Diagnostic Potential of Circulating MicroRNA-21 in Hepatocellular Carcinoma.

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Abstract:- Background & Aims:- Several studies have reported the significance of circulating microRNA as a biochemical marker of cancer. However, there are no reports on the significance of circulating microRNA in hepatocellular carcinoma. The aim of this study was to evaluate the significance of plasma microRNA-21 level as a biochemical marker for hepatocellular carcinoma. Materials & Methods: To identify the causal role of (MicroRNA-21) in hepatocarcinogenesis we used a human model in which 30 diagnosed HCC cases of different stages, 20 HCV positive cases and 20 healthy controls were tested for circulating microRNA-21 using whole blood samples taken from mentioned individuals at National Liver Institute Menoufiya University. MicroRNA extraction, Amplification & RT-PCR were done for all samples with other various biochemical analysis. Results: Real-time RT-PCR analysis demonstrated up-regulation of oncogenic miR-21 at different stages of hepatocarcinogenesis. On the other hand, there were no significant miRNA-21 changes neither in HCV nor Control groups. ROC study showed that the best cutoff value for miR-21 was 3.93 (Fold expression) and the sensitivity was 93% while the specificity was 90%. Compared to the cutoff value for AFP which was 91.7 (ng/mL) and the sensitivity was 75.2% while the specificity was 92.3%. Conclusion: Circulating MiRNA-21 level is more sensitive than AFP and highly specific as a biological marker for HCC, also it is proved to be beneficial in early diagnosis of HCC. Targeting of microRNA-21 is sufficient to limit tumor cell proliferation and invasion in a manner that is likely to involve associated changes in multiple targets, suggesting that suppression of microRNA-21 may be an approach for the treatment of hepatocellular carcinoma.

Key words: Hepatocellular Carcinoma – MiRNA-21 – PTEN – AFP.

1 INTRODUCTION

Hepatocellular carcinoma is the fourth most common cancer in the world and third common cause of death related to cancer (1). Chronic liver disease caused by hepatitis C virus (HCV) is an established significant risk factor for development of HCC and HCV related cirrhosis is the primary reason for the drastic increase in HCC (2). In Egypt, according to National Cancer Institute, HCC was reported to account for about 4.7% of chronic liver disease patients (3). In another study, in 2005, a remarkable increase from 7-14% was reported over a decade. Patients with advanced liver disease, particularly cirrhosis, are those at risk for HCC and should be screened every six months for its development (4). A new emerging area of HCC therapeutics is based on miRNAs. miRNAs are small (~21–23 nucleotides long), non-coding RNAs that regulate post-transcriptional gene expression of their target genes either by inducing translational repression via their binding to partially complementary sequences or by directing mRNA degradation through their binding to perfectly complementary sequences in the 3’ untranslated region (UTR) of messenger RNAs (mRNAs) (5). Each mature miRNA potentially controls many gene targets, and each mRNA is regulated by multiple miRNAs. To date, more than 17,000 distinct mature miRNA sequences have been identified from over 140 species (6). A number of recent studies have documented the involvement of miRNAs in HCC in tumor progression and metastasis (7) (8). In humans, more than 50% of miRNA genes are located at fragile sites or in cancer-associated genomic regions that are frequently involved in chromosomal abnormalities, such as loss of heterozygosity, amplification and breakpoints (9), indicating that they might be useful as novel diagnostic and/or prognostic markers and could constitute potential molecular targets in cancers. Thus, miRNAs modulate various cellular signaling pathways involved in cell growth, proliferation, motility and survival. miRNAs are also subjected to regulation by epigenetic mechanisms, and mutations in their promoter and coding regions were shown to contribute to tumorigenesis (10).

2 Materials and Methods

2.1 Study Design:
The study was conducted on 70 subjects divided into 3 groups, selected from inpatient wards and outpatient clinic, National Liver Institute, Menoufiya University in the period from January 2014 to September 2104. The Institution’s ethics committee approved the study protocol, and enrolment of the individuals to the study was conditioned by an obtained written informed consent.

The patients were grouped as following:

Group 1 (HCC group): This group included 30 newly diagnosed patients before receiving therapy. Their ages ranged from 35 to 51 years, they were 26 males and 4 females. The diagnosis was based on clinical examination, laboratory tests, ultrasonography, spiral CT & biopsy in some cases.

Group 2 (Chronic Viral Hepatitis C - HCV Group): This group included 20 patients with HCV. Their ages ranged from 30 to 52 years, they were 15 males and 5 females. They were diagnosed by ultrasonographical findings (shrunken liver, coarse echopattern, attenuated hepatic vein and fine nodular surface), PCR and biochemical evidence of parenchymal damage as well as liver biopsy in some cases.

Group 3 (Control group): Included 20 apparently healthy subjects served as a control group, their ages ranged from 32 to 53 years, they were 18 males and 2 females.

All patients and control groups were subjected to the following:

Biochemical tests as (Liver function tests, prothrombin concentration (PC%), Viral markers, serum AFP, CBC, Kidney

2.2 Extraction:
For the real-time PCR MirRNAs were extracted (Qiagen miRNA Extraction kit) from whole blood on EDTA using QIAzol (Lysis solution) according to the manufacturer’s instruction. Single-stranded cDNAs were generated using the RT kit (TaqMan Reverse Transcriptase kit) according to the manufacturer’s directions. The RNA purity was assessed by the RNA concentration and quantified by NanoDrop ND-1000 (Nanodrop, United States).

2.3 Quantification:
PCR quantification experiments were performed with PCR (Applied Biosystems; Real Time 7500 Fast PCR system) using the TaqMan miRNA Assay (MiRNA-21) and Universal TaqMan master mix according to the manufacturer’s protocol. The primers for microRNA-21 and housekeeping gene were supplied by Qiagen Germany. Fluorescence measurements were made in every cycle and the cycling conditions used were: 95 °C for 30 s, and 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Expression reported as ΔCt values, where: -
-ΔCt = Ct (Target) – Ct (Reference)
ΔΔCt = ΔCt (Target) – ΔCt (Mean control)
Normalized Target Gene Expression Level (RQ) = 2(−ΔΔCT)

2.4 Statistical analysis:
Data were collected, tabulated and statistically analyzed by SPSS version 23.0 statistical package (SPSS, Inc, Chicago, IL, USA). ANOVA (followed by LSD post-hoc test), Kruskal-Wallis, Student’s t, Chi-square and Spearman rank correlation tests were performed at 5% level of significance. The diagnostic performance for MiRNA-21 & AFP to discriminate HCC cases from normal cases was evaluated using Receiver Operating Characteristic (ROC) curve analysis. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for each marker were determined.

3 Results
The results of the present study are summarized, statistically analyzed and presented in the following tables and figures.

Table (1): Pearson's correlation matrix between Focal lesion size, MiRNA-21 and AFP in HCC Group

Table (3): Comparison between MiRNA-21 and focal lesion size largest diameter in hepatocellular carcinoma

Table (4): Validity of the cutoff value of the Circulating MiRNA21 for prediction of HCC.

P3 between HCC and HCV
This shows:
1) Highly significant increase of the mean serum level of MicroRNA-21 RQ (Fold expression) in HCC group compared to each of HCV and control groups (P < 0.01). Also, it showed no significant elevation in HCV group compared to controls (P > 0.05).
2) Highly significant increase of the mean serum level of AFP in HCC group compared to each of HCV and control groups (P < 0.01). However there is significant elevation in HCV group compared to Controls (P<0.05).
of 3.93 (fold expression) for diagnosis of HCC, sensitivity was 93 \% but the specificity was 90\%.

Table (5) shows that, when we used AFP at a cutoff point of 91.7 (ng/mL) for diagnosis of HCC, sensitivity was 75.2 \% but the specificity was 92.3\%.

Table (6): miRNA21 HCC group versus non-HCC groups (HCV and control groups)

<table>
<thead>
<tr>
<th>miRNA21 As a diagnostic test</th>
<th>Clinical diagnosis</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCC</td>
<td>Non-HCC</td>
</tr>
<tr>
<td>Positive (≥ Cutoff 3.93)</td>
<td>28</td>
<td>3</td>
</tr>
<tr>
<td>Negative (&lt; Cutoff)</td>
<td>2</td>
<td>37</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>40</td>
</tr>
</tbody>
</table>

- Sensitivity of miRNA-21 in HCC group = 93 \%.
- Specificity of miRNA-21 in HCC group = 90\%.
- Positive predictive value miRNA-21 in HCC group = 94.4\%.
- Negative predictive value miRNA-21 in HCC group = 87.5\%.
- Accuracy of miRNA-21 in HCC group = 92.5\%.

Figure (1): ROC curve showing the predictive value and the cutoff point of the MicroRNA-21 as a predictor marker for hepatocellular carcinoma.

In this study, our findings show that the Circulating miRNA-21 was significantly overexpressed in blood of HCC patients compared to HCV patients and controls Table (1) which shows Statistically significant increase of the mean serum level of MicroRNA-21 RQ (Fold expression) in HCC group compared to each of HCV and control groups (P < 0.01). Also, it showed no significant elevation in HCV group compared to controls (P > 0.05). These results agreed with Esau C et al., 2009 (12) who said (We show that miR-21 expression is increased in malignant hepatocytes, and in human HCC compared with matching nontumoral tissue) and Esquela et al., 2006 (15) who stated (MiR-21 has been shown to be overexpressed in many different solid tumors, including breast, colon, lung, pancreas, prostate, stomach, as well as in cholangiocarcinoma cell lines). Targeting miR-21 could modulate the expression of downstream mediators of tumor growth and metastasis.

Figure (2): ROC curve showing the predictive value and the cutoff point of the plasma α-fetoprotein as a predictor marker for hepatocellular carcinoma.

Figure (2) the smallest cutoff point for the plasma α-fetoprotein as a predictor marker for HCC is 91.7 (ng/mL). The area under the curve is 0.83 (p-value < 0.01).

4 Discussion
Although a role for miRNAs in cancer has been proposed, the molecular mechanisms by which miRNA can modulate tumor growth or metastases are unknown (11). Previous studies show that miR-21 expression is increased in malignant hepatocytes, and in human HCC compared with matching nontumoral tissue (12). Moreover, miR-21 promotes cell invasion, migration, and growth via repression of tumor suppressor genes expression and downstream effects involving the phosphorylation of FAK and Akt, and the expression of MMP-2 and MMP-9 (13). The identification of miR-21 as an important regulator of tumor cell proliferation, migration, and invasion in vitro emphasizes an essential role of this miRNA in mediating hepatic oncogenesis and tumor behavior, and provides insight into the contribution of altered miRNA expression in contributing to the tumor phenotype (14). In this study, our findings show that the circulating MicroRNA-21 was significantly overexpressed in blood of HCC patients compared to HCV patients and controls table (1) which shows statistically significant increase of the mean serum level of MicroRNA-21 RQ (Fold expression) in HCC group compared to each of HCV and control groups (P < 0.01). Also, it showed no significant elevation in HCV group compared to controls (P > 0.05). These results agreed with Esau C et al., 2009 (12) who said (We show that miR-21 expression is increased in malignant hepatocytes, and in human HCC compared with matching nontumoral tissue) and Esquela et al., 2006 (15) who stated (MiR-21 has been shown to be overexpressed in many different solid tumors, including breast, colon, lung, pancreas, prostate, stomach, as well as in cholangiocarcinoma cell lines). Targeting miR-21 could modulate the expression of downstream mediators of tumor growth and metastasis.
Therapeutic strategies to decrease miR-21 therefore potentially may be useful to limit HCC growth and metastasis. Further work is warranted to evaluate the role of miR-21 and the identified downstream targets and to develop therapeutic strategies targeting miR-21 in vivo. The ability to therapeutically manipulate miRNA expression is feasible, and recent proof-of-concept studies have shown that miRNA antagonists targeted to the liver can modulate expression of downstream genes (12). We come to an important clue, when doing the experiment on whole blood taken on EDTA. Assessment of the Circulating MiRNA-21 level Expression in the studied groups (HCC, HCV & Control), the results and findings were approximately identical to those done on tissue by variable techniques. And since this experiment was delivered in National Liver Institute on Egyptian people, it is not a role to give the same findings in other countries or circumstances. However, the accuracy of the obtained results compared to tissue samples could be very beneficial in avoiding invasive techniques like biopsies to obtain tissue samples. Another very important diagnostic value is the relation of MiRNA-21 and focal lesion size in HCC, Table (3) where it shows a highly significant correlation between MiRNA-21 and focal lesion size largest diameter. Which could be implemented in early diagnosis of HCC. This finding agreed with Qibin Liao et al., 2015(20) who confirmed that as a potential diagnostic biomarker for HCC, circulating miR-21 possesses several unique advantages. First, serum or plasma microRNA is characterized by minimal invasion and convenience compared with histopathological examination. Second, serum microRNA expression levels are stable and reproducible (21); Third, plasma miR-21 level cannot be influenced by both cirrhosis and viral status. Fourth, Significant overexpression of plasma miRNA-21 was observed even in patients with early-stage HCC (22) On the other hand, AFP level of 400 ng/ml is considered as an indicator of HCC in general, which does not occur at an early HCC stage. As a result, about one-third of all HCC case with small lesions (<3cm) were not diagnosed in the early tumor stage (23) Therefore, circulating miR-21 may serve as a novel co-biomarker to AFP to improve the diagnostic accuracy of early-stage HCC. ROC curve Fig (1 and 2) for both MiRNA-21 and AFP in HCC to compare sensitivities, specificities and cutoffs. We found that the smallest cutoff point for the plasma α-fetoprotein as a predictor marker for HCC is 91.7 (ng/mL). The area under the curve is 0.83 (p-value < 0.01). Sensitivity was 75.2 %but the specificity was 92.3%. The smallest cutoff point for the Circulating MicroRNA-21 as a predictor marker for HCC is 3.93(fold expression), the area under the curve is 0.97(p-value < 0.01). Sensitivity was 93 %but the specificity was 90%. Which clarifies that as a screening tool for HCC the Circulating MicroRNA-21 was more sensitive than AFP (23). All the previous findings potentiate the idea of the beneficial therapeutic implementation of MiRNAs Inhibitors (small, double-stranded RNA molecules that regulate gene expression by binding to and inhibiting a specific mature miRNA. The miRNA inhibitors were designed using appropriate algorithm and the mature miRNA sequence information from miRBase. The algorithm computes all possible sequence parameters, selects the one predicted to best maintain the TuD structure, providing maximum miRNA recognition and binding. Each synthetic TuD (S-TuD) is stabilized by incorporating 2’-O-methylated nucleotides, and provides two miRNA binding sites. Optimal miRNA inhibition is provided after transfection due to the robust secondary structure of the inhibitor that is based upon the synthetic ‘Tough Decoy’ (TuD) 2 molecule (24) in various gene therapy cancer control programs, which give promising results.

5 Conclusions
- Targeting of microRNA-21 is sufficient to limit tumor cell proliferation and invasion in a manner that is likely to involve associated changes in multiple targets, suggesting that suppression of microRNA-21 may be an approach for the treatment of hepatocellular carcinoma.
- Oncomirs and their tumor suppressor targets implicate their role in hepatocarcinogenesis and suggest their use in the diagnosis and prognosis of liver cancer.
- Circulating MiRNA-21 level is more sensitive Than AFP, yet highly specific as a biological marker for HCC and it is proved to be beneficial in early diagnosis.

6 References


