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Identification of hepatitis B virus encoding/affecting microRNAs

Key Messages

1. We have discovered a profile of human miRNAs expression from the hepatitis B virus (HBV)–producing cell line HepG2 2.2.15, which can provide more information for the study of the HBV life cycle or HBV-related hepatocellular carcinoma.
2. No HBV-encoded miRNAs were found in the HepG2 2.2.15 cell line.

Introduction

In Hong Kong, hepatitis B virus (HBV) is the major cause of liver cancer, accounting for over 70% of all cases. Over 10% of our population are chronic carriers of HBV, and the risk of these people eventually developing cirrhosis and liver cancer is 100-fold higher than in non-carriers.[1] Many of the factors governing viral latency remain unresolved, and current antiviral treatment regimens are largely ineffective at eliminating cellular reservoirs of latent virus.

MicroRNA (miRNA) is recognised as one of the major regulatory gene families in eukaryotic cells. Virus-encoded miRNAs, which could rapidly accumulate in infected cells, would be a powerful means of modulating viral and cellular gene expression.[2] Several DNA viruses that enter the nucleus during their life cycles have been found to encode miRNAs. Using computational methods, it was predicted that the HBV genome could reasonably encode one candidate pre-miRNA. Experimental evidence also suggests that hepatocytes are the only confirmed site of replication for all members of the hepatitis virus family. This is in line with the characteristics of miRNAs, which are usually expressed in a cell-type and tissue-specific manner.

Based on the insight of miRNA and the fact that regulation of gene expression is not limited to the expression of one or more proteins, we hypothesised that HBV achieved its infection by encoding miRNA(s) or affecting the hosts' miRNA(s). We aimed at identifying HBV-encoded miRNA(s) and HBV-affected host miRNA(s). We report on the analysis of small RNA libraries derived from an HBV-producing cell line and suggest several potential novel miRNAs which are of special interest for future studies.

Methods

This study was conducted from November 2007 to November 2008.

Cell culture of HBV-producing cell line

The cell line HepG2 2.2.15, which was stably transfected with a head-to-tail dimer of HBV DNA (strain ayw), was maintained in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad [CA], USA) and supplemented with 10% heat-inactivated foetal bovine serum and 100 mg/mL penicillin/streptomycin (Invitrogen) at 37ºC under 5% CO_2.

Detection of HBV genes expression in the cell line model

Total DNA was extracted from a HepG2 2.2.15 cell lysate. PCR primers were used to confirm expression of the X, C, Pre-S, and S genes in the cell line. The PCR products were identified by electrophoresis in 2% agarose gels and stained with SyberSafe gel stain (Invitrogen).

RNA isolation and construction of cDNA library of small RNA

According to the manufacturer's protocol, total RNA was extracted from a HepG2 2.2.15 cell lysate using Trizol reagent (Invitrogen). Then, 12% denaturing (7M Urea) polyacrylamide gel electrophoresis was used to size fractionate and enrich the small RNAs to 18 nucleotide (nt) to 26 nt size fractions. Subsequently, small RNAs were cloned using the miRCat-33 microRNA cloning kit (IDT DNA Technologies, Coralville IA). Briefly, 5' and 3' adaptors were ligated

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to the enriched small RNAs. The 5' and 3' ligated small RNAs were then converted into cDNA using SuperScript III reverse transcriptase (Invitrogen) and a specific primer. After reverse transcription, the cDNA was amplified by PCR, and the PCR products were then gel-purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA) and cloned into the pCR2.1 TOPO vector (Invitrogen). The positive insert-carrying plasmids were then sequenced using the ABI auto sequencing kit. Sequencing reactions were undertaken with the BigDye Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, USA).

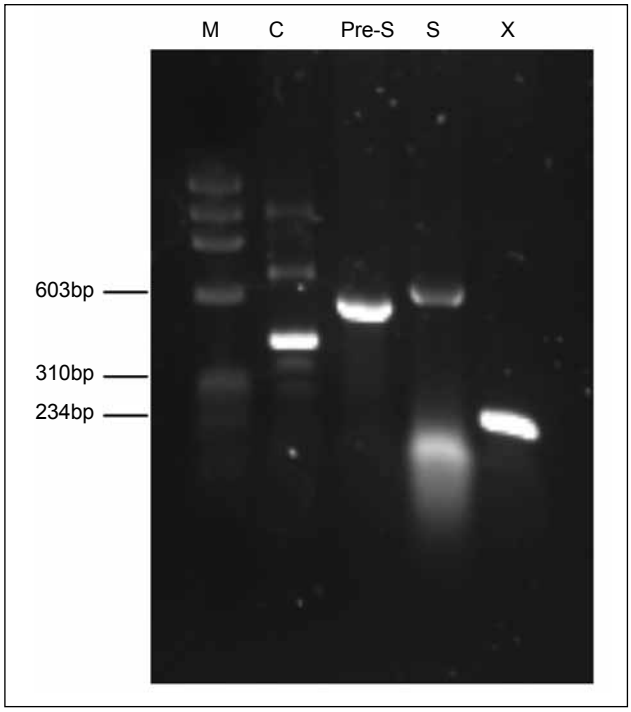

Fig 1. PCR amplification of hepatitis B virus genes (C, Pre-S, S, and X)

Small RNA sequence analyses

Sequence data obtained from cloning were analysed with Bioedit v7.0.4.1 software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) to identify inserts and orientation. Cloned small RNA sequences of length >17 nt were matched against the published mature miRNAs and stem-loop sequence databases (miRbase - Sanger miRNA database version 12.0, Sep 2008). Then, all the novel nucleotide sequence alignments were carried out by BLASTN searching against non-redundant (nr) and human genome databases (Human build 36.3 version) of the National Center for Biotechnology Information (NCBI). Secondary structures of RNA precursors were predicted from longer genomic sequences of the cloned RNAs using the Mfold software (http://www.bioinfo.rpi.edu/applications/mfold/old/ma/forml.cgi).

Results

Confirmation of HBV genes expression in HepG2 2.2.15 cells

At the beginning of the miRNA cloning experiment, PCR with specific primers were used to confirm that the cell line was HBV producing. All four genes (X, C, Pre-S, and S) were expressed in the cell line (Fig 1). These results ensured that the cell line was a relevant model of chronic HBV infection that could constitutively produce infectious HBV particles.

Analysis of sequence data from the HBV-producing hepatoblastoma cDNA library of small RNAs

A total of 233 clones were subsequently sequenced and databases searched. There were several kinds of RNA fragments. More than 76% of the cloned RNAs represented breakdown products of abundant coding and non-coding RNAs such as mRNA, tRNA, rRNA, sn/snoRNA and other unknown short fragments. About 21% of the cloned RNAs represented known human miRNAs. About 3% of

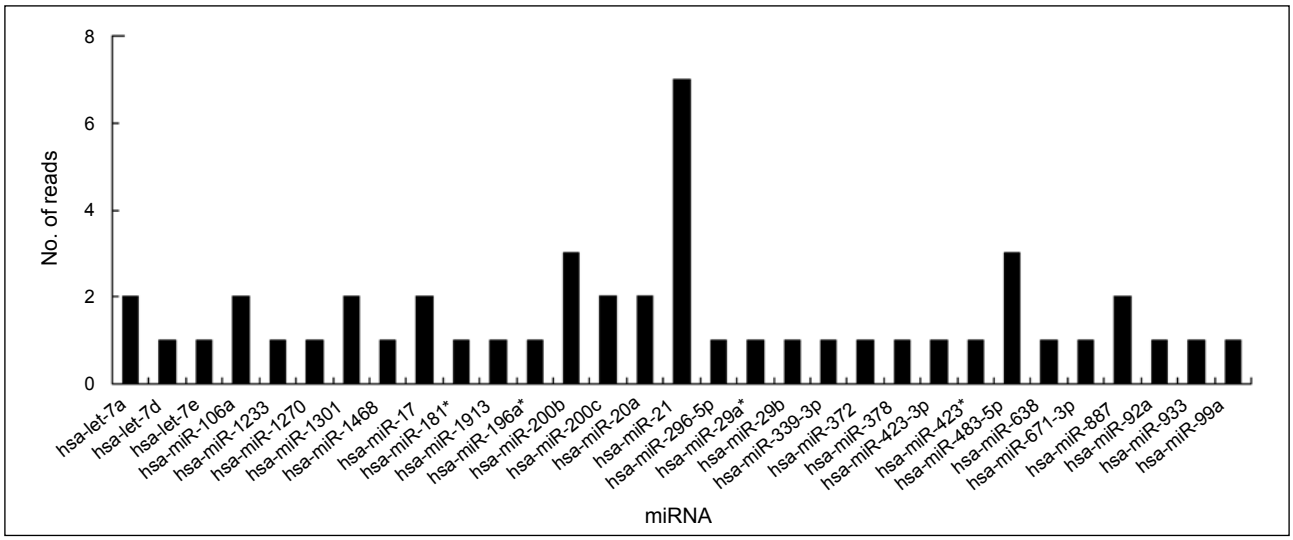

Fig 2. Frequency distribution of cloned and sequenced miRNAs that matched human miRNAs

the cloned sequences were matched with the miRNA of other species or stem-loops of known human miRNA or only matched with human chromosomes. These small RNA sequences might represent novel miRNAs identified in this study. However, no sequence of miRNA was found to be completely identical to that of HBV genome.

Expression pattern of cloned miRNAs

In the chronic HBV infection cell line model, a profile of human miRNAs was obtained (Fig 2). Among the known human miRNAs, hsa-let-7a, hsa-miR-106a, hsa-miR-1301, hsa-miR-17, hsa-miR-200b, hsa-miR-200c, hsa-miR-20a, hsa-miR-21, hsa-miR-483-5, and hsa-miR-887 were the miRNAs that had been sequenced twice or more. The most abundant miRNA was hsa-miR-21, which had been cloned and sequenced seven times. The second most abundant human miRNAs were hsa-miR-200b and hsa-miR-483-5, which were sequenced three times.

Discussion

miRNAs are involved in diverse biological functions, including development, life span, cell proliferation, differentiation, signalling pathways, apoptosis and metabolism.[3] Since the discovery of miRNAs in 2000, more than 6000 miRNA entries across 72 virus, plant, and animal species have been recorded in the miRBase release (version 12.0).

HepG2 2.2.15 miRNA profile

Recent studies have established a crucial role of miRNAs in cancer. We therefore used the miRNA profiling data in HepG2 (without HBV) cells for comparison. As we had used cloning and sequencing methods for miRNA profiling, we assumed that our results (clone count data) could be used for comparison with those of other hepatocellular carcinoma (HCC) miRNA-profiling studies, which might obtained by sequencing or by miRNA microarray profiling.

In one study, the expression of a group of miRNAs (including human miR-21, miR-20a, and miR-34a) was increased more than 2-fold in a HepG2 cell line (without harbouring HBV) when compared with that of normal human hepatocytes. In our study, high-level synthesis of miR-21 and miR-20a were also detected in HepG2 2.2.15 cells.

MiR-21 has been shown to be over-expressed in many different solid tumours, including those of breast, colon, lung, pancreas, prostate, stomach, as well as in cholangiocarcinoma cell lines. Therefore, it is highly

(a)

Clone name	Sequence	Similarity to Stem-loop of miRNAs	Alignment to hairpin miRNAs	Similarity to Chromosome location
P4-615	AAGGATGGCGGAGTGGTCT	rno-mir-349	Our Seq 1 AAGGAUGGCGGAGUGGUCU 19 rno-mir-349 17 AAGGUUGGGGGAGGGGGCU 35	Ch17,
190	AGGATGTCGGAGTGGTCT	hsa-mir-494	Our Seq 1 AGGAUGUCGGAGUGGUCU 18 hsa-mir-494 15 AGGUUGUCCGUGUUGUCU 32	Ch9, 19
128	GAGGATGGCGGAGCGGTCT	hsa-mir-935	Our Seq 18 GACCGCUCCGCCAUCCUC 1 hsa-mir-935 31 GGCCCCUCGGCCAUCCUC 48	Ch1
939	CTCAGGATGGCCCGAGCGGTCT	hsa-mir-212	Our Seq 20 ACCGCUCGGGCCAUCCUGAG 1 hsa-mir-212 51 ACUGCCCGGGCCGCCCUCAG 70	Ch4
P16-704	GGATGGATGGCCGATCG	hsa-mir-412	Our Seq 1 GGAUGGAUGGCCGAUC 16 hsa-mir-412 12 GGAUGGAUGGUCGACC 27	Ch11

(b)

(c)

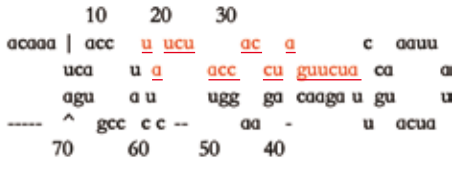

Fig 3. (a) Potential novel miRNA stem-loop sequence alignment, (b) sequence alignment of miRNA clone 311 and mouse mmu-miR-672 precursor miRNA, and (c) fold back precursor sequence of a potential novel miRNA as predicted by the MFold software, with underlined bases representing the mature miRNA sequences

likely that miR-21 plays a fundamental role in tumour cell behaviour and malignant transformation. Our findings were comparable with those of the other tumours in which miR-21 was over-expressed. Moreover, miR-21 has been reported to have anti-apoptotic properties in glioblastoma and cholangiocarcinoma. Thus, altered expression of miR-21 can have several diverse effects in tumour cells.[4]

Another up-regulated miRNA (miR-20a) can induce senescence in mouse embryonic fibroblasts. Senescence has been revaluated as a tumour suppressor mechanism (an alternative to apoptosis). MiR-20a has been suggested to play an anti-apoptotic role via the E2F/miR-20a auto-regulatory feedback loop. The E2F transcription factors play an essential role in the proper regulation of cellular proliferation, and cell cycle progression is critical for the normal development of organisms and the prevention of cancer.

Interestingly, no miR-34a expression was detected in our study. In one study, two different cell lines—SNU-449 (which has HBV DNA integration) and PLC/PRF-5 (which can produce HBsAg but not infectious HBV particles)—also showed a down-regulated miR-34a synthesis.[4] These results were in line with those in another study involving feeding rats with a methyl-deficient diet in order to induce hepatocarcinogenesis.[5] Down-regulation of miR-34a in rat liver during hepatocarcinogenesis was observed.[5] Therefore, HBV production in HCC cell lines might cause miR-34a down-regulation. As miR-34a has been reported to have a tumour suppression function, the relationship between down-regulation of miR-34a in HBV-producing hepatocytes is worth studying.

Another affected miRNA profile was the miR-483-5p, which was sequenced three times in our study, but it was not up-regulated in HepG2 cells in previous studies. As yet, there is no report on their functions in liver cells. Therefore, the effect of HBV on regulating miR-483-5p expression and functions in HCC remains to be discovered.

Moreover, in the miRNA family, let-7 (such as hsa-let-7a, hsa-let-7d, and hsa-let-7e) was relatively up-regulated in HBV-producing HepG2 cells as compared with non-HBV-producing HepG2 cells.[4] An example of the function of the let-7 miRNA family was displayed by hsa-let-7a that modulates interleukin-6-dependent STAT-3 survival signalling in human malignant cholangiocytes by targeting the tumour suppressor gene NF2.[4]

Furthermore, in the miRNA family, miR-17 was up-regulated in both HBV-producing and non-HBV-producing HepG2 cells. Concerning miR-17 miRNA family members, miR-17-5p, miR-20a, miR-93, and miR-106a can regulate mouse STAT3 mRNA in vitro. It has been suggested that STAT3, a known embryonic stem cell regulator, is a target mRNA responsible for the effects of these miRNAs on cellular differentiation in the mouse. Also, temporal regulation of cell cycle progression can be exerted by two miRNAs (miR-17 and miR-20a). Disrupting miR-17 or miR-20a during cell cycle G1 phase progression resulted in premature E2F accumulation, leading to a DNA damage-induced G1 phase checkpoint.

Analysis of potential novel human miRNAs

Among the 3% of potential novel clones, five clones matched the stem-loop sequences of identified human miRNAs (Fig 3a). One of the clones named 311 matched a mouse miRNA (Fig 3b), and another named P6-544 did not match any known miRNAs, but an identical sequence exists in human chromosomes 1. It contains a typical hairpin consisting of approximately 70 nt in length as folded by Mfold software, which is the characteristic for miRNA precursors (Fig 3c).

Conclusions

In this pilot study, we discovered a profile of human miRNAs and identified several potential novel miRNAs, which can provide more information for HBV-related HCC studies. Moreover, no HBV-encoded miRNA was found in the HBV-producing cell line HepG2 2.2.15.

Acknowledgements

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