INTRODUCTION

Hepatocellular carcinoma (HCC), an aggressive malignancy, is the fifth common malignancy worldwide and the second leading cause of malignancy related deaths [1]. In Egypt, HCC represents around 70.5% of all liver tumors and 23.8% of all malignancies [2-3], and is associated with the high prevalence of hepatitis C virus (HCV) which is considered a well-known risk factor for the development of hepatic disorders including chronic hepatitis, cirrhosis and HCC [4-5]. Early diagnosis of hepatic complications of HCV has a pivotal role in the improvement of the prognosis with improving survival for patients with HCC [6].

ABSTRACT

Background. The goals of this study were to elucidate the use of the expression of microRNA-30e (miR-30e) and microRNA-223 (miR-223) as diagnostic biomarkers for early diagnosis of hepatocellular carcinoma (HCC) associated with hepatitis C virus (HCV) infection. Methods. The study included three groups, the first group included thirty patients with HCC associated with HCV, the second group included thirty patients with cirrhosis with HCV and the third group included thirty healthy control subjects. Blood samples were obtained for determination of serum expression of miR-30e and miR-223 by real time polymerase chain reaction. Results. There was significant decrease of miR-30e expression in patients with HCC (0.16 ± 0.1) compared to both patients with cirrhosis (0.4 ± 0.2, P<0.01) and healthy control subjects (1.2 ± 0.4, P<0.001). There was also significant reduction of miR-223 expression levels in patients with HCC (0.2 ± 0.1) compared to patients with cirrhosis (0.5 ± 0.2, P<0.01) and healthy control subjects (1.0 ± 0.1, P<0.001). There was also significant decrease of miR-30e expression in late stage versus early stage of HCC (0.1 ± 0.0 vs. 0.22 ± 0.1, P<0.001), while there was no significant difference of alpha-fetoprotein (AFP) between patients with late and early HCC. Also, values of miR-223 had significantly reduced levels in late HCC compared to its expression values in early HCC (0.1 ± 0.0 vs. 0.23 ± 0.2, P<0.001). Conclusion. There was significant reduction of expression of miR-30e and miR-223 in serum of HCC patients compared to either patients with cirrhosis or healthy subjects. These results show that the combined use of both biomarkers had better sensitivity in the diagnosis of HCC compared to AFP.

Key words: hepatocellular carcinoma, microRNA-30e, microRNA-223, hepatitis C virus, alpha-fetoprotein.

several screening methods for patients with HCV for early detection of the development of HCC through radiological and laboratory methods. The screening schedule depends mainly on the combined use of serum alpha-fetoprotein (AFP) and abdominal ultrasound for patients with HCV every six months. However, this method of screening has many disadvantages as it has many positive rates and does not detect small HCC below 2 cm in diameter. Moreover, AFP may have normal levels in up to 30% of patients with HCC and may be elevated in liver cirrhosis without HCC. The replacement of abdominal ultrasound by computed tomography (CT) and magnetic resonance imaging (MRI) for HCC screening yielded more accurate results but also in lesions greater than 2 cm [7]. Therefore, there is still a need for the use of non-invasive biomarker(s) that can detect early HCC and differentiate between benign hepatic lesions and HCC, especially in patients with chronic HCV.

The progress in the understanding of the molecular basis of oncogenesis has led to the use of serum microRNAs (miRNAs) as biomarkers. MicroRNAs constitute a family of small non-coding RNAs produced endogenously and has about 21–25 nucleotides that act in RNA silencing and post-transcriptional regulation of gene expression [8]. MicroRNAs have regulatory functions in cellular proliferation and differentiation [9] while aberrant expression of miRNAs has been confirmed to be associated with many types of cancers [10]. Most types of miRNAs circulate in serum and plasma protected within exosomes to prevent them from degradation by RNase [11]. MicroRNAs in serum are stable and easy to be determined by molecular techniques affording them as surrogate non-invasive biomarkers for early detection of malignancies [12]. Among the suggested miRNAs that can be used as biomarkers for HCC diagnosis are the microRNA-30e and microRNA-223 [12-13].

MicroRNA-30e (miR-30e) has a physiological role in the development of the hepatobiliary system in the embryo and in the lipid biosynthesis [14-15]. MiR-30e has been shown to play a protective role in experimental liver fibrosis through the transforming growth factor-mediated pathway [16]. MiR-30e has been also shown to be a prognostic marker for breast cancer [17-18] and HCC [12]. MicroRNA-223 (miR-223) has known roles in healthy subjects for differentiation of granulocytes and suppresses erythrocytes differentiation [19]. In cancer, it participates in tumor growth through cellular transformation and angiogenesis [20]. MicroRNA-223 was also reported as a potential biomarker for diagnosis of HCC [21]. The goal of the present study, however, was to boost evidence of the diagnostic value of miR-30e and miR-223 expression levels as valid and sensitive biomarkers for early diagnosis of HCC associated with HCV.

**MATERIALS AND METHODS**

**Patients, ethics, and design**

The present study is a case-control study that included 60 patients classified into two groups: the first group included 30 patients with HCC diagnosed by clinical, abdominal ultrasound and CT according to Barcelona Clinic Liver Cancer (BCLC) classification [22], and the second group included 30 patients with liver cirrhosis associated with HCV diagnosed by clinical examination and laboratory investigations, and the stages of cirrhosis were recorded according to Child-Pugh score. Inclusion criteria were patient age >18 years with HCV associated HCC and/or cirrhosis. Exclusion criteria were patients with hepatitis B virus, HIV, and patients with malignancies other than HCC. In addition, a third control group consisted of 30 healthy subjects was included. All the study participants are those admitted to Mansoura University Hospitals between December 2017 and December 2018. The study was approved by Mansoura University Faculty of Medicine ethical committee, and an informed approval consent was obtained from each subject. Patients with HCC were further classified into patients with early HCC if the size of the tumor was less than 3 cm and the total number of the masses was equal or less than 3, while patients with larger tumor size or more number of tumors were classified as late HCC according to Barcelona classification [22].

**Laboratory investigations**

Ten milliliters of blood was obtained from each subject and divided into two aliquots. One aliquot was used for serological studies for hepatitis B surface antigen (HBsAg) and complete liver tests including alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, direct bilirubin and albumin by autoanalyzer system (Dialab GmBH, Austria). HCV antibodies were performed by immunoassay with the use of Elecsys system (Roche Diagnostics). Alpha-fetoprotein was measured by ELISA (DRG International Inc., USA). The second aliquot of blood
samples was used for serum separation and RNA extraction for miR-30e and miR-223 expression assay by real time polymerase chain reaction (real-time-PCR).

**Serum extraction of miR-30e and miR-223, and reverse transcription real-time-PCR**

RNA was isolated from serum samples by the use of miRNA isolation kit (Ambion®, Life Technologies). The amplification SYBR Green Master mixture was supplied from Applied Biosystems (Applied Biosystems, CA, USA) with 20 ng of the cDNA and volumes 400 and 600 nM forward and reverse primers respectively in a final volume of 20 μL. TaqMan® MicroRNA Assays (Applied Biosystems, CA, USA) were used to assess miR-30e and miR-223 expression levels on two steps: reverse transcription and real-time-PCR. The extracted RNA from each sample was subjected to the reverse-transcription with specific looped RT primers. The reverse transcription reaction included incubation for 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C. The steps of real-time-PCR were performed by the use of 4.5 μL of 1:5 diluted cDNA samples as templates in 10 μL reactions containing primers and probes for miR-30e and miR-223 according to the manufacturer’s instructions. The reactions were performed by the use of an ABI7900 Sequence Detection System (Applied Biosystems) by the use of the following steps: 95°C for 10 min followed by 40 cycles at 95°C for 15 sec, and 60°C for 1 min. Total RNA input was normalized based on the cycle threshold (Ct) values obtained for RNU6B, which is a nucleolar RNA used as an endogenous control in this type of analysis. Relative expressions levels of RNAs were calculated by ∆Ct.

**Statistical analysis**

Data were collected, revised, coded and analysed by the statistical package for social science (SPSS) v24 (SPSS Inc., Chicago, IL, USA). The quantitative data were presented as mean ± standard deviations (SD) and ranges. The comparison between the studied groups was done as appropriate by using the unpaired t-test or the one-way ANOVA. Descriptive statistics were expressed as percentage and comparison between percentages was performed by the use of chi-square test. Statistical significance was assumed when P-values are less than 0.05.

**RESULTS**

The study included 30 patients with HCC, 30 patients with cirrhosis associated with HCV, and 30 healthy control subjects. There was statistically significant difference in mean age between the studied subjects with high mean age for patients with HCC (52.7 ± 4.6, P<0.001). The liver functions tests had statistically significant difference between the groups with high values for ALT, AST, AFP, total bilirubin and direct bilirubin and lower albumin among patients with cirrhosis (P<0.001), Table 1.

There was significant decrease of miR-30e folds of expression in patients with HCC (0.16 ± 0.1) compared to patients with cirrhosis and healthy control subjects (0.4 ± 0.2 and 1.2 ± 0.4 respectively, P<0.001 for both).

**Table 1. Demographic and laboratory data of the studied groups**

<table>
<thead>
<tr>
<th></th>
<th>Control (n=30)</th>
<th>Liver cirrhosis (n=30)</th>
<th>HCC (n=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender (n [%])</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>21 (70%)</td>
<td>22 (73.3%)</td>
<td>24 (80%)</td>
</tr>
<tr>
<td>Female</td>
<td>9 (30%)</td>
<td>8 (26.7%)</td>
<td>6 (20%)</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>37.9 ± 2.7</td>
<td>49.1 ± 4.7‡</td>
<td>52.7 ± 4.6‡</td>
</tr>
<tr>
<td><strong>Albumin (g/l)</strong></td>
<td>40.0 ± 2.0</td>
<td>31.0 ± 10.0‡</td>
<td>37.0 ± 10.0</td>
</tr>
<tr>
<td><strong>Total bilirubin (µmol/l)</strong></td>
<td>17.0 ± 1.7</td>
<td>71.4 ± 28.9‡</td>
<td>17.6 ± 6.8</td>
</tr>
<tr>
<td><strong>Direct bilirubin(µmol/l)</strong></td>
<td>3.4 ± 1.7</td>
<td>40.8 ± 10.2‡</td>
<td>8.5 ± 3.4‡</td>
</tr>
<tr>
<td><strong>ALT (IU/ml)</strong></td>
<td>27.3 ± 7.2</td>
<td>119.2 ± 8.5‡</td>
<td>39.4 ± 17.1‡</td>
</tr>
<tr>
<td><strong>AST (IU/ml)</strong></td>
<td>19.4 ± 2.5</td>
<td>105.8 ± 10.4‡</td>
<td>63.2 ± 19.6‡</td>
</tr>
<tr>
<td><strong>AFP (ng/ml)</strong></td>
<td>4.1 ± 1.4</td>
<td>94.7 ± 11.6‡</td>
<td>414.3 ± 63.6‡</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SD or number and %. Significance levels: ‡P<0.001 vs. control group (unpaired t-test).
there was significant reduction of miR-223 expression levels in patients with HCC (0.2 ± 0.1) compared to either patients with cirrhosis or healthy control subjects (0.5 ± 0.2 and 1.0 ± 0.1 respectively, P<0.001 for both). Meanwhile, there was significant increase in AFP level in HCC patients (414.3 ± 63.6) compared to patients with cirrhosis (94.7± 11.6) and healthy control (94.7 ± 11.6 and 4.1 ± 1.4 respectively, P<0.001 for both, Table 1 and Fig 1). The receiver operating characteristic (ROC) curve demonstrated that areas under the curve (AUC) for miR-30e and miR-223 were 0.87 and 0.88 respectively which represented a good discrimination for HCC from cirrhosis in both biomarkers, while the AFP had low AUC (0.1). The cut-off values for miR-30e and miR-223 were 0.25 and 0.35 folds with sensitivities 83.3% and 73.3% respectively and specificities 87% and 83% respectively, Fig 2. There was marked increase in the AUC to 0.9 when both miR-30e and miR-223 were combined for discrimination between cirrhosis and HCC, with increased sensitivity to 93%, Fig 3.

There was significant low value for miR-30e in late stage of HCC compared to its value in early HCC (0.1 ± 0.0 vs. 0.22 ± 0.1, P<0.001), while there was no significant difference of AFP readings between patients with late and early HCC.
Also, values of miR-223 had significantly reduced levels in late HCC (0.1 ± 0.0) compared to its expression in early HCC (0.1 ± 0.0 vs. 0.23 ± 0.2, P<0.001, Fig 4). The ROC of miR-30e and miR-223 in differentiation between early and late HCC had good AUC (0.93 and 0.96 respectively) with cut-off values of 0.95 and 0.98 respectively for both biomarkers and 96% sensitivity and 96% specificity, Fig 5.

DISCUSSION

Hepatocellular carcinoma is a significant health problem in Egypt and is associated with high prevalence of HCV infection. The prognosis of HCC depends mainly upon the early detection. The approach for the use of miRNAs has been proved to be successful for diagnosis of multiple diseases especially in cancer. MicroRNAs are deregulated in neoplasms with a role in the development and prognosis of cancer. In the present study, serum miR-30e and miR-223 were found to have reduced expression in patients with HCC compared to both healthy control subjects and patients with liver cirrhosis associated with HCV. Similar results were reported previously by Bhattacharya and co-workers [12] in patients with HCC due to different etiologies. Other investigators described deregulation of miR-30e in other types of malignancies such as breast cancer [17], ovarian cancer [23] and pancreatic adenocarcinoma [24]. The role of miR-30e in HCC development is thought to be through enhancement of autophagy leading to the development of HCC [25]. Recent studies have shown that miR-30e regulates the genes ATG5 and ATG12 which control the autophagy process [26]. Moreover, HOXA1, which has been recognized as a new miR-30e target, plays significant roles in regulating cell proliferation, carcinogenesis and metastasis [27].

The other biomarker, miR-223 down-regulates insulin-like growth factor 1 receptor which is up-regulated in patients with cirrhosis and HCC [28]. Other mechanism by which miR-223 controls progression of cirrhosis and HCC is suggested to be through Stathmin1, a microtubule-regulatory protein that controls the microtubule dynamics, cellular proliferation, and S phase of the cell cycle [29]. Accordingly, down-regulation of miR-30e and miR-223 can contribute to the development of HCC associated with HCV and hence our results show that they can be used as biomarkers for the differentiation of cirrhosis from HCC with good sensitivities (83.3% and 73.3%) and specificities (87% and 83%) for miR-30e and miR-223 respectively. Moreover, the use of combined miR-30e and miR-223 assays increases the sensitivity to 93%, thus, the remarkable findings of the present study show that miR-223 cannot be only used as a therapeutic target [30], but also as a good diagnostic biomarker, either solely or combined with miR-30e, for the diagnosis of HCC and differentiating it from cirrhosis.

The development of HCC includes many biomarkers which can be used for diagnosis. Previous studies have reported that miR-92-3p, miR-107, and miR-3126-5p biomarkers can discriminate between early and late HCC [13]. In our study, in addition to the differentiation between HCC and cirrhosis, the combined use of miR-30e and miR-223 as biomarkers has also differentiated between early and late stage of HCC which has critical prognostic value as the delayed diagnosis carries bad prognosis with median survival period varies from 4 to 15 months even with aggressive and combined therapies [31-32]. This distinguished finding was shown by the significant decrease of miR-30e and miR-223 values in late stage compared to early stage of HCC. The ROC curve of miR-30e and miR-223 in differentiation between early and late HCC had good AUC (0.93 and 0.96 respectively) with cut-off values of 0.95 and 0.98 respectively for both biomarkers, and with 96% sensitivity and 96% specificity. The early diagnosis of HCC represents a
miR-30e and miR-223 for early diagnosis of HCC associated with HCV

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challenge as it may not be detected by radiological methods with limited value of AFP as shown in the present study. However, one important limitation of this study is the small number of the studied patients and the study of only two miRNAs, therefore, additional studies are needed to validate these findings and to build up substantial knowledge of the use of these biomarkers for the diagnosis and/or staging of HCC.

In conclusion, this study reported significantly reduced expressions of miR-30e and miR-223 biomarkers in serum of patients with HCC as compared to either patients with liver cirrhosis or healthy subjects. The combined use of both biomarkers showed good sensitivity in the diagnosis of HCC compared to AFP. Both biomarkers showed also remarkable reduced levels in late HCC compared to early HCC. Despite the limitations of this study, we think this little piece of research might provide useful information on the use of miR-30e and miR-223 expressions as non-invasive biomarkers for early diagnosis of HCC. Additional multicenter studies with larger number of patients would be indeed more useful to bridge the gap of data limitation at hand and their context in comparison with other similar studies.

Fig 4. Comparison of miR-30e (A), miR-223 (B) expression and AFP (C) for patients with early vs. late HCC.

Fig 5. Receiver operative curve (ROC) for miR-30e and miR-223 in the diagnosis of early vs. late HCC.
Conflict of interest

The authors declare that they have no conflict of interest.

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enhances the cell proliferation, invasion and metastasis of prostate cancer cells. *Oncol Rep.* 2015;34:1203–1210.


