Biochemical and Molecular Alterations of Micro RNA 125b and Micro RNA 489-3p as diseases biomarkers in Hepatitis C Virus (HCV)

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1. INTRODUCTION

Because the liver is the primary organ for metabolism, detoxification, and secretory functions, it is susceptible to a wide range of disorders (Kalra et al., 2023). Chronic liver diseases are defined as progressive hepatic dysfunction over six-month (Shah et al., 2020). Chronic hepatitis C patients are at a significant risk of developing serious consequences, such as cirrhosis, in cirrhotic individuals HCC (WHO, 2020). HCV is the only species member that causes substantial cirrhosis i third developing fibr, and cirrhosis after 20 years of infection; the majority develop various stages of hepatocellular carcinoma (Thrift et al., 2017). The potential treatment for the CHC virus has undergone a significant metamorphosis in recent years; human viral infection discoveries in humans have revealed surprising results which have expanded our knowledge of microRNA role inside the body (Laurence, 2016). The role of various miRNAs in controlling the viral infection response was investigated, which explains the causes of CHC progression in the majority of infected patients, as well as the consequences of infection in the risk of cirrhosis and HCC development (Cazanave et al., 2011). Several studies have shown that miRNAs act as tumor suppressors by down-regulating oncogenic targets or tumor promoters by negatively regulating tumor-suppressive target miRNAs (Shenouda and Alahari, 2009). MiRNAs may be employed as biomarkers for the early detection of cancer and other disorders in either case (Wang D et al., 2010). Furthermore, miRNAs and their target genes may be used in anticancer therapy (Hong et al., 2020). Among miRNA families with crucial activities, the miR-125b family has been linked to a range of carcinomas as a tumor suppressor or promoter (Sun et al., 2013). Hepatitis C virus infection modifies a number of cellular microRNAs, one of which, miR-122, is critical for the HCV replication cycle (Roberts et al., 2011). Other miRNAs prevalent in the liver, such as miR-125b, a highly conserved homolog of lin-4, are being investigated for their involvement in important cellular pathways such as inflammation, fibrogenesis, and hepatocellular oncogenesis (Kim et al., 2013; Shrivastava et al., 2015). Exosomal miR-125b levels have been linked to the prognosis of hepatocellular carcinoma (Liu et al., 2017), and plasma miR-125b levels have been recommended as a non-invasive biomarker in chronic viral hepatitis (Akamatsu et al., 2015). MiR-489-3p levels were shown to be less in late recurrent HCC patients than in early recurrent instances (You et al., 2018). Low levels of miR-489-3p were linked to malignant clinical characteristics and worse survival rates in HCC patients. This study objected to exploring MicroRNA 125b and MicroRNA 489-3p potential as precise diagnostic biochemical markers for the identification of chronic HCV infection.

2. MATERIALS AND METHODS

2.1 Materials:
The current investigation follows a cross-sectional design that received approval from the Gastroenterology Hospital in Mansoura and the Oncology Institute in Mansoura. The

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study cohort encompassed 20 individuals diagnosed with chronic liver disease and under treatment at Mansoura Gastroenterology Hospital. Out of these, 5 patients exhibited positive PCR results for chronic HCV infection. The age range of the patients fell between 30 and 50 years, with body weights ranging from 60 to 90 kg. To serve as a comparison, 5 serum samples were obtained from a control group of healthy individuals.

2.2 Methods:
2.2.1 Biochemical Analysis:
Five HCV patients and the control group had serum samples taken for analysis:
A) Liver function variables involved ALT (Murray, 1984), AST (Murray, 1984), TBil (Pagana et al., 2019), and DBil (Pagana et al., 2019). GGT (Beleta and Gella, 1990) ALP (John 1987) and ALB (Young, 2001). In addition to the analysis of AFP as a tumor marker detector (Johns Hopkins Medicine (2022). 3.1 Biochemical analysis
DNA extraction:
The RNeasy Mini Kit Catalogue no.74104
For total RNA extraction and purification derived from initial crude RNA preparations and diverse enzymatic procedures (including proteinase digestion, DNase digestion, labeling reactions, and RNA ligation), 96% ethanol from AppliChem was utilized. Prior to use, this ethanol was diluted to a concentration of 70% by incorporating distilled and deionized water (DDW) (Yuan et al., 2006).

Equipment and apparatuses used for extraction of RNA
1. Epipendorf Tubes with a capacity of 1.5 ml
2. Biohit monochannel micropipettes in the range of 20-200 µl and 100-1000 µl.
3. Sterile filter tips with capacities of 200 µl and 1000 µl.
4. A centrifuge (Sigma Sartorius, USA).
The material used for mastermix preparation for SYBR Green real-time PCR
a) Quantitect SYBR green PCR kit Cat. No. 204141
It comprises 1 ml of 2x QuantiTect SYBR Green PCR Master Mix and 2 ml of RNase-Free Water.
The primer and the sequence were illustrated in Table (1).

Table (1): Oligonucleotide primers and probes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5'-3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>U6 (housekeeping)</td>
<td>GCCCTGCCGACGACATATACAAAT GCTCTCAGAGATTGTCGTCGAT</td>
<td>Chen et al., 2003</td>
</tr>
<tr>
<td>miR-125b</td>
<td>CCCCCCTAAGCTTTCTGTTTTGCTTTGCTC CCCCAGATTCACAAATTTTGCTGAGCAA</td>
<td>Chen et al., 2019</td>
</tr>
<tr>
<td>miR-489-3p</td>
<td>CTC AACG TGG TCT CGT GCA GGT AAG CTC AAT TCA GTG GAG GAC TGC COT</td>
<td>Zheng and Chen., 2020</td>
</tr>
</tbody>
</table>

Source: Biobasic (Canada).

Molecular analysis:

Real time PCR calculation equipment and apparatuses
1. Real time PCR machine (Stratagene MX3005P)
MX3005P QPCR system represents a comprehensive solution for quantitative polymerase chain reaction (QPCR) detection and subsequent data analysis. This system seamlessly integrates an advanced thermal cycler, a cutting-edge optical system featuring a quartz-tungsten halogen lamp along with a singular photomultiplier tube (PMT), and an exceptionally robust analysis software. Inclusive of the package are five carefully chosen filter sets, enhancing the capabilities of the system. The strategic design of the scanning optics ensures unparalleled differentiation between dyes as well as between individual samples, further solidifying its performance.

2. Unichannel micropipettes (100-1000), (2-20), (0.5-10) and (20-200) µl (Biohit)
3. Filter tips of different sizes.
4. Optical tubes (0.2 ml) (Applied biosystem).
5. Optical caps (Applied biosystem).
SYBR green rt-PCR results were evaluated as follows:
To produce amplification curves and determine Ct values, stratagene MX3005P programme was used. The Ct value of each sample was compared with the control using the "ΔΔ Ct" technique published by (Yuan et al., 2006) to examine changes in gene expression across various RNA samples. This entailed using the following ratio: (2-DDct).
Whereas ΔΔCt = CTt reference – CTt target
ΔCt target = Ct control – Ct treatment and ΔCt reference = Ct control- Ct treatment.

3. RESULTS
3.1 Biochemical analysis:
The results of the serum biochemical examination are mentioned in Table (2).
Liver function parameters:
The HCV group demonstrated a substantial rise in ALT, AST, GGT, and ALP activity, as well as a significant increase in total bilirubin and direct bilirubin and a noteworthy drop in albumin concentration (Alb) compared to control. However, the HCV group had a substantial elevation (7.64 ± 0.26) in AFP concentration compared to healthy ones (3.28 ± 0.21).

Table (2): The biochemical and Molecular changes of HCV and control groups.

<table>
<thead>
<tr>
<th>Patients’ groups</th>
<th>ALT (U/L) ± S. E.</th>
<th>AST (U/L) ± S. E.</th>
<th>ALP (U/L) ± S. E.</th>
<th>GGT (U/L) ± S. E.</th>
<th>Albumin (g/dl) ± S. E.</th>
<th>Total Bilirubin (mg/dl) ± S. E.</th>
<th>Direct Bilirubin (mg/dl) ± S. E.</th>
<th>AFP (ng/ml) ± S. E.</th>
<th>PCR for HCV RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control healthy group</td>
<td>23.79 ± 1.60</td>
<td>31.12 ± 1.30</td>
<td>52.20 ± 4.45</td>
<td>27.59 ± 2.85</td>
<td>3.91 ± 0.21</td>
<td>9.00 ± 0.21</td>
<td>0.23 ± 0.04</td>
<td>3.28 ± 0.21</td>
<td>2.65 X 10⁶ ± 0.92</td>
</tr>
<tr>
<td>HCV group</td>
<td>97.83 ± 7.48</td>
<td>147.82 ± 6.85</td>
<td>78.25 ± 4.61</td>
<td>68.48 ± 4.96</td>
<td>5.55 ± 0.14</td>
<td>1.96 ± 0.16</td>
<td>0.62 ± 0.08</td>
<td>7.76 ± 0.26</td>
<td>189.71 X 10⁶ ± 3.48</td>
</tr>
</tbody>
</table>

Data are presented as (Mean ± S.E); Mean values with different superscript letters at the same column are significantly different at (P<0.05).
3.2. Molecular analysis:

Molecular analysis results in (Table 3) The relative expression level of "miRNA-125b" and "miRNA-489-3P" genes, in liver tissue of control and HCV groups. Concerning miR-125b gene expression of HCV group revealed a substantial elevation (10.07 ± 0.19) (P < 0.05) in mRNAl125b more than healthy group (1.00 ± 0.05).

However, miR-489-3p Gene Expression: revealed that HCV group had a substantial reduction in mRNA489 average ct (32.46) compared to healthy group, average ct (25.94).

Table (3): Relative expression level of "miRNA-125b" and "miRNA-489-3P" genes, in liver tissue of control and Hepatitis C Virus (HCV) groups of patients.

<table>
<thead>
<tr>
<th>Patients groups</th>
<th>miRNA-125b Fold change Mean ± SE</th>
<th>miRNA-125b Average Ct</th>
<th>miRNA-489-3p Fold change Mean ± SE</th>
<th>miRNA-489-3p Average Ct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control healthy group</td>
<td>1.00 ± 0.005</td>
<td>23.04</td>
<td>1.00 ± 0.06</td>
<td>25.94</td>
</tr>
<tr>
<td>HCV group</td>
<td>10.07 ± 0.19</td>
<td>19.10</td>
<td>0.36 ± 0.24</td>
<td>32.46</td>
</tr>
</tbody>
</table>

Data were statistically analyzed as (Mean ± SE), the difference in mean values between superscript letters in the same column is significant at (P < 0.05).

4. DISCUSSION

Hepatitis C virus is a risk factor for chronic liver failure and liver cirrhosis progression. Chronic hepatitis C (CHC) is commonly undiagnosed, with normal serologic or biochemical tests diagnosing the majority of cases (Gupta et al., 2014).

In this study, noteworthy high alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-Glutamyltransferase (GGT), and alkaline phosphatase (AlkP) values, significant increase in Total bilirubin (TBIL), Direct bilirubin (DBIL) and alpha-fetoprotein (AFP) concentration with significant decrease in albumin concentration were recorded in HCV group compared to control. These results were supported by Alazawzy (2018). Additionally, Hyder et al. (2013) demonstrated a notable increase in enzymatic functions between viral hepatitis patients compared to the control group. The higher aminotransferase values in the positive HCV marker group were also reported by Munir et al. (2011). A wide range of ALT and AST values, from normal to permanently raised, was recorded in chronic HCV, despite studies showing that individuals with normal ALT had a slower development and a reduced occurrence of cirrhosis Hussein. (2016). When hepatocytes are injured in HCV infection, aminotransferases from the liver leak into the bloodstream, leading to high levels of them in the blood. In addition, (Lee et al., 2001), reported that Patients with chronic HCV who have high HCV RNA titers and abnormal ALT levels have active HCV replication with a higher risk of liver damage. ALT levels are used as a marker for monitoring antiviral therapy response in CHC infection (Dufour et al., 2000). ALP and GGT levels rise in chronic liver disorders. As the bile ductules’ cell is encouraged to generate ALP, it reaches the bloodstream; this may come from enhanced production and release of the enzyme into serum rather than reduced biliary secretion (Lowe et al., 2017).

Total bilirubin levels were reported to be considerably greater in HCV individuals, revealed to be an indicator of hepatic damage (Min Du et al., 2016). This rise is due to suppression of the conjugation process and the release of unconjugated bilirubin from hepatocytes. A low serum albumin concentration, on the other hand, has been linked to hepatic dysfunction. As albumin concentrations take several weeks to decline after decreased albumin synthesis, albumin reductions begin early in the disease’s progression, and the short albumin half-life of about 18-20 days is induced by hepatitis C infection. (Ashraf et al., 2010).

In cirrhosis follow-up, there was a considerable increase in AFP levels, which operate as a marker for HCC development. In HCV patients, there was a link between AFP levels and long-term virological response. This was consistent with the findings of (Masetti et al., 2018). HCV protein expression has been demonstrated to affect multiple potentially carcinogenic pathways through cell signaling transcription modulation, apoptosis, transformation, translational control, and interaction with the translational machinery and post-translational modification system. Aside from the potential effects of the HCV virus on the host genome, HCC may lead to a cycle of inflammation, necrosis, and regeneration in the liver as a result of chronic hepatitis C-induced liver cell injury. In this setting of inflammation, increased cell turnover, as well as oxidative DNA damage, may assist in the accumulation of genetic and epigenetic alterations like activation of cellular oncogenes and telomerase, proliferative signaling pathways, with inactivation of tumor suppressor genes and overexpression of angiogenic and growth factors (You et al., 2018). Elevated AFP levels are also reported in individuals with viral hepatitis without HCC, particularly in those with chronic HCV and liver cirrhosis (Chu et al., 2001).

According to our findings, chronic hepatitis C is an important risk factor for HCC. Given that mRNA dysregulation has been associated with the development and progression of HCC, HCC-associated mRNA signature discovery is of considerable significance for HCC early detection in positive HCV patients prior to disease onset. HCV modifies mRNA expression to promote hepatocyte development toward tumor formation by modulating several signaling pathways (Kanwal et al., 2011). miRNAs represent a significant focal point in addressing viral hepatatis, presenting a promising avenue for targeting HBV and HCV infections. These infections, in some manner, contribute to the progression of HCC and eventual mortality during persistent infection. By targeting these miRNAs, there’s a potential to prevent the progression of these infections, subsequently mitigating the risk of HCC incidence. This approach involves the regulation of numerous oncogenic miRNAs, such as miR125b and miR489-3p, both of which serve as viable biomarkers for HCC (Elemeery et al., 2017). In this study, a noticeable elevation in mRNA125b gene expression was observed in HCV individuals, while gene expression of mRNA489-3p exhibited a marked reduction. Correspondingly, serum levels of miR125b were heightened in chronic HCV genotype 1 infection in contrast to NAFLD individuals, thus, hepatic miR-125b expression remained consistent among chronic HCV infection and NAFLD individuals) Choudhuri et al., 2016). Our investigation revealed a noteworthy augmentation in miR-125b expression within HCV replicon cells and in the serum of HCV infection patients. This miR-125b upregulation was consistently seen in human samples obtained from infected individuals. (Bala et al.,
mediated the biological roles of miR-489-3p in HCC. Chen et al. (2013) disclosed that miR489-3p suppressed the expression of MMP7 in HCC cells. The levels of MMP7 in HCC tissues were negatively correlated with the expressions of miR-489. Moreover, we found that miR 489-3p could directly interact with the 3'-UTR of MMP7. These experiments suggest that MMP7 is a downstream molecule of miR 489-3p. Furthermore, we found that restoration of MMP7 could abrogate the anti-metastatic effects of miR-489 on HCC cell migration and invasion. These suggest that miR 489-3p inhibits the migration and invasion of HCC cells, possibly by targeting MMP7.

(Chen et al., 2013) demonstrates that miR489-3p expression is significantly decreased in HCC. The low level of miR489-3p correlates with adverse clinical parameters of HCC patients and shortened survival. And miR489-3p inhibits the metastasis of HCC cells. Furthermore, MMP7 is a direct target of miR489-3p in HCC. Altogether, miR489-3p exerts its inhibitory effects on HCC metastasis, at least in part, by targeting MMP7. Finally, miR-489-3p was considerably down-regulated in liver fibrosis compared to controls.

5. CONCLUSIONS

MiRNA125b expression was dramatically elevated in HCV patients, but the miRNA489-3p expression was significantly reduced, suggesting that miRNA125b and miRNA489-3p expression, in addition to other biochemical parameters, may be employed as helpful indicators for chronic HCV diagnosis.

6. REFERENCES


15. Hsi, E., Huang, CF., Dai, CY., Joo, SH., Chou, WW., Huang, JF. 2014. Peripheral blood mononuclear cells microRNA predicts treatment outcome of hepatitis C virus genotype 1 infection. Antiviral Res. 42(9);105-135.


