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Unraveling the Role of MicroRNA-21 and Mitogen-Activated Protein Kinase Kinase-3 in the Diagnosis of Hepatocellular Carcinoma

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Abstract: As promising noninvasive biomarkers of diverse disease stages, circulating miRNAs have emerged as promising biomarkers. Micro RNA 21 expression is also correlated with the proliferation, invasion, and metastatic properties of malignant cells. Therefore, mitogen-activated protein kinases (MAPKs), including a group of serine and threonine kinases, have been identified. They are vital signaling components that alter the external stimuli of several responses at the level of the cell , like proliferation, survival, differentiation, and migration. Hence, because of their essential roles in these cellular functions, decotrol MAPKs contribute to the development of many cancers as hepatocellular carcinomas (HCCs). A study was performed here to investigate miR-21 expression profiles and mitogen-activated protein kinase-kinase 3 (MAPKK3) in Hepatitis C virus (HCV)-related HCC patients. The expression of miR-21 was assessed in the plasma of 10 healthy subjects, 20 patients with cirrhosis, and 20 patients with HCC using RT-PCR. Then, MAPKK3 was detected using enzyme-linked immunosorbent assay (ELISA) for all subjects. Compared to cirrhosis, HCC showed significantly higher levels of micro RNA 21 and MAPKK3. Additionally, a significant difference was observed in micro RNA 21 and MAPKK3 expression between the HCC and cirrhotic groups. Correlation analysis also revealed a significantly positive correlation between micro RNA 21 and MAPKK3 concentrations. Likewise, AFP correlation along with the micro RNA 21 or MAPKK panel was significantly correlated. In particular, results showed that miR-21 and MAPKK levels were higher in patients with similar number and tumor size of focal lesions. Thus, these results revealed that micro RNA 21 and MAPKK3 were biomarkers that can be accustemed to improve the diagnosis of HCC patients and that micro RNA-21 can be used as a separate biomarker for enhancing diagnostic precision.

keywords: MicroRNA-21, Hepatocellular Carcinoma, Mitogen-Activated Protein Kinase 3.

Introduction

Hepatocellular carcinoma (HCC) considered to be the sixth most common malignancy and the third cause of mortality among all cancer-related deaths worldwide (11). HCC could be diagnosed by ultrasonography, computerized tomography, Magnetic Resonance Imaging and serum alpha-fetoprotein (AFP) which is the most often utilized tumor marker. However, AFP levels have been found to be higher in patients with chronic hepatitis B and C who do not have HCC (2). Nevertheless, non-liver malignancies, such as stomach cancer and cholangiocarcinoma, can also have increased AFP levels. Therefore, new biomarkers with higher accuracy and the ability to complement hepatic imaging should improve the diagnostics of HCC.

Micro RNA 21 was one of the first carcinogenic micro RNAs discovered, with overexpression found in a variety of human malignancies among HCCs (26). Malignant cell proliferation, invasion, and metastasis have all

been related to increased expression of micro RNA 21. Furthermore, it influences cellular transformation and the inhibitory impact of micro RNA 21 on apoptotic signaling is strongly supported by the hypothesis that it acts like an onco-micro RNA (19). Patients with chronic hepatitis or colorectal cancer were found to have elevated serum micro RNA 21 levels in a Chinese study. This finding made the authors hypothesize that micro RNA 21 can be used as a new biomarker for detecting liver damage and not especially HCC (29). On the other side, a study comparing HCC patients and chronic hepatitis patients found that micro RNA 21 expression was greater in sera of patients having HCC compared to chronic hepatitis patients (7).

Alternatively, changes in the expression and activity of the MAPK component cascades could be demonstrated in human tumors (18). Studies concerning human HCC have also reported that MAPK expression and activities were increased in cancerous lesions over noncancerous adjacent lesions (24). However, little has been revealed on the involvement of MAPK/ERK in human HCC. Furthermore, phosphorylating MAPKK3 activates the P38 cascade, which is primarily regulated by miR-21. Hence, an established link has been observed between MAPKK3 and miR-21 via the P38 cascade. This micro RNA 21 has also been shown to target the sprouty protein (17) and is considered a crucial suppressor of the ERK/MAPK signaling cascade (9). Additionally, miR-21 participates in the hepatic fibrosis process by coincidentally regulating ERK1 signaling in stellate cells and EMT in the liver tissue (31). Moreover, the overexpression of micro RNA 21 and the downregulation of the sprouty protein have been associated with hepatic fibrosis (22).

Therefore, we examined the micro RNA 21 and expression of MAPKK3 levels in Egyptian healthy controls, including patients diagnosed with cirrhosis and Hepatitis C virus (HCV)-related HCC. We also analyzed their AFP levels to figure out their potential role among the tested population.

Materials and methods

Ethics statement

The institutional review board committee of the Egyptian Liver Research Institute and Hospital (ELRIAH), including Mansoura University (serial numbers: 10–2019 & 2019–2020-179, respectively), approved this study. Written informed consent was taken from all patients and healthy participants.

Materials

Our research was conducted from 2019 to 2020 on the following Egyptian subjects: 20 patients with HCV-induced cirrhosis, 20 patients with HCV-induced HCC, and 10 healthy controls. The 10 healthy individuals had no history of viral or nonviral hepatic disorders. They also had negative serological outcomes for all pathological liver diseases, including normal liver function tests, thereby contributing to this study as negative HCV controls. However, all unhealthy patients had HCV-positive antibodies and were diagnosed with HCV genotype 4 viral titers as detected by HCV Ab plus Rapid Test (Spectrum) and RT-PCR (GeneXpert, California, USA), respectively. The study excluded patients with HIV infection, hepatitis B virus antigen, or antibody, or liver diseases not related to HCV infection. HCC patients who had undergone surgery or begun antiviral therapy were also excluded. Samples were collected from ELRIAH. Five milliliters of blood was obtained from all participants. Then, their plasma samples were separated for further biochemical assessments. Moreover, all HCV-mediated categories were stratified using a triphasic CT assessment and an AFP assessment.

CDNA synthesis and the extraction of RNA

Total RNA was extracted from plasma samples by using a miRNeasy extraction kit (Qiagen, Germany) according to the manufacturer's protocol. Subsequently, the total RNA obtained was further used in reverse transcription, followed by quantitative real-time PCR (RT-qPCR). In brief, cDNA products from the RT reaction were added to the SYBER Green PCR master mix (Qiagen, Germany). The reactions were then amplified at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 55°C for 30 s, and 70°C for 30 s (25).

RT-PCR

Samples of cDNA expressing different genes were enumerated on a PCR system (GeneXpert, California, USA). The designed primer was then validated to ensure perfect identity with minimal cross-reactivity and to allow exact as well as reproducible quantitation of the understudied micro RNAs. Next, 20 µl of PCR reaction comprising 0.4 µM primer, 10 µl of qPCR Master Mix, and 2 µl of 5× diluted cDNA was prepared. Delta Ct (ΔCt) values resembling relative expression changes for micro RNA and equating to that of the spike in miR-21 controls of the same sample were then calculated by deducting the ΔCt value for each sample from the total ΔCt value. Then, fold change was considered $2^{\wedge-\Delta\Delta Ct}$ (15).

MAPK/ERK assay

MAPK/ERK activities were analyzed by enzyme-linked immunosorbent assay (ELISA). 100 µL of the sample was added to each well and incubated for 90 min at 37°C. After liquid removal was, 100 µL of biotinylated detection Anti-MAPK (anti – p38 antibody) was added and incubated for 1 h at 37°C. Subsequently, 100 µL of HRP (Horseradish peroxidase) conjugate was added, incubated for 30 min at 37°C, aspirated, and washed five times. Next, 90 µL of substrate reagent was added and incubated for 15 min at 37°C. At that point, 50 µL of stop solution was added and read at 450 nm immediately (4).

Statistical analysis

Data were analyzed using SPSS statistical package version 21.0 (IBM Corporation Software Group, USA). Additionally, qualitative data were presented as frequency and percentage; normally distributed data, as mean ± SD; and nonnormal distributed data, as median and IQR. Furthermore, comparison between the three groups was conducted using the chi-square test, ANOVA, or Kruskal–Wallis test, whereas comparison for any two groups was conducted using the Mann–Whitney U test. All *p*-values were two-tailed, and $p \leq 0.05$ was considered statistically significant. Subsequently, the ROC (Receiver operating characteristic) curve was used to evaluate the diagnostic performance of miR-21, MAPKK3, and AFP in the different groups. Then, the correlation between the understudied biomarkers was examined using Pearson correlation.

Results

Study subjects' demographic data and clinical characteristics

A summary of demographic and clinical data is provided in **Tables 1, 2**. Age and gender distributions were found to differ significantly between the four groups (P = 0.006 and P = 0.021). Significant differences in gender were also noticed between the cirrhotic group and the HCC group. Compared to the control group, we found no significant differences in ALT levels for cirrhotic patients or patients who had HCC (P = 0.39 and P = 0.07, respectively). Furthermore, elevated levels of AST were significant in all groups compared to the healthy control (P < 0.001). When compared to healthy control patients, total bilirubin levels were significantly considerably higher in the diseased groups (P < 0.001 and P < 0.001, respectively).

Alternatively, in comparison to the control group, albumin levels were significantly lower in the diseased groups. (P < 0.001). Results also showed no significant differences in creatinine levels between the groups (P = 0.67). Furthermore, MAPKK3 and AFP were significantly deregulated around the diseased groups (P < 0.001 and P = 0.004, respectively). However, elevated levels of AFP were non-significant in CHC patients compared to the HCC group (P = 0.079). From the results, Hgb, total leukocyte count, and platelet counts were non-significantly deregulated compared to the control group (P = 0.089 and P = 0.53, respectively). By contrast, platelet counts and FibroScan (kPa) were significantly deregulated among the groups (*P* < 0.001 and *P* < 0.001, respectively). Platelet counts and FibroScan also showed non-significant results in patients diagnosed with HCC in comparison to the cirrhotic group (P = 0.973 and P = 0.659).

Data were expressed as either mean ± SD, median. ALT: alanine transaminase, AST: aspartate transaminase, MAPKK3: mitogen-activated protein kinase-kinase 3, AFP: alpha-fetoprotein, Hgb: hemoglobin, WBCS: white blood cells, Plt: platelets, kPa: kilopascal.

Plasma miRs expression in healthy controls, cirrhotic participants, and HCC patients.

MiR-21 and MAPKK3 were scrutinized in cirrhotic and HCC patients (Table 3). Fold differences in miR-21 expression levels were statistically and significantly upregulated (P < 0.001) between the HCC and cirrhotic groups. Furthermore, the median levels of MAPKK3 between the understudied groups revealed a statistically significant upregulation in HCC patients (49.5) compared to the liver cirrhosis group (13.95) (with P-value < 0.001). However, no significant change in AFP was found between the HCC and cirrhotic groups (P = 0.079).

Table (1). Comparison of demographic data between patient and control groups

Group Variable	I. Control N = 10	II. Cirrhosis N = 20	III. HCC N = 20	*P*-value			
				All	I vs. II	I vs. III	II vs. III
Age, yrs., mean ± SD	48.1 ± 7.2	57.4 ± 9.0	58.6 ± 704	0.006	0.008	0.002	0.665
Gender, male, n (%)	6 (60.0%)	10 (50.0%)	18 (90.0%)	0.021	0.605	0.053	0.006
Smoking, n (%)	3 (30.0%)	2 (10.0%)	8 (40.0%)	0.092	0.166	0.592	0.028
DM, n (%)	3 (30.0%)	11 (55.0%)	4 (20.0%)	0.064	0.196	0.542	0.022
Hypertension, n (%)	2 (20.0%)	7 (35.0%)	7 (35.0%)	0.661	0.398	0.398	1.000

Table (2). Comparison of laboratory data between the cirrhotic, control, and HCC groups

Group Variable	I. Control N = 10	II. Cirrhosis N = 20	III. HCC N = 20	P-value			
				All	I vs. II	I vs. III	II vs. III
ALT, U/L, median IQR)	16.5 (40.0)	27.50 (29.0)	41.50 (54.0)	0.115	0.397	0.074	0.127
AST, U/L, median (IQR)	15.5 (12.0)	27.50 (20.0)	48.00 (69.0)	<0.001	0.015	<0.001	<0.001
Albumin, g/dL, median (IQR)	4.6 (0.45)	3.95 (0.87)	2.90 (1.35)	<0.001	<0.001	<0.001	0.002
T. Bilirubin, mg/dL, median (IQR)	0.5 (0.20)	0.80 (0.47)	1.60 (1.43)	<0.001	0.002	<0.001	0.001
Creatinine, mg/dL, median (IQR)	0.9 (0.09)	0.80 (0.30)	0.86 (0.22)	0.670	0.448	0.746	0.512
MAPKK3, median (IQR)	1.4 (3.22)	13.95 (31.03)	49.5 (62.83)	<0.001	<0.001	<0.001	<0.001
AFP, ng/mL, median (IQR)	2.6 (1.75)	5.95 (5.46)	13.79 (71.90)	0.004	0.006	0.005*	0.079
Hgb, g/dL, mean ± SD	12.72 ± 0.78	13.50 ± 1.95	12.29 ± 1.75	0.089	0.239	0.469	0.051
WBCs, ×103/µL, mean ± SD	4.8 ± 0.92	5.80 ± 2.15	5.11 ± 3.29	0.530	0.170	0.771	0.437
Plt, ×103/µL, mean ± SD	198.50 ± 24.34	99.44 ± 28.60	99.15 ± 24.44	<0.001	<0.001	<0.001	0.973
FibroScan, kPa, median (IQR)	6.55 (3.0)	27.05 (15.6)	29.05 (35.0)	<0.001	<0.001	<0.001	0.659

Table (3). Comparison of patient's groups regarding the miR-21, MAPKK3 and AFP to differentiate between Cirrhotic subjects and HCC group

Group Variable	Cirrhosis N = 20	HCC N = 20	*P*-value
Fold difference relative to control	1.77 (3.14)	12.94 (22.78)	<0.001
miR-21, median (IQR)			
MAPKK3, median (IQR)	13.95 (31.03)	49.5 (62.83)	<0.001
AFP, median (IQR)	5.95 (5.46)	13.79 (71.90)	0.079

Data were expressed in median (IQR). miR-21: miRNA-21, MAPKK3: mitogen-activated protein kinase-kinase 3, AFP: alpha-fetoprotein.

Correlation between miR-21, MAPKK3, and AFP of HCC groups

Regression analysis was conducted to prove the correlation between the expression levels of the understudied markers. It was found that miR-21 expression levels were significantly and positively correlated with AFP levels (r = 0.882, P < 0.001) **(Fig. 1)**. It was also shown that MAPKK3 levels were significantly and positively correlated with AFP (r = 0.621, P < 0.001) (Fig. 2). Therefore, a positive correlation existed between miR-21 and MAPKK3 (r = 0346, P = 0.014) (Fig. 3).

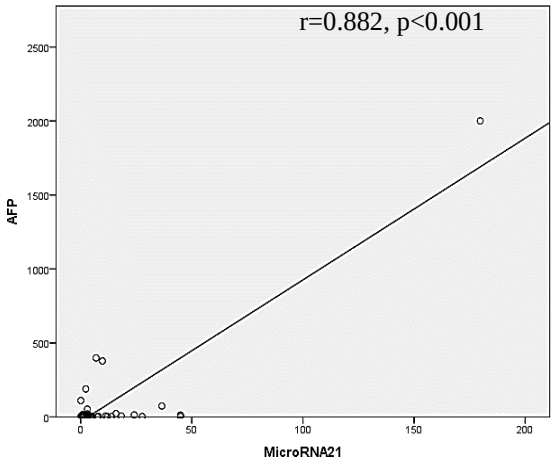

Fig 1. Correlation between miR-21 and AFP

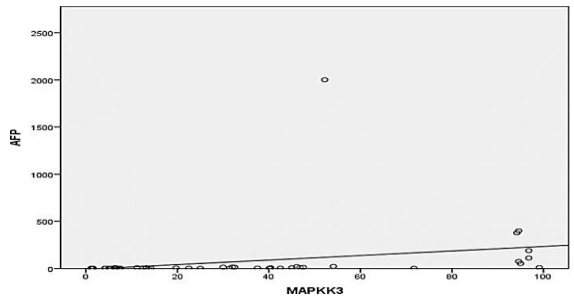

Fig 2: Correlation between MAPKK3 and AFP

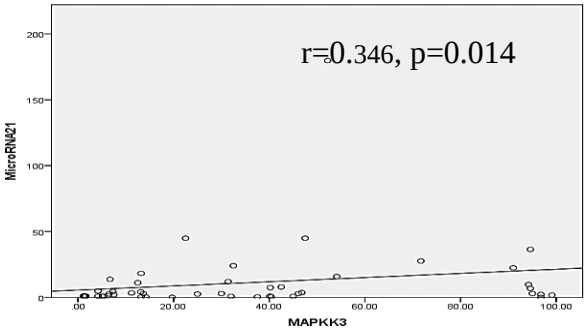

Fig 3: Correlation between MAPKK3 and miR-21

Evaluation of miR-21 and MAPKK3 as potential diagnostic markers

To investigate the possibility that miR-21 and MAPKK3 would serve as new and potential biomarkers for HCC, ROC analysis was conducted. The diagnostic performances of miR-21 and MAPKK3 were then assessed. As shown in Table 4 and Fig. 4, the AUC(Area Under Curve) values for miR-21 and MAPKK3 were 0.892 (95% CI, 0.794-0.99) and 0.805 (95% CI, 0.665-0.946), with cut-off values of 6.916 and 22.5, respectively. This result also demonstrated that miR-21 and MAPKK3 had specificity and sensitivity to discriminate HCC from the cirrhotic group ($P < 0.001$ and $P > 0.001$, respectively). However, AFP failed to differentiate between the cirrhotic and HCC groups ($P = 0.077$).

Table (4). Cut-off levels with their sensitivities and specificities using MAPKK3, AFP, and miR-21 as predictors in HCC cases

Marker	miR-21	MAPKK3	AFP
Cut-off	**6.916**	**22.5**	**9.65**
Sensitivity(%)	75	90	63.2
Specificity(%)	95	60	85
PPV (%)	93.8	69.2	80
NPV (%)	79.2	85.7	70.8
AUROC	0.892	0.805	0.666
95% C.I.	0.794–0.990	0.665–0.946	0.480–0.852
Sig.	<0.001**	0.001**	0.077

miR-21: miRNA-21, MAPKK3: mitogen-activated protein kinase-kinase 3، AFP: alpha-fetoprotein.

Relation of miR-21 and MAPKK3 with the tumor features

As shown in Table 5, miR-21 and MAPKK3 showed a highly significant increase according to the number of focal lesions and tumor size. However, MAPKK3 levels decreased significantly with tumor sizes of at least >3 cm. In the case of portal vein invasion, miR-21 and MAPKK3 showed no statistically significant difference among the HCC group.

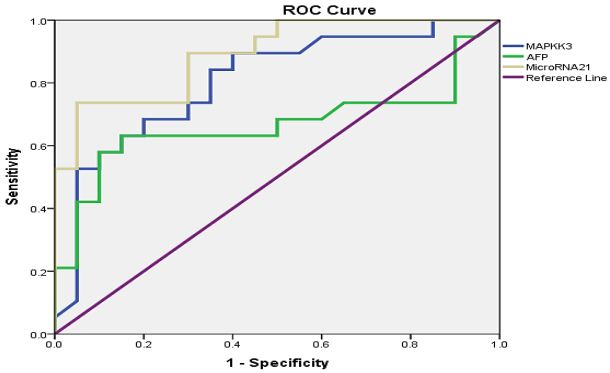

Fig 4. ROC curve for MAPKK3, AFP, and miR-21 as a predictor in HCC cases

Table (5): Relation of miR-21/MAPKK3 with the tumor features

HCC group	miR-21	MAPKK3
Number of focal lesions Multiple (N = 7) Single (N = 13) P-value	21.83 (8.41) 14.45 (6.44) 0.015	86.20 (68.45) 52.96 (62.30) 0.02
Tumor size <3 cm (N = 11) ≥3 cm (N = 9) P-value	10.85 (18.59) 14.05 (7.55) 0.042	48.78 (39.87) 29.32 (81.02) 0.038
Portal vein Normal (N = 6) Dilated Patent (N = 9) Thrombosed (N = 5) *P*-value	9.4 (12.45) 0.61 (3.22) 5.74 (11.43) 0.098	52.00 (43.8) 17.20 (15.53) 14.80 (11.21) 0.099

Discussion

The infection of HCV is a shattering health problem that affects more than 185 million people worldwide (12). Unsettled chronic HCV infection develops into more deteriorating stages, for instance cirrhosis or HCC. The latter is the fifth most prevalent malignancy in men that results in mortality (20). However, a significant difficulty is the absence of accurate biomarkers for early detection and prognosis of

HCC, necessitating the development of novel sensitive and specific diagnostic methods. Therefore, our research investigated the expression of plasma micro RNA 21 and MAPKK3 as potential and dependable noninvasive biomarkers in the Egyptian population that can distinguish between many stages of HCV infection, thereby serving as a critical and definite biomarkers for HCC.

Our study of demographic data revealed no significant differences in AFP levels between diseased groups (cirrhotic and HCC groups) that were reclassified based on triphasic CT. Thus, we confirmed that AFP was not a perfect biomarker for diagnosing HCC (16). Nevertheless, circulatory micro RNAs have newly pinched considerable responsiveness as auspicious noninvasive biomarkers for diagnosing many diseases (3).

The observed upregulation of micro RNA-21 in HCC patients was reported to be significant compared to that of the cirrhotic group (with a *P*-value of 0.001). This finding also coincided with a study that has revealed elevated expression levels of miR-21 in HCC plasma (29). Furthermore, the results of this paper were in partial agreement with an Egyptian study conducted on similar groups (1). Results from their data also publicized that micro RNA 21 was significantly upregulated in HCC plasma samples compared to CHC, which was detected in cirrhotic samples. However, our results showed upregulated levels with a marginal nonsignificant value. Additionally, our data contradicted with those of Oliveira et al., who validated that miR-21 upregulation levels in HCC tissues and plasma were insignificant compared to those of CHC samples obtained from German patients (21). However, the previously cited study elaborated chronic hepatitis and HCC patients of dissimilar etiologies.

In terms of micro RNA -21's diagnostic performance in HCC patients, this study publicized that miR-21 had a good diagnostic power to discriminate HCC patients from cirrhotic patients. This outcome was consistent with the findings of the previous study held by Tomimaru et al., who confirmed that miR-21 expression was able to distinguish between HCC and chronic hepatitis patients using a ROC curve analysis (27). The previous study showed ROC analysis results with AUC = 0.773, sensitivity = 61.61%, and specificity = 83.3%. However, our data revealed a powerful indicator to classify cirrhotic patients from those with HCC.

One more important consequence that can support the diagnosis is the relationship between miRNA-21 and focal lesion size in HCC. Therefore, a highly significant relationship was observed between miRNA-21 and the largest focal lesion size. However, this result can only be applied at the early diagnosis stage of HCC. The finding agreed with that of Liao et al., who confirmed that micro RNA 21 possessed several unique advantages (14). First, minimal invasion and convenience characterize plasma miRNA for diagnosis equated with histopathological inspection. Second, plasma micro RNA expression levels are unwavering and reproducible (13). Third, both cirrhosis and viral status do not influence plasma miR-21 levels. Fourth, substantial overexpression of plasma micro RNA 21 was perceived even in patients with early-stage HCC (27). Hence, AFP remains the most frequently used serological biomarker for HCC screening. However, although AFP levels of 400 ng/mL are deliberated an indicator of HCC, approximately one-half of all HCC cases with small lesions (<3 cm) in our study were not established in the early tumor stage (5).

In our study, micro RNA-21 expression was significantly higher in the HCC group compared to the cirrhotic group ($p < 0.001$). It had sensitivity and specificity of 75 and 95% in detecting HCC with a cut off value of 9.65. Moreover, it showed a significant positive correlation with both AFP and MAPKK.

This study demonstrated that MAPKK3 activities in the HCC group were significantly higher compared to those in the control group, which is in agreement with a previous report (24). MAPKK3 levels in our study were also significantly elevated in the HCC and cirrhotic groups compared to the control group ($p < 0.001$) (13.95-fold difference relative to control). These results agreed with those of Ito et al., who found that a 1.1- to 3.1-fold difference improved the activation of MAPKK3 in human HCC compared to healthy subjects

(6). Similarly, Rovida et al. found that ERK, a member of the mitogen-activated protein kinase family, was relevant for the appearance and progression of several types of cancer and was reported to be amplified in HCC (23). Furthermore, when it comes to MAPKK3's diagnostic ability in HCC, results showed that MAPKK3 had a good diagnostic power to discriminate HCC from cirrhotic patients (AUC = 0.892, sensitivity = 90%, and specificity = 60%). Xie et al. found an explanation for the high expression of ERK. He proposed that this elevated expression was associated with the aggressive behavior of HCC cells (28). Recently, some studied established a relationship between zinc and MAPKK3 expression. It was reported that zinc deficiency has been observed in patients with chronic hepatitis C, as zinc inhibits MAPKK3 expression (8).

Therefore, our results showed a highly significant relationship between MAPKK3 and tumor size, which was explained by the fact that the MAPKK3 gene was expressed in the liver and amplified in human HCC cell lines with overexpression in primary human HCC. However, no data currently exist regarding its functional role in HCC (30).

Conclusion

Our findings proposed that miR-21 can be utilized as a stand-alone biomarker or in combination with MAPKK3 to accurately diagnose HCV-associated HCC development with high meticulousness. In particular, miR-21 levels were higher in patients with tumor size <3 cm. Therefore, the panel based on these two biomarkers along with AFP levels should enhance the investigative surveillance of HCC patients diagnosed with normal AFP levels or those with HCC having small tumor sizes.

Affirmation of Competing Interest

The authors affirm that they have no recognized competing financial interests or individual relationships that would have swayed the work described in this paper

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List of abberreviations

AFP	Alpha fetoprotein
AGO	Protein Argonaute
ALT	Alanine aminotransferase
AST	Aspartate transaminase
ATP	Adenosine triphosphate
AUC	Area under the curve
AUROC	Area under roc curve
CBC	Complete blood count
CLD	Chronic liver disease
CT	Computed tomography
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
GIT	Gastrointestinal tract
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
INR	International normalized ratio
IU	International unit
MAP	Mitogen-activated protein kinase
MAPK	Mitogen-activated protein kinase
MKP	MAP kinase phosphatases
MRI	Magnetic resonance imaging
MAPKK3	Mitogen-activated protein kinase kinase 3
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
SD	Standard deviation
STE	Serine/threonine
TACE	Transcatheter Arterial Chemotherapy and Embolization

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