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Basic Study

Assessment of circulating levels microRNA-326, microRNA-424, and microRNA-511 as Biomarker for hepatocellular carcinoma in Egyptians

Youssef S *et al*. MicroRNAs as biomarkers in HCC

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Abstract

BACKGROUND

Hepatocellular carcinoma (HCC) ranks as the fifth most common cancer. Differential expression of microRNAs (miRNAs)-326, miRNA-424, and miRNA-511 has been associated with the diagnosis and prognosis of HCC in different populations. However, limited information is available regarding their expression in Egyptian HCC patients.

AIM

To assess the role of circulating miRNAs-326, miRNA-424, and miRNA-511 in Egyptian HCC patients.

METHODS

This prospective observational study was conducted on 70 HCC patients and 25 healthy controls. The circulating level of these three miRNAs was evaluated by real-time PCR.

Receiver operating characteristic curve analysis was used to test the diagnostic accuracy of microRNA expression levels.

RESULTS

All miRNAs were differentially expressed in HCC patients; miRNAs326 and miRNA-424 were upregulated, while miRNA-511 was downregulated. Both miRNA-326 and miRNA-424 showed sensitivity and specificity of 97%, 71.4%, and 52%, 60%, respectively, to differentiate HCC from controls. Moreover, miRNA-326 was associated with survival and could differentiate between CHILD grades (A *vs* B); miRNA-424 significantly differentiated early *vs* intermediate stages of HCC; while miRNA-511 significantly correlated with response to modified Response Evaluation Criteria in Solid Tumors (mRECIST).

CONCLUSION

We conclude that miRNA-326, miRNA-424, and miRNA-511 have diagnostic and prognostic roles in Egyptian patients with HCV-related HCC and should be considered for better disease management.

Key Words: Hepatocellular carcinoma; miRNAs-326; miRNA-424; miRNA-511; Modified response evaluation criteria in solid tumors

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Core Tip: Hepatocellular carcinoma ranks as the sixth most common malignancy worldwide. Recent cancer incidence data confirmed that the global age normalization rate (ASR) of primary liver cancer is 10.1/100000, with a male/female ratio of 3:1.

Hepatocellular carcinoma (HCC) diagnosis usually occurs in the late stages, resulting in an elevated death rate; this makes HCC the third deadliest malignancy. We examine the role of circulating miRNAs326, miRNA-424, and miRNA-511 could serve as promising candidate non-invasive biomarker for HCC. Such biomarkers could prove for target gene therapy research and may prove to assist with monitoring the severity of HCC.

INTRODUCTION

Liver cancer is the fifth most common cancer in men and is the second reason for cancer-related deaths worldwide[1]. Hepatocellular carcinoma (HCC) is a primary liver cancer and constitutes 80%-90% of all primary tumors of the liver[2,3]. It has a high incidence and mortality rate, with approximately 662.000 mortality cases every year[4]. Most HCC patients are diagnosed at advanced stages, contributing to the high rates of recurrence and development of metastasis[5]. In addition, the commonly used biomarker for HCC screening and diagnosis is alpha-fetoprotein (AFP), which has modest sensitivity and specificity[6] and is actually influenced by tumor size and cancer stage[7]. Due to these factors, it is crucial to look for more efficient biomarkers that can be practically used for the early diagnosis and prognosis of HCC.

MicroRNAs (miRNAs) are short, non-coding RNAs that regulate the transcription or degradation of certain target mRNAs[8-10]. They mediate important physiological processes such as cell differentiation, proliferation, and survival[11,12]. Aberrant regulation of miRNAs is associated with the initiation and progression of numerous cancers, including HCC[13]. Even more, survival and response to chemotherapeutic drugs have been found to be linked to miRNAs[14-16]. Experimentally, a number of miRNAs have been proven to be related to HCC. They have been proposed as diagnostic and prognostic markers of HCC[17,18].

Aberrant expression of miRNA-424 has been documented in HCC tissues and cell lines[19,20]. It plays a tumor suppressor role[19]. A similar correlation was found with miRNA-326[21] and miRNA-511[22], and both were significantly associated with survival[23]. Depending on the Cancer Genome Atlas, Lu and colleagues studied the

prognostic and diagnostic roles of 33 miRNA signatures and found 11 down-regulated and 22 upregulated miRNAs through comparison between cancerous and non-cancerous samples. They clearly declared the good role of miRNA-326, -424, and -511[24].

Most miRNA studies in HCC are experimental and are based on HCC tissues and cell lines. They were mainly performed to discover the etiopathogenetic role of miRNAs in HCC. However, scarce studies declare their fundamental role in the diagnosis and prognosis of HCC. Hence, we aimed to perform this cross-sectional study with prospective follow-up of HCC patients and peculiarly searched for three miRNAs (326, 424, and 511) to highlight their potential role in the diagnosis and prognosis of HCC while testing them in blood.

MATERIALS AND METHODS

Patients and study desing

This prospective observational study was conducted on 70 adult Egyptian patients who developed HCC on top of HCV-related liver cirrhosis and 25 healthy age- and sex-matched participants who were seronegative for HCV and HBV, serving as controls. HCC was diagnosed according to the American Association for the Study of Liver Diseases (AASLD) updated practice guidelines[25], or it was histo-pathologically confirmed. All the patients were recruited from the multidisciplinary HCC clinic, the Endemic Medicine Department, Kasr al Ainy School of Medicine, Cairo University, and they were HCC-treatment naïve at the time of enrollment. Exclusion criteria included; HBV co-infection, any cause of chronic liver disease other than HCV, any associated malignancies other than HCC, and prior treatment for HCV or HCC.

Data collection

All patients were subjected to clinical assessment, laboratory investigations (complete blood picture, prothrombin time and concentration, liver & kidney function tests, AFP, hepatitis markers), ultrasound examination, and triphasic CT with documentation of HCC site, size, and number as well as the presence of portal vein thrombosis (PVT). At

the time of enrolment into the study, blood samples were obtained from each participant, and Child-Pugh-score[26] and BCLC stage[27] were assessed based on the clinical, laboratory, and imaging data obtained.

Collection of blood samples

Whole blood samples were collected from each participant in 5-ml sterile RNAase-free vacutainer tubes containing EDTA. Blood samples were collected on ice and processed within 30 minutes of collection. To separate the plasma, each blood sample was centrifuged for 10 min at 1900 g at 4 0C (Heraeus, Labofuge 400 R; Germany). Plasma samples were transferred carefully into sterile RNase-free tubes. Plasma samples were then centrifuged for 10 minutes at 12000 g at 4 ^{o}C to remove cellular nucleic acid contamination, and haemolysed plasma samples were excluded. Samples were separated into aliquots and kept immediately at 80 ^{o}C until processed.

RNA extraction

Total RNA was extracted from 200 µL plasma using the miRNeasy Serum/plasma cell lysates kit (Qiagen, Germany), according to the manufacturer's instructions. RNA concentration and purity were monitored using a nanodrop spectrophotometer device (ThermoScientific, Wilmington, DE).

Quantitative RT-PCR

The reverse transcription reaction was performed using TaqMan™ MicroRNA Reverse Transcription Kit, Cat number # 4366596, (Applied Biosystems, Foster City, CA, United States) according to the manufacturer's instructions. miRNA-326, miRNA-511, and miRNA-424 quantifications were carried out using quantitative real-time PCR (qRT-PCR) (Stratagene Mx3000p; Agilent Technologies, Germany). The qRT-PCR for each sample was carried out in duplicate using TaqMan 2x universal master mix II (Applied Biosystems, Foster City, CA, United States) and TaqMan microRNA Assay Mix containing PCR primers and TaqMan probes for each miRNA. The RNU6B expression

level was used as an endogenous control for normalization. To determine miRNA relative expression, it was reported as a fold change (ΔCt and ΔΔCt calculations).

Patient management and follow-up

After blood sample withdrawal, all the patients were managed according to the Barcelona Clinic Liver Cancer guidelines[27] after a case-by-case discussion. All HCC patients were followed up for a period of 24 mo. The response of HCC to treatment was evaluated according to the Modified Response Evaluation Criteria in Solid Tumors (mRECIST)[28]. The overall survival time of the patients was defined as the period from the initial presentation to the last follow-up or death.

Statistical analysis

Microsoft Excel 2016 and the Statistical Package for Social Science (SPSS; Windows, version 26, IBM Corp., Armonk, N.Y., United States) were used to analyze the data. Continuous, normally distributed variables were represented as mean ± SD, with a 95% confidence interval (CI), while non-normally distributed variables were summarized as the median and interquartile range (IQR). Categorical data were presented using frequencies and percentages. A *P* value of less than 0.05 was considered statistically significant. The Student *t*-test was performed to compare the means of normally distributed variables between groups, and the Mann-Whitney *U* test was used for non-normal variables. The Chi-square test and Fisher's exact test were used to determine the distribution of categorical variables between groups. The diagnostic performance of the studied miRNAs was assessed by receiver operating characteristic (ROC) curves. The area under the ROC (AUROC) was used as an index to compare the accuracy of tests. The optimal cut-off point value was taken from the maximum combined sensitivity and specificity. The sensitivity and specificity of relevant cut-offs were also displayed. The survival analysis was done by the "Log Rank (Mantel-Cox) Kaplan-Meier test."

RESULTS

Demographic results

The mean age of the studied cohort was 62.0 ± 7.6 years. Most of the patients were males (68.8%), non-diabetic (74.3%), non-smokers (80%), and Child A (82.9%). According to the BCLC staging system, most patients were in the early stage (48.6%). Most of the HCC lesions were single (70%), present in the right hepatic lobe (84.3%), and not associated with portal vein thrombosis (92.9%). That is why most of the patients were subjected to hepatectomy and Microwave ablation (MWA), and most of them showed complete response according to modified Response Evaluation Criteria in Solid Tumors (mRECIST). All HCC patients were followed up for a period of 24 mo or until death. The mean survival time was 367.1±173.9 days. The median values of miRNA-326, miRNA-511, and miRNA-424 were 35, 1.2, and 5.1, respectively (Table 1).

Correlation of miRNAs with different parameters in HCC patients

There was no significant association between the studied miRNAs levels and gender, smoking, or the patients' performance status. The serum level of miRNA-424 was significantly elevated in diabetic patients. There was a significant difference in miRNA-326 between Child grades A and B. Moreover, there was a significant difference in miRNA-424 between early and intermediate HCC (Table 2).

Diagnostic efficiency of miRNAs in our patients

On comparing the miRNAs levels between healthy participants and HCC patients, it was found that HCC patients showed significantly higher levels of miRNA-326 (P = 0.001) and significantly lower miRNA-511 (P = 0.02) (Table 3).

ROC analysis revealed the diagnostic performance of the studied miRNAs. miRNA-326 showed the best diagnostic performance, diagnosing HCC at a cut-off value of 1.165 with a sensitivity of 97.1%, specificity of 52%, PPV of 85%, NPV of 86.7%. The AUC was 78.4% (67.7- 89.1), and the overall accuracy was 85.3% (P < 0.001) (Table 4, Figure 1).

Association of miRNAs with response to treatment and survival

Despite finding no statistically significant differences in the studied miRNAs between the alive and dead HCC patients, we found that miRNA-326 >1.165 was significantly associated with overall survival (P = 0.001). (Table 5, Figure 2). Moreover, there was a significant association between the BCLC stage as well as the response to treatment according to mRECIST and overall survival (Figure 2). It was also found that miRNA-511 was significantly associated with the response to treatment according to mRECIST (Table 6).

DISCUSSION

Several experimental studies have shown that miRNAs play a regulatory role in the progression of HCC by controlling cell cycle progression, cell growth, and apoptosis. Our study focused on three important miRNAs: miRNA-326, miRNA-424, and miRNA-511. miRNA-326 upregulation is known to inhibit cell proliferation and colony formation and influence the invasiveness and migratory properties of HCC. It has a tumor suppressor role, and its low expression was found to be associated with TNM staging, tumor differentiation, and lymph node metastases in HCC patients. Regarding miRNA-424, experimental studies found that it is downregulated in HCC and has a role in inhibiting tumor migration, proliferation, and invasion. It was associated with AFP, TNM, multinodularity, vascular invasion, and intrahepatic metastases, as well as poor survival[29,30] and predicted tumor recurrence in HCC patients following liver transplantation[31]. Few studies have mentioned miRNA-511's role in HCC proliferation and invasiveness.

In our study, miRNA-326 was upregulated, contrasting the aforementioned studies in which miRNA-326 was down-regulated. Importantly, these studies were on HCC tissues and cell lines, while our study adopted an easier methodology of testing miRNA-326 in the blood. It seems that blood tests could give paradoxical results. The Moya *et al* study assessed different miRNAs as biomarkers for prostate cancer and found a similar paradox. This difference is attributed to the possibility of the preferential retaining of

oncomirs (these are microRNAs that are overexpressed in cancers) and the release of tumor suppressor miRNAs into the circulation to promote oncogenesis[32]. Added to this part, they highlighted the physiological cancer conditions that can cause leakage of molecules.

In our study, a statistically significant difference was noticed while revising the MiRNA signature of miRNA-326 and MiRNA-424 between our HCV-related HCC patients and healthy controls. A clear diagnostic role is identified for both markers. We would like to document another crucial issue. We found no single miRNA to correlate with the different HCC parameters. For example, miRNA-424 differentiated early *vs* intermediate HCC significantly, miRNA-326 succeeded as a major factor related to survival, and miRNA-511 significantly correlated with response to treatment. miRNA-326 cut-off level > 1.165 showed statistically significant sensitivity and specificity while upregulated, while miRNA-511 cut-off < 2.063 showed statistically significant sensitivity and specificity while downregulated. This provides the main clue that no single miRNA is to be used for the diagnosis and prognosis of HCC. Wang and Lei identified an eleven LncRNA signature for predicting HCC prognosis and incorporated seven MiRNAs (including miRNA-326 and -424)[33]. In another study, the authors mentioned seven miRNAs that were found to have different expressions between tumors and in-vicinity non-tumorous tissues and found that they were significantly associated with survival (including miRNA-326 and -511)[23]. Similarly, a multidimensional signature stratified the HCC patients into low-risk and high-risk groups ($P < 0.001$) in the training set, validation set, and independent set, and all were statistically significant. It showed better survival prediction power when compared to TNM staging and included three MiRNAs (-149, -424, and -579)[34]. In the Lu *et al* study, five biomarkers (among 33 MiRNAs) significantly correlated with patient survival. These markers were miRNA-326, -421, -511, -3677, and -424. They divided patients into lower-risk and high-risk groups according to the miRNA signatures. None of the high-risk patients survived for 5 years[24] So, it seems that using a combination of miRNAs gives better results than using one biomarker.

The mechanism of action of MiRNA in HCC has been proposed in several experimental studies. Concerning miRNA-326, different mechanisms of action were proposed, including NF-kB expression[35], targeting MAPK1 and CSF1 as regulated by circASAP1[21], and targeting LIM and SH3 protein 1 (LASP1)[36]. Also, it acts by suppression of PDK1 in the PDK1/AKT/C-myc axis[37]. It acts as a sponge for circular RNA circ-0000517[38] and a regulator for the SMAD 6 axis[39]. HCC proliferation was found to be promoted *via* CircRNA-PTN that also sponges miRNA-326 and affects ErbB/PI3K in HCC cells[40]. Finally, HOXD-AS1 binds directly to miRNA-326, thereby targeting gene SLC27A4, which also plays a role in HCC progression and metastasis promotion[41]. Proposed mechanisms of action of miRNA-424 include the direct targeting of C-Myb[19], AKT3 and E2F3[30], E2F7 expression[42], and acts as a sponge to different LncRNAs such as LncRNA CASC9[43], LINL 00657[44], LncRNA CDKN2B-AS1[45], and LncRNA LINC00511[34]. All these targets are correlated with poor prognosis, promotion of cell viability, and migration. Also, it affected angiogenesis by activating the VEGFR-2 signaling pathway[46]. Serum clinical samples from HCC patients and healthy volunteers showed that miRNA-424 significantly decreased in HCC patients and correlated with poor overall survival and disease-free survival[47]. As for MiRNA-511, studies proposed its action through sponging LINC01559[22], regulation of the AKT1 axis[48], and targeting PIK3R3 in the PIK3R3/AKT/mTOR signaling pathway[49].

Finally, we would like to highlight the aggressive behavior of HCC. Our patients were predominantly BCLC-A, Child-Pugh score A, performance status 0-1, and the majority had single lesions without evidence of vascular invasion or lymph node metastases. Moreover, we had good overall response rates (complete and partial response rates were nearly 60%). Nearly half of patients died during the follow-up period despite all these facts. This urges the importance of primary prevention of HCV-related HCC through early management of its risk factors rather than managing HCC after its development.

Nonetheless, our study has limitations. We could not study more miRNAs that are correlated with HCC, and we could not correlate blood tests with tissue tests. However,

our study is a clinical (not an experimental) one, and for sure, this helped to better correlate miRNA with different co-existing potentials and factors that could be present in actual life scenarios. In conclusion, we found a good diagnostic and prognostic role for our studied biomarkers (MiRNA-326, -424, and -511) in HCC patients.

CONCLUSION

We conclude that miRNA-326, miRNA-424, and miRNA-511 have diagnostic and prognostic roles in Egyptian patients with HCV-related HCC and should be considered for better disease management.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) is lethal and ranks as the fifth most common cancer. Differential expression of microRNAs (miRNAs)-326, miRNA-424, and miRNA-511 has been associated with the diagnosis and prognosis of HCC in different populations. However, limited information is available regarding their expression in Egyptian HCC patients.

Research motivation

This study aimed to assess the role of circulating miRNAs-326, miRNA-424, and miRNA-511 from plasma as a non invasive route of detection and to explore the impact of these miRNA in Egyptian HCC patients.

Research objectives

This study objectives are to assess the role of circulating miRNAs-326, miRNA-424, and miRNA-511 in Egyptian HCC patients by a non invasive method using plasma samples.

Research methods

This prospective observational study was conducted on 70 adult Egyptian patients who developed HCC on top of HCV-related liver cirrhosis and 25 healthy age- and sex-matched participants who were seronegative for HCV and HBV, serving as controls. HCC was diagnosed according to the American Association for the Study of Liver Diseases (AASLD) updated practice guidelines[25], or it was histo-pathologically confirmed. All the patients were recruited from the multidisciplinary HCC clinic, the Endemic Medicine Department, Kasr al Ainy School of Medicine, Cairo University, and they were HCC-treatment naïve at the time of enrollment. Exclusion criteria included; HBV co-infection, any cause of chronic liver disease other than HCV, any associated malignancies other than HCC, and prior treatment for HCV or HCC. Data collection: All patients were subjected to clinical assessment, laboratory investigations (complete blood picture, prothrombin time and concentration, liver & kidney function tests, alpha-fetoprotein (AFP), hepatitis markers), ultrasound examination, and triphasic CT with documentation of HCC site, size, and number as well as the presence of PVT. At the time of enrolment into the study, blood samples were obtained from each participant, and Child-Pugh-score[26] and BCLC stage[27] were assessed based on the clinical, laboratory, and imaging data obtained. Whole blood samples were collected from each participant in 5-ml sterile RNAase-free vacutainer tubes containing EDTA. Blood samples were collected on ice and processed within 30 minutes of collection. To separate the plasma, each blood sample was centrifuged for 10 minutes at 1900g at 4 0C (Heraeus, Labofuge 400 R; Germany). Plasma samples were transferred carefully into sterile RNase-free tubes. Plasma samples were then centrifuged for 10 minutes at 12,000g at 4 °C to remove cellular nucleic acid contamination, and haemolysed plasma samples were excluded. Samples were separated into aliquots and kept immediately at 80 °C until processed. RNA extraction: Total RNA was extracted from 200 µl plasma using the miRNeasy Serum/plasma cell lysates kit (Qiagen, Germany), according to the manufacturer's instructions. RNA concentration and purity were monitored using a nanodrop spectrophotometer device (ThermoScientific, Wilmington, DE). Quantitative RT-PCR: The reverse transcription reaction was performed using TaqMan™ MicroRNA Reverse

Transcription Kit, Cat number # 4366596, (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. miRNA-326, miRNA-511, and miRNA-424 quantifications were carried out using quantitative real-time PCR (qRT-PCR) (Stratagene Mx3000p; Agilent Technologies, Germany). The qRT-PCR for each sample was carried out in duplicate using TaqMan 2x universal master mix II (Applied Biosystems, Foster City, CA, USA) and TaqMan microRNA Assay Mix containing PCR primers and TaqMan probes for each miRNA. The RNU6B expression level was used as an endogenous control for normalization. To determine miRNA relative expression, it was reported as a fold change (ΔCt and ΔΔCt calculations). Patient management and follow-up: After blood sample withdrawal, all the patients were managed according to the Barcelona Clinic Liver Cancer guidelines[27] after a case-by-case discussion. All HCC patients were followed up for a period of 24 mo. The response of HCC to treatment was evaluated according to theModified Response Evaluation Criteria in Solid Tumors (mRECIST)[28]. The overall survival time of the patients was defined as the period from the initial presentation to the last follow-up or death.

Research results

The mean age of the studied cohort was 62.0 ± 7.6 years. Most of the patients were males (68.8%), non-diabetic (74.3%), non-smokers (80%), and Child A (82.9%). According to the BCLC staging system, most patients were in the early stage (48.6%). Most of the HCC lesions were single (70%), present in the right hepatic lobe (84.3%), and not associated with portal vein thrombosis (92.9%). That is why most of the patients were subjected to hepatectomy and Microwave ablation (MWA), and most of them showed complete response according to modified Response Evaluation Criteria in Solid Tumors (mRECIST). All HCC patients were followed up for a period of 24 mo or until death. The mean survival time was 367.1 ± 173.9 days. The median values of miRNA-326, miRNA-511, and miRNA-424 were 35, 1.2, and 5.1, respectively (Table 1). Correlation of miRNAs with different parameters in HCC patients: There was no significant association between the studied miRNAs levels and gender, smoking, or the patients' performance status. The

serum level of miRNA-424 was significantly elevated in diabetic patients. There was a significant difference in miRNA-326 between Child grades A and B. Moreover, there was a significant difference in miRNA-424 between early and intermediate HCC (Table 2). Diagnostic efficiency of miRNAs in our patients: On comparing the miRNAs levels between healthy participants and HCC patients, it was found that HCC patients showed significantly higher levels of miRNA-326 (P = 0.001) and significantly lower miRNA-511 (P = 0.02) (Table 3). ROC analysis revealed the diagnostic performance of the studied miRNAs. miRNA-326 showed the best diagnostic performance, diagnosing HCC at a cut-off value of 1.165 with a sensitivity of 97.1%, specificity of 52%, PPV of 85%, NPV of 86.7%. The AUC was 78.4% (67.7- 89.1), and the overall accuracy was 85.3% (P < 0.001) (Table 4, Figure 1). Association of miRNAs with response to treatment and survival: Despite finding no statistically significant differences in the studied miRNAs between the alive and dead HCC patients, we found that miRNA-326 > 1.165 was significantly associated with overall survival (P = 0.001). (Table 5, Figure 2). Moreover, there was a significant association between the BCLC stage as well as the response to treatment according to mRECIST and overall survival (Figure 2). It was also found that miRNA-511 was significantly associated with the response to treatment according to mRECIST (Table 6).

Research conclusions

We conclude that miRNA-326, miRNA-424, and miRNA-511 have diagnostic and prognostic roles in Egyptian patients with HCV-related HCC and should be considered for better disease management.

Research perspectives

miRNA miRNA-326, miRNA-424, and miRNA-511 can be detected from plasma and have diagnostic and prognostic roles in Egyptian patients with HCV-related HCC and should be considered for better disease management.

8 ACKNOWLEDGEMENTS

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