



## Evaluation of miR-222 Expression in HBV Infected Patients in Comparison with HDV and HBV Co-infected Patients

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### Abstract

**Background:** Liver disease is more severe in HDV+HBV co-infected patients than HBV infected patients which seems to be related to differences in the expression of genes and other factors such as MicroRNAs (miRNAs). The aim of this study was to investigate miR-222 expression in HBV infected patients in comparison with HDV+HBV co-infected patients.

**Methods:** First, total RNA was extracted from the serum samples and then, complementary DNA (cDNA) was produced using cDNA synthesis kit. Finally, miR-222 gene expression was measured using U6 as the internal control by quantitative PCR (qPCR).

**Results:** The level of miR-222 expression in HDV+HBV co-infected samples was significantly up regulated. The fold change of the miR-222 expression between two groups was 3.3 (95% CI; 0.011- 17.63) with  $p < 0.001$ .

**Conclusion:** The expression of miR-222 was higher in HBV+HDV co-infected patients than HBV infected patients. Further studies should be conducted to confirm whether miR-222 can be a biomarker for prognosis of severe liver diseases.

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**Keywords:** Co-infection with hepatitis B and D, Hepatitis B virus, Hepatitis D virus, MiR-222

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### Introduction

Hepatitis B virus (HBV) infection remains an important public health issue<sup>1</sup> and the most important causes of liver diseases are the acute or chronic types of the infection. Today, about 350 million people are infected with hepatitis B virus<sup>2</sup>. Approximately 257 million people have chronic HBV infection which resulted in 887,000 deaths in 2015<sup>3,4</sup>.

Hepatitis D virus (HDV) requires the presence of HBV for proliferation and survival. About 15 million people worldwide are infected with HDV<sup>5</sup>. Patients who have both HBV and HDV infections usually present with a more severe form of the HBV<sup>6</sup>, probably due to differences in the expression of genes and other factors such as MicroRNAs<sup>7</sup>.

MicroRNAs are small RNAs with an average length of 22 nucleotides with conserved sequences and they play important roles in regulating gene expression. This regulation can be associated with post-transcriptional levels, mRNA degradation, or blocked translation<sup>8-10</sup>.

In this study, the Micro RNA-222 (miR-222) expression in HBV infected samples was compared with HBV+HDV co-infected samples.

### Materials and Methods

#### Study subjects

Totally, 40 serum samples were collected from Keyvan Virology Laboratory (Tehran, Iran) including 20 HBV samples and 20 HBV+HDV co-infected samples. The mean age of HBV and HDV+HBV co-infected patients was  $35.3 \pm 6.9$  and  $38 \pm 7.2$  years, respectively with 65% being males and 35% females. Also, informed consent was obtained from all subjects to be included in the study.

#### RNA extraction

Total RNA was extracted based on manufacturer's instruction of the Biosol kit (Bon Yakhteh Company, Iran). Spectrophotometry was used to check the quality of Ribonucleic Acid (RNA) extraction. Also, the concentration of samples was measured by NanoDrop. Then, Complementary Deoxyribonucleic Acid (cDNA) was produced using the cDNA synthesis kit (Bon Yakhteh Company, Iran). First, a poly (A) tail was added to the extracted RNA (miR-222) by poly (A) adenyating enzyme at 75 °C for 5 min. Then, cDNA was synthesized by 1  $\mu$ l of RT enzyme, 2  $\mu$ l of dNTP, and 4  $\mu$ l of RT buffer up to 20  $\mu$ l, at 25 °C for 10 min, 42 °C for 60 min, and 72 °C for 10 min.

**Real-time quantitative PCR (qPCR)**

By using a high specificity miRNA qPCR core Bon-miR reagent kit and a specific primer for miR-222, gene expression was assessed. U6 small nuclear RNA (U6 RNA) was used as a reference gene. Forward primers for miR-222 and U6 were 5'- ACC GAG CTA CAT CTG G-3' and 5'-AAC GAT GAC ACG CAA A-3', respectively. The real-time PCR was carried out in triplicate according to the manufacturer's instruction with thermal cycling at 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and melt curve analysis by heating from 50 °C to 95 °C at the rate of 0.1 °C.

**Statistical analysis**

For comparison of expression of miR-222 in two groups, chi-square test was used. Fold change of the miR-222 expressions between two groups was determined by the  $2^{-\Delta\Delta Ct}$  method and p-value less than 0.05 was considered as the significance level.

**Results**

**Curve analysis of the real-time PCR**

In figure 1 (1A and 1B), the melt curve analysis indicated that the sequence-specific amplification of the miR-222 and U6 (At 83 °C and 86 °C, respectively) was performed. As shown in figure 1A, the presence of two similar peaks from two different samples indicates the specificity of the chosen primers and also, confirms the specific amplification of the miR-222 in real-time PCR with SYBR Green method. In addition, in figure 1b, the denaturation of the PCR product of U6 gene at 86 °C showed sequence- specific amplification of U6 gene.

**Ct value of samples**

The miR-222 expression rate was normalized based on the U6 expression, and then the mean of cycle threshold (Ct) of triplicate repeats of all the samples of HBV and co-infected samples (HDV+HBV) was calculated (Table 1).

**Relative expression software tool (REST) analysis**

Finally, the fold change was calculated by using relative expression software tool (REST, version 2009). The results showed that in co-infected samples with

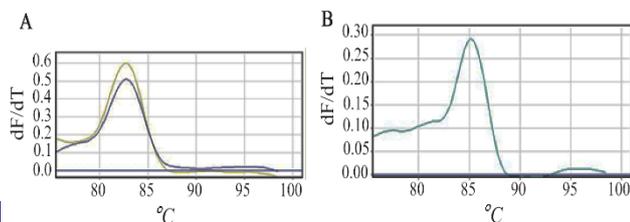


Figure 1. Melt curve analysis of miR-222 (2A) and U6 (1B). Melting curves were obtained from the continuous monitoring of fluorescence of SYBR Green dye during temperature changes and denaturation of PCR products occurred at 83 °C and 86 °C, indicating specific amplification of miR-222 and U6, respectively in real-time PCR.

Table 1. The mean and standard deviations (SD) of the Ct values for the miR-222 and the U6 genes obtained from the mean of Ct (Triplicate repeats) of 40 samples of the HBV infected patients and HBV+HDV co-infected patients

Ct of miR-222		Ct of U6	
Patients	Mean±SD	Patients	Mean±SD
(HBV)	18±0.20	(HBV)	34±0.17
(HBV+HDV)	17±0.22	(HBV+HDV)	33±0.12

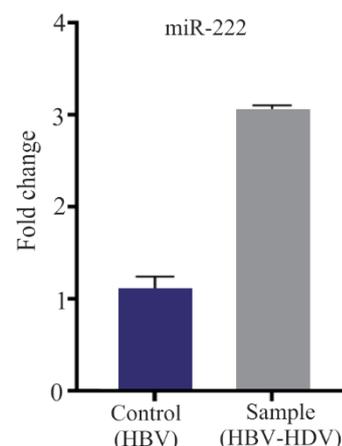


Figure 2. The changes in rate of miR-222 expression in HBV infected patients and co-infected patients with HDV+HBV (Average repetitions as triplicate). The difference of fold change of the miR-222 expressions between two groups was 3.3 p<0.001.

HBV and HDV, the level of miR-222 expression was significantly up regulated. The difference of fold change of the miR-222 expression between two groups was 3.3 (95% CI; 0.011-17.63) with p<0.001 (Figure 2).

Also, there was no significant association between miR-222 overexpression with age and gender of patients based on the chi-square test (p>0.1 and p>0.09, respectively).

**Discussion**

The results of this study showed that the rate of miR-222 expression in HBV+HDV co-infected patients increased about 3.3 times more than the patients that were exclusively infected with HBV.

The rate of miR-222 expression is significantly higher in malignant liver cells resulted from chronic HBV and hepatitis C virus (HCV) infections. Bandyopadhyay and Bharadwaj identified the expression of MicroRNAs in HBV infection and found that the expression of miR-222 was increased<sup>11</sup>. Tao *et al* suggested an increase in the expression of miR-222 in HCC could be due to hepatitis B virus infection<sup>12</sup>. Hepatitis B virus X protein (HBx) of HBV is associated with development of Hepatocellular Carcinoma (HCC) and HBx modulates the cellular processes such as reduction of DNA repair and inhibition of p53-mediated apoptosis by direct interaction with p53<sup>13</sup>.

Also, it was reported that HBx caused up regulation of target protein of miR-222, p27, in HepG2 cells which results in lower expression of miRNA-222. Overexpression of miR-222 directly results in down regulation of the tumor suppressor and cell cycle regulator p27<sup>14</sup>. However, the exact mechanism and pathways of miR-222 overexpression in HBV+HDV co-infected patients have not been fully elucidated; based on the results of this research, it can be concluded that miR-222 overexpression in HBV+HDV co-infection may be due to HDV co-infection and suppression of HBV replication and transcription. Since HDV can interfere with HBV replication, HDV superinfection with chronic HBV infection is associated with a decrease in HBsAg, HBV virion secretion, intercellular HBV DNA, and pregenomic RNA<sup>15,16</sup>.

### Conclusion

The expression of miR-222 was higher in HBV+HDV co-infected patients than HBV infected patients. Further studies should be conducted to confirm whether miR-222 can be a biomarker for prognosis of severe liver diseases.

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### Conflict of Interest

The authors declare that they have no competing interests.

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