MicroRNA expression profiles of LO2 cells expressing the wild-type and mutant HBx gene

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Received July 21, 2012; Accepted November 7, 2012

DOI: 10.3892/mmr.2012.1203

Abstract. Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Although numerous studies have suggested the potentially oncogenic roles of wild-type or mutant hepatitis B virus X (HBx) protein in hepatocarcinogenesis, their exact mechanism remains unclear. Increasing evidence suggests that microRNAs (miRNAs) play essential roles in embryogenesis, cell differentiation and carcinogenesis. This study aimed to investigate the effect of HBx on the miRNA expression profile of LO2 cells. We established the LO2 cell line transfected with recombinant plasmid pcDNA3.0/HBx-d382, pcDNA/HBx and plasmid pcDNA3.0 using Lipofectamine™ 2000, which was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) and western blotting. We then demonstrated the miRNA expression profiles in the stably transfected LO2 cells using a mammalian miRNA microarray containing whole-human mature and precursor miRNA sequences. The results were confirmed by real-time quantitative PCR (qPCR). RT-PCR and western blot analysis showed that a stably HBx-transfected LO2 cell line had been successfully established. According to the microarray, compared to LO2/pcDNA3.0 cells, 6 miRNAs were shown to have higher expression and 5 were shown to have decreased expression in LO2/HBx-d382 cells, while 4 up- and 12 downregulated miRNAs were observed in LO2/HBx cells. There were 8 different expression patterns of miRNAs between LO2/HBx and LO2/HBx-d382 cells. All the chip results were consistent with the real-time PCR data. Consequently, the HBx gene may influence the miRNA expression profile of LO2 cells. Thus, it may be helpful to further investigate the role of HBx in hepatocarcinogenesis and clarify the underlying molecular mechanisms involved.

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Key words: microRNA, hepatitis B virus X protein, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, accounting for 500,000-600,000 mortalities per year (1). It has been estimated that approximately 53% of HCC cases are related to hepatitis B virus (HBV) (2). HBV, which belongs to the Hepadnaviridae family, causes both acute and chronic infection of the liver. HBV is a partial double-stranded DNA virus with a 3.2-kb genome containing four known open reading frames, namely, S, C, P and X genes. The X gene, which encodes hepatitis B virus X protein (HBx), is most closely associated with HCC (3). Evidence suggests that HBx is a multifunctional regulator that modulates the cell cycle, genetic stability, transcription, protein degradation, signal transduction and apoptosis by directly or indirectly interacting with host factors (4). The protein inhibits cell proliferation by inducing late G1 arrest and induces apoptosis in a p53-dependent and -independent manner (5). Several transgenic mouse models reveal that the X gene is capable of inducing HCC (6). Notably, a number of studies have demonstrated that mutations and deletions of HBx, particularly the COOH-terminal deletion of HBx, are frequent events in HBV-associated HCC tissues (7). It has been reported that mutations of the HBx gene may cause uncontrolled growth and contribute to multistep hepatocarcinogenesis (8). Therefore, mutations of the HBx gene are very important in hepatocarcinogenesis. However, the exact molecular mechanisms involved in hepatocarcinogenesis remain unclear.

MicroRNAs (miRNAs) are a class of short (~22 nucleotides), endogenous, single-stranded noncoding RNAs. They are responsible for the post-transcriptional expression regulation of targeted genes. Growing evidence shows that miRNAs may play a key role in the regulation of cellular differentiation, proliferation, apoptosis, gene expression and cancer development (9). HCC is a complex disease involving epigenetic and chromosomal instability, as well as expression abnormalities of both coding and noncoding genes; the latter include miRNAs (10). It has been found that miR-18 and miR-224 are significantly overexpressed and miR-195, miR-199a, miR-200a and miR-125a are underexpressed in HCC tissues (10). Furthermore, several miRNAs downregulated in HCC, such as miR-21, miR-221, miR-223 and miR-224, have been identified as modulators of cell growth, apoptosis, migration or inva-

sion (11). miR-152 has also been found to be downregulated in HBV-related HCC tissues (12).

In a previous study, our data showed that a mutant of the HBx gene with a deletion from 382 to 400 bp (HBx-d382) was a common event and potentially related to the development of HCC (13). However, the molecular mechanisms of HBx involved in HCC development are still not well understood. Moreover, given the importance of miRNAs in HCC development, we investigated whether miRNAs play a role in the hepatocarcinogenic effect of HBx. The present study investigated the miRNA expression profiles of the non-tumor human hepatic LO2 cells stably transfected with HBx and HBx-d382 using miRNA microarray.

Materials and methods

Cells culture and establishment of the stably transfected cell lines. The plasmid pcDNA3.0, recombinant plasmid pcDNA3.0/HBx-d382 (a mutant of the HBx gene with a deletion from 382 to 400 bp) and pcDNA/HBx (the restructuring of HBx genetic fragments originates from liver cell line HepG2.215) were all from our laboratory and were stably established previously (13).

The hepatocyte cell line LO2 (Chinese Academy of Science, Cell Biology of Shanghai Institute, Shanghai, China) was cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) containing 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (FBS; Gibco) in a humidified atmosphere of 5% CO_2 and 95% air at 37˚C. We attempted to establish a stable LO2 cell line transfected with HBx and HBx-d382. LO2 cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and selected with G418 (Geneticin®; Gibco). The plasmids pcDNA3.0, pcDNA3.0/HBx-d382 and pcDNA/HBx were used in the transfection experiments. Empty pcDNA3.0 vector plasmid was used as a control. The stable transfection of pcDNA3.0/HBx-d382 (termed LO2/HBx-d382), pcDNA/HBx (termed LO2/HBx) or the empty vector (termed LO2/pcDNA) was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) and western blot analysis. The study was approved by the ethics committee of Xiangya Hospital, Central South University, Changsha, China.

RT-PCR analysis. Total RNA of the LO2 cell line was extracted using TRIzol (Invitrogen). The primers used in the PCR were as follows: HBx, F, 5'-AAGGTACCATGGCT GCTAGGCTGTGCT-3' and R, 5'-CTGGGCCCTTAGGCA GAGGTGAAAAAGTTG-3' (481 or 462 bp amplified fragment); β-actin, F, 5'-CTCCATCCTGGCCTCGCTGT-3' and R, 5'-GCTGTCACCTTCACCGTTCC-3' (242 bp amplified fragment). The cycling conditions for HBx amplification were 94˚C for 3 min, followed by 35 cycles of 94˚C for 30 sec, 68˚C for 30 sec and 72˚C for 1 min, followed by an elongation cycle of 72˚C for 5 min. The resultant PCR products were analyzed using electrophoresis on 1.5% Tris/Borate/EDTA (TBE) agarose gel with β-actin as an internal control.

Western blot analysis. Total protein was extracted from transfected cells using the RIPA lysis buffer (Beyotime, Nantong, China). Equal amounts of protein samples were separated by SDS-PAGE and electroblotted onto PVDF membranes (Millipore, Billerica, MA, USA). Blots were blocked with 5% skimmed milk, followed by incubation with antibodies specific for rabbit anti-HBx (dilution, 1:1,500; Abcam, Cambridge, UK), and mouse anti-β-actin (dilution, 1:10,000; Sigma, St. Louis, MO, USA). Blots were then incubated with goat anti-rabbit or anti-mouse secondary antibody conjugated to horseradish peroxidase (dilution, 1:5,000; Jackson ImmunoResearch, Inc., West Grove, PA, USA) and visualized by enhanced chemiluminescence (ECL) (Amersham Biosciences, Piscataway, NJ, USA).

Table I. Corresponding absorbance at 260 and 280 nm.

Cells	A260/A280	2100 RIN	28s/18s
L02/pcDNA3.0	1.99	10	2.2
L02/HBx	2.11	10	2
L02/HBx	1.97	10	2.2

miRNA isolation. Total RNA of LO2 cells transfected with wild-type and mutant HBx as well as empty plasmid pcDNA3.0 was extracted using TRIzol. The concentration and quality of RNA was measured by ultraviolet (UV) absorbance at 260 and 280 nm and checked by gel electrophoresis individually. miRNA isolation was performed from all pooled total RNA using an miRNA isolation kit (Ambion, Austin, TX, USA). RNA quality was measured using the Small RNA kit (Agilent, Santa Clara, CA, USA) on the Agilent 2100 Bioanalyzer and analyzed by capillary electrophoresis individually. The average RNA integrity number (RIN) value of all samples was ≥6.0 and 28s/18s >0.7, indicating high-quality RNA with minimal degradation products.

miRNA microarray analysis. The Human miRNA Microarray version 3.0 (Agilent) contains approximately 15,000 probes including 866 human and 89 human viral miRNAs from the Sanger database v12.0. This chip not only has high sensitivity and specificity but can also distinguish between mature and precursor miRNAs. Microarray analysis was performed according to the manufacturer's instructions (Agilent). Four clones of each cell line were analyzed with microarray analysis. Briefly, first strand cDNA was synthesized from 200 ng RNA, followed by cRNA amplification and labeling with Cy3 or Cy5. Equal amounts of Cy3- or Cy5-labeled cRNA from the cells of the LO2/HBx-d382, LO2/HBx or LO2/pcDNA3.0 fraction were mixed and hybridized using the Agilent Human Whole Genome Oligo Microarray in a dye swap design at 65˚C overnight. On the following day, slides were washed and signals were scanned with GenePix™ 4000B (Agilent). There were four spot replicates for each probe on the chip.

Signal intensities from scanned images were analyzed using Agilent Feature Extraction software version 10.5 and GeneSpring GX. The abundance of probes in the cells of the LO2/HBx-d382, LO2/HBx or LO2/pcDNA3.0 fraction was compared with the abundance in the total fraction. For each probe, the signal intensity from the cells of the LO2/HBx-d382, LO2/HBx or LO2/pcDNA3.0 fraction was divided by the

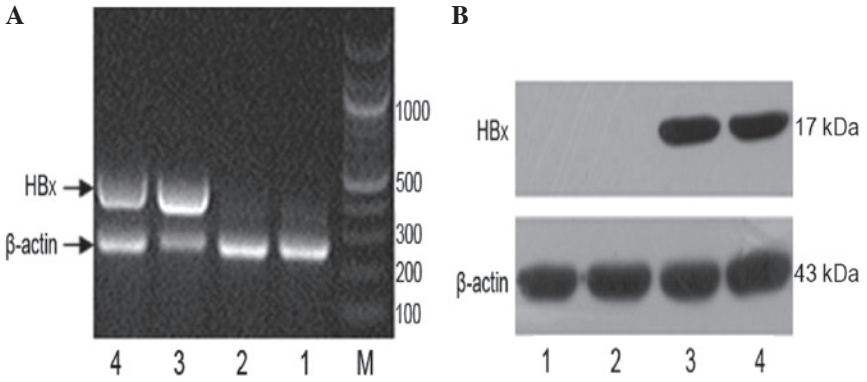

Figure 1. Identification of stable HBx transfection in LO2 cells. (A) Integration of the HBx gene was identified by RT-PCR. (B) Western blot analysis showed the expression of HBx in LO2 cells. 1, LO2; 2, LO2/pcDNA3.0; 3, LO2/HBx-d382; 4, LO2/HBx; M, marker. HBx, hepatitis B virus X protein.

signal intensity from the total cell lysate RNA fraction. For each dye swap, the average LO2/HBx-d382/T, LO2/HBx/T or LO2/pcDNA3.0/T ratio was calculated. Fluorescence intensity was normalized with the background subtracted, and all scanned images were processed and converted into normalized data using a quantile method. LO2/HBx-d382/T and LO2/HBx/T of each probe were compared with each other as well as with LO2/pcDNA3.0/T. After standardization, we output the log2 value of the primary signal. Two criteria were set to identify the abnormal expression of miRNAs: when compared with LO2/pcDNA3.0, a different value of each probe present in the LO2/HBx-d382 or LO2/HBx >1 (corresponding to the ratio of the original signal is >2) was considered to be enriched in the LO2/HBx-d382 or LO2/HBx fraction; while a different value <-1 (corresponding to the ratio of the original signal is <0.5) was considered to be reduced in LO2/HBx-d382 or LO2/HBx fraction.

Real-time PCR. Total RNA of transfected cells was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized with the RevertAid™ First Strand cDNA Synthesis kit (MBI Fermentas, Inc., Burlington, ON, Canada) in a total volume of 20 μl. The cDNA sample was analyzed by real-time PCR using the THUNDERBIRD® SYBR qPCR mix (Toyobo, Osaka, Japan). All the primers of miRNAs that were detected as differentially expressed in the microarray were from a Bulge-Loop™ miRNA qPCR Primer Set (RiboBio, Guangzhou, China) including the human U6 snRNA. The experiment consisted of two steps: i) it was based on the stem-loop structure protruding primer reverse transcription reaction and ii) real-time fluorescence quantitative PCR (qPCR). The stem-loop structure of the RT-convex primers combined with miRNA 3' terminal end and the reverse transcriptase leads to the reverse transcription reaction, while the specific primers and SYBR Green fluorescent dye participating in the quantitative PCR reaction system realizes quantitative detection of reverse transcription products. Each cDNA sample was run on a 96-well optical plate in a total volume of 20 μl/well including 2 μl RT product, 4 μl specific primers, 4 μl sterile double-distilled water (ddw) and 10 μl SYBR qPCR Mix. qPCR was performed on the 7500HT Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the following profile: 95˚C for 1 min, and 40 cycles of 95˚C for 15 sec and 60˚C for 35 min. A dissociation step was performed following the qPCR amplification for melting curve analysis. All reactions were performed in triplicate. The whole miRNA expression of LO2/HBx-d382 or LO2/HBx cells were compared to each other as well as to LO2/pcDNA3.0 cells and normalized using U6 snRNA. Since a cycle threshold (CT) value of 35 represents single molecule template detection, CT values >35 were considered to be below the detection level of the assay. Therefore, only the miRNAs with CT ≤35 were included in the analyses.

The relative amount of miRNAs studied in the samples was determined with the $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta CT = (CT_{target}\text{-}CT_{U6})_{sample} - (CT_{target}\text{-}CT_{U6})_{control}$. Briefly, the CT values for the U6 snRNA were subtracted from CT values of the target gene to achieve the ΔCT value. $2^{-\Delta CT}$ was calculated for each sample and then each of the values was then divided by a control sample to achieve the relative miRNA levels ($2^{-\Delta\Delta CT}$).

Target genes selected. Target genes were selected from the following websites: miRBase (http://microrna.sanger.ac.uk/sequences/), TargetScan (http://www.targetscan.org/), PicTar (http://pictar.mdc-berlin.de/) and miRanda (http://www.microrna.org/microrna/home.do).

Results

Establishment of a stably HBx-transfected LO2 cell line. RT-PCR was used to identify the integrated HBx gene in the cDNA of the engineered LO2 cells. β-actin was used as a loading control (Fig. 1A). The data showed that the HBx gene had been successfully introduced into the host genome of LO2 cells. Western blot analysis showed that expression of the HBx protein was detectable in LO2 cells (Fig. 1B). Our data suggest that a stably HBx-transfected LO2 cell line was successfully established.

RNA quality check. The mirvana miRNA isolation kit was used to extract and purify miRNA and the quality of RNA was analyzed using capillary electrophoresis individually. There were 3 visible electrophoresis strip peaks representing 5s, 18s and 28s, respectively (Fig. 2). Our data showed that the quality of RNA was high without degradation, and it was not polluted by genomic DNA (Table I).

Table II. Differential miRNA expression in HBx-d382-transfected LO2 cells.

miRNA name	L02/PCDNA3.0 mean	L02/HBx-d382 mean	Fluorescence difference value (log2 value)	Up- or downregulation in L02/HBx-d382 cells
hsa-miR-338-3p	4.629886	1.577917	-3.05196969	Downregulated
hsa-miR-551b	4.000756	1.198526	-2.802229496	Downregulated
hsa-miR-1	3.541191	1	-2.541190817	Downregulated
hsa-miR-455-3p	5.435329	4.10132	-1.334009537	Downregulated
hsa-miR-200c	3.309559	2.003005	-1.306553869	Downregulated
hsa-miR-501-5p	2.094981	3.116949	1.021967456	Upregulated
hsa-miR-1307	1.764127	2.865505	1.101377863	Upregulated
hsa-miR-1180	1.697469	2.909304	1.211835771	Upregulated
hsa-miR-497	1.653463	2.965216	1.31175287	Upregulated
hsa-miR-1246	4.304869	5.661847	1.356977617	Upregulated
hsa-miR-623	1.815217	3.272093	1.456875568	Upregulated

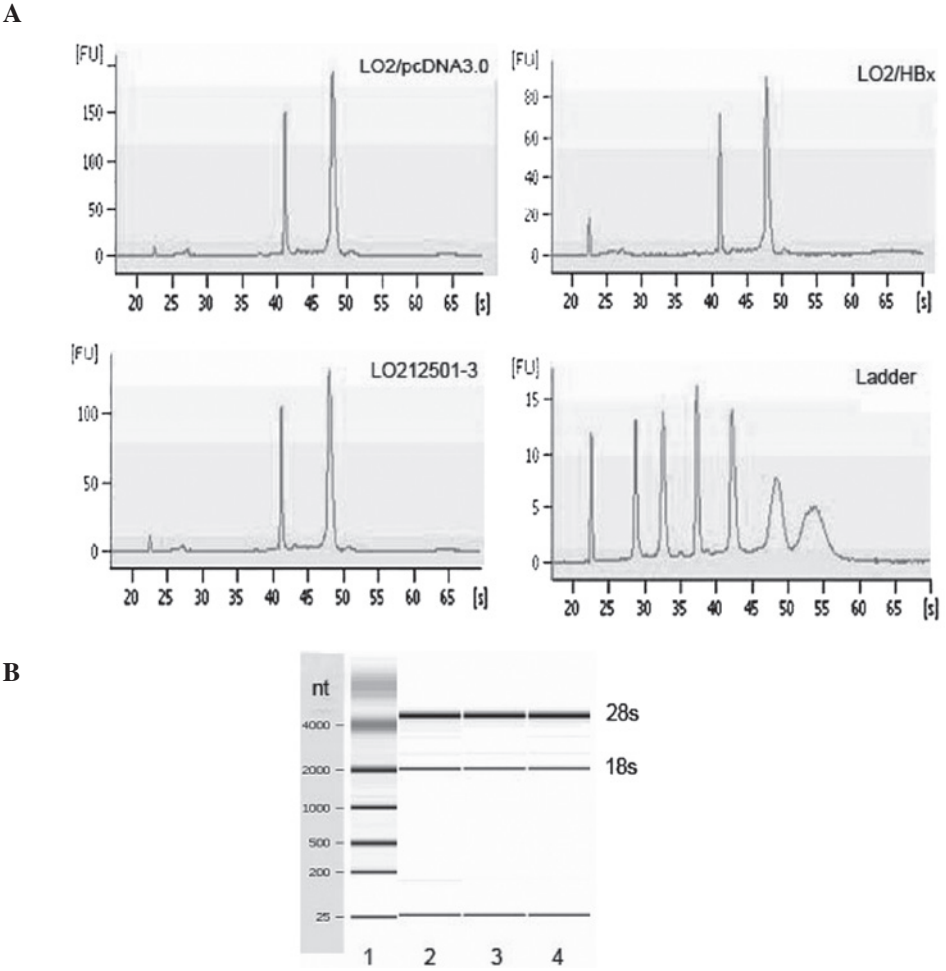

Figure 2. (A) The capillary electrophoresis of RNA in transfected cells. There were 3 visible electrophoresis strip peaks representing 5 (left), 18 (middle) and 28s (right), respectively. The ladder was RNA marker. (B) Representative images of RNA electrophoresis. 1, LO2; 2, LO2/pcDNA3.0; 3, LO2/HBx-d382; 4, LO2/HBx.

miRNA microarray scanning. Fig. 3 shows images scanned with GenePix™ 4000B. The detection rate of all chips was 22.10±3.08%, while the coefficient of variation (CV) of one single sample was 8.93±2.11%, which indicated that different measures of hybridization quality and consistency were high.

miRNA microarray analysis. Eleven miRNAs were differentially expressed in LO2/HBx-d382 compared to LO2/pcDNA3.0 cells. The normalized hybridization signal of miRNAs identified in the microarray analysis is listed in Table II. The 6 upregulated miRNAs were miR-501,

Table III. Differential miRNA expression in HBx-transfected LO2 cells.

miRNA name	L02/PCDNA3.0 mean	L02/HBx mean	Fluorescence difference value (log2 value)	Up- or downregulation in L02/HBx cells
hsa-miR-338-3p	4.629886	2.557788	-2.0721	Downregulated
hsa-miR-24-1	3.602923	1.588591	-2.01433	Downregulated
hsa-miR-200c	3.309559	1.359513	-1.950046	Downregulated
hsa-miR-29c	8.110281	6.527961	-1.58232	Downregulated
hsa-miR-744	3.99746	2.519724	-1.47774	Downregulated
hsa-miR-455-3p	5.435329	4.121858	-1.31347	Downregulated
hsa-miR-324-5p	5.94758	4.638507	-1.30907	Downregulated
hsa-miR-551b	4.000756	2.719708	-1.28105	Downregulated
hsa-miR-340	3.018177	1.751115	-1.26706	Downregulated
hsa-miR-590-5p	4.962567	3.78986	-1.17271	Downregulated
hsa-miR-660	4.119494	2.947035	-1.17246	Downregulated
hsa-miR-193a-3p	5.329816	4.214547	-1.11527	Downregulated
hsa-miR-7	6.283198	7.473472	1.190274	Upregulated
hsa-miR-1274a	6.674666	7.974562	1.299896	Upregulated
hsa-miR-137	1.788507	3.308352	1.519845	Upregulated
hsa-miR-663	1.124881	3.014342	1.889461	Upregulated

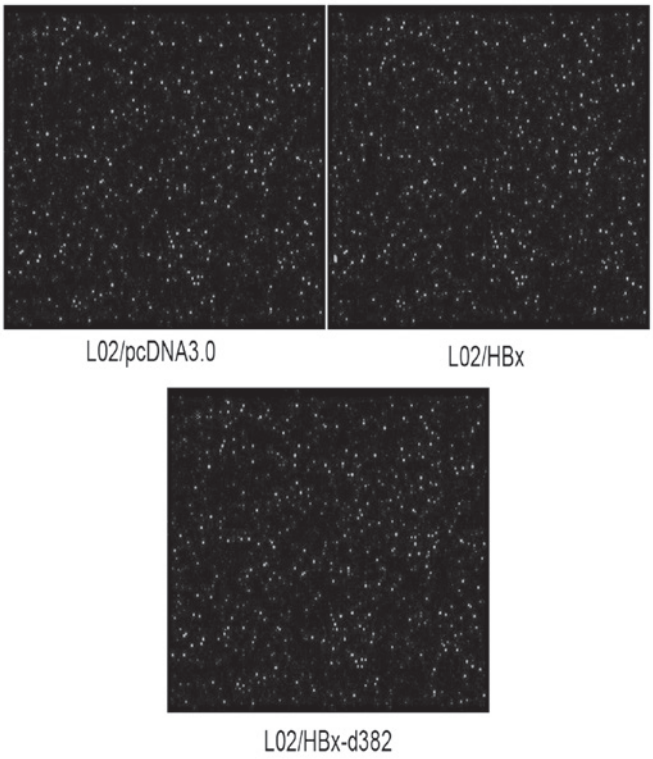

Figure 3. miRNA microarray of LO2 cells.

miR-1307, miR-1180, miR-497, miR-1246 and miR-623. The 5 downregulated miRNAs were miR-1, miR-338-3p, miR-551b, miR-455-3p and miR-200c.

Four upregulated and 12 downregulated miRNAs were observed in LO2/HBx compared to LO2/pcDNA3.0 cells. Normalized hybridization signals of miRNAs identified in the microarray analysis are listed in Table III. miR-7, miR-1274a, miR-137 and miR-663 were upregulated in LO2/HBx cells, while miR-338-3p, miR-24-1, miR-200c, miR-29c, miR-744, miR-455-3p, miR-324, miR-551b, miR-340, miR-590, miR-660 and miR-193a-3p were downregulated.

Eight miRNAs were differentially expressed in LO2/HBx compared to LO2/HBx-d382 cells. Normalized hybridization signals of miRNAs identified in the microarray analysis are listed in Table IV. miR-551b, miR-663, miR-7 and miR-1 were upregulated and miR-1307, miR-501-5p, miR-29c and miR-24-1 were downregulated in LO2/HBx cells.

Real-time PCR analysis. The microarray data were confirmed by real-time PCR with the $2^{-\Delta\Delta CT}$ method. The whole miRNA expression of the HBx-expressed cell lines (LO2/HBx-d382 or LO2/HBx) were compared to each other as well as to LO2/pcDNA3.0 cells and normalized using U6 snRNA. The relative quantity of the control was normalized as 1 and, therefore, $2^{-\Delta\Delta CT}$ showed the relative miRNA levels. Our data revealed that the real-time PCR results were consistent with the chip results (Table V).

As shown in Table VI, the targets of miRNAs were predicted on several websites such as miRBase (http://microrna.sanger.ac.uk/sequences/), TargetScan (http://www.targetscan.org/), PicTar (http://pictar.mdc-berlin.de/) and miRanda (http://www.microrna.org/microrna/home.do).

Discussion

HCC is one of the most common malignancies and most of the cases are attributable to persistent HBV infection (14). Comparative studies of mammalian and avian hepadnaviruses, transgenic animal studies, and cell culture transformation studies imply that the 17.0-kDa X protein of HBV plays an important role in HCC development by influencing transcription, cell proliferation, apoptosis and signal transduction (15,16). It has been reported that in tumor tissues, the majority of HBx natural mutants have lost their capacity for controlling cell

Table IV. Eight miRNAs were differentially expressed in LO2/HBx compared to LO2/HBx-d382 cells.

miRNA name	L02/HBx-d382 mean	L02/HBx mean	Fluorescence difference value (log2 value)	Up- or downregulation in L02/HBx cells
hsa-miR-551b	1.198526	2.719708	1.521182	Upregulated
hsa-miR-1307	2.865505	1.072718	-1.79279	Downregulated
hsa-miR-501-5p	3.116949	1.199773	-1.91718	Downregulated
hsa-miR-29c	7.846427	6.527961	-1.31847	Downregulated
hsa-miR-663	1.415526	3.014342	1.598816	Upregulated
hsa-miR-7	6.44578	7.473472	1.027692	Upregulated
hsa-miR-24-1	3.565019	1.588591	-1.97643	Downregulated
hsa-miR-1	1	2.906131	1.90613	Upregulated

proliferation, viability and transformation and, therefore, may cause uncontrolled growth and contribute to the multistep development of HCC (17). A recent study demonstrated that a number of miRNAs have multiple functions including cell cycle control, cancer development, diagnosis and treatment (18-21). Given the importance of miRNAs in carcinogenesis, we hypothesized that miRNAs may also be involved in the mechanism of wild-type or mutant HBx in hepatocarcinogenesis.

In the present study, we established LO2 cell lines transfected with HBx-d382 and HBx. RT-PCR and western blot analysis showed that expression of the HBx gene was detectable in LO2 cells, a fact suggesting that stably HBx-transfected LO2 cell lines were successfully established. The miRNA expression profile was detected using the Agilent miRNA microarray. The results showed that 6 miRNAs exhibited higher expression and 5 miRNAs exhibited lower expression in LO2/HBx-d382 compared to LO2/pcDNA3.0 cells. Meanwhile, 4 upregulated and 12 downregulated miRNAs were observed in LO2/HBx compared to LO2/pcDNA3.0 cells. While of the above-mentioned miRNAs, miR-551b, miR-663, miR-7 and miR-1 were upregulated and miR-1307, miR-501-5p, miR-29c and miR-24-1 were downregulated in LO2/HBx cells compared with LO2/HBx-d382 cells. Microarray data were consistent with the real-time qPCR analysis. Our data showed that the wild-type or mutant HBx had an impact on the miRNA expression of hepatic LO2 cells.

Of the above-mentioned miRNAs, the function of some has been revealed, while the function of others remains unknown. For example, miR-1 regulated aspects of both pre- and post-synaptic function at neuromuscular junctions. It regulated the muscle transcription factor MEF-2, which results in altered pre-synaptic acetylcholine (Ach) secretion (22). Other studies also found that miR-1 was involved in many types of cancer such as lung, colon and liver cancer, where it was expressed at a lower level (23,24). Datta *et al* (25) suggested that ectopic expression of miR-1 in HCC cells inhibited cell growth, reduced replication potential and clonogenic survival by regulating Foxp1 and MET and it was suggested to be one of the mechanisms by which DNA hypomethylating agents suppress hepatocarcinoma cell growth. From several predicted target websites, we found that there were many predicted targets of miR-1 and some of them were involved in cell proliferation or controlling the cell cycle, such as cyclin D1, cyclin D2, cdk-6 and cdk-9. A study showed that HBx induced DNA hypermethylation of the p16INK4a promoter to repress its expression, which subsequently led to activation of G1-CDKs, phosphorylation of pRb, activation of E2F1, transcriptional activation of DNMT1 and finally induced development of HCC (26). Hence, we hypothesized that the significantly low expression of miR-1 in LO2/HBx-d382 cells may regulate cyclin D1 and induce cell growth.

Another widely-investigated miRNA is miR-200c. Yu *et al* (27) reported that miR-200c is an independent prognostic factor in pancreatic cancer and its upregulation inhibits pancreatic cancer invasion. They also found that the patients with high levels of miR-200c expression had significantly better survival rates than those with low levels of miR-200c expression. Several studies have shown that miR-200 family miRNAs (miR-200a, -200b, -200c-141 and -429) prevent epithelial-to-mesenchymal transition (EMT) by directly suppressing the expression of ZEB1 and ZEB2, causing degradation of the mRNA, and resulting in an upregulation of E-cadherin (28,29). Previous studies have demonstrated that HBx regulated EMT in the SMMC-7721 hepatoma cell line *in vivo* (30). Moreover, our data indicated that a decrease of miR-200c expression was detected in both LO2/HBx-d382 and LO2/HBx cells, so we suggest that miR-200c is possibly linked to the mechanism of HBx in EMT. It has been recently reported that loss of miR200c expression is linked to aberrant DNA methylation and histone modifications, while HBx regulates DNA methylation (31). Therefore, further investigation is required to clarify whether miR-200c plays a key role in the upregulation of DNA methylation influenced by HBx.

miR-29c, a different isoform of miR-29, has been reported to target the expression of TCL1, a critical oncogene in aggressive chronic lymphocytic leukemia (CLL), a fact strongly suggesting that miR-29 may function as a tumor suppressor in CLL (32). A recent study showed that miR-29 directly targeted DNA methyltransferases Dnmt3A and -3B55 and that it activated P53 by targeting P85α and CDC42 (33). Although there are insufficient data regarding its function, miR-338-3p has been detected by using a bead-based microarray analysis to be significantly downregulated in HCC tissue and this has been suggested to be related to the development of HCC (21,34). A number of miRNAs affect the growth of cancer cells *in vitro* and *in vivo* when overexpressed or inhibited. Therefore, cancer

Table V. Relative miRNA expression levels.

miRNA name	ΔCT_{sample}	$\Delta CT_{control}$	$2^{-\Delta\Delta CT}$[a]
$\Delta CT_{sample} = (CT_{target}\text{-}CT_{U6})_{LO2/HBx\text{-}d382\ cells}$			
$\Delta CT_{control} = (CT_{target}\text{-}CT_{U6})_{LO2/pcDNA3.0\ cells}$			
hsa-miR-338-3p	10.89	8.079	0.14
hsa-miR-551b	12.83	10.18	0.16
hsa-miR-1	13.89	11.69	0.22
hsa-miR-455-3p	13.22	11.94	0.41
hsa-miR-200c	9.83	7.82	0.24
hsa-miR-501-5p	10.99	12.14	2.2
hsa-miR-1307	8.50	9.64	2.2
hsa-miR-1180	8.64	9.84	2.3
hsa-miR-497	13.47	14.92	2.73
hsa-miR-1246	11.07	12.85	3.43
hsa-miR-623	9.1	11.38	4.85
$\Delta CT_{sample} = (CT_{target}\text{-}CT_{U6})_{LO2/HBx\ cells}$			
$\Delta CT_{control} = (CT_{target}\text{-}CT_{U6})_{LO2/pcDNA3.0\ cells}$			
hsa-miR-338-3p	9.87	7.60	0.21
hsa-miR-24-1*	16.71	15.56	0.45
hsa-miR-200c	10.10	8.78	0.4
hsa-miR-29c	8.43	6.49	0.26
hsa-miR-744	12.72	10.91	0.28
hsa-miR-455-3p	13.22	11.94	0.41
hsa-miR-324-5p	13.83	11.70	0.23
hsa-miR-551b	11.57	10.43	0.45
hsa-miR-340	14.98	13.90	0.47
hsa-miR-590-5p	13.17	11.26	0.27
hsa-miR-660	8.56	7.45	0.46
hsa-miR-193a-3p	15.63	13.87	0.30
hsa-miR-7	15.24	16.55	2.48
hsa-miR-1274a	8.49	10.15	3.16
hsa-miR-137	7.13	8.86	3.32
hsa-miR-663	11.23	13.69	5.5
$\Delta CT_{sample} = (CT_{target}\text{-}CT_{U6})_{LO2/HBx\ cells}$			
$\Delta CT_{control} = (CT_{target}\text{-}CT_{U6})_{LO2/HBx\text{-}d382\ cells}$			
hsa-miR-551b	11.78	13.10	2.50
hsa-miR-1307	10.13	8.82	0.40
hsa-miR-501-5p	11.69	10.41	0.41
hsa-miR-29c	7.97	6.81	0.45
hsa-miR-663	11.06	12.95	3.71
hsa-miR-7	14.96	16.02	2.08
hsa-miR-24-1*	17.00	15.28	0.30
hsa-miR-1	12.42	14.06	3.11

[a]When $2^{-\Delta\Delta CT} > 2$ or < 0.5, the difference was considered to be significant.

cell growth may be controlled by manipulating miRNAs. For example, miR-7 and miR-137 are considered as cancer therapeutic tools (35). Silber *et al* (36) reported that the targeted delivery of miR-137 to glioblastoma multiforme tumor cells, which inhibited the proliferation of glioblastoma multiforme cells and induced differentiation of brain tumor stem cells, may be therapeutically effective for the treatment of this disease.

Our data showed that miRNA expression was different between the wild-type and mutant HBx-transfected LO2 cells. For example, miR-338-3p was detected to have a lower expression level in LO2/HBx-d382 compared to LO2/HBx cells. On the contrary, miR-29c and miR-24-1 were detected to have a lower expression level in LO2/HBx compared to LO2/HBx-d382 cells. miR-1 had a low expression level in

Table VI. Predicted targets of miRNAs.

miRNA name	Putative target associate with HBx	Description
hsa-miR-338-3p	CCND (cyclin D1)	G1/S-specific cyclin D1
hsa-miR-551b	BCL2	Apoptosis regulator Bcl-2
hsa-miR-1	CCND1	
	CCND2	G1/S-specific cyclin D1
	cdk-6	Cell division protein kinase 6
	cdk-9	Cell division protein kinase 9
hsa-miR-200c	JUN	Transcription factor AP-1 (activator protein 1)
	MYC	Myc proto-oncogene protein (c-Myc)
hsa-miR-501-5p	HBXIP	Hepatitis B virus X-interacting protein
	RSF1	Remodeling and spacing factor 1 (Rsf-1) (Hepatitis B virus X-associated protein)
hsa-miR-497	CCNE1	G1/S-specific cyclin E1
	BCL2	Apoptosis regulator Bcl-2
	CCND2	G1/S-specific cyclin D2
hsa-miR-623	GCK	Glucokinase
	CCRK	Cell cycle-related kinase
hsa-miR-24-1	BCL2L2	Apoptosis regulator Bcl-W (Bcl-2-like 2 protein)
hsa-miR-29c	PTEN	Phosphatase and tensin homolog deleted on chromosome 10
	PPP1R13B	Apoptosis-stimulating of p53 protein 1
hsa-miR-324-5p	GCK	
	TMEM16J	Tumor proteinp 53 inducible protein 5
	CCND3	G1/S-specific cyclin D3
hsa-miR-340	CTNNB1	Catenin β-1 (β-catenin)
hsa-miR-193a-3p	RSF1	Remodeling and spacing factor 1 (Rsf-1)
	PTEN	Phosphatase and tensin homolog deleted on chromosome 10
	APOA2	Apolipoprotein A-II precursor (Apo-AII)
hsa-miR-7	APOA2	
	TP53INP2	Tumor protein p53-inducible nuclear protein 2
	CKS2	Cyclin-dependent kinases regulatory subunit 2 (CKS-2)
hsa-miR-137	CDK6	Cell division protein kinase 6
	CDC42	Cell division control protein 42 homolog precursor
hsa-miR-663	CDK3	Cell division protein kinase 3

LO2/HBx-d382 cells and no difference in LO2/HBx cells when compared to LO2/PCDNA3.0. As mentioned above, miR-1 had several predicted targets such as cyclin D1 and D2 which could promote cell proliferation, a fact which may explain why mutant HBx led to uncontrolled growth. Meanwhile, miR-551b, which demonstrated a lower expression level in LO2/HBx-d382 compared to LO2/HBx cells, may directly or indirectly regulate BCL2 (Table V), which is well-known as an anti-apoptotic protein (37). Therefore, the difference in miRNAs between the HBx-expressing cell lines may be one of the reasons for the different functions and biological characteristics of the wild-type and mutant HBx transfected cells.

Due to the fact that miRNA expression may differ from cells to tissues, and from hepatitis and cirrhosis to HCC, it also may be dissimilar in cell lines of different types of cancer as well as in cancer tissues resulting from different etiopathogenesis. Therefore, not all differential expression of miRNAs in our study could be the same as that in HCC tissue. However, due to the stable organization that is less variable than in tissue and unaffected by the immune system and the surrounding environment of stroma cells, we used cell lines to study the miRNA expression, which has certain advantages and is flexible. Our data showed that some of the differentially expressed miRNAs in LO2 cells transfected with wild-type or mutant HBx were similar to that in malignant tissues, suggesting that HBx may influence miRNA and, therefore, directly or indirectly induce hepatocarcinogenesis. Although the function of certain miRNAs found in this study has not been described, we could not exclude their potential involvement in oncogenesis. For example, although limited data were available regarding the function of miR-497, it was found to possess several predicted targets associated with HBx using public prediction algorithms, such as BCL2 and CCND2.

In conclusion, our study showed that the X gene is capable of influencing the miRNA expression profile in LO2 cells. Further investigation is required to clarify the roles of the identified miRNAs and, therefore, it may be helpful to inves-

tigate the role of HBx in hepatocarcinogenesis as well as to clarify the underlying molecular mechanisms involved.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation (no. 30872228), the National Key Program in the 11th Five Year Plan of China (no. 2008ZX10002-007), the Natural Science Foundation of Hunan (no. 10JJ5034) and Projects in the Science and Technology Pillar Program of Hunan (no. 2010SK3093).

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