Original Article

Hepatitis B Virus X Protein Induces Expression Changes of miR-21, miR-22, miR-122, miR-132, and miR-222 in Huh-7 Cell Line

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ABSTRACT

Hepatitis B virus (HBV) X protein (HBx) plays a key role in hepatocellular carcinoma (HCC). HBx may alter the expression of multiple microRNAs (miRs), which are important in hepatocarcinogenesis. This study aimed to investigate the importance of HBx protein in the expression of miR-21, miR-22, miR-122, miR-132, and miR-222. A recombinant vector expressing HBx was developed. The Huh-7 cell line was transfected with the HBx-pcDNA3.1+ recombinant plasmid. A Real-Time Polymerase Chain Reaction was used to evaluate the expression of miR-21, miR-22, miR-122, miR-132, and miR-222 in the cell line. It was found that the expression of miR-21 and miR-222 was upregulated at all points of time after HBx transfection. The expression of miR-21 was 4.24-fold 72 h after transfection. The miR-22 had a 7.69-fold downregulation after 24 h, and the miR-122 had a significant downregulation after 48 h (10-fold). The miR-132 expression reached its lowest rate 12 h after HBx transfection (8.33-fold), and the miR-222 expression was upregulated in transfected cells but was not significantly different (1.18- to 2.45-fold). The significant downregulation of miR-22, miR-122, and miR-132 implicates their inhibitory roles in the progression of HBV-associated HCC. The expression of these microRNAs could be used as a prognostic marker for the progression of HBV-associated liver disease.

Keywords: HBx, Hepatitis B virus X protein, MicroRNA

1. Introduction

Despite the availability of hepatitis B virus (HBV) vaccines, the worldwide incidence of hepatitis B cases is estimated at 296 million, with over 1.5 million new infections occurring annually (1). Hepatocellular carcinoma (HCC) is recognized as one of the most deadly malignant cancers and ranks third among all annual cancer mortality rates (2). Chronic HBV infection represents a major etiological risk for HCC progression (3). Malignant transformation processes are still the focus of extensive research. Recent studies have shown that the HBV-encoded X (HBx) protein, which is important in virus replication, plays a key role in hepatocarcinogenesis (4). According to the latest epidemiological data from the Globocan 2020 report, newly diagnosed liver cancer cases account for over 900,000 cancer cases annually (4.7% of all cancers), and there are approximately 830,000 deaths linked to HCC annually (2). In HBx-induced HCC, signaling pathways that control the normal physiological functions of the host cells are disrupted. For instance, HBx protein can modify the expression of microRNAs (miRNAs), which has been demonstrated to contribute to HCC pathogenesis (5). The miRNAs can affect the expression of oncogenes or tumor-suppressor genes. There is significant data on host miRNA profile alterations during HBV infection. For instance, it has been reported that HBx can suppress miRNA-148a expression, which results in the activation of AKT and the kinase signaling pathway controlled by extracellular signals (6). The stimulation of these pathways results in rapamycin activation and the subsequent promotion of cancer cell proliferation and metastasis. The mammalian target of the rapamycin pathway has recently emerged as a chronic modulator of insulin-mediated glucose metabolism. The HBx-induced inhibition of miRNA-15b expression has been demonstrated in a recent study, targeting the Fucosyltransferase2 enzyme, increasing the tumor-induced Globo-H antigen's level, and increasing HCC cell proliferation (7). The inhibiting effect of miRNA-205 on carcinogenesis was also shown to be reduced by HBx through decreasing miRNA-205 expression in the livers of transgenic HBx mice (8). Although several reports have provided evidence of a relationship between HBx, specific miRNAs, and target genes (5, 7), the regulation of the global miRNA profile in liver cells by HBx in relation to the development and progression of HCC remains to be fully clarified. Therefore, this study was designed to investigate the role of HBx in the variation of the gene-expression profiles of miR-21, miR-22, miR-122, miR-132, and miR-222 in the Huh-7 cell line.

2. Materials and Methods

HBx-Expressing Vector Development

The HBx-coding sequence was derived from a patient with acute HBV infection (GenBank accession number KX544797) (9). A pair of primers were designed with HindIII and Nhe1 cutting sites at their 5' ends. A Polymerase Chain Reaction (PCR) was performed as previously reported (9). The PCR product was cloned to the TA vector (pTZ57R/T, Thermo Fisher Scientific, USA), and cloning was double controlled by the transformation of DH-5alpha and double digestion. The digested HBx-coding sequence was further sub-cloned to pcDNA3.1+ from a previous study (10), and cloning was controlled as described above. The pcDNA3.1+ vector containing the desired HBx sequence (named HBx-pcDNA3.1+) was also sent for DNA sequencing (Macrogen, Korea).

Preparation for Cell Culture

Huh-7, a well-differentiated hepatocyte-derived cell line (11) with no evidence of the HBV genome, was propagated in a complete medium containing Dulbecco's Modified Eagle's Medium (Sigma, USA) supplemented with 10% Fetal Bovine Serum (Gibco, Rockville, MD) and 1% Pen/Strep antibiotic (Sigma, USA). Following the melting of the cryovials, the cells were seeded in T-75 flasks and incubated at 37°C, 5% CO_2, and 10% humidity for propagation. The viability of cells was evaluated

using Trypan Blue Staining. For further study, flasks containing 80%-90% confluence cells and >90% viability were used.

Transfection and *in-vitro* Expression Study of HBx

In this stage, 10^6 Huh-7 cells were seeded in 6-well plates containing complete medium and incubated at 37°C, 5% CO_2, with a humidity of 10% until a confluency of approximately 60% was observed. Cell supernatants were discarded after 12 h and substituted with the antibiotic-free medium containing HBx-pcDNA3.1+ and the reagent Lipofectamine® 2000 (Thermo Fisher Scientific, USA). Transfection optimization was carried out with fluorescent microscopy, as previously described (12). Transfection was performed in duplicate. Wells that contained empty vector pcDNA3.1+ and untransfected mock cells were used as controls.

HBx cDNA synthesis and Real-Time PCR

RNA was collected at different time points (12, 24, 78, and 72 h after treatment) from either transfected cell lines or the control ones with the RNX-Plus solution (SinaClon, Iran). Treatment with DNaseI (SinaClon, Iran) was used to increase the quality of the RNA. A spectrophotometer (DeNovix, USA) was also used to measure the quality of the RNA. A total of 100 ng of the extracted RNA was used for cDNA synthesis. The cDNA synthesis was performed using the cDNA synthesis kit, RevetAidK1622 (Thermo Scientific, USA), according to the manufacturer's protocol. The accuracy of RNA extraction and cDNA synthesis was checked by GAPDH amplification, as described elsewhere (13). Real-time PCR (RT-PCR) was also used to confirm HBx expression in transfected cells 12, 24, 48, and 72 h after transfection. For this purpose, RNA was extracted, and cDNA was synthesized as described earlier in this thread. The RT-PCR was performed with a master mix of qPCR (Yekta Tajhiz Azma, Iran) according to the provided protocol. A cycle threshold (Ct) of below 33 was considered a positive expression.

cDNA Synthesis of miRs and Real-Time PCR

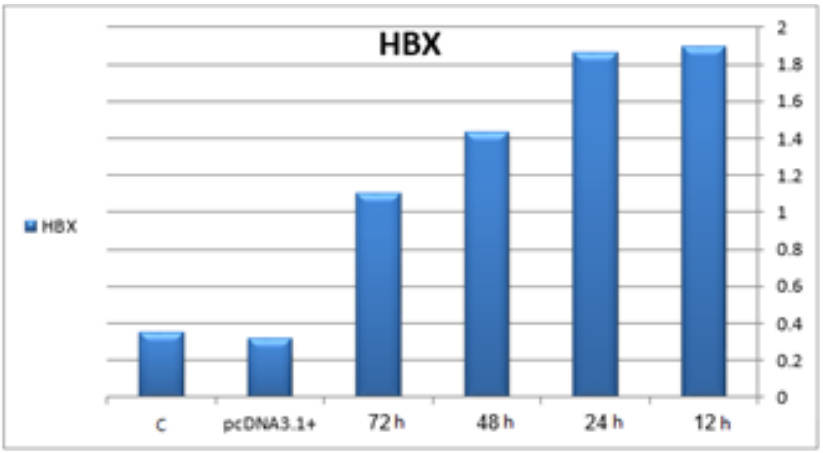
After total RNA extraction, the cDNA of miRs was synthesized using the BONmiR High Sensitivity MicroRNA 1st Strand cDNA Synthesis Kit (Stem Cell Technology Research Center, Tehran, Iran) according to the manufactured protocol. A relative RT-PCR was used to evaluate the expressions of miR-21, miR-22, miR-122, miR-132, and miR-222 using BON-miR QPCR (Stem Cell Technology Research Center, Tehran, Iran). Thermal amplification was performed in one cycle at 95°C for 2 min, followed by 41 cycles at 95°C for 5 sec and at 60°C for 30 sec in the ABI7300 RT-PCR machine (Applied Biosystems, USA). The data was also calibrated with miR SNORD as an internal control. Although 19 significantly different expressed miRNAs were found, only when the overlap coefficient was ≥0.5 and the overlap number of the pathways of the miRNA targeted gene was >3, were the eligible miRNAs selected for inclusion in the analysis of the miRNA pathway network. In this study, we chose the previously mentioned miRNAs for the following reasons. It has been reported that miR-122 accounts for about 50%-70% of the total liver miRNAs. Previous studies reported that miR-122 targets cyclin G1 (CCNG1) expression, which abolishes p53-mediated inhibition of HBV replication. Further investigation showed that miR-132 could inhibit the growth of hepatoma cells via the Akt-signaling pathway. HBx-mediated upregulation of TRERNA1 (Translation Regulatory Long Non-Coding RNA 1) promotes cell proliferation in hepatocellular carcinoma by the sponge action of miR-22 targeting NRAS. A high level of miR-21 results in a significant increase in proliferation and a decrease in IL-12 expression. It has been reported that the overexpression of miR-222 directly results in the down-regulation of the tumor suppressor and cell cycle regulator p27.

Statistical Analyses

Data on gene expression are reported as a fold-change relative to the internal control. Accordingly, $2^{-\Delta\Delta ct}$ was utilized for investigating fold-change expression in miRs in cells transfected with HBx at different post-transfection times. The reduced expression ($2^{-\Delta\Delta ct}<1$) was transformed and reported as $\frac{1}{2^{-\Delta\Delta ct}}$. There are three types of normalization methods commonly used for miRNA analysis by qPCR: endogenous controls, exogenous controls, and mean expression value normalization, or "global mean normalization". Normalization using endogenous control genes is currently the most accurate method to correct potential differences in RNA input or RT efficiency biases. Exogenous controls, or "spike-ins", are typically used to monitor extraction efficiency or sample input amount for difficult samples, such as plasma/serum or other biofluids. Large-scale miRNA expression profiling studies may utilize global mean normalization, which uses the calculated mean of all miRNAs in a given sample as the normalizer. In the present study, as mentioned above, the SNORD gene was our endogenous control as a normalizer.

3. Results

RT-PCR showed successful transfection of the HBx-coding vector pcDNA3.1+. The effectiveness of qPCR was also assessed. The expression of HBx, miRs, and internal control at different time points is demonstrated in figure 1. The RT-PCR analysis of miRs revealed that miR-21 expression was 2.86-fold higher than the control group 12 h after HBx transfection. Interestingly, 24 and 48 h after HBx transfection, miR-21 expression was 1.89- and 1.49-fold, respectively. On the other hand, the expression of miR-22 was reduced at the four transfection time points. It was 2.5-fold lower than that of the control 12 h after HBx transfection and dropped to its lowest rate (7.69-fold) 24 h after HBx transfection. The reduction continued, and it dropped to 2.78- and 2.33-fold lower 48 and 72 h after HBx transfection, respectively. Similar to miR-22, miR-122 had decreased levels of expression. The expression was 3.57-fold lower 12 h after HBx transfection. It decreased significantly after 24 h to 10-fold and then to 6.25- and 3.7-fold lower than the control 48 h and 72 h after HBx transfection, respectively. Besides miR-22 and miR-122, miR-132 showed decreased levels of expression after HBx transfection. Its expression was at its lowest rate (8.33-fold) 12 h after HBx transfection. It was then as low as 6.67-, 5-, and 4.35-fold 24, 48, and 72 h after HBx transfection, respectively. The miR-222 showed higher expression (2.45-fold) in the HBx-transfected cells 12 h after HBx transfection than 24 h (1.18-fold) and 48 h (1.96-fold). Its expression was constant and did not change significantly afterward.



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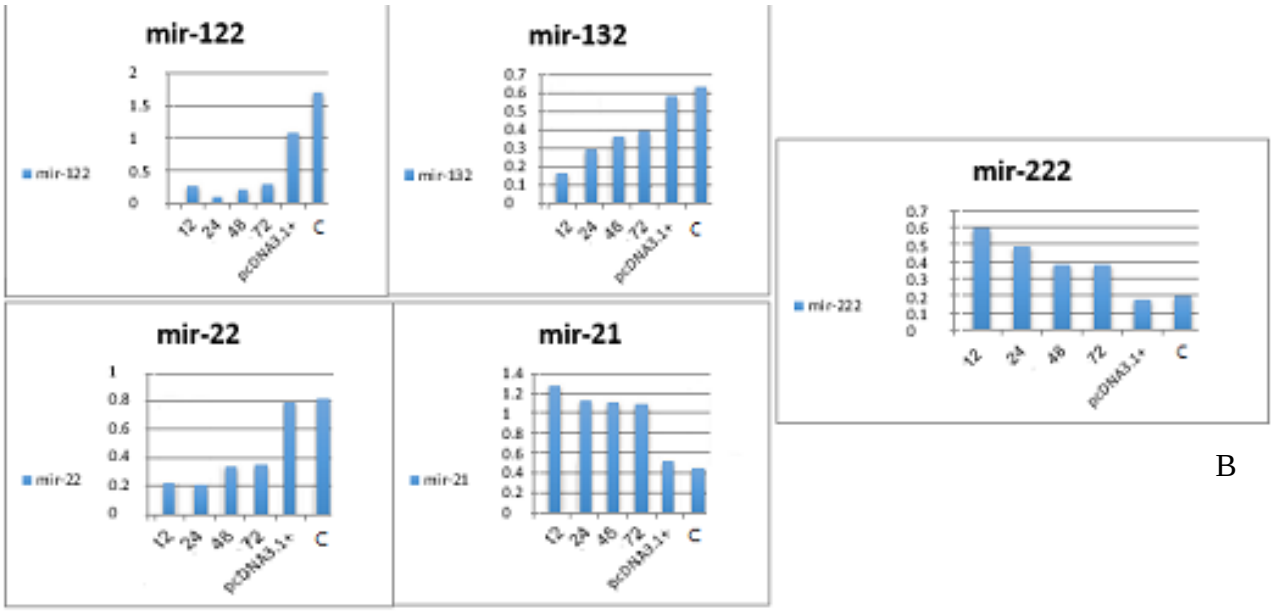

Figure 1. The expression of HBx, miRs, and internal control in different time points of 12hr, 24hr, 48hr, 72hr

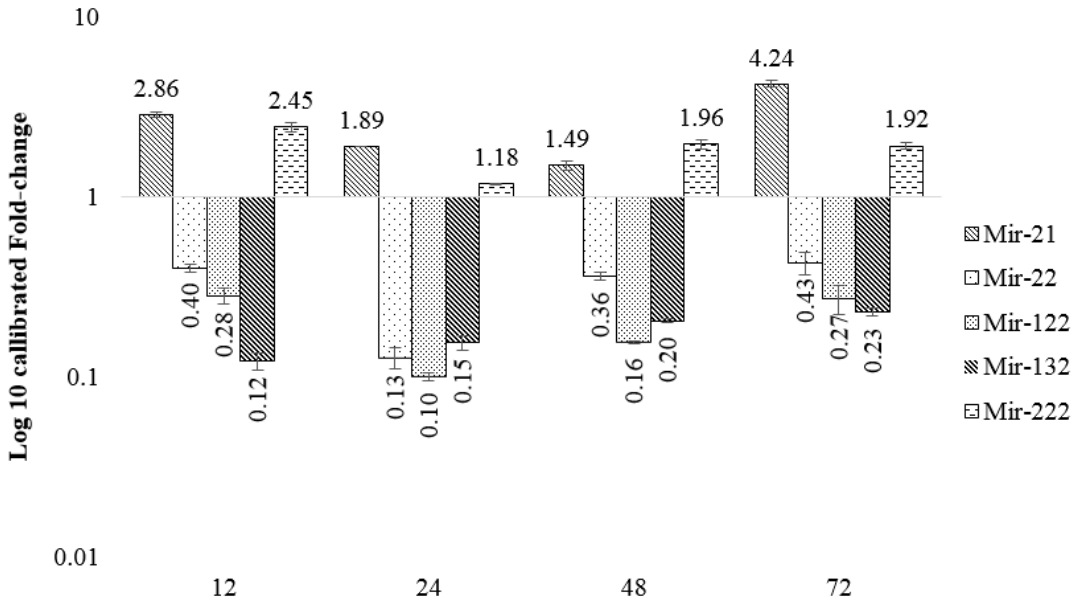

Figure 2. Different miRs expression fold-change in various time points.

4. Discussion

HBx is a multifunctional protein that modulates the expression of viral genes by its trans-activator function, which may lead to HCC (14-16). In addition, HBx may affect the expression of certain cell transcripts. Non-coding transcripts, such as miRs, are known to be targeted by HBx (17,18). Different modifications, such as mutations or epigenetic changes at the nucleotide level of miRs, may be involved in the development of cancer (19-21). HBx-induced apoptosis contributes to HCC carcinogenesis in two contradictory ways, depending on the factors HBx interacts with. Under certain conditions, multiple mechanisms are involved in HBx-induced apoptosis. In the present study, we assessed the differential expression of miRs in Huh-7 (an HBV-replicating human hepatoma cell line), and the outcomes showed the upregulated expression of miR-21 and miR-222 and the downregulated expression of miR-22, miR-122, and miR-132 in the presence of HBV replication. Some of the HBx-induced miRNAs and their target genes were mapped for their putative cooperation with each other in livercells. MiR-122 is highly expressed in various tumors, including renal cell carcinoma, ovarian cancer, liver cancer, and pancreatic cancer. It accounts for about 50%-70% of the total liver miRNAs. MiR-132 is a preserved chromosome 17 non-coding transcript that is reduced in HCC (22). Several studies have revealed the inhibiting role of miR-132 in various cancers with different mechanisms, including suppressing CCNE1 expression, targeting hematological and neurological expressed 1 (HN1), zinc finger E-box-binding homeobox 2 (ZEB2), Sox5, or ZEB2, and being methylated, in addition to the stimulation of apoptosis (21, 23). Some previous studies have reported that the expression levels of miR-132 have an indirect relationship with cancer progression (24). Cote *et al*. suggested that miR-132 expression can be used as a diagnostic marker for pancreatic adenocarcinoma in association with other miRNAs (15). The results of the present study revealed the downregulated level of miR-132 expression at all four time points after HBx transfection in the Huh-7 cell line, and the reduced expression level of miR-132 was associated with increased expression of HBx in Huh-7. These outcomes could be due to the hypermethylation of the miR-132 promoter by the activity of HBx-associated DNMTs (25). Furthermore, miR-122 has been demonstrated to indirectly affect HBV replication through the downregulation of cyclin G1, which leads to the p53-related restriction of HBV transcription. On the other hand, in hepatoma cell lines, miR-122 was detected to indirectly induce HBV replication by decreasing HMOX1, which can restrict HBV replication by decreasing the stability of the core protein (26). Reduced expression of miR-122 could significantly reduce the expression of p53 and tumor cell death (26). Our findings suggest that HBx may boost the development of tumorigenesis by decreasing the miR-122 level and the subsequent aberrant expression of p53 and apoptosis. B-cell lymphoma 2 (Bcl-2) and myeloid cell leukemia-1 (Mcl-1) are two apoptosis inhibition genes. HBx is found to increase Bcl-2 and Mcl-1 expressions and downregulate their counterpart, Bcl-2-associated X protein (Bax), in hepatic progenitor cells. A decrease in caspase-9 and caspase-3 and an increase in β-catenin were also observed. This shows that HBx inhibits apoptosis in hepatic progenitor cells. Considering all its effects, HBx has both an apoptosis and an anti-apoptosis function (20). Similar to miR-132 and miR-122, the expression level of miR-22 was reduced 12, 48, and 72 h after HBx transfection at Huh-7. It has been shown that the increased expression of the miR-22 transcript is correlated with the increased levels of liver function tests (such as AST and ALT levels) (27). The results of the present study suggest that miR-22 may play a role in the progression of carcinogenesis under the influence of the HBx protein. However, further studies are needed. In the present study, two additional miRs, miR-21 and miR-222, were also investigated. In contrast to the other studied miRs, miR-21 and miR-222 expression levels increased. The expression of miR-21 increased in constant mode at

four time points after HBx transfection. Yin et *al*. showed that the upregulation of miR-21 leads to a significant increase in proliferation and a reduction in the expression of IL-12 which is a therapeutic candidate in cancer treatment (14). HBx can also upregulate miR-21, which inhibits programmed cell death protein-4 and PTEN, leading to increased cell proliferation (16). Meanwhile, miR-222 increased in a time-dependent manner and reached its peak (4.24-fold increase) 72 h after HBx transfection. Galardi *et al*. showed that the upregulation of miR-222 can directly lead to a decrease in p27, a tumor suppressor and the regulator of the cell cycle (28). It has been shown that both miR-21 and miR-222 are involved in the development of HCC and are labeled oncomiRNA (29). These miRs modulate the mechanism of the cell cycle by disrupting the p27 protein (30). As a result, the upregulated expression of miR-21 and miR-222 under the effect of HBx by targeting cell cycle checkpoints may have a key role in the progression of carcinogenesis. Significant non-conservative substitutions were observed in the HBx gene, suggesting that these mutations are likely to change the property/structure of the HBx protein, thereby influencing the tumorigenic potential or facilitating the escape of the HBx protein from host immune surveillance (29). Our findings revealed the possible role of HBx-induced non-coding miRs expression in carcinogenesis. In addition, the results suggest the potential advantage of using miR expression levels as a prognosis tool for HBV-induced carcinogenesis.

Conclusion

The results of this study showed that the expressions of miR-21, miR-22, miR-122, miR-132, and miR-222 were influenced by HBx. Significantly decreased expression levels of miR-22, miR-122, and miR-132 and increased levels of miR-21 and miR-222 may implicate their inhibitory and enhancing roles in the progression of HBV-associated HCC. The expression of these microRNAs could be used as a prognostic marker for the progression of HBV-associated liver disease. The majority of HCC cases in Asia are associated with HBV infection. Due to its high morbidity and mortality worldwide, we may be able to probe deeper into how the various mutated forms of HBx observed in HCC patients can modulate hepatocarcinogenesis. This may then lead to better therapeutic strategies to manage HBV-associated HCC patients.

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Authors' Contribution

Conceive and design of the experiments: A.T; data analysis: H.R, E.B; writing of the paper: E.B, M.K, H.R; performance of the experiments: M.K; Read and confirm of final version of article: all of authors; Revise: E.B, A.T. M.K and E.B are equally the first author.

Ethics

All experimental protocols were used according to Ethics Committee of Golestan University of medical sciences (code: IR.GOUMS.REC.1395.44).

Conflict of Interest

The authors declare that this research was conducted in the absence of any relationships that could be construed as a potential conflict of interest.

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