 26 miRNAs were identified as being downregulated in Huh7 cells: *let-7b*, *let-7g*, *let-7i*, *miR-127-3p*, *miR-214*, *miR-376a*, *miR-381*, *miR-409-3p*, *miR-376c*, *miR-493**, *miR-432*, *miR-487b*, *let-7d*, *let-7a*, *let-7f*, *let-7c*, *miR-200a*, *let-7e*, *miR-134*, *miR-503*, *miR-34a*, *miR-638*, *miR-150**, *miR-1225-5p*, *miR-21**, and *miR-223*. Among them, *in silico* analysis revealed that only the *let-7* family is capable of potentially targeting the 3′UTR of the *bcl-xl* mRNA. To confirm the results of the microarray analysis, quantitative real time RT-PCR was performed to evaluate the expression of *let-7c* and *let-7g* (Fig. 1B). After normalisation to endogenous RNU6B expression levels, the expression levels of both miRNAs were substantially lower in Huh7 cells than in normal hepatocytes. These results were consistent with the results of microarray analysis. Furthermore, the expression levels of both miRNAs were found to be downregulated in another human hepatoma cell line, HepG2, compared to normal hepatocytes.

let-7c and let-7g downregulate Bcl-xL expression by directly targeting the 3′UTR of bcl-xl mRNA

To examine whether *let-7* miRNAs are capable of suppressing translation of Bcl-xL, hepatoma cell lines were transfected with *let-7c* or *let-7g* or the negative control. Three days after transfection, Huh7 cells showed a decrease in Bcl-xL protein levels in both the *let-7c*-transfected group and the *let-7g*-transfected group in comparison with the negative control group (Fig. 2A). The transfection of *let-7c* and *let-7g* showed suppression of Bcl-xL protein levels in HepG2 cells as well (Fig. 2B). It did not affect expression of Bak and Bax, but increased Mcl-1 expression, which may be a secondary phenomenon of suppression of Bcl-xL. Normal hepatocytes were also transfected with *let-7c* or *let-7g* (Suppl. Fig. 1). The transfection led to a decrease in Bcl-xL expression in normal hepatocytes, but the decline was lesser than that observed in hepatoma cells. This finding may be explained by the observation that endogenous expression of *let-7c* and *let-7g* was extremely high in normal hepatocytes.

To examine whether the downregulation of Bcl-xL by *let-7c* or *let-7g* is caused by direct binding to a putative targeting site in the *bcl-xl* mRNA, we constructed the luciferase reporter plasmid pMIR-Bcl-xL-3′UTR containing the putative *let-7* binding site of *bcl-xl* 3′UTR downstream of the luciferase open reading frame (Fig 3A). The pMIR-Bcl-xL-3′UTR construct was cotransfected with the control pMIR-REPORT β-gal vector into Huh7 cells together with *let-7c* or *let-7g* or the negative control. When *let-7c* or *let-7g* Pre-miR was cotransfected with pMIR-Bcl-xL-3′UTR, the expression of firefly luciferase was significantly reduced compared to the negative control cotransfected group. There was no difference in firefly luciferase expression levels when pMIR-REPORT, which does not contain the putative *let-7* binding site, was cotransfected with *let-7c*, *let-7g* or the negative control (Fig. 3B). We also generated a pMIR-Bcl-xL-3′UTR mutant with a single base mutation in the seed region of the putative binding sequence to investigate whether the downregulation of firefly luciferase can be attributed to the insert (Fig. 3A). A single base mutation prevented the downregulation of firefly luciferase

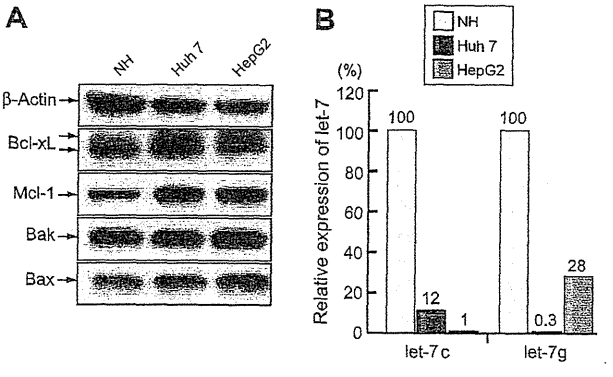

Fig. 1. Expression of Bcl-xL and *let-7* miRNAs in cultured human hepatocytes and hepatoma cells. Human hepatoma cell lines, Huh7 and HepG2, and normal hepatocytes (NH) were cultured and then lysed. (A) Western blot analysis for Bcl-2 family proteins. Bcl-xL migrates as a doublet band (see text). (B) Real time RT-PCR analysis for *let-7c* and *let-7g* expression. After normalisation to endogenous RNU6B expression, the expression of each miRNA in hepatoma cells was expressed in comparison to the levels observed in normal hepatocytes.

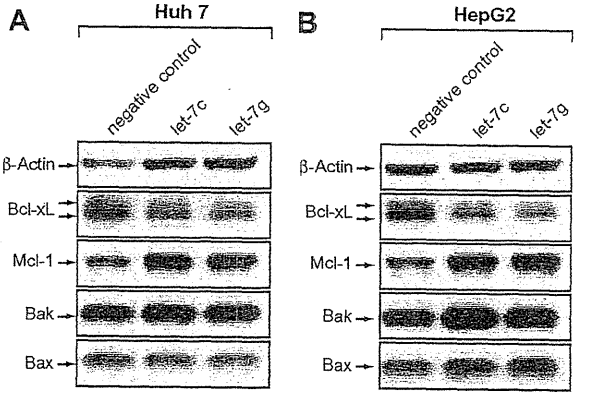

Fig. 2. Over-expression of *let-7* miRNAs downregulates Bcl-xL expression in hepatoma cells. Hepatoma cell lines Huh7 (A) and HepG2 (B) were transfected with *let-7c*, *let-7g*, or negative control miRNA at 50 nM and cultured for 3 days. Expression levels of Bcl-2 family proteins were determined by Western blot analysis.

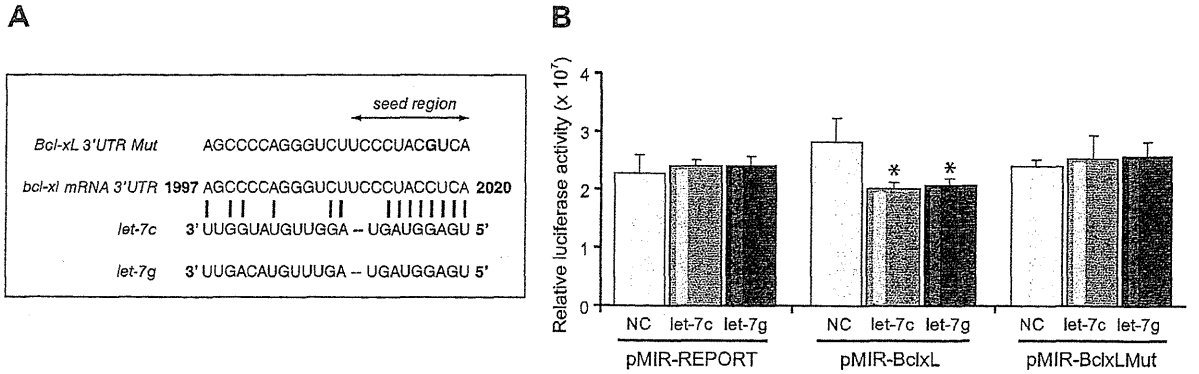

Fig. 3. Sequence-specific suppression of *bcl-xl* gene expression by *let-7c* or *let-7g* miRNAs. (A) The putative target site of *bcl-xl* mRNA 3'UTR determined by computational predictions. The target sequence was cloned into pMIR-REPORT vector (pMIR-Bcl-xL-3'UTR). pMIR-Bcl-xL-3'UTR mutant was also generated with a single mutation (indicated by a bold character) in the target site. (B) Each of these constructs was transfected into Huh7 cells together with *let-7c*, *let-7g* or negative control miRNA (NC). At 24 h after transfection, the activity of firefly luciferase was measured and normalised to β-galactosidase expression levels ($n = 3$). $^*p < 0.05$.

induced by *let-7c* or *let-7g*, which strongly suggests a direct inhibitory effect of *let-7* on Bcl-xL expression (Fig. 3B).

Downregulation of let-7c miRNA in human HCC tissues overexpressing Bcl-xL but not bcl-xl mRNA

To investigate the relationship between *let-7* expression levels and Bcl-xL protein levels in human HCC samples, we used 22 pairs of surgically resected human HCC tissue samples and adjacent non-tumour tissue samples with highly preserved RNA. Compared to the non-tumour counterparts, *bcl-xl* mRNA was found to be over-expressed in HCC tissue samples in only two cases; Bcl-xL was also over-expressed at the protein level in these cases. To assess the significance of *let-7* in post-transcriptional regulation of Bcl-xL *in vivo*, we selected 20 pairs of HCC tissue samples that did not over-express *bcl-xl* mRNA. When these samples were divided into two groups according to relative *let-7c* expression levels, the relative expression of Bcl-xL protein was significantly higher in the *let-7c* low expression group than in the *let-7c* high expression group (Fig. 4). By contrast, there was no significant difference in *bcl-xl* mRNA expression between the two groups. We also examined the relationship between relative *let-7g* expression and Bcl-xL expression. The *let-7g* low expression group tended to over-express Bcl-xL protein compared to the *let-7g* high expression group, although the difference did not reach statistical significance (data not shown). These results are consistent with the hypothesis that *let-7* miRNAs negatively regulate Bcl-xL expression independent of transcriptional regulation.

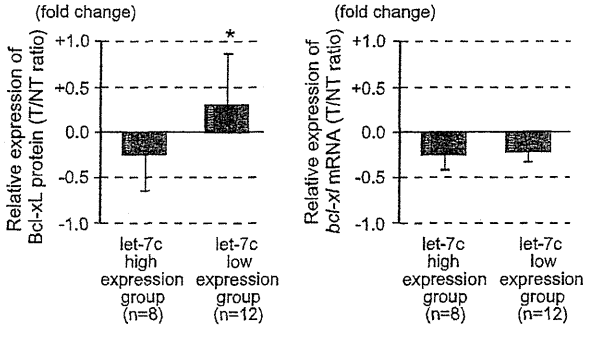

Fig. 4. Expression of Bcl-xL, *bcl-xl* mRNA and *let-7* miRNAs in human HCC tissue. Relationship between *let-7* and Bcl-xL expression in human HCC tissue samples. HCC tissue samples that did not show transcriptional upregulation of *bcl-xl* mRNA were divided into two groups according to relative *let-7c* expression levels with the threshold set at a 0.4-fold change in the tumour to non-tumour (T/NT) ratio. Relative expression of Bcl-xL protein and *bcl-xl* mRNA was calculated as the optical densities of the Bcl-xL blots normalised with the β-actin blots and those of real time RT-PCR assays, respectively, and are shown as the ratio of expression in the tumour to non-tumour expression in $\log_{10}$ scale. $^*p < 0.05$.

let-7c miRNA sensitises human Huh7 cells to sorafenib, which downregulates Mcl-1 expression

To investigate the effect of *let-7* in the resistance of hepatoma cells to apoptosis, we transfected Huh7 hepatoma cells with *let-7c* miRNAs and then subjected them to apoptosis analysis and a cell viability assay. There was no significant difference in caspase-3/7 activation or cell viability between *let-7c* miRNA-transfected Huh7 cells and control miRNA-transfected Huh7 cells (represented by the DMSO-treated group of Fig. 5A and B). These results are in agreement with our previous finding that anti-sense oligonucleotide-mediated knockdown of Bcl-xL sensitised hepatoma cells to apoptotic stimuli, such as serum starvation and *p53* activation, but did not induce apoptosis by itself [13]. Next, we exposed miRNA-transfected Huh7 cells to staurosporine, which is a well-established apoptosis inducer. Staurosporine treatment induced apoptosis, as determined by caspase-3/7 activation and decreased the viability of Huh7 cells by itself, but *let-7c* miRNA-transfected Huh7 cells were more susceptible to staurosporine treatment than control miRNA-transfected cells. *let-7c* miRNA-transfected Huh7 cells showed a significant decrease in cell viability, even upon exposure to low-dose of staurosporine at which control miRNA-transfected Huh7 did not show a significant difference in cell viability (Fig. 5B). In addition, the activation of caspase-3/7 was more intense in *let-7c* miRNA-transfected Huh7 cells than in control miRNA-transfected Huh7 cells (Fig. 5A). Thus, suppression of *let-7* expression leading to over-expression of Bcl-xL, may be a mechanism by which hepatoma cells resist apoptotic stimuli. While normal hepatocytes were more sensitive to staurosporine than hepatoma cells, transfection of *let-7* miRNA did not affect sensitivity to staurosporine

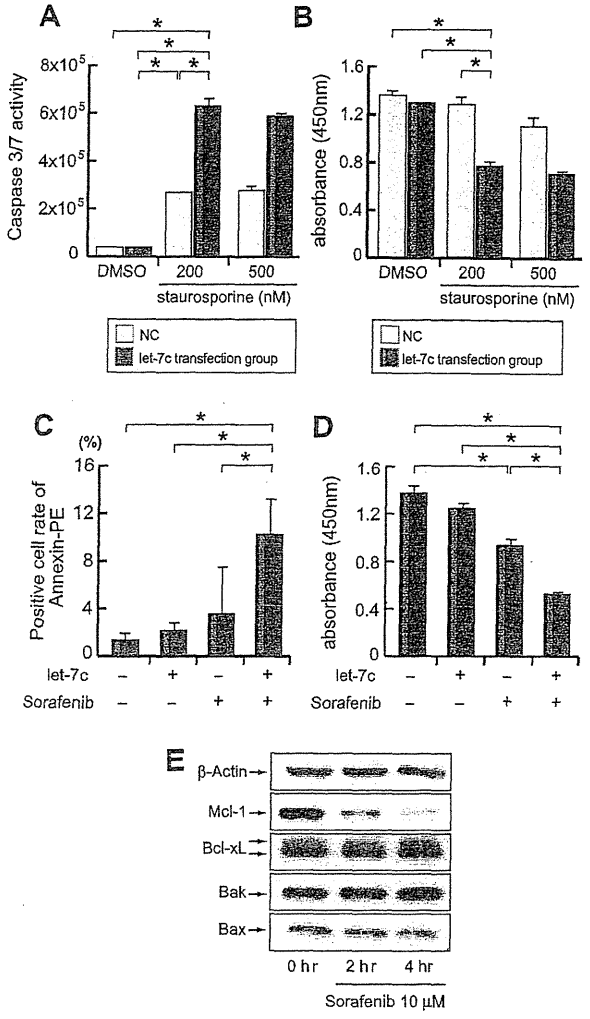

Fig. 5. Introduction of *let-7* miRNAs sensitises hepatoma cells to apoptotic stimuli. (A and B) Response to staurosporine treatment. Huh7 cells were transfected with *let-7c* (grey bars) or control miRNA (white bars) for 48 h and then further treated with staurosporine or DMSO alone for 12 h. The activities of caspase-3 and -7 were determined by luminescent substrate assays for caspase-3 and -7 ($n = 4$) (A). Cell viability was determined by the WST assay ($n = 4$) (B). *$p < 0.05$. (C and D) Response to sorafenib treatment. Huh7 cells were transfected with *let-7c* or control miRNA for 48 h and then further treated with sorafenib (5 μM) or DMSO alone for 48 h (C) or 72 h (D) 7-AAD negative cells were gated and the positive cell rate for annexin V-PE was determined ($n = 4$) (C). Cell viability was determined by the WST assay ($n = 4$) (D). *$p < 0.05$. E. Western blot analysis for Bcl-2 family proteins in lysates of Huh7 cells treated with sorafenib.

in normal hepatocytes (Suppl. Fig. 2), which is in agreement with the modest decline of Bcl-xL expression described earlier.

To examine the impact of *let-7* family miRNAs as a therapeutic tool, we investigated the effect of *let-7* miRNAs on apoptosis resistance to sorafenib, a recently approved anti-cancer drug for HCC. It has been reported that sorafenib was capable of downregulating Mcl-1 expression in tumour cells [24], and HCC has been reported to over-express Mcl-1, which is another anti-apoptotic Bcl-2 protein capable of conferring resistance to apoptosis [24–27]. In agreement with these findings, sorafenib treatment clearly downregulated Mcl-1 expression in hepatoma cells, but did not affect Bcl-xL expression (Fig. 5E). In contrast, sorafenib treatment did not affect Mcl-1 expression in normal hepatocytes (Suppl. Fig. 3). We hypothesised that *let-7* miRNA targeting Bcl-xL may induce apoptosis of hepatoma cells in cooperation with sorafenib. Apoptosis determined by Annexin V staining did not increase in *let-7c* miRNA-treated Huh7 cells compared to control miRNA-treated cells (represented by the DMSO-treated group in Fig. 5C). Sorafenib treatment of Huh7 cells led to a slight increase in the annexin V-positive cell rate, although the difference did not reach statistical significance levels under our experimental conditions (Fig. 5C). Of importance is the finding that sorafenib-induced apoptosis was markedly enhanced in *let-7c* miRNA-transfected cells. In addition, sorafenib treatment significantly reduced the viability of Huh7 cells and this decrease was markedly enhanced in cells transfected with *let-7c* miRNA (Fig. 5D). This finding implies that *let-7* miRNA transfection potentiates sorafenib-induced apoptosis and toxicity in hepatoma cells.

Discussion

Anti-apoptotic members of the Bcl-2 family, which consists of five members, Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and Bfl-1, are critically involved in the mitochondrial pathway of apoptosis [28]. Cancer cells frequently over-express one or more members of this family to acquire a survival advantage [29]. These proteins are over-expressed in a variety of ways, including genetic translocation, particularly in the case of Bcl-2, and transcriptional regulation. Unlike the case of the *bcl-2* gene, mutations or amplification of the *bcl-x* gene have not been demonstrated in tumour cells. With regard to miRNA regulation, previous research clearly demonstrated that Bcl-2 is a direct target of *miR-15* and *miR-16*. The expression levels of *miR-15* and *miR-16* inversely correlate with Bcl-2 expression in chronic lymphocytic leukaemia [30]. More recently, Mcl-1 was reported to be suppressed by *miR-29* [31]. Our present study is the first demonstration of miRNA-mediated regulation of Bcl-xL expression. Since Bcl-xL is over-expressed not only in HCC but also in other tumours, the present findings may shed light on the mechanisms of Bcl-xL over-expression in other malignancies.

While more than 500 human miRNAs have been identified, *let-7* is a prototype of human miRNA and was first identified in 2001 [32]. *let-7* miRNAs are downregulated in several malignancies. A highly characterised example is non-small cell lung cancer in which downregulation of *let-7* miRNAs is well correlated with poor prognosis in patients [33]. In HCC, some reports showed downregulation of *let-7*, while others did not [7]. In the present study, *let-7c* miRNA was under-expressed at less than 40% of the normal level in approximately half of the HCC tissues. Further study is needed to determine the clinical significance of *let-7* miRNA in HCC. Several target genes have been identified for *let-7* miRNA, including Ras [34], Myc [35], HMGA2 [36], CDC25A, and CDK6 [37]. The major function of this miRNA is to promote cell proliferation. Since these proteins could act as oncogenes in tumour cells, *let-7* miRNA is believed to serve as a tumour suppressor [38]. In the present study, we have demonstrated that *bcl-xl* is a direct target for *let-7* miRNA, implying that this well-known tumour suppressor miRNA directly regulates apoptosis, another important process in malignancy.

Sorafenib is a recent FDA-approved anti-cancer drug for HCC [21]. It functions as a multi-kinase inhibitor and can induce

apoptosis at least in part by downregulating Mcl-1 in tumour cells [24]. Like Bcl-xL, several reports have identified Mcl-1 as being over-expressed in some HCCs [25–27]. Since Bcl-xL and Mcl-1 share a similar structure and functions, we reasoned that downregulation of both proteins would efficiently kill hepatoma cells. To verify this hypothesis, we treated hepatoma cells with sorafenib and *let-7* miRNA. As expected, sorafenib treatment downregulated Mcl-1 expression as early as 2 h post-treatment; however, it did not efficiently induce apoptosis. Transfection of *let-7* miRNA itself was not capable of inducing apoptosis of hepatoma cells despite a clear reduction in Bcl-xL expression. Importantly, *let-7* miRNA substantially increased sensitivity to sorafenib. Since both *let-7* miRNA and sorafenib may have pleiotropic effects on gene expression and cellular processes, downregulation of Bcl-xL and Mcl-1 may not be a single mechanism for killing hepatoma cells. However, our study revealed that Bcl-xL-targeting miRNA, *let-7*, controls resistance of hepatoma cells to this novel class of anti-HCC drug.

In conclusion, we have demonstrated that *let-7* miRNA negatively regulates Bcl-xL expression in HCCs. Reconstitution of *let-7* miRNA may reduce apoptosis resistance to anti-cancer drugs targeting Mcl-1 in HCC. Further study is needed to examine the significance of *let-7* miRNA expression for predicting responses to sorafenib therapy in patients with HCC.

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Disclosures

All authors have nothing to disclose.

Conflicts of interest

All authors have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2009.12.024.

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Research Article

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Expression of CD133 confers malignant potential by regulating metalloproteinases in human hepatocellular carcinoma

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Background & Aims: Although CD133 expression is identified as a cancer stem cell marker of hepatocellular carcinoma (HCC), the detailed characteristics of HCC cells expressing CD133 remain unclear.

Methods: We examined the malignant characteristics of CD133-expressing HCC cells.

Results: CD133-expressing cells could be detected with low frequency in 5 HCC tissues. We derived two different HCC cell lines by (1) transfection of CD133 siRNA in PLC/PRF/5 cells (CD133si-PLC/PRF/5), and (2) by a magnetic cell sorting method that allowed to divide Huh7 cells into two CD133 positive (+) and negative (−) groups. CD133 knockdown in PLC/PRF/5 cells resulted in a decrease of the mRNA and protein expressions of matrix metalloproteinase (MMP)-2 and a disintegrin and metalloproteinase (ADAM)9. We next examined the malignant characteristics related to decreasing MMP-2 and ADAM9 in HCC cells. In CD133si-PLC/PRF/5 cells and CD133− Huh7 cells, invasiveness and vascular endothelial growth factor (VEGF) production, which are both related to the activity of MMP-2, were inhibited compared to CD133-expressing HCC cells. We previously demonstrated that ADAM9 protease plays critical roles in the shedding of MHC class I-related chain A (MICA) which regulates the sensitivity of tumor cells to natural killer cells (NK). Decreasing ADAM9 expression in CD133si-PLC/PRF/5 cells and CD133− Huh7 cells resulted in an increase in membrane-bound MICA and a decrease in soluble MICA production. Both CD133si-PLC/PRF/5 cells and CD133− Huh7 cells were susceptible to NK activity, depending on the expression levels of membrane-bound MICA, but CD133-expressing HCC cells were not.

Conclusion: These results demonstrate that CD133 expression in HCC cells confers malignant potential which may contribute to the survival of HCC cells.

Cancer

Introduction

Chronic liver disease caused by hepatitis virus infection and non-alcoholic steatohepatitis leads to a predisposition for hepatocellular carcinoma (HCC) [1]. With regard to treatment, surgical resection, percutaneous techniques such as ethanol injection and radiofrequency ablation and transcatheter arterial chemoembolization (TACE) are well established and improve the prognosis of HCC patients [2]. HCC is an aggressive tumor with early vascular invasion and metastasis, and the expression of angiogenic factors such as vascular endothelial growth factor (VEGF), may help predict the prognosis of HCC patients [3]. Elucidation of the tumor biology of HCC is important to develop better ways to treat it.

The existence of cancer stem cells (CSCs) has been reported for many cancers [4], including those of the breast, brain, colon, pancreas, and blood. CSCs have been characterized in solid tumors using a variety of stem cell markers including CD133 [5]. CD133+ HCC cells isolated from human HCC cell lines and xenograft tumors possess a greater colony forming efficacy, a higher proliferative output, and a greater ability to form tumors in vivo [6–8]. Preliminary studies have demonstrated that the expression of ATP-binding cassette drug transporters is high in CD133+ cells, and that it increases the resistance of CD133+ CSCs to chemotherapeutic agents [9]. Chemo-resistant CD133+ HCC cells displayed preferential activation of the Akt/PKB and Bcl-2 pathway, promoting cell survival by suppressing apoptosis [10]. Hence, the significance of CD133 expression in cancer cell seems to be important for the development of cancer. A clearer understanding of the characteristics of CD133-expressing HCC is therefore required to establish new cancer therapies against HCC.

The metalloprotease family includes various types of proteases, such as the matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinases (ADAMs), and have been reported

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Abbreviations: HCC, hepatocellular carcinoma; VEGF, vascular endothelial growth factor; CSCs, cancer stem cells; MMP, matrix metalloproteinase; ADAM, a disintegrin and metalloproteinase; MICA, MHC class I-related chain A; Ab, antibody; siRNA, small interfering RNA.

to be involved in tumor-induced angiogenesis, tumor invasion, and tumor escape mechanisms from immune cells in various cancers [11,12]. Although the expression of metalloproteinase has been reported in human HCC [13], the significance of their presence in CD133-expressing cells remains unclear.

In the current study, we evaluated the malignant characteristics of CD133-expressing human HCC cells, which have a greater ability of invasion and VEGF production via MMP-2 activation. In addition, these cells were resistant to the cytolytic activity of NK cells by acting upon the ADAM9/MHC class I-related chain A (MICA) pathway. The present study shed light on the significance of CD133 expression on HCC cells and led to a new strategy for developing treatments against human HCC.

Materials and methods

HCC cell lines

Human HCC cell lines, PLC/PRF/5 cells, Huh7, HepG2, and Hep3B cells were cultured with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco/Life Technologies, Grand Island, NY) in a humidified incubator at 5% CO_2 and 37 °C.

Immunohistochemistry

Five human HCC liver tissue samples (Table 1) were surgically resected after informed consent, under an Institutional Review Board-approved protocol, had been obtained. The liver sections were subjected to immunohistochemical staining by the ABC procedure (Vector Laboratories, Burlingame, CA) using the anti-CD133 antibody (Ab) (ABGENT, San Diego, CA) and the anti-CD90 Ab (AbD Serotec, Oxford, UK). The expressions of CD133 were quantified as previously described [14].

Flow cytometry

For the detection of CD133 or MICA in HCC cells, the cells were incubated with PE-conjugated anti-CD133 Ab (Miltenyl Biotech, Auburn, CA) or anti-MICA Ab (2C10, Santa Cruz Biotechnology, Santa Cruz, CA), and then subjected to flow cytometric analysis performed using a FACscan flow cytometer (Becton–Dickinson, San Jose, CA). We analyzed positive cell rates by using the control staining as previously described [15].

RNA silencing

The small interfering RNA (siRNA) method was used to knockdown CD133, MMP-2 and ADAM9 expressions of PLC/PRF/5 cells as previously described [16]. The following types of siRNA were used: CD133 knockdown PLC/PRF/5 (CD133si-PLC/PRF/5), 5′-UUUCUGUGGAUGUAACUUUCAGUGU-3′; MMP-2 knockdown PLC/PRF/5 (MMP-2si-PLC/PRF/5), 5′-UAGUGUGUCCUUCAGCACAAACAGG-3′; ADAM9 knockdown PLC/PRF/5 (ADAM9si-PLC/PRF/5), 5′-UGUCCAAACACAUUAAUCCCGCCUG-3′; control-PLC/PRF/5, negative universal control.

Table 1. Clinical background of HCC patients.

Sex	Age	Eology	Non-cancerous tissue
male	73	HCV	LC
male	67	HCV	LC
male	76	NBNC	normal
female	51	HBV	LC
female	45	HBV	LC

HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, patients without HBV and HCV; LC, liver cirrhosis.

Cell separation

CD133+/− Huh7 cells were isolated by magnetic cell sorting using CD133 MicroBeads according to the manufacturer's instructions (Miltenyl Biotech). The isolated CD133+/− Huh7 cells were injected subcutaneously into nude mice to evaluate the tumorigenicity of each cell. We found that CD133+ Huh7 cells were more tumorigenic than CD133− Huh7 cells (Fig. 1A).

Western blotting

Cells were lysed and immunoblotted as previously described [16]. For immunodetection, the following Abs were used: anti-MMP-2 Ab (Thermo Fisher Scientific, Fremont, CA) and anti-ADAM9 Ab (R&D Systems, Minneapolis, MN). To assess the protein levels, optical densities of bands in each blot were analyzed using ImageJ 1.40 g.

MMP-2 Zymography

Proteolytic activity of supernatant was examined by gelatin zymography according to the manufacturer's protocol (Cosmo-Bio, Tokyo, Japan). The supernatants of cells were subjected to 10% SDS–polyacrylamide gel electrophoresis using gels containing 0.3% gelatin. The proteolytic band of 62 kDa corresponding to the active form of MMP-2 was scanned using a Photo scanner.

Real-time RT-PCR

Total RNA extraction and reverse transcription were performed as previously described [16]. Ready-to-use assays (Applied Biosystems, Foster city, CA) were used for the quantification of MMP-2,9,14, ADAM8,9,10,12,17, TIMP-1,2,3, MICA, and β-actin 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. β-Actin mRNA from each sample was quantified as an endogenous control of internal RNA.

Invasion assays

The invasion activity was measured by Boyden-chamber assay using BD BioCoat Matrigel Invasion Chamber (BD Biosciences) as previously described [17]. To assess the involvement of MMP-2 protein in the invasion activity, active-form MMP-2 proteins (20 μM, R&D systems) were added to the culture of CD133si-PLC/PRF/5 cells or CD133− Huh7 cells.

ELISA

HCC cells were cultured for 24 h and the supernatants were subjected to enzyme-linked immunosorbent assay (ELISA). Concentrations of VEGF and soluble MICA were evaluated by QuantiGlo human VEGF Immunoassay (R&D Systems) and by the DuoSet MICA eELISA kits (R&D Systems) according to the manufacturer's recommendations.

WST-8 assay

Cell growth of CD133si-PLC/PRF/5 or control-PLC/PRF/5 cells was determined by WST-8 assay (Nacalai tesque, Kyoto, Japan) as previously described [16].

NK cell analysis

NK cells were isolated from peripheral blood mononuclear cells by magnetic cell sorting using CD56 MicroBeads according to the manufacturer's instructions (Miltenyl Biotech). More than 95% of the cells were $CD56^{+}CD3^{-}$ lymphocytes. The cytolytic ability of NK cells was assessed by 4-h ^{51}Cr-releasing assay with or without MICA/B-blocking Ab (R&D systems).

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 the two-sample *t* test with Welch correction after each group had been tested with equal variance and Fisher's exact probability test. We defined statistical significance as $p < 0.05$.

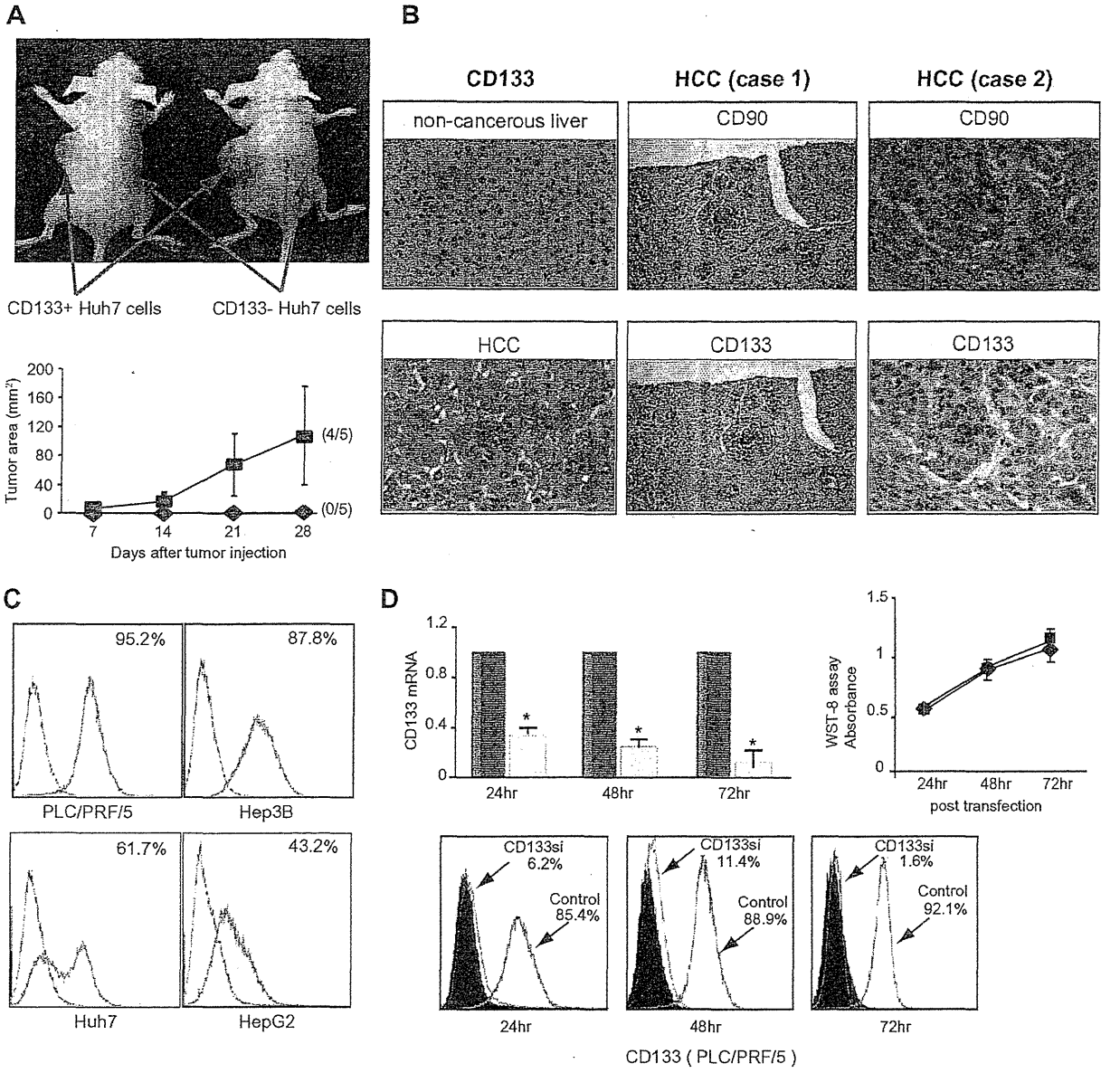

Fig. 1. Tumorigenicity of CD133+/− Huh7 cells in nude mice and the expressions of CD133 in human HCC tissues and cell lines, and CD133 knockdown in PLC/PRF/5 cells. (A) Evaluation of the phenotypes of CD133+/− Huh7 cells in a xenograft growth model. We separated CD133+/− cells using magnetic beads and injected subcutaneously 2.5 × 10^6 CD133+ (■, left flank) or CD133− (◆, right flank) Huh7 cells into BALB/c nu/nu mice ($N = 5$/each group). Representative macroscopic view of each group, 28 days after tumor injection, is shown (upper panel). The tumor size was monitored every week (lower panel). The fraction of mice of each group, bearing tumor after 28 days, is given in parentheses. (B) Immunohistochemical detection of CD133 in human HCC tissues and non-cancerous liver tissues ($N = 5$). Immunohistochemical detection of CD133 and CD90 in human HCC tissues ($N = 5$). Representative images are shown. (C) Human HCC cell lines were stained with anti-CD133 Ab and subjected to flow cytometry. Black lines, control IgG staining; green lines, CD133 staining. Positive cell rates are shown in the figure. (D) PLC/PRF/5 cells were transfected with CD133 siRNA (CD133si-PLC/PRF/5) or control siRNA (control-PLC/PRF/5). The mRNA and protein expressions of CD133 were evaluated by real-time PCR (left upper panel, white bar, CD133si-PLC/PRF/5 cells; black bar, control-PLC/PRF/5 cells) and flow cytometry (lower panel). Black curves, control IgG staining; red line, CD133 staining of CD133si-PLC/PRF/5 cells; blue line, CD133 staining of control-PLC/PRF/5 cells. Positive cell rates are shown in the figure. The viability of siRNA-transfected cells was evaluated by WST-8 assay (right upper panel). CD133si-PLC/PRF/5 cells (◆) and control-PLC/PRF/5 cells (■).

Results

Detection of CD133+ cells in human HCC tissues and CD133 expressions in human HCC cell lines

We examined the presence of CD133+ cells in HCC tissues. As shown in Fig. 1B, CD133+ cells could be detected with low frequency in HCC tissues (2.2 ± 1.6%) and was undetectable in non-cancerous liver tissues (0.0%). The expression of CD90, another HCC CSC marker [18], could also be detected in CD133+ cells. Similar results were observed in all five HCC tissues. We examined CD133 expressions in four human HCC cell lines. All cells were CD133 positive in both PLC/PRF/5 and Hep3B cells, and about half of the cells were CD133 positive in Huh7 and HepG2 cells (Fig. 1C).

Expression of MMP-2 and ADAM9 in CD133si- and control-PLC/PRF/5 cells and CD133+/− Huh7 cells

We established CD133si-PLC/PRF/5 cells by transfection of CD133 siRNA (Fig. 1D). The expression of CD133 mRNA and protein in CD133si-PLC/PRF/5 cells was inhibited within 72 h after transfection. The cell viability of CD133si-PLC/PRF/5 cells was similar to that of control-PLC/PRF/5 cells within 72 h after transfection (Fig. 1D). Next, we examined the mRNA expression of MMPs, ADAMs, and TIMPs. The mRNA expression of MMP-2 and ADAM9 in CD133si-PLC/PRF/5 cells was significantly lower than in control-PLC/PRF/5 cells (Fig. 2A and B); however, the expression of other metalloproteinases such as MMP-9,14, ADAM8,10,12,17, and TIMP-1,2,3 was not affected (data not shown). The protein (pro-form and active form) expressions of both MMP-2 and ADAM9 decreased in CD133si-PLC/PRF/5 cells compared to control-PLC/PRF/5 cells (Fig. 2A and B). A protein zymography assay for MMP-2 was consistent with this result in that the activity of MMP-2 protein in CD133si-PLC/PRF/5 cells also decreased compared to control-PLC/PRF/5 cells (Fig. 2A).

In Huh7 cells CD133 expression was positive in half of the cell population, as shown in Fig. 1B. We separated the Huh7 cells into CD133+ and − cells by magnetic cell sorting (Fig. 2C). The mRNA of MMP-2 in CD133− Huh7 cells was significantly lower than that of CD133+ Huh7 cells (Fig. 2C). The proteins (pro-form and active form) expressions of both MMP-2 and ADAM9 decreased in CD133− Huh7 cells as compared to CD133+ Huh7 cells (Fig. 2C and D).

The invasion ability of CD133si- and control-PLC/PRF/5 cells, and CD133+/− Huh7 cells

Human HCC cells require MMP-2 activity for invasion [19]. The invasion of PLC/PRF/5 cells transfected for siRNA against MMP-2 (MMP-2si-PLC/PRF/5 cells) was significantly lower than that of control cells (Fig. 3A). Of note, is the finding that the invasion of CD133si-PLC/PRF/5 cells was significantly lower than that of control cells (Fig. 3B). In order to examine the involvement of MMP-2 protein in the invasion activity, MMP-2 protein was added to the culture of CD133si-PLC/PRF5 cells. Although there was a tendency for MMP-2 to promote the invasion ability of CD133si-PLC/PRF/5 cells, this effect was not statistically significant (Fig. 3B).

In Huh7 cells, the invasion of CD133− Huh7 cells was significantly lower than that of CD133+ Huh7 cells (Fig. 3C). The

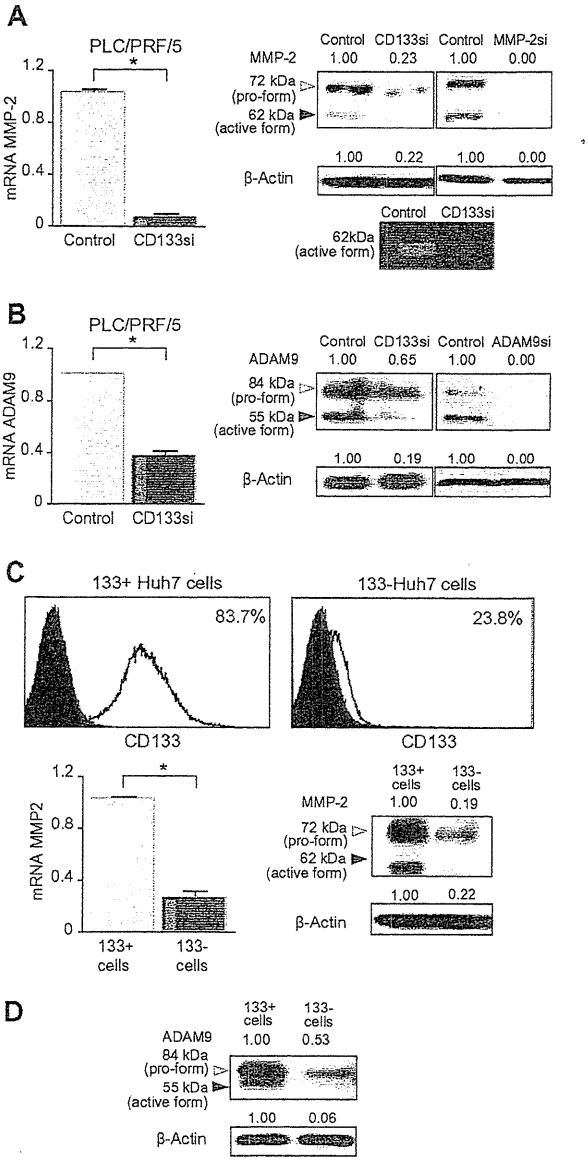

Fig. 2. Expression levels of MMP-2 and ADAM9 in CD133si-PLC/PRF/5, control-PLC/PRF/5 cells, and CD133+/−Huh7 cells. (A and C) MMP-2 mRNA and protein levels were detected by real-time PCR and Western blotting respectively, and the activity of MMP-2 was assayed by protein zymography in CD133si PLC/PRF/5si cells or control-PLC/PRF/5 cells, and CD133+/− Huh7 cells. To confirm the specificity of the anti-MMP-2 antibody used in Western blotting, the expression of MMP-2 (pro-form 72 kDa, active form 62 kDa) was evaluated in MMP-2si-PLC/PRF/5 and control-PLC/PRF/5 cells (Fig. 2A, right). (B and D) ADAM9 mRNA and protein levels, in CD133si-PLC/PRF/5 cells or control-PLC/PRF/5 cells, and CD133+/−Huh7 cells, were analyzed by real-time PCR and Western blotting respectively. To confirm the specificity of the anti-ADAM9 antibody used in Western blotting, the expression of ADAM9 (pro-form 84 kDa, active form 55 kDa) was analyzed in ADAM9si-PLC/PRF/5 and control-PLC/PRF/5 cells (Fig. 2B, right). (C) Huh7 cells were separated into CD133+ and − cells by magnetic cell sorting and the expression levels of CD133 were detected by flow cytometry. White curves, CD133 staining; black curves, control IgG staining. Positive cell rates are shown in the figure. Representative flow cytometry data are shown. Similar results were obtained from three independent experiments. Western blotting data were obtained from three independent experiments. Representative data are shown. The ratios of the protein levels (pro-form, upper side; active form, lower side) compared to control (1.00) are shown. $p < 0.05$.

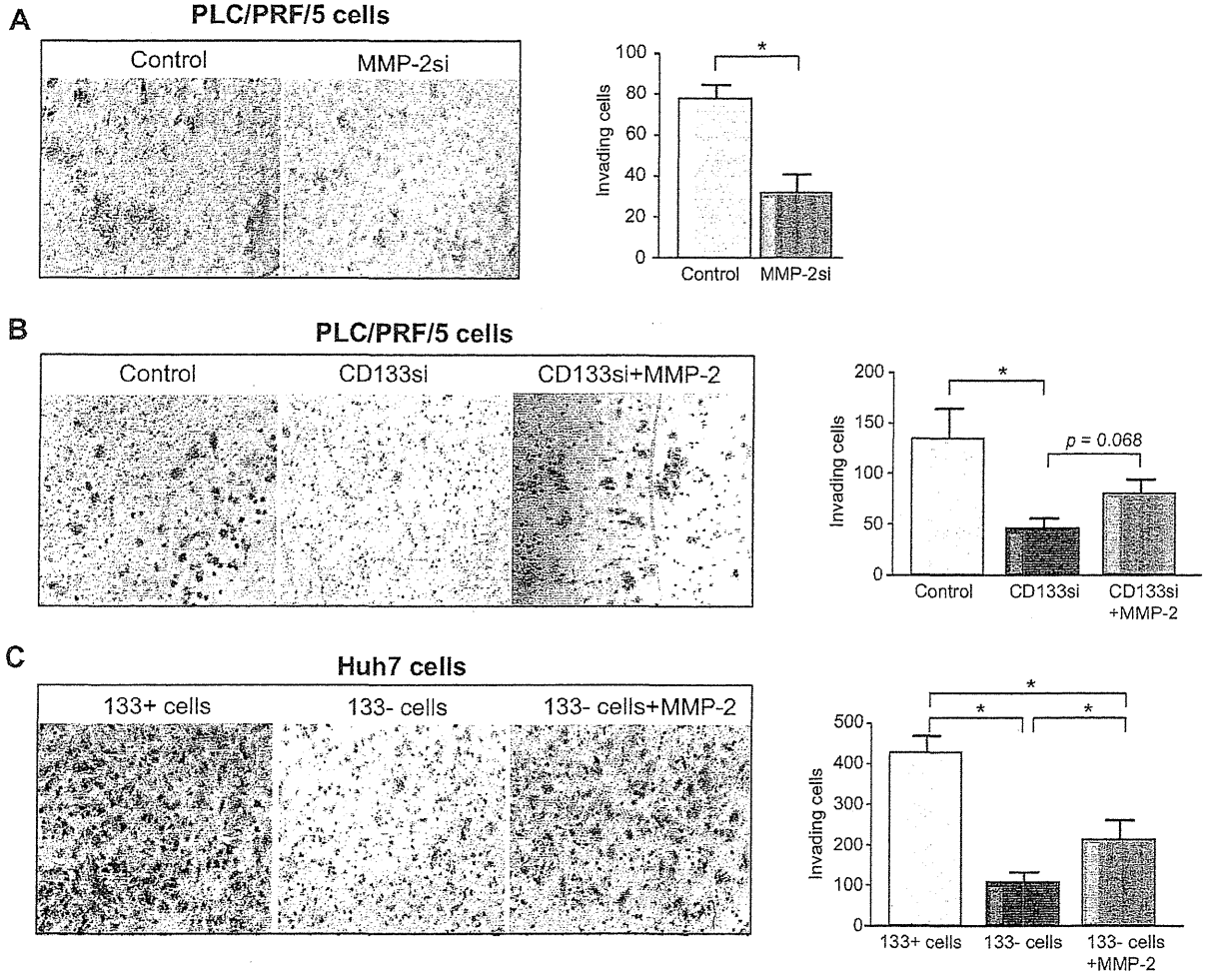

Fig. 3. The capability of invasion of CD133si-PLC/PRF5 cells, control-PLC/PRF/5 cells, and CD133+/–Huh7 cells. (A) The invasion activity of MMP-2si-PLC/PRF/5 cells or control-PLCPRF/5 cells was assayed by Boyden-chamber assay. Representative data of invading cells are shown on the left, and the quantification of the number of invading cells is shown on the right. (B) The invasion activity of CD133si-PLC/PRF/5 cells or control-PLCPRF/5 cells was evaluated by Boyden-chamber assay. Representative data of invading cells are shown on the left and the quantification of the number of invading cells is shown on the right. To assess the involvement of MMP-2 protein in the invasion activity, MMP-2 protein (20 μM) was added to CD133si-PLC/PRF/5 cultured cells. (C) The invasion activity of CD133+ and – Huh7 cells was evaluated by Boyden-chamber assay. MMP-2 protein (20 μM) was added to CD133 – Huh7 cultured cells. Representative data of invading cells are shown on the left and the quantification of the number of invading cells are shown on the right. Similar results were obtained from two independent experiments. $^{*}p < 0.05$.

MMP-2 protein was also added to the culture of CD133– Huh7 cells and this led to a significant increase in the invasiveness of CD133– cells (Fig. 3C). These results demonstrated that CD133-expressing HCC cells had higher abilities of invasion, and MMP-2 could promote the invasiveness of CD133 negative HCC cells.

The VEGF production ability of CD133si- and control-PLC/PRF/5 cells, and CD133+/– Huh7 cells

It has previously been reported that MMP-2 is associated with an increased bioavailability of VEGF [20]. In agreement with this, we observed that in MMP-2si-PLC/PRF/5 cells, the levels of VEGF were significantly lower than in control-PLC/PRF/5 cells (Fig. 4A). We then investigated the production of VEGF in CD133si- and control-PLC/PRF/5 cells, as well as in CD133– Huh7 cells. The VEGF levels of CD133si-PLC/PRF/5 cells were significantly lower than in control-PLC/PRF/5 cells (Fig. 4B), and in CD133– Huh7 cells, the VEGF levels were significantly lower than in CD133+ Huh7 cells (Fig. 4C). To examine the involvement of MMP-2 in the production of VEGF, MMP-2 was added to the culture of CD133– Huh7 cells. This led to a significant increase in the VEGF production of CD133– Huh7 cells (Fig. 4C). These results demonstrated that CD133-expressing HCC cells had an increased ability of angiogenesis because of higher expression levels of MMP-2.

CD133 knockdown resulted in increasing membrane-bound MICA expression, decreasing soluble MICA, and enhancing the cytolytic activity of NK cells in PLC/PRF/5 cells

MICA, a ligand of human NKG2D receptor, is frequently expressed in HCC and determines its sensitivity to NK cells

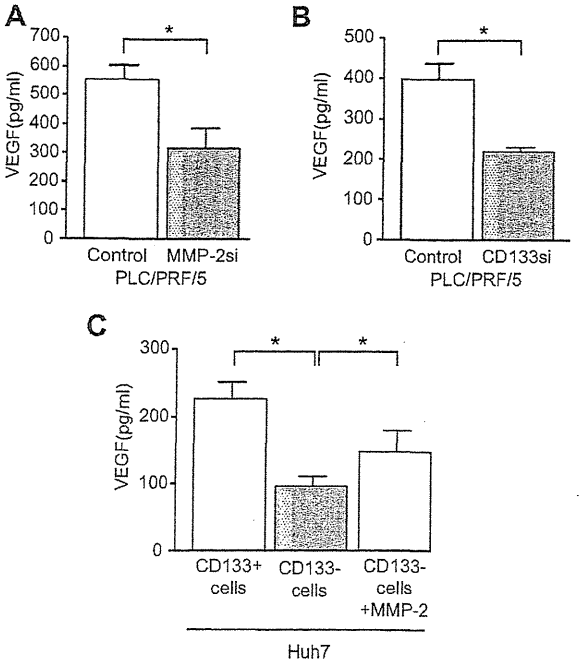

Fig. 4. Analysis of VEGF expression in CD133si-PLC/PRF/5 cells, control-PLC/PRF/5 cells, and CD133+/–Huh7 cells. (A) We measured the VEGF expression in PLC/PRF/5 cells, in presence or absence of MMP-2 protein. MMP-2si-PLC/PRF/5 or control-PLC/PRF/5 cells were cultured for 24 h and the supernatants were subjected to VEGF ELISA. (B) We next assayed the expression of VEGF in CD133si-PLC/PRF/5 cells or control-PLC/PRF/5 cells. These cells were cultured for 24 h and the supernatants were subjected to VEGF ELISA. (C) CD133+/– Huh7 cells were cultured for 24 h and the supernatants were subjected to VEGF ELISA. 133+ cells, CD133+ Huh7 cells; 133– cells, CD133– Huh7 cells; 133– cells+ MMP-2, CD133– Huh7 cells were cultured with MMP-2 protein (25 μM). Similar results were obtained from two independent experiments. $p < 0.05$.

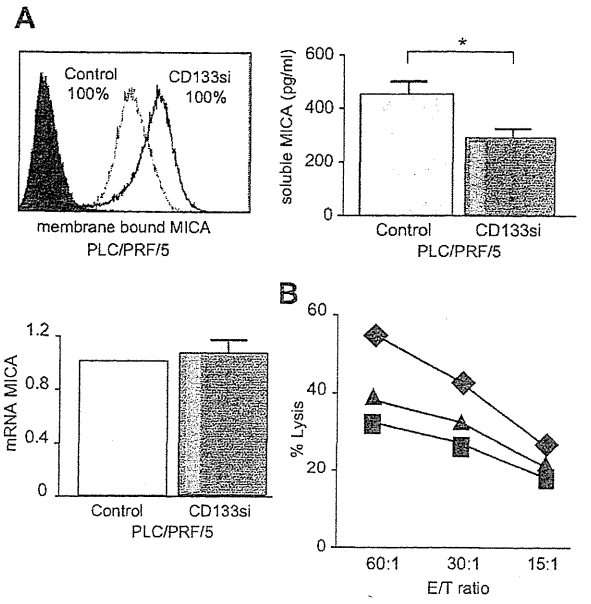

Fig. 5. Expression levels of MICA, soluble MICA production, and NK sensitivity in CD133si-PLC/PRF/5 cells and control-PLC/PRF/5 cells. (A) Expression levels of membrane-bound MICA (upper left panel) and soluble MICA production (upper right panel) in CD133si-PLC/PRF/5 cells or control-PLC/PRF/5 cells were evaluated by flow cytometry and specific ELISA respectively. Positive cell rates are shown in the figure. mRNA levels of MICA in CD133si-PLC/PRF5 or control-PLC/PRF/5 cells detected by real-time RT-PCR (lower panel). (B) We examined the cytolytic activity of NK cells against CD133si-PLC/PRF/5 cells or control-PLC/PRF/5 cells. CD133si-PLC/PRF/5 cells or control-PLC/PRF/5 cells were subjected to ^{51}Cr-release assay against NK cells. Cytolytic activity of NK cells against control-PLC/PRF/5 cells (■) or CD133si-PLC/PRF/5 cells without (♦) or with blocking antibody of MICA (▲). Similar results were obtained from two independent experiments. $p < 0.05$.

[21]. However, MICA is also proteolytically cleaved from HCC, which is an important mechanism for tumor evasion from host immunity [22]. We previously found that ADAM9 protease plays essential roles in the shedding of MICA from HCC cells [23]. The expression of membrane-bound MICA in CD133si-PLC/PRF5 cells increased, and soluble MICA production from CD133si-PLC/PRF/5 cells significantly decreased compared to the control-PLC/PRF/5 cells (Fig. 5A). In contrast, mRNA levels of MICA were equal in both CD133si-PLC/PRF/5 cells and control-PLC/PRF/5 cells (Fig. 5A). These results suggested that CD133 knockdown induced inhibition of MICA shedding in PLC/PRF/5 cells. We next evaluated the NK sensitivity of CD133si-PLC/PRF/5 cells or control-PLC/PRF/5 cells by ^{51}Cr-release assay. The cytolytic activity of NK cells against CD133si-PLC/PRF/5 cells was higher than against control-PLC/PRF/5 cells (Fig. 5B). The blocking MICA/NKG2D signal resulted in a decrease in the cytolytic activity of NK cells against CD133si-PLC/PRF/5 cells to the levels of the control-PLC/PRF/5 cells (Fig. 5B). These results were similarly observed in CD133 knockdown Hep3B and HepG2 cells (data not shown). These results suggested that control-HCC cells were more resistant than CD133 knockdown HCC cells to the cytolytic activity of NK cells due to MICA/NKG2D signals.

The expressions of membrane-bound MICA and the production of soluble MICA of CD133+/– Huh7 cells and the cytolytic activity of NK cells against CD133+/– Huh7 cells

We examined whether CD133+/– Huh7 cells exhibited similar characteristics as those observed in CD133si-PLC/PRF/5 and control-PLC/PRF/5 cells. The expression of membrane-bound MICA in CD133– Huh7 cells tended to be higher than in CD133+ Huh7 cells, and soluble MICA production from CD133– Huh7 cells was significantly lower than in CD133+ Huh7 cells (Fig. 6A). We examined the NK sensitivity of CD133+ or – Huh7 cells. The cytolytic activity of NK cells against CD133+ Huh7 cells was lower than that against CD133– Huh7 cells (Fig. 6B), as observed for CD133si- and control-PLC/PRF/5 cells.

Discussion

In order to establish new cancer therapy against HCC, the significance of CD133 expression on HCC cells needs to be elucidated. We demonstrated that CD133+ cells were observed only in HCC tissues and not in non-cancerous liver tissues, which is consistent with previous reports [8,14]. Although, all four HCC cell lines tested expressed CD133, some variations in expression was observed between cell lines; this may reflect the differences in the ability of CSCs to differentiate into progeny cells.

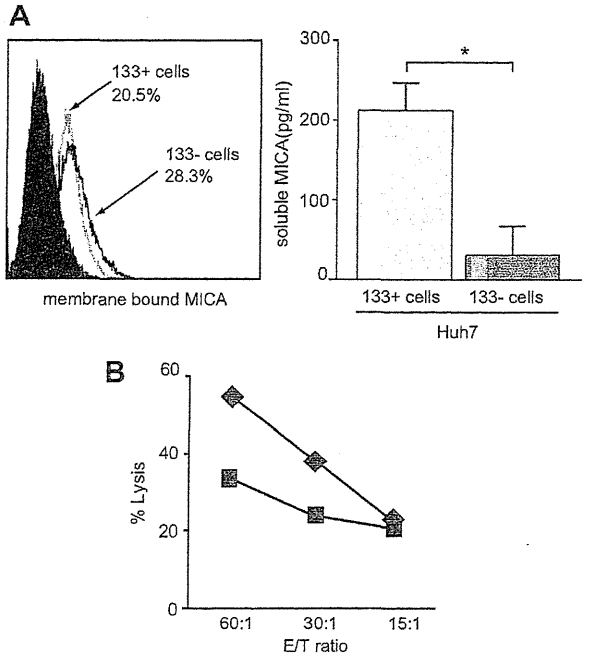

Fig. 6. Expression levels of MICA, soluble MICA production, and NK sensitivity in CD133+/− Huh7 cells. (A) Expression level of membrane-bound MICA (left panel) and soluble MICA production (right panel) in CD133+/− Huh7 cells were evaluated by flow cytometry and specific ELISA, respectively. Positive cell rates are shown in the figure. (B) We examined the cytolytic activity of NK cells against CD133+/− Huh7 cells. CD133+/− Huh7 cells were subjected to ^{51}Cr-release assay against NK cells. Cytolytic activity of NK cells against CD133+ Huh7 cells (■) or CD133− cells (♦). Representative results are shown. Similar results were obtained from two independent experiments. $p < 0.05$.

Song et al. reported that HCC patients with increased CD133 expressions in HCC tissues had shorter overall survival and higher recurrence rates compared to patients with low CD133 expression [14]. These results suggested that CD133 may have oncogenic roles in human HCC, which led us to investigate the molecular characteristics of CD133-expressing HCC cells.

We found that MMP-2 expression at mRNA and protein levels and MMP-2 activity in CD133si-PLC/PRF/5 cells decreased. This agrees with findings for CD133+/− Huh7 cells which could be divided into two groups by the expression of CD133. At present, there has been no report on the detailed relationship between the expression of CD133 molecules and MMP-2. Other MMPs (MMP-1,3,7,9,10,12,13) have an activator protein-1 (AP-1) binding site in the proximal promoter [11], while MMP-2 does not. Dai et al. demonstrated that FoxM1 directly binds to the MMP-2 promoter and regulates MMP-2 expression in glioma cells [24]. Although the detailed mechanism of inhibition of MMP-2 in CD133 negative HCC cells remains unclear, newly identified transcriptional factors may contribute to this mechanism.

A previous study demonstrated that MMP-2 knockdown mice exhibited reduced angiogenesis and impaired tumor growth [25]. CD133 knockdown resulted in the inhibition of MMP-2 activity in PLC/PRF/5 cells and the invasive ability of CD133si-PLC/PRF/5 cells was significantly impaired. These results thus suggested that the decreased activity of MMP-2 impaired the invasive ability of HCC cells. MMP-2si-PLC/PRF/5 cells also lost this ability, which supports the essential role of MMP-2 in the invasion by PLC/PRF/5 cells.

MMP-2 activity is also associated with an increased bioavailability of VEGF [20] – this latter being an important factor in the process of angiogenesis. The production of VEGF in CD133si-PLC/PRF/5 cells significantly decreased compared to that in control-PLC/PRF/5 cells, suggesting that the existence of CD133-expressing HCC cells might offer circumstances conducive to HCC survival by angiogenesis. These results were also consistent with findings for CD133+/− Huh7 cells. If the phenotype of the CD133− Huh7 cells could be reversed by transfection of the CD133 gene in CD133− Huh7 cells, this would be an evidence for the direct implication of CD133. However, transfection into CD133− Huh7 cells is technically very difficult because the reagent of transfection was toxic to CD133− Huh7 cells isolated by magnetic cell sorting. Our present results suggested that CD133 expression in HCC cells may reflect the malignant potential of HCC cells which, in turn, may influence the clinical outcomes of HCC patients.

MICA shedding is thought to be the principle mechanism by which tumor cells escape from NKG2D-mediated immunosurveillance [26]. We previously found that both ADAM9 and ADAM10 proteases were cooperatively associated with the shedding of MICA in human HCC [16,23]. Thus, it would be interesting to examine the activity of ADAMs protease in CD133-expressing HCC cells to understand how HCC tumor cells escape from innate immunity. In the present study, we demonstrated that CD133 knockdown in PLC/PRF/5 cells resulted in the decrease of ADAM9 mRNA and protein, but not of ADAM10, and was associated with the increase in expression of membrane-bound MICA as well as the decrease in production of soluble MICA in human HCC. The mRNA expression of MICA did not change between CD133si-PLC/PRF/5 cells and control-PLC/PRF/5 cells, suggesting that decreasing ADAM9 activity played an essential role in regulating MICA in CD133-expressing cells. At present, the detailed association between CD133 molecule and ADAMs proteases remains unclear. However, CD133-expressing HCC cells may be escaping from NKG2D-mediated immunosurveillance by promoting MICA shedding via ADAM9 protease.

Consistent with the results of the expressions of MICA in CD133-expressing cells, CD133 positive HCC cells are resistant and CD133 negative HCC cells are susceptible to the cytolytic activity of NK cells. Cai et al. demonstrated that the number of CD56$^+$ NK cells was reduced in HCC tissues compared to healthy donors, and CD56$^+$ NK cells in HCC patients displayed impairments in cytotoxicity and IFN-γ production [27]. This suggests that the immunological microenvironment in HCC tissues may be favorable to the survival of HCC cells, especially of CD133+ HCC cells. The specific targeting and eradication of CSCs is the most important challenge for cancer treatment. We previously demonstrated that some anti-HCC chemotherapy drugs regulated the ADAM family proteins, resulting in the enhancement of NK sensitivity [16,23]. Thus, by controlling the expression of CD133 and/or ADAM9 protease with new reagents, it may be possible to develop a new therapeutic strategy against CD133-expressing CSCs.

In spite of recent progress and early successes reported for HCC treatment, there is significant room for improvement. In

the present study, we demonstrated that CD133-expressing HCC cells have a higher ability for invasion and for producing the angiogenetic factor VEGF, and they are resistant to NK activity. These findings suggest that CD133 expression in HCC cells confers malignant potential in human HCC that may induce the growth and metastasis of HCC cells. We believe that understanding the detailed characteristics of CD133-expressing cells will pave the road for the development of new treatments for human HCC.

Conflicts of interest

The Authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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