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Hepatitis B surface antigen inhibits MICA and MICB expression via induction of cellular miRNAs in hepatocellular carcinoma cells

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Hepatitis B surface antigen (HBsAg) seropositivity is an important risk factor for hepatocellular carcinoma (HCC), and HBsAg-transgenic mice have been reported to spontaneously develop HCC. The major histocompatibility complex class I-related molecules A and B (MICA and MICB) are NKG2D ligands that play important roles in tumor immune surveillance. In the present study, we found that HBsAg overexpression in HepG2 cells led to upregulation of 133 and downregulation of 9 microRNAs (miRNAs). Interestingly, several HBsAg-induced miRNAs repressed the expression of MICA and MICB via targeting their 3′-untranslated regions. In addition, the expression of MICA and MICB was significantly reduced upon HBsAg overexpression, which was partially restored by inhibiting the activities of HBsAg-induced miRNAs. Moreover, HBsAg-overexpressing HCC cells exhibited reduced sensitivity to natural killer cell-mediated cytolysis. Taken together, our data suggest that HBsAg supresses the expression of MICA and MICB via induction of cellular miRNAs, thereby preventing NKG2D-mediated elimination of HCC cells.

Introduction

Hepatitis B virus (HBV) is a DNA virus, containing a partial double-stranded circular genome of ~3.2 kb in length. The HBV genome is transcribed into four major RNAs, including the 3.5 kb precore and pregenome RNA, the 2.4 kb pre-S1 messenger RNA (mRNA), the 2.1 kb pre-S2/S mRNA and the 0.7 kb X mRNA (1,2). The 2.1 kb pre-S2/S mRNA is translated into hepatitis B surface antigen (HBsAg) and the pre-S2 protein (1,2). HBV-infected cells secrete virions as well as subviral particles. HBsAg is a component of the outer shell of HBV virions. It also forms subviral particles that comprise almost exclusively of this antigen and the associated host-derived lipids. Lacking viral nucleic acid, these subviral lipoprotein particles are non-infectious (1). The function of these 'dummy' particles remains unclear. It is speculated that they serve as a high-dose tolerogen to adsorb neutralizing antisurface antibodies, and suppress immune elimination of infected cells during the progression of HBV infection (1,3–6). HBV infection is a major cause of hepatocellular carcinoma (HCC) (7). Seropositivity for HBsAg is an important risk factor for HCC (8), and HBsAg seroclearance is associated with a low risk for HCC development (9). HBsAg-transgenic mice have been reported to develop HCC 15 months after birth (10).

The major histocompatibility complex class I-related molecules A and B (MICA and MICB) are innate immune system ligands for the NKG2D receptor that are expressed on natural killer (NK) cells, $CD8^+$ T cells and γδ T cells (11–13). NKG2D engagement is sufficient to stimulate NK cell cytolysis and cytokine production. It also provides an enhancing or costimulatory signal for the activation of $CD8^+$ T cells, and probably other T cells (11–13). In contrast to the classical major histocompatibility complex class I molecules, MICA and MICB are not expressed in most normal cells, but highly expressed in transformed cells (14–16). MICA and MICB may play important roles in tumor immune surveillance by promoting antitumor NK and T-cell responses (14–16). Nevertheless, tumor cells have also developed strategies to escape NKG2D immunity by downregulation of NKG2D ligands (17–19). We have demonstrated that inhibition of HBV replication induces MICA expression, which leads to increased NK cell-mediated cytolysis of HBV-expressing HCC cells (20). This finding suggests that HBV compromises the innate immune system by downregulating NKG2D ligands. However, the molecular mechanism underlying HBV-mediated expressional regulation of MICA remains to be elucidated.

MicroRNAs (miRNAs) are small, non-coding regulatory RNA molecules that influence a wide range of biological and pathological processes (21,22). They bind to the 3′-untranslated region (3′-UTR) of target mRNA to inhibit translation or promote mRNA degradation (21,23). Mounting evidence has shown that the expression of MICA and MICB is regulated by a variety of miRNAs, including miR-20a, miR-93, miR-106b, miR-372, miR-373 and miR-520d (24–26). We also found that MICA and MICB expression was upregulated upon knockdown of Dicer, an enzyme essential for miRNA biogenesis (27). In addition, HBV and HBx, an HBV viral protein, were reported to be involved in the regulation of cellular miRNA expression (28–33). Therefore, it is interesting to address whether the effect of HBV on MICA expression in HCC cells is mediated by miRNAs.

In this study, we aimed to investigate whether HBsAg can regulate the expression of MICA and MICB in HCC cells. Our results showed that overexpression of HBsAg in HepG2 and Huh7 cells led to downregulation of MICA and MICB. In addition, using Solexa sequencing technology, we identified HBsAg-regulated miRNAs and found that some of them repressed the expression of MICA and MICB via targeting the 3′-UTRs of their mRNAs. Moreover, we found that the downregulation of MICA and MICB in HBsAg-overexpressing HCC cells was partially restored by inhibiting the activities of HBsAg-induced miRNAs.

Materials and methods

Cell culture

Human HCC cell lines HepG2 and Huh7 were purchased from American Type Culture Collection (ATCC, Manassas, VA) and Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China), respectively. HepG2.2.15 cells were developed by transfection of HepG2 with a plasmid containing two head-to-tail dimers of the HBV genome, and they can constitutively secrete viral proteins and Dane-like particles (34). HepG2 and HepG2.2.15 cells were grown in RPMI 1640 medium (Hyclone, Logan, UT) and Huh7 cells were grown in Dulbecco's modified Eagle's medium (Hyclone), supplemented with 10% fetal bovine serum. The NK-92MI cell line (ATCC) was grown in MEM α-medium (Gibco, Grand Island, NY) with 12.5% horse serum and 12.5% fetal bovine serum. All cells were incubated at 37°C in a humidified incubator with 5% CO_2.

Immunohistochemical analysis

The primary human HCC tissues and their adjacent non-tumor liver tissues were obtained from 38 patients who underwent surgery in The First Affiliated

Abbreviations: HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; MICA, major histocompatibility complex class I-related molecule A; MICB, major histocompatibility complex class I-related molecule B; miRNA, microRNA; mRNA, messenger RNA; NK, natural killer; RT–PCR, reverse transcription–PCR; 3′-UTR, 3′-untranslated region.

Hospital of Wenzhou Medical College (Wenzhou, China). None of the patients received chemotherapy before surgery, and the detailed characteristics of the patients are shown in Supplementary Table 1, available at *Carcinogenesis* Online. The specimens of all patients in our study were examined by three board-certified pathologists who were blinded to the results of the pathological examination by former pathologists. Informed consent was obtained from all the patients, and the study was approved by the Scientific Ethics Committee of Wenzhou Medical College.

Tissue specimens were fixed in 10% formalin, dehydrated in graded ethanol and embedded in paraffin before preparation of slide tissue sections. After deparaffinization and rehydration, specimens were incubated with 3% H_2O_2 to quench endogenous peroxidase activity; then, the slide sections

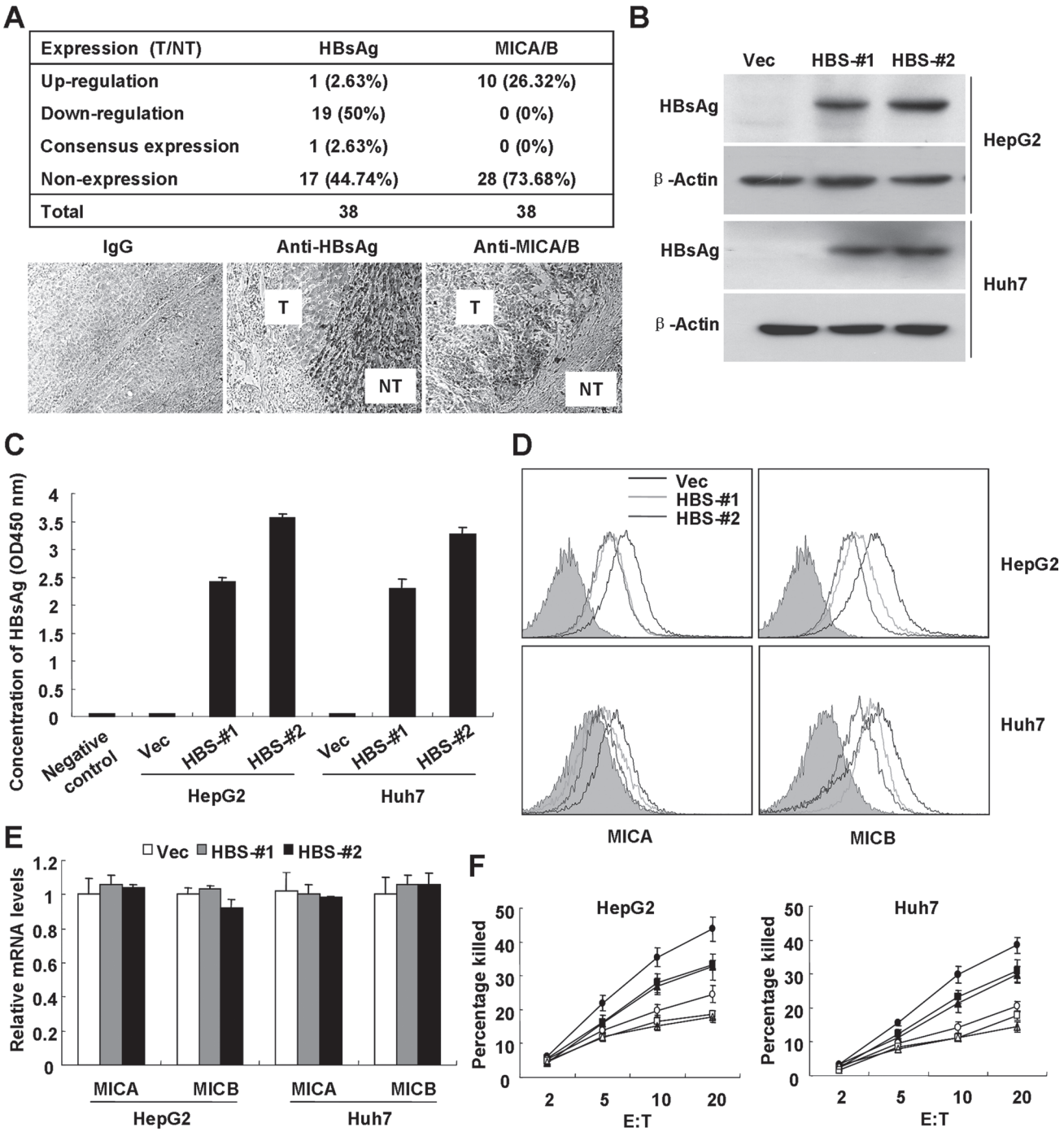

Fig. 1. Downregulation of MICA and MICB in HBsAg-overexpressing HCC cells. (**A**) The expression of HBsAg and MICA/B in 38 human HCC and the adjacent non-tumor liver tissues by immunohistochemical staining. (Top panel) Statistic summary of the staining and (bottom panel) representative staining micrographs (T: tumor tissue, NT: non-tumor tissue). (**B**) The intracellular HBsAg was determined by western blotting, and β-actin was used as the loading control. (**C**) The secreted HBsAg in the culture medium was quantified by ELISA. It is worth noting that the mean absorbance value of the empty vector transfectant (Vec) is similar to that of the negative control, indicating that it does not secrete HBsAg. (**D**) Cell surface MICA and MICB expressions were measured by flow cytometry in HBsAg-overexpressing cells and control cells. The filled histograms represent isotype control antibody staining. Horizontal and vertical axes denote intensity of fluorescence and number of events, respectively. (**E**) The relative MICA and MICB mRNA levels were determined by real-time RT–PCR in HBsAg-overexpressing cells. (**F**) Reduced lysis of HepG2 and Huh7 cells by NK-92MI cells upon HBsAg overexpression. NK-92MI cells were incubated with HBsAg-overexpressing cells (HBS-#1, squares; HBS-#2, triangles) or the control (circles) cells in the absence (closed) or presence (open) of anti-NKG2D antibody. The effector-to-target ratio is as indicated.

were microwaved in citrate buffer for 15 min and incubated with 5% serum blocking buffer at room temperature for 15 min, followed by incubation with anti-HBsAg mAb (sc-53299; Santa Cruz Biotechnology, Santa Cruz, CA), anti-MICA/B mAb (MAB13001; R&D Systems, Minneapolis, MN) or the isotype control (R&D Systems) at 4°C overnight. Antibody staining was visualized using peroxidase-conjugated goat anti-mouse immunoglobulin G and diaminobenzidine. The slides were counterstained with hematoxylin, dehydrated in ascending graded ethanol and cleared in xylene before light microscopy examination.

Plasmid transfection and establishment of stable HBsAg-overexpressing transfectants

The pT-HBS plasmid was constructed by cloning the HBsAg-coding sequence into the pTarget vector (Promega, Madison, WI) with the following primers: forward (ACGAACATGGAGAACATCAC) and reverse (GGTTTAAATGTATACCCAGAGAC). Both pT-HBS and the empty pTarget plasmids were transfected into HepG2 or Huh7 cells using Lipofectamine 2000 (Invitrogen, Grand Island, NY), and the transfectants were selected in the presence of 800 μg/ml G418. The resistant clones were further validated for HBsAg expression by western blotting and ELISA kits (Kehua Bio-engineering, Shanghai, China) according to the manufacturer's instructions and maintained in a medium containing 400 μg/ml G418 for subsequent studies.

The plasmids were transiently transfected into HepG2.2.15 cells by using Lipofectamine 2000; the cells were then collected 48 h after transfection and analyzed for the expression of miRNA, cell surface MICA and MICB and NK cell-mediated cytolytic assay as described below. The HBsAg secretion in the culture medium was measured using the ELISA kits.

Small RNA sequencing and bioinformatic analysis

Small RNAs ranging from 16 to 30 nucleotide were gel purified, and those with 5′-phosphate and 3′-hydroxyl group were ligated with 3′- and 5′-adaptors. The ligation products were gel purified, reversely transcribed and PCR amplified. The PCR fragments were then sequenced using an Illumina GAII platform. Low-quality sequencing reads were discarded, and the adaptor sequences were removed before analysis of small RNA sequences. The sequences were mapped to the reference human genome (hg19) by using the SOAP2 program without mismatches. For annotation, small RNA sequences were aligned to miRBase 16.0, Rfam 9.1 and GenBank using BLAST and SOAP2 software.

miRNAs and transfection

micrON™ miRNA mimics, micrOFF™ miRNA inhibitors and the negative controls were obtained from RiboBio (Guangzhou, China). Small RNAs were transfected using siPORT NeoFX (Ambion, Austin, TX) according to the manufacturer's instructions, and cells were collected for flow cytometry or real-time reverse transcription–PCR (RT–PCR) analysis 48 h later.

Flow cytometry

Cells (5×10^5) were incubated with anti-MICA mAb (MAB1300; R&D Systems), anti-MICB mAb (MAB1599; R&D Systems) or the isotype control (R&D Systems) at 4°C for 1 h. Then, cells were washed three times with phosphate-buffered saline, followed by staining with phycoerythrin-conjugated goat anti-mouse immunoglobulin G (F0102; R&D Systems) at 4°C for 30 min. The fluorescence was finally detected with FACSAria (BD Biosciences, San Jose, CA) and analyzed with WinMDI software (http://facs.scripps.edu/software.html).

Real-time RT–PCR

Total RNA was prepared using Trizol reagent (Invitrogen) and incubated with RNase-free DNase I (Fermentas, Glen Burnie, MD) for 30 min. To quantify the levels of mRNAs, the DNA-free RNA was reverse transcribed using the M-MLV reverse transcription kit (Promega) according to the manufacturer's instructions. Samples prepared in the absence of reverse transcriptase served as negative controls. Real-time RT–PCR was carried out using SYBR premix Ex Taq (TaKaRa, Dalian, China) with the ABI 7300 Sequence Detection System (Applied Biosystems, Foster, CA). The level of GAPDH mRNA served as an internal control. Primer sequences (forward and reverse) were as follows: MICA, CTTGGCCATGAACGTCAGG and CCTCTGAGGCCTCRCTGCG; MICB, ACCTTGGCTATGAACGTCACA and CCCTCTGAGACCTCGCTGCA and GAPDH, ACCCACTCCTCCACCTTTG and CACCACCCTGTTGCTGTAG. For miRNAs detection, reverse transcription and real-time RT–PCR were performed using the bulge-loop miRNA qPCR primer set (RiboBio) according to the manufacturer's instructions, and a human U6 small nuclear RNA was used for normalization.

Luciferase reporter assay

To construct the firefly luciferase reporters, the 3′-UTRs of MICA or MICB were amplified by PCR and inserted into the pGL3-control vector (Promega) at the Xba1 site immediately downstream the stop codon of firefly luciferase. Primer sequences (forward and reverse) were as follows: MICA-3′-UTR, CTAGTCTAGAGAAGAAAACATCAGCTGCAGA and CTAGTCTAGATCTACTAACATTTTGCAGCCTC; MICB-3′-UTR, CTAGTCTAGAACTCTACAGCCAGGCGGC and CTAGTCTAGATTTAAA GAGTAAACATTTACGGT. Cells were cotransfected with 0.4 μg firefly luciferase reporter vector (Luc-MICA 3′-UTR, Luc-MICB 3′-UTR or pGL3-control) and 0.02 μg Renilla luciferase control vector (pRL-CMV), with or without 25 pmol miRNAs, using Lipofectamine 2000 in 24-well plates. Luciferase activities were measured 24 h after transfection using a dual-luciferase reporter assay system (Promega) in which firefly luciferase activity was normalized to Renilla luciferase activity.

Western blotting

Cells were lysed in radioimmunoprecipitation assay buffer, and total protein was measured using the Bradford protein assay (Bio-Rad, Hercules, CA). Samples were denatured at 100°C for 5 min. Thirty micrograms of denatured total protein was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). The blots were then incubated with a primary antibody for 2 h, followed by incubation with horseradish peroxidase-linked secondary antibody for 1 h at room temperature and detected with ECL plus reagents (Millipore). The primary antibodies, anti-HBsAg (IMG-4023) and anti-β-actin (sc-81178) antibodies were obtained from Imgenex (San Diego, CA) and Santa Cruz Biotechnology, respectively.

NK cell-mediated cytolytic assay

The *in vitro* cytolytic activity of NK-92MI cells against various targets was determined using the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega) according to the manufacturer's instructions. Briefly, NK-92MI

Table I. Differential expression of miRNAs that target MICA and/or MICB in HepG2/vec and HepG2/HBS-#2 cells

miRNA name	Relative count		Fold change ($\log_2$HBS-#2/vec)	*P* value	Target	References
	HepG2/vec	HepG2/HBS-#2				
hsa-miR-373	0.01	2.86	8.16	1.51E-19	MICA/B	V (24)
hsa-miR-29b	33.83	345.85	3.35	0	MICB	P
hsa-miR-15b	145.97	1310.79	3.17	0	MICB	P
hsa-miR-195	0.18	1.60	3.16	1.75E-07	MICB	P
hsa-miR-16	414.90	3167.32	2.93	0	MICB	P
hsa-miR-424	35.93	250.48	2.80	0	MICB	P
hsa-miR-29c	4.47	16.61	1.89	8.13E-37	MICB	P
hsa-miR-106a	3.58	11.28	1.66	1.84E-21	MICA/B	P
hsa-miR-107	24.49	75.43	1.62	5E-130	MICB	P
hsa-miR-20a	763.03	1914.53	1.33	0	MICA/B	V (24)
hsa-miR-29a	139.54	347.26	1.32	0	MICB	P
hsa-miR-17	607.09	1475.53	1.28	0	MICA/B	V (24)

P, predicted by TargetScan; V, validated by experiments (24).

cells were incubated with target cells (5×10^3) at different effector-to-target (E:T) ratios in a U-bottomed 96-well plate for 6 h. The assay was terminated by centrifugation at 250*g* for 4 min at room temperature, and the supernatant was collected for lactate dehydrogenase measurement. Spontaneous release was determined by incubating the target cells only. Maximum release was determined as lysis of target cells in 1% Triton X-100. In blocking experiments, anti-NKG2D (MAB139; R&D Systems) was added in the medium to a final concentration of 10 μg/ml. The percentage of lysis was calculated as follows: % cytotoxicity = 100 × (experimental release − NK spontaneous release − target spontaneous release)/(target maximum release − target spontaneous release), and the experiment was performed in triplicate.

Statistical analysis

Each experiment was independently performed at least three times. All data are shown as mean ± SD. Differences between samples were analyzed by independent samples *t*-test using SPSS V17.0 software. Association between categorical variables was analyzed by chi-square test, and the results were considered significant at $P < 0.05$.

Results

Downregulation of MICA and MICB in HBsAg-overexpressing HCC cells

To address whether HBsAg can regulate MICA/B expression in HCC cells, we first measured HBsAg and MICA/B expression in 38 human HBV-related HCC specimens by immunohistochemical staining. Consistent with a previous finding obtained by Jinushi *et al.* (35), the expression of MICA/B was detected in 10 of 38 (26%) HCC tissues, but not in the surrounding non-cancerous tissues (Figure 1A; Supplementary Table 1, available at *Carcinogenesis* Online). Compared with the adjacent non-tumor liver tissues, 19 of 38 (50%) HCC tissues showed reduced HBsAg expression. Interestingly, 7 of the 10 MICA/B-positive tumor tissues showed significantly lower HBsAg levels as compared with the adjacent non-tumor tissues (Supplementary Table 1, available at *Carcinogenesis* Online), suggesting that HBsAg regulates the expression of MICA/B in HBV-associated human HCC tissues. Further statistical analysis indicated that the expression of HBsAg and MICA/B was not significantly associated with different clinical characteristics, including sex, age and stage of tumor (Supplementary Table 2, available at *Carcinogenesis* Online).

We then developed stable HBsAg-overexpressing transfectants from HepG2 and Huh7 cells and selected two (HBS-#1 and HBS-#2) from each cell line for further study. For validation, the intracellular and the secreted HBsAg proteins were measured by western blotting and ELISA, respectively (Figure 1B and 1C). The flow cytometry results showed that the cell surface MICA and MICB proteins were significantly reduced in HBsAg-overexpressing cells as compared with the control cells (Figure 1D). However, real-time RT–PCR data indicated that HBsAg overexpression did not significantly alter MICA and MICB mRNA levels (Figure 1E), suggesting that the expression of MICA and MICB was regulated by HBsAg at post-transcriptional level. Consistent with reduction of MICA and MICB, HBsAg-overexpressing cells exhibited lower sensitivity to lysis by NK-92MI cells, an interleukin-2-independent human NK cell line (36) (Figure 1F).

Identification of HBsAg-regulated miRNAs

Previous studies have shown that the expression of MICA and MICB was repressed by several miRNAs via targeting the 3′-UTRs of their

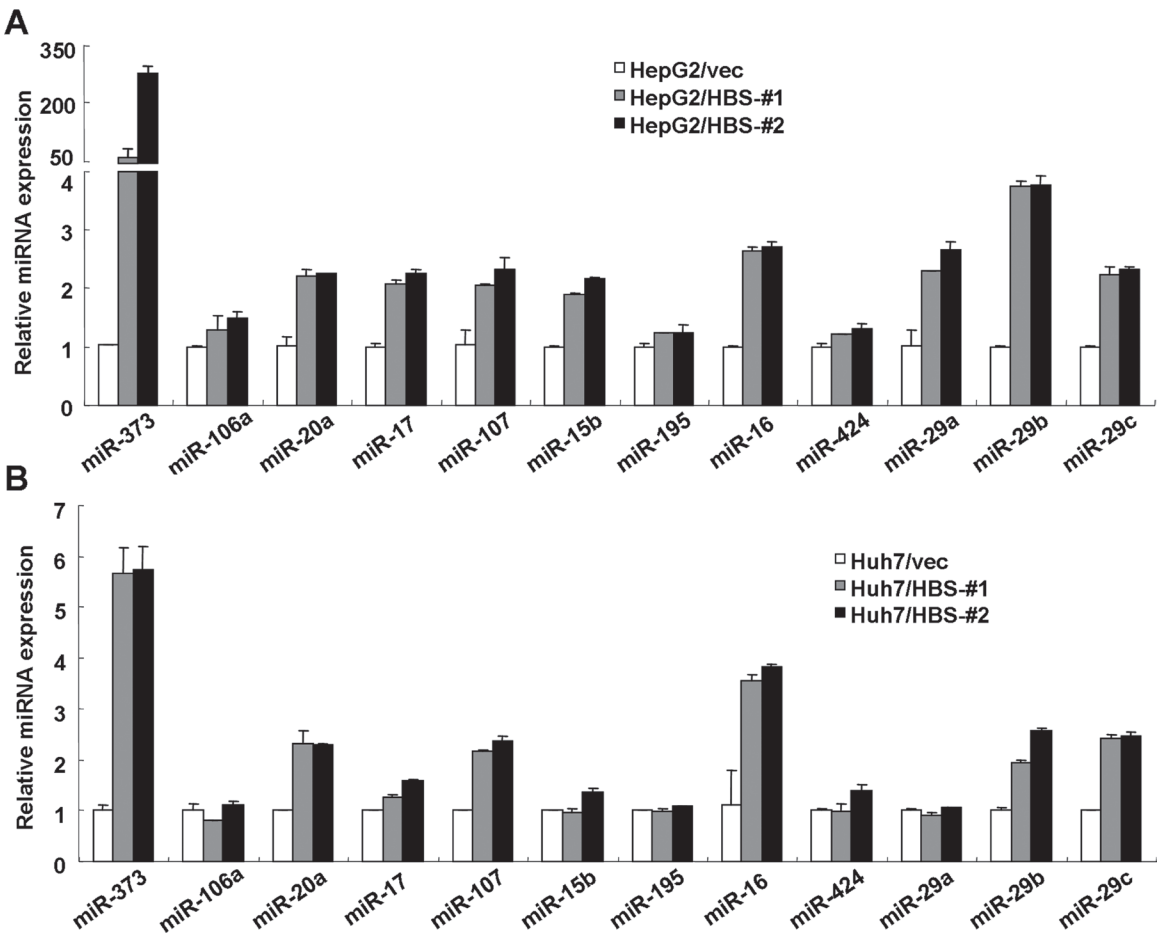

Fig. 2. Upregulation of miRNAs in HBsAg-overexpressing HepG2 and Huh7 cells. The relative miRNA levels in HBsAg-overexpressing HepG2 (**A**) and Huh7 (**B**) cells were determined by real-time RT–PCR.

mRNAs (24,25). Our data suggest that HBsAg inhibits the expression of MICA and MICB at the posttranscriptional level (Figure 1D and 1E). To address whether HBsAg regulates the expression of cellular miRNAs, small RNAs of HBsAg-overexpressing transfectant (HepG2/HBS-#2) and the control transfectant (HepG2/vec) were sequenced using Solexa sequencing technology. In total, 16 429 452 genome-matching sequence reads were identified from HepG2/HBS-#2, and 18 382 283 from HepG2/vec. The small RNAs in both libraries showed a length distribution peak at 22–23 nucleotide and first nucleotide bias for uridine (U) (Supplementary Figure 1A and B, available at *Carcinogenesis* Online). These characteristics suggest that the small RNAs are Dicer-processed products rather than random degradation products (37). Consistent with these characteristics, the most abundant small RNAs (>60%) were found to be derived from annotated miRNA loci (Supplementary Figure 1C, available at *Carcinogenesis* Online).

Further analysis revealed that 133 miRNAs were upregulated and 9 miRNAs were downregulated in HBsAg-overexpressing HepG2 cells (Supplementary Table 3, available at *Carcinogenesis* Online). Interestingly, based on the literature and miRNA target prediction, we found that 12 HBsAg-induced miRNAs may repress the expression of MICA and/or MICB via targeting the 3′-UTRs of their mRNAs (Table I; Supplementary Table 4, available at *Carcinogenesis* Online). We then validated the expression of these miRNAs by real-time RT–PCR and found that nine of them (miR-373, miR-20a, miR-17, miR-107, miR-15b,

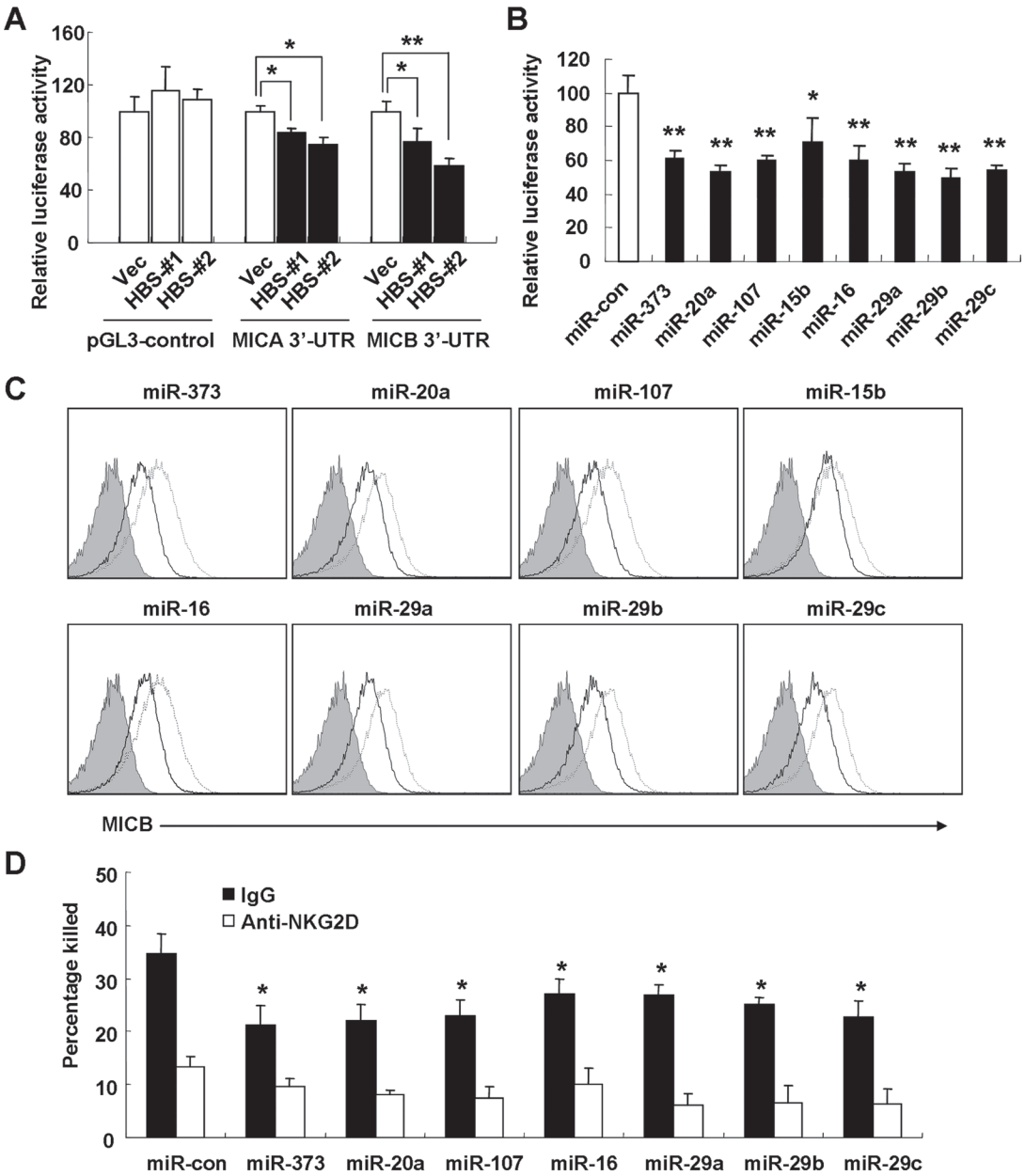

Fig. 3. Downregulation of MICA and MICB by HBsAg-induced miRNAs. (**A**) Luciferase activities of Luc-MICA 3′-UTR and Luc-MICB 3′-UTR were decreased in HBsAg-overexpressing HepG2 cells. *$P < 0.05$; **$P < 0.01$. (**B**) Luciferase activities of Luc-MICB 3′-UTR were repressed in HepG2 cells transfected with different miRNA mimics. *$P < 0.05$; **$P < 0.01$. (**C**) Cell surface MICB expression was measured by flow cytometry in HepG2 cells 48 h after transfection with different miRNA mimics (thick lines) or the negative control (dashed lines). The filled histogram represents isotype control antibody staining. Horizontal and vertical axes denote intensity of fluorescence and number of events, respectively. (**D**) Transfection of miRNA mimics reduced the sensitivity of HepG2 cells to lysis by NK-92MI cells. NK-92MI cells were incubated with miRNA-transfected HepG2 cells at an effector-to-target ratio of 10:1 in the presence of anti-NKG2D or isotype control monoclonal antibody. *$P < 0.05$.

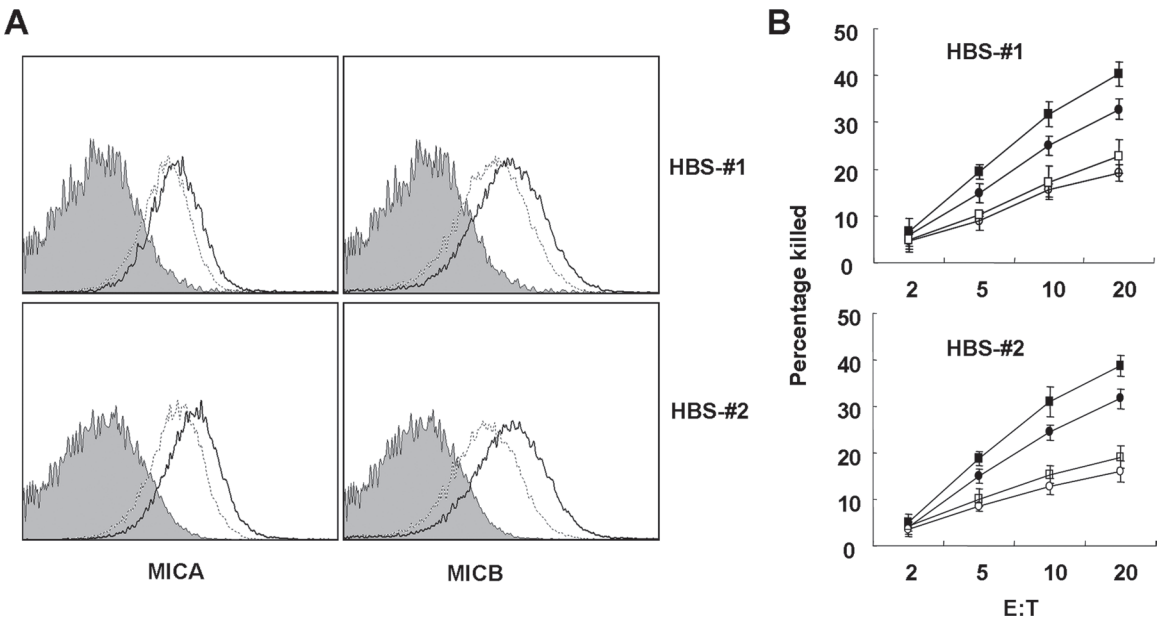

Fig. 4. Partial restoration of MICA and MICB expression by miRNA inhibitors. HBsAg-overexpressing HepG2 cells were transfected with a mixture of eight miRNA inhibitors or the negative control. (**A**) Cell surface MICA and MICB levels were measured by flow cytometry 48 h after transfection with the miRNA inhibitor mixture (thick lines) or the negative control (dashed lines). The filled histograms represent isotype control antibody staining. Horizontal and vertical axes denote intensity of fluorescence and number of events, respectively. (**B**) The cytolytic assay was performed 48 h after transfection. NK-92MI cells were incubated with cells transfected with the miRNA inhibitor mixture (squares) or the negative control (circles) in the absence (closed) or presence (open) of anti-NKG2D antibody. The effector-to-target ratio is as indicated.

miR-16 and miR-29a/29b/29c) were in fact upregulated in HBsAg-overexpressing HepG2 cells (Figure 2A). To ensure that the observed effect of HBsAg on miRNA expression was not cell line specific, we quantified the expression of these miRNAs in HBsAg-overexpressing Huh7 cells and found that most of them were also upregulated (Figure 2B).

Downregulation of MICA and MICB by HBsAg via induction of miRNA expression

As shown in Table I, three out of the nine HBsAg-regulated miRNAs (miR-373, miR-20a and miR-17) have been reported to repress the expression of both MICA and MICB (24), whereas the remaining six miRNAs (miR-107, miR-15b, miR-16, miR-29a, miR-29b and miR-29c) were predicated to target MICB (Supplementary Table 4, available at *Carcinogenesis* Online). To verify these results, we constructed two luciferase reporters consisting of the 3′-UTRs of MICA (Luc-MICA 3′-UTR) or MICB (Luc-MICB 3′-UTR) and performed luciferase assays. Our results showed that the luciferase activities of Luc-MICA 3′-UTR and Luc-MICB 3′-UTR in HBsAg-overexpressing cells were lower than those in the control cells (Figure 3A). And cotransfection of different miRNA mimics with Luc-MICB 3′-UTR dramatically repressed the luciferase activity (Figure 3B). In addition, flow cytometry analysis revealed that the expression of MICB was significantly inhibited when miR-373, miR-20a, miR-107, miR-16, miR-29a, miR-29b or miR-29c mimic were transfected into HepG2 cells, whereas transfection of the miR-15b mimic only showed a modest effect (Figure 3C). Furthermore, in agreement with their effect on MICB expression, transfection of these miRNA mimics led to decreased sensitivity of HepG2 cells to NK-92MI cell-mediated cytolysis (Figure 3D). The transfection efficiency and quantification of miRNAs are shown in Supplementary Figure 2, available at *Carcinogenesis* Online.

To address whether the reduction of MICA and MICB in HBsAg-overexpressing cells is the consequence of miRNA upregulation, we transfected a mixture of miRNA inhibitors (inhibitors of miR-373, miR-20a, miR-17, miR-107, miR-16 and miR-29a/29b/29c) into HBsAg-overexpressing HepG2 cells. As shown in Figure 4A, inhibition of these miRNAs partially restored the expression of MICA and MICB. Consistent with this, cells transfected with the miRNA inhibitor mixture exhibited greater sensitivity to lysis by NK-92MI cells, which was partially inhibited by an anti-NKG2D antibody (Figure 4B). These results indicate that the elevated sensitivity of miRNA inhibitor-transfected cells to NK cell-mediated cytolysis results from upregulation of the NKG2D ligands.

Downregulation of MICA and MICB by HBV via induction of miRNA expression

Others and we have reported that inhibition of HBV replication induces MICA expression, which leads to increased NK cell-mediated cytolysis of HBV-expressing HCC cells (20,38). Here, we found that HBsAg represses the expression of MICA and MICB via induction of cellular miRNAs, thereby preventing NKG2D-mediated elimination of HCC cells. Therefore, we wondered whether HBV expression also represses the expression of MICA and MICB via induction of cellular miRNAs. Based on a published microarray data set (39), we found that 30 of the 133 HBsAg-induced miRNAs were upregulated, and two of the nine HBsAg-repressed miRNAs were downregulated to some extent in HepG2.2.15 cells as compared with HepG2 cells (Supplementary Table 3, available at *Carcinogenesis* Online). Real-time RT–PCR results confirmed that miR-373, miR-107 and miR-29a/29b were upregulated in HepG2.2.15 cells (Figure 5A). To further investigate whether HBV inhibits MICB expression, we compared the expression of MICB in HepG2.2.15 and HepG2 cells and found that although the mRNA level of MICB was not significantly downregulated in HepG2.2.15 cells, the cell surface MICB protein expression was lower in HepG2.2.15 cells than that in HepG2 cells (Figure 5B and 5C).

To address whether the reduction of MICA and MICB in HBV-expressing cells is due to miRNA upregulation, we transfected a mixture of miRNA inhibitors (inhibitors of miR-373, miR-107 and miR-29a/29b) into HepG2.2.15 cells. As shown in Figure 5D, inhibition of these miRNAs partially restored the expression of MICA and MICB. Consistent with this, cells transfected with the miRNA inhibitor mixture exhibited greater sensitivity to lysis by NK-92MI cells, which was partially inhibited by anti-NKG2D antibody (Figure 5E).

Enhanced HBsAg expression in HepG2.2.15 cells does not inhibit the expression of MICA and MICB

To investigate whether forced HBsAg expression in HBV-expressing cells leads to a further reduction of MICA and MICB, we transiently transfected the pT-HBS or pTarget into HepG2.2.15 cells (Supplementary Figure 3A, available at *Carcinogenesis* Online). The flow cytometry results showed that the cell surface MICA and MICB protein levels were not significantly decreased in pT-HBS-transfected cells compared with the control cells (Supplementary Figure 3B, available at *Carcinogenesis* Online). Real-time RT–PCR indicated that miRNAs targeting MICA/B were only slightly induced by forced HBsAg expression in HepG2.2.15 cells (Supplementary Figure 3C, available at *Carcinogenesis* Online). Consistently, we found that forced HBsAg expression in HepG2.2.15 cells only slightly reduced the sensitivity to lysis by NK-92MI cells (Supplementary Figure 3D, available at *Carcinogenesis* Online).

Discussion

Seropositivity for HBsAg is an important risk factor for HCC (8), and HBsAg seroclearance is associated with a low risk of HCC development (9). HBsAg-transgenic mice were reported to spontaneously develop HCC (10,40). These observations indicate that HBsAg plays an important role in HCC development. It was postulated that chronic HBsAg-induced hepatocellular injury triggers the development of HCC (40,41). In the present study, we investigated the role of HBsAg in HCC cells and found that HBsAg regulates the expression of a variety of cellular miRNAs. Interestingly, several HBsAg-induced miRNAs repressed the expression of MICA and MICB via targeting the 3′-UTRs of their mRNAs. We also found that HBsAg represses the expression of MICA and MICB and enhances the resistance of HCC cells to NK cell-mediated cytotoxicity. In addition, HBsAg-induced reduction of MICA and MICB was partially restored by inhibiting the activities of HBsAg-induced miRNAs. Taken together, these data indicate that HBsAg represses the expression of MICA and MICB via induction of cellular miRNAs in HCC cells and thereby reduces the sensitivity of HCC cells to NKG2D-mediated cytolysis.

It has been reported that HBV replication in the tumor tissues appeared to be reduced as compared with the adjacent non-tumor liver tissues (42). We showed here that HBsAg was decreased in 50% of the HCC tissues as compared with the adjacent non-tumor tissues. Furthermore, we found that MICA/B were expressed in a subset of human HCC tissues, but not in the surrounding non-cancerous

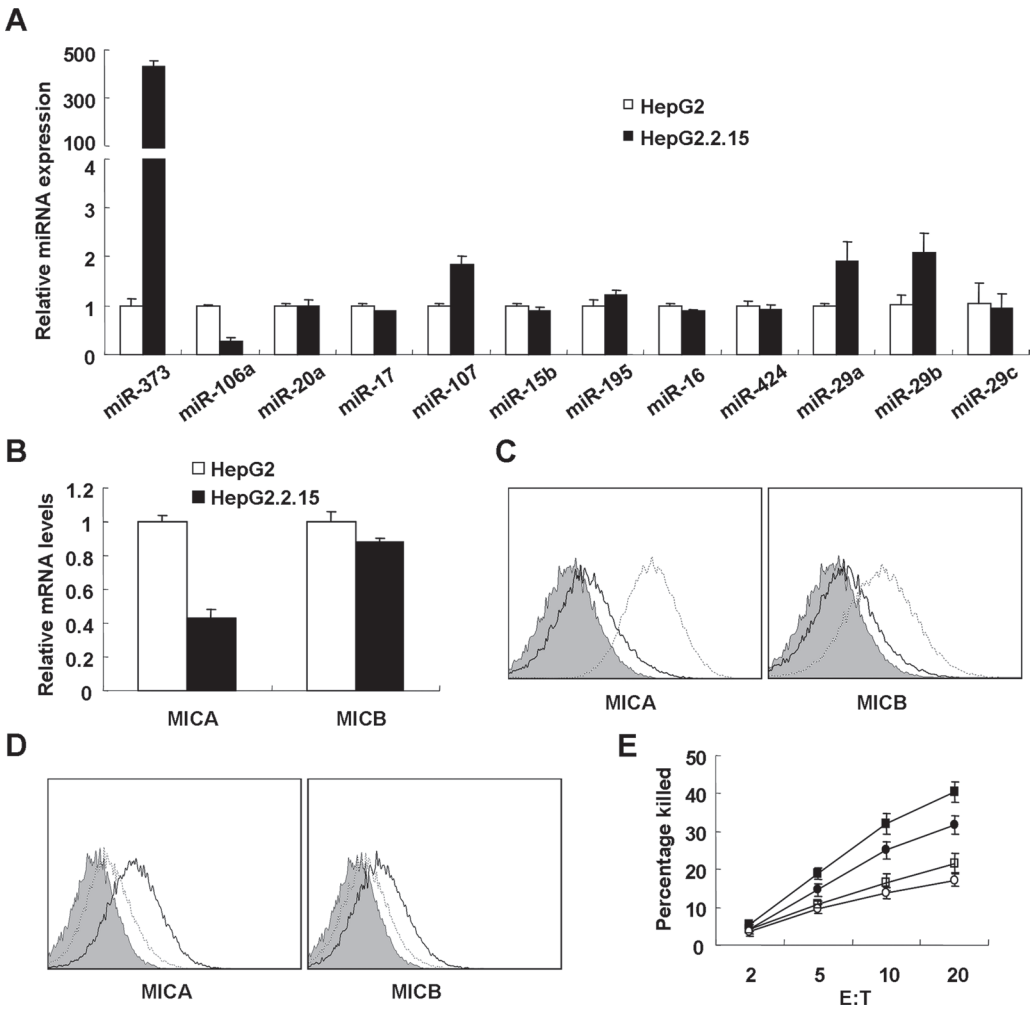

Fig. 5. Downregulation of MICA and MICB expression by HBV via induction of miRNA expression in HepG2.2.15 cells. (**A**) The relative miRNA levels in HepG2 and HepG2.2.15 cells were determined by real-time RT–PCR. (**B**) The relative MICA and MICB mRNA levels in HepG2 and HepG2.2.15 cells. (**C**) Cell surface MICA and MICB levels were measured by flow cytometry in HepG2.2.15 (thick lines) and HepG2 cells (dashed lines). The filled histograms represent isotype control antibody staining. Horizontal and vertical axes denote intensity of fluorescence and number of events, respectively. (**D**) Cell surface MICA and MICB expressions were measured by flow cytometry 48 h after transfection with the miRNA inhibitor mixture (thick lines) or the negative control (dashed lines). (**E**) Cytolytic assay performed 48 h after transfection. NK-92MI cells were incubated with cells transfected with the miRNA inhibitor mixture (squares) or the negative control (circles) in the absence (closed) or presence (open) of anti-NKG2D antibody. The effector-to-target ratio is as indicated.

tissues, and that the levels of HBsAg in 7 of 10 MICA/B-positive tumor tissues were significantly reduced, compared with those in the adjacent non-tumor tissue. However, the *in vivo* effect of HBsAg on the expression of MICA and MICB in HBV-related HCC tissues needs further investigation for the following reasons. First, the levels of HBsAg-regulated miRNAs in the paraffin-embedded tissues from patients were not determined. Second, as shown in Supplementary Table 3, available at *Carcinogenesis* Online, although some miRNAs are differentially regulated by HBsAg and HBV, some are commonly regulated by HBsAg and HBV. We showed here that HBV can also repress the expression of MICA and MICB via cellular miRNAs. Therefore, we cannot rule out the possibility that increased MICA/B expression in HCC tissues is the consequence of downregulation of other HBV-encoded proteins, such as HBx, which has been reported to regulate the expression of cellular miRNAs (31,33,43–45). Of note, because HepG2.2.15 cells constitutively express high levels of HBsAg, enhanced HBsAg expression in this cell line does not inhibit the expression of MICA and MICB, suggesting that forced expression of HBsAg in HBV-expressing cells may not lead to a further reduction of MICA and MICB.

Our sequencing data showed that 142 miRNAs are regulated by HBsAg in HepG2 cells. Besides regulating MICA and MICB expression, these miRNAs may play important roles in hepatocarcinogenesis via other mechanisms. For example, miR-373 was shown to promote HBV expression by targeting nuclear factor I/B (NFIB) (39), and tumor migration and invasion via targeting CD44 (46). The role of the miR-29 family in carcinogenesis is more complicated. The members of the miR-29 family suppress DNA methylation by targeting DNMT3A and DNMT3B (47,48), and deregulation of DNA methylation has been postulated as a driving force for carcinogenesis (49). miR-29 also suppresses innate and adaptive immune responses by targeting interferon-γ (50). Moreover, Bandyopadhyay *et al.* (51) reported that miR-29 is important for hepatitis C virus replication and hepatic stellate cell activation. miR-15b and miR-16 were reported to regulate tumor necrosis factor-mediated hepatocyte apoptosis by inhibiting BCL2 expression in acute liver failure (52). In addition, miR-107 promotes metastasis of colorectal cancer by targeting DAPK and KLF4 (53).

In summary, our data indicate that HBsAg plays a pivotal role in HCC cells. HBsAg may repress the expression of MICA and MICB via induction of cellular miRNAs, thereby preventing elimination of HCC cells. Moreover, some HBsAg-induced miRNAs may regulate the expression of HBV, or they may regulate the apoptosis, migration and invasion of HCC cells.

Supplementary material

Supplementary Tables 1–4 and Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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