CIRCULATING miR-210 AND miR-1246 AS POTENTIAL BIOMARKERS FOR DIFFERENTIATING HEPATOCELLULAR CARCINOMA FROM METASTATIC TUMORS IN THE LIVER

miR-210 I miR-1246 U CIRKULACIJI KAO POTENCIJALNI BIOMERKERI ZA DIFERENCIRANJE HEPATOCELULARNOG KARCINOMA KOD METASTATSKIH TUMORA JETRE

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Summary

Background: To date few reports have pointed out the role of circulating miRNAs in discriminating metastatic liver tumors from primary hepatocellular (HCC) tumors. Such discrimination will have significant therapeutic and prognostic implications. The purpose of this study was to evaluate the potential value of a panel of HCC-related circulating miRNAs (miR-142, miR-182, miR-200a, mir-210, miR-211, miR-302b, miR-324, miR-338, miR-340 and miR-1246) as noninvasive biomarkers for discriminating primary HCC from metastatic tumors in the liver.

Methods: The expression level of the selected miRNAs was quantified by quantitative real time PCR in 33 patients with HCC, 22 patients with metastatic tumors in the liver, and 30 healthy volunteers as control. Mann-Whitney U test was used to evaluate the difference in miRNAs expression between primary and metastatic liver tumors and to study the associations between their relative expression levels and the clinicopathological factors. Receiver operating characteristic curve was used to evaluate the diagnostic value of the individual miRNAs.

Results: Statistical analyses revealed a differential expression in the level of serum miR-210 and miR-1246 between the two groups of patients. The sensitivity and specificity of miR-210, for differentiating HCC from metastatic malignancies in the liver were found to be 73.7% and 64.28%, respectively. Whilst, of miR-1246 were 72.2% and 67.8%, respectively. In addition, the differential expression of the two miRNAs was also found to be associated with clinicopathological parameters in the two studied groups.

Kratak sadržaj

Uvod: Danas ima nekoliko podataka o ulozi cirkulišućeg miRNK za razlikovanje metastskih tumora jetre od primarnih hepatocelularnih tumora (HCC). Takva prepoznavanje imalo bi značajan terapeutski i prognostički značaj. Svrha ovog izučavanja je bila da se proceni potencijalna vrednost panela miRNK, (miR-142, miR-182, miR200a, mir-210, miR-211, miR-302b, mR-324, mR-338, mR-340 i miR-1246) koji se nalaze u cirkulaciji, a u vezi su sa HCC kao neinvanzivnih biomarkera za razlikovanje primarnog HCC od metastatskih karcinoma jetre.

Metode: Ekspresija nivoa selektovanih miRNK je određena primenom kvantitativne real time PCR u 33 pacijenta sa HCC, 22 pacijenta sa metastatskim tumorima jetre i 30 zdravih osoba kao kontrolne volonterske grupe. Mann-Whitney U test je korišćen za procenu razlika u miRNK ekspresiji između primarnih i metastskih tumora jetre kao i za procenu između nivoa njihove relativne ekspresije i kliničkopatioloških faktora. ROC kriva je korišćena za procenu dijagnostičke vrednosti pojedinih miRNK.

Rezultati: Statistička analiza je ukazala na diferencijalnu ekspresiju nivoa serumske miR-210 i miR-1246 između dve grupe pacijenata. Osetljivost i specifičnost miR-210 za razlikovanje HCC kod metastatskog maligniteta jetre nađeno je da iznosi 72,7% odnosno 64,28%, dok je u slučaju miR-1246 iznosila 72,2% odnosno 67,8%. Osim toga nađena je diferencijalna ekspresija dve miRNK u vezi sa kliničko-patološkim parametrima u obe proučavane grupe.

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Conclusions: Serum miR-210 and miR-1246 have some diagnostic value for discriminating patients with metastatic tumors to patients with primary HCC

Keywords: circulating miRNAs; biomarkers; HCC; liver metastasis

Zaključak: Serumska miR-210 i miR-1246 imale su izvesnu dijagnostički značajnu vrednost za metastatske tumore pacijenata sa primarnim HCC.

Ključne reči: cirkulišuća miRNK, HCC, metastaza jetre

Introduction

Hepatocellular carcinoma (HCC) is one of the most aggressive human malignancies worldwide, accounting for 90% of primary liver cancers (1). Despite the advances in HCC treatment, the high frequencies of postsurgical recurrence and metastasis are the main causes of the dismal therapeutic outcome of HCC (2). One of the main obstacles in HCC therapy is to differentiate hepatocellular carcinoma from metastatic tumors in the liver. This distinction may not be easy because of the high degree of similarity between hepatocellular carcinoma and metastatic liver cancers, especially adenocarcinoma, on the level of morphology and the immunoexpressed markers (3). At the present time, identification of the origin of these metastases and their differential diagnosis from primary HCC requires precise pathological investigation using a panel of immunostains (4). In addition, the use of advanced imaging techniques and their correlation with the clinical state of the patient is also needed. In most of the cases, these previous methodologies have failed in the detection of the primary site of cancer (5) and in turn, misled the choice of the best treatment regime (6) and hence, poor prognosis in the majority of patients (7). Therefore, searching for novel diagnostic markers for hepatic cancers will improve both the diagnosis and treatment.

MicroRNAs (miRNAs) are special class of small non-coding RNAs that play critical roles in the regulation of gene expression. They are characterized by their remarkable tissue specificity and are nearly involved in the regulation of all aspects of cellular activity including metabolism, cell proliferation, apoptosis, differentiation, cellular response to viral infection, and tumorigenesis (8). Several studies have pointed out the value of miRNAs as potential markers for tissue classification and identification (9–11). In addition, circulating miRNAs which are protected from RNAase-mediated degradation in body fluids have been emerged as candidates of non-invasive biomarkers in the diagnosis of many diseases, including liver diseases (12–14).

Recently, Tan Y et al. (15) have identified a panel of serum miRNAs as biomarkers to be used in the diagnosis of hepatitis B virus-related HCC, as well as, in differentiating HCC patients from healthy and cirrhotic patients. Moreover, have identified miRNAs signature that significantly predicted metastasis-free HCC and HCC from venous metastasis, as well as, tumor recurrence. A related study has used miRNA expression profile in the segregation of non-tumorous livers from primary HCC and venous metastasis (16). Using miRNA expression profiling, it was also possible to differentiate primary from metastasized brain tumors (17).

The goal of the present study was to explore the potential use of serum miRNAs as biomarkers for discriminating Egyptian patients with primary HCC from patients with metastatic tumors in the liver. To fulfill this, a panel of cancer-associated miRNAs namely miRNA-142, 182, 200a, 210, 211, 302b, 324, 338, 340, and 1246, was chosen on the basis of their reported relevance to HCC (18-24) and were detected by qRT-PCR among 33 primary HCC patients, 22 patients with metastatic liver tumors of different origins, and 30 healthy volunteers used as controls.

Materials and Methods

Patients and samples

This study was performed according to the guidelines for the use of human subjects' materials according to the 'Declaration of Helsinki' and approved by the Institutional Review Board. A written informed consent was obtained from all the participants involved. Serum samples were obtained from 33 patients with primary hepatocellular carcinoma, 22 patients with metastatic tumors in the liver, in addition to, 30 healthy volunteers, during the period 2013–2014. The HCC patients were diagnosed by abdominal ultrasonography, triphasic CT abdomen, serum alfa-fetoprotein (AFP), and were confirmed histopathologically with no evidence of local invasion or distant metastasis. The control group had no clinical or biochemical evidence of liver disease or known medical illness at recruitment and with normal abdominal ultra-sonography. All controls were negative for HBV and HCV infection as evidenced by negative serological markers and negative PCR for HBV and HCV. The relevant clinico-pathological characteristics of the studied subjects are shown in Table I. Five ml blood samples were collected from patients and controls for serum separation, and all the samples were processed on the same day within few hours of collection.

Quantitative RT-PCR for miRNA expression

Total RNA including miRNA was extracted from serum samples using miRNeasy Mini kit (Qiagen,

Table I Demographic and laboratory data of the participants.

Clinical variable	Primary HCC (n=33)	Metastatic HCC (n=22)	Healthy controls (n=30)
Age (Years) no. (%)			
≤60y	19 (57.6)	10 (45.5)	20 (66.7)
>60y	14 (42.4)	12 (54.5)	10 (33.3)
Gender, no. (%)			
Male	24 (72.7)	12 (54.5)	18 (60)
Female	9 (27.2)	10 (45.4)	12 (40)
HCV, no. (%)			
Yes	24 (72.7)	7 (31.8)	0 (0)
No	9 (27.2)	15 (68.1)	30 (100)
Cirrhosis, no. (%)			
Yes	15 (45.4)	10 (45.4)	0 (0)
No	18 (54.5)	12 (54.5)	30 (100)
Laboratory values			
Median (Interquartile)			
AFP (ng/mL)	180 (1–1413)	23 (1–306)	4.9 (3–8)
ALT (U/L)	60 (10–609)	21.60 (9–67)	23 (17–32)
AST (U/L)	51 (15–185)	31 (8–105)	26 (0–33)
Origin(n)			
Liver	33	---	---
Adenocarcinoma	---	13	---
Bladder	---	1	---
Colon	---	1	---
Pancreas	---	1	---
Ovary	---	1	---
Unknown	---	5	---

Germany). Frozen serum samples were incubated at 37 °C in a water bath until they were completely thawed, then 200 µL of serum were added to 1000 µl of Qiazol lysis reagent. The extraction was performed according to the instruction manual of the manufacture. cDNA was synthesized from the isolated RNA using miScript II RT kit (Qiagen) according to the manufacturer instructions. The relative expression of miRNAs was quantified by miScript SYBER Green PCR kit (Qiagen), which includes miScript universal primer and quantiTect SYBER Green PCR master mix. The reactions were performed in 384 well plates with 10 µL total volume/well and contained 1X SYBER green master mix, 200 nmol/L forward primer (miRNA specific primer) and 200 nmol/L universal primer, using 3 ng cDNA/well. The conditions included initial denaturation at 95 °C for 15 min., followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. All the samples were performed in duplicates on ViiA 7 real time PCR system (Applied Biosystem, Foster city, CA, USA). miR-16 was used as an endogenous control (25, 26). The relative gene expression analysis of the target miRNAs was performed using the delta-delta-Ct method as described previously (27).

Statistical analysis

Statistical analysis was performed using SPSS 20 software (SPSS, Inc., Chicago, IL, USA). Results of miRNAs expression were analyzed and were found to demonstrate an abnormal distribution. All miRNA expression data are shown as the median and interquartile range (IQR). The nonparametric Mann-Whitney U test was used to study the different associations between the relative expression levels of circulating miRNA and the clinic-pathological factors as well as to evaluate the differences in the miRNA expression between primary HCC and metastatic liver tumor samples. Diagnostic values for individual miRNAs were determined by calculating the area under the receiver operating characteristic (ROC) curves. $P < 0.05$ was considered statistically significant.

Results

Patients' characteristics

A total of 85 participants including 33 primary HCC patients, 22 patients with metastatic liver tumors, and 30 normal volunteer were recruited into this study. The characteristics of the study subjects, as well as, the

Table II Expression levels of the studied miRNAs.

miRNA	Primary HCC (n=33)	Metastatic H CC (n=22)	p value
miR-142	0.215 (0.074–2.526)	0.360 (0.068–0.939)	0.902
miR-182	0.212 (0.090–0.701)	0.138 (0.054–0.546)	0.605
miR-200a	0.321 (0.049–1.175)	0.244 (0.057–1.954)	0.983
miR-210	0.233 (0.029–5.768)	2.008 (0.799–8.417)	0.03*
miR-211	0.341 (0.096–5.168)	1.355 (0.192–4.467)	0.405
miR-302b	0.407 (0.030–4.086)	0.437 (0.047–1.041)	0.776
miR-324	0.606 (0.066–9.755)	1.437 (0.153–11.007)	0.480
miR-338	0.255 (0.031–1.302)	0.208 (0.053–2.268)	0.562
miR-340	0.425 (0.335–1.253)	0.282 (0.038–0.796)	0.396
miR-1246	2.409 (0.063–168.764)	42.839 (4.674–125.990)	0.048*

Results are expressed as Median fold change (Interquartile range: 25–75%)
*significant (Significance level at P <0.05)

subjects' data including Laboratory values are summarized in *Table I*. The age distribution between patients with either primary or metastatic liver tumors was almost similar; however, the percentage of male patients was higher (72.7%) in primary HCC group than in the metastatic liver tumors group (54.5%).

Serum miR-210 and miR-1246 differential expression in the two studied groups:

Using qRT-PCR, the expression levels of 10 miRNAs in the sera of patients with primary and metastatic liver tumors were measured (*Table II*). Our data showed that of the 10 studied miRNAs, only miR-210 and miR-1246 were significantly deregulated between the two patients' groups. The expression level of miR-1246, as well as, miR-210 were significantly higher in metastatic liver tumors compared to patients with primary HCC patients (P <0.05).

miR-210 and miR-1246 expression levels and clinicopathological characteristics

To further investigate the effect of the clinicopathological factors on the differential expression of miR-210 and miR-1246 between the primary and metastatic HCC patients, patients' groups were subdivided into subgroups according to the clinicopathological factors, as shown in *Table III*. The expression levels of miR-210 and miR-1246 were then compared between the two groups using the non-parametric Mann-Whitney U test. Results revealed the presence of significant association between miR-210 differential expression and age (> 60 years), as well as, the presence of HCV infection. In addition, the expression of miR-210 was also significantly associated with the level of serum ALT (≤ 40 U/L). On the other hand, miR-1246 differential expression was observed among females, patients ≤60 years old, as well as patients with cirrhosis and low level of serum AFP (≤200 ng/mL).
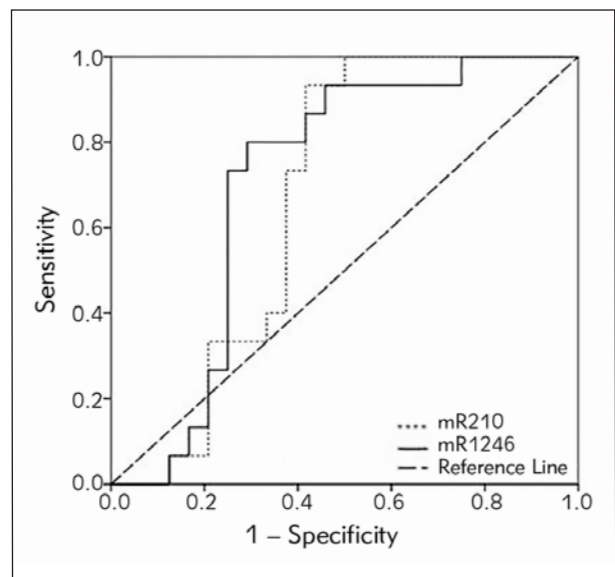

Figure 1 The ROC curve of miR-210 and miR-1246 for discriminating hepatocellular carcinoma from metastatic liver tumors. The AUC of miR-210 was 0.67 (p=0.073) at a cut off value of 0.916, the sensitivity was 73.68% and the specificity was 64.28%. The AUC of miR-1246 was 0.708 (p=0.03) at a cut off value of 9.951, the sensitivity was 72.2% and the specificity was 67.8%.

Differential Diagnostic potential of circulating miR-210 and miR-1246

ROC curves were used to study the potential of serum miR-210 and miR-1246 to discriminate patients with primary HCC from patients with metastatic liver tumors. The ROC curve for serum miR-210 expression levels had an AUC of 0.67, with 73.7% sensitivity and 64.28% specificity, at a cutoff value of 0.92 (fold change). The ROC curve for serum miR-1246 expression levels had an AUC of 0.708, with 72.2% sensitivity and 67.8% specificity, at a cutoff value of 9.95 (fold change) (*Figure 1*). SPSS

Table III Association of miR-210 and miR-1246 expression levels with clinicopathological factors.

Parameter	Primary HCC	Metastatic HCC	Mann-Whitney P-value
Age (year)			
miR-210			
≤60	0.880(0.134–10.045)	1.388(0.681–5.997)	0.531
≤60	0.056(0.027–7.131)	3.691(1.007–10.391)	0.018*
miR-1246			
≤60	1.261(0.129–10.429)	93.441(29.010–724.479)	0.013*
>60	5.704(0.015–213.449)	19.041(3.230–88.945)	0.951
Gender			
miR-210			
Male	0.324(0.033–7.131)	1.480(0.681–7.726)	0.159
Female	0.056(0.038–28.616)	3.691(1.519–22.112)	0.165
miR-1246			
Male	5.544(0.067–213.449)	81.281(18.642–165.666)	0.147
Female	0.191(0.029–0.861)	12.811(3.230–63.807)	0.007*
HCV			
miR-210			
Yes	0.324(0.028–5.840)	8.140(2.834–22.112)	0.024*
No	0.143(0.081–32.794)	1.007(0.681–5.977)	0.346
miR-1246			
Yes	1.654(0.030–213.449)	12.811(3.230–76.405)	0.341
No	2.980(0.109–10.509)	68.683(18.642–165.666)	0.075
Cirrhosis			
miR-210			
Yes	0.125(0.051–41.369)	0.930(0.733–6.371)	0.206
No	0.336(0.030–5.697)	2.849(1.208–7.689)	0.139
miR-1246			
Yes	0.201(0.030–6.371)	192.552(82.646–6.371)	0.032*
No	2.095(0.238–1232.561)	15.926(2.700–54.366)	0.488
AFP (ng/mL)			
miR-210			
≤200	0.134(0.045–7.714)	2.008(0.799–8.417)	0.069
>200	0.330(0.032–8.911)	0.952(0.182)	#
miR-1246			
≤200	1.057(0.029–8.106)	61.018(12.811–126.368)	0.029*
>200	4.262(0.147–1071.488)	17.446(2.170)	#
ALT (U/L)			
miR-210			
≤40	0.083(0.034–3.823)	2.008(1.062–7.456)	0.014*
>40	0.608(0.057–14.237)	8.417(0.711)	#
miR-1246			
≤40	0.264(0.028–1.556)	76.347(34.409)	#
>40	6.397(0.193–561.932)	51.268(4.290)	#
AST (U/L)			
miR-210			
≤40	0.051(0.028–5.448)	3.730(0.937–6.963)	0.160
>40	0.336(0.125–14.196)	5.074(0.974–15.264)	0.105
miR-1246			
≤40	1.261(0.021–63.177)	47.714(12.811–109.652)	0.088
>40	5.544(0.067–678.09)	88.945(39.523–165.666)	0.266

Results are expressed as Median fold change (Interquartile range: 25–75%)
*significant (Significant level at P <0.05)
In-valid P – value because of the small sample size in the groups

analysis for the combined classifier (miR-210 and miR-1246) resulted in a better sensitivity (86.36%) but lower specificity (43.75%) than either classifier alone.

Discussion

Previous studies identified miRNAs in several body fluids, including plasma and serum (28), and several members of miRNAs have been proposed as potential biomarkers for several pathological conditions, including human cancer (29–31). One of the advantages that make miRNAs ideal biomarkers is their accessibility and high stability in the circulatory system (32). Several reports have pointed out the diagnostic and prognostic potential of circulating serum miRNAs in different types of cancers (32–37). However, to date, there have been few reports on the role of circulating miRNAs in discriminating metastatic liver tumors from primary HCC tumors. Using the AFP level is known to be of poor sensitivity and specificity to discriminate primary HCC from metastatic liver, where it can be negative in 30–40% of the primary stages of HCC, in addition, its elevation has also been detected in the presence of acute and chronic viral hepatitis, patients with cirrhosis that is caused by hepatitis as well as with conditions other than the liver, including AFP secreting gastric tumors, colorectal carcinoma, and pancreas (38–40). Moreover, AFP levels may be elevated initially in the early stages of HCC, then drops before rising again as the disease progresses (41). Therefore, this study aimed at investigating the possibility of using some HCC-related miRNAs as noninvasive biomarkers for discriminating primary HCC from metastatic malignancies in the liver, which will be of therapeutic and prognostic significance.

The current study showed that the levels of circulating miR-210 and miR-1246 were statistically different between patients with primary HCC and those with metastatic tumors in the liver. The median fold change of the relative expression level of miR-210 was significantly higher in patients with metastatic liver tumors (60% of cases), compared to patients with primary HCC (30% of cases). In fact, several studies reported that miR-210, so-called master hypoxamir, is upregulated by hypoxia in most human solid tumors including hepatocellular carcinoma (42–45). Furthermore, Ying et al. (42) showed that the hypoxia-induced miR-210 can promote migration and invasion of HCC cells. miR-210 can be regarded as a (versatile) molecule that regulates cell cycle and affects many aspects of tumor cell in response to hypoxia. It was demonstrated that miR-210 can help tumor cells to adapt the hypoxic stress, through decreasing the mitochondrial function, up-regulating glycolysis, and stimulating angiogenesis (46). The increased level of circulating miR-210 in patients with metastatic tumors in our study, could be due to its increased biogenesis in metastasized tumor tissues and hence, its secretion

into circulation. ROC curve analysis showed that miR-210 could be used as a marker for patients with metastatic liver tumors from primary HCC patients, with an AUC of 0.67. At the cut-off value of 0.916, the optimal sensitivity and specificity were 73.68% and 64.28%, respectively. Recently, Wang et al. (47) have demonstrated that circulating miR-210 can be used as a diagnostic and prognostic biomarker for colorectal cancer. Several previous studies also described an elevated circulating miR-210 in some other cancers, such as pancreatic cancer (37), kidney cancer (48), breast cancer (49), and glioma (50).

In the present study, a higher miR-1246 expression was detected in patients with metastatic liver tumors (77%) compared to 45% in primary HCC. The increased level of circulating miR-1246 in the sera of patients with metastatic tumors is consistent with the findings of Sun et al. (24), who demonstrated that miR-1246 was highly expressed in metastatic HCC cells, and its inhibition effectively reduced migration and invasion of HCC cells, through the down-regulation of its target CADM1. In fact, accumulating evidence from the literature supported the tight link of miR-1246 with several types of tumors. For example, Takeshita et al. (51) have reported that miR-1246 has strong potential as a diagnostic and prognostic biomarker in esophageal squamous cell carcinoma. Further, miR-1246 was recently demonstrated to play an important role in the migration and invasion of non-small lung cancer cells (52) as well as in the metastasis of colorectal cancer (53). The detection of miR-1246 in cancer-derived exosomes has strongly reinforced the potential role of miR-1246 in tumor progression and metastases (54, 55). An interesting study has recently reported that colon cancer cells carrying specific mutant p53 proteins promote the formation of a distinctive population of reprogrammed tumor-associated macrophages by releasing exosomes containing miR-1246 (56). In the present study, ROC curve analysis of miR-1246 for discriminating patients with metastatic liver tumors from primary HCC patients, showed similar results to those obtained for miR-210. Where the AUC was 0.708, with an optimal sensitivity and specificity of 72.2% and 67.8%, respectively, at a cut-off value of 9.95. These findings suggest a relative potential of circulating miR-210 and miR-1246 for discrimination of liver cancer from metastatic origins from those of primary type.

Conclusions

Our findings suggest that serum miR-210 and miR-1246 have some diagnostic value for discriminating Egyptian patients with metastatic tumors to the livers from patients with primary HCC tumors. The use of these two miRNAs for discrimination would be more effective if combined with a complementary microRNA biomarker for primary HCC tumors, since their expression is more related to metastasis process-

es. Future studies including large patient populations and patients with metastatic liver tumors of different origins are needed to confirm the potential discriminating value of miR-210 and miR-1246.

Authors' contributions

The authors declare that each one has contributed equally to every phase of the research and approved the final manuscript.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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