RESEARCH ARTICLE

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RNA Interference based Midkine Gene Therapy for Hepatocellular Carcinoma

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Abstract

Background: Hepatocellular carcinoma (HCC) arises from hepatocytes and accounts for 90% of primary liver cancer. Reasons for HCC prognosis remaining dismal are that HCC is asymptomatic in its early stages, leading to late diagnosis, and it is markedly resistant to conventional chemo- and radiotherapy. In this study, we investigated RNA interference (RNAi)–based treatment for HCC by targeting *MDK*. **Aim:** The present study aimed to evaluate *MDK* serum levels as a diagnostic biomarker for HCC detection and the effect of *MDK* silencing by RNAi on HCC. **Subjects and methods:** A total of 140 participants, including 120 patients diagnosed with HCC and 20 healthy volunteers were enrolled in this study, all patients who underwent liver resection were sampled for tumor and adjacent non-tumor liver tissues, in addition to 5 ml of blood sample. Midkine expression levels were evaluated by ELISA and by qRT-PCR. The in vitro transfection and gene knockdown efficiency of midkine by *MDK*-siRNA was detected by qRT-PCR and ELISA. Gene knockdown effect at the molecule level on the proliferation of HepG2 in vitro was determined by cell counting. **Results:** The results showed that the expression of *MDK* was significantly increased in the serum of HCC patients compared to control serum samples with $P<0.001$ and significant elevated expression levels of *MDK* in tumor tissues compared to non-tumor ones with $P<0.001$. It also showed that down-regulation of *MDK* using RNAi can significantly inhibit HepG2 cells. **Conclusion:** Molecular targeting of *MDK* using RNAi interference decreases proliferation and could be a therapeutic target.

Keywords: HCC- Midkine- gene therapy- RNAi.

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Introduction

Liver cancer ranks third as a leading cause of cancer-associated deaths worldwide [1]. The most common form of primary liver malignancy is hepatocellular carcinoma (HCC). HCC, evolving in the setting of chronic liver disease (CLD) and cirrhosis, with a rapidly increasing trend which annually have over 906,000 new cases and 830,000 deaths in 2020 [2–4]. According to the World Health Organization forecasts, the number of deaths from liver cancer will exceed 1 million by 2030, posing a critical threat to global health, and ranking it as the sixth most commonly diagnosed cancer [5, 6]. More than one million people die from HCC annually in Western countries [7]. In Egypt, it represents the fourth common cancer and the most common cause of mortality-related and morbidity- related cancer due to the high prevalence of cirrhosis related to chronic HCV, accordingly, the health authorities consider HCC as the most challenging health problem as the number of HCC patients increased twofold over a decade [8–10]

Alpha-fetoprotein (AFP) serves as a widely utilized serum biomarker for hepatocellular carcinoma (HCC) detection. However, approximately 30% of HCC patients exhibit no AFP expression [11]. Elevated AFP levels may occur in patients with cirrhosis or aggravated chronic hepatitis, limiting its diagnostic accuracy, especially in detecting small or early-stage HCC. Consequently, there's an urgent need for streamlined and effective methodologies aiding in HCC surveillance, diagnosis, treatment assessment, and prognosis evaluation [12]. Midkine (*MDK*) was discovered in the course of retinoic-acid-mediated differentiation of murine embryonic carcinoma cells during early stages of embryogenesis [13]. It is a 13-kDa heparin-binding cytokine and growth factor with anti-apoptotic, pro-angiogenic, pro-inflammatory and anti-infective functions, that enable it to partake in a series of physiological and pathophysiological processes

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[14]. The human *MDK* gene is found on chromosome 11 at p11.2 [15]. Midkine is implicated in various physiological processes such as development, reproduction, and repair, thus playing key roles in the pathogenesis of malignancies and other diseases [16, 17]. Early diagnosis has been considered as the most important factor to achieve long-term survival for HCC patients [18]. *MDK* has emerged as a promising serum marker for HCC and ranks among the five potential novel biomarkers for early detection of this cancer [19].

Hepatocellular carcinoma (HCC) presents a global challenge amid its rising incidence and evolving causative factors, due to its heterogenous etiology [20]. A wide range of novel HCC therapeutics have been developed and tested over the last two decades, including molecular targeted therapy [21], immunotherapy, immune-cell therapy, differentiation strategy and RNA interference (RNAi) [22]. Central to the problem of HCC management is that this cancer remains largely asymptomatic until it is very advanced [23]. Gene therapy refers to the delivery of therapeutic nucleic acids into a person's cells to cure a genetic condition. In the context of cancer, a small interfering RNA (siRNA) is considered a way to correct abnormal gene expression by introducing nucleic acids that code for a downregulated tumor suppressor gene, siRNA prevents the translation of oncogenic mRNAs or suicide genes that produce molecules causing cancer cells to undergo apoptosis. Though there are in vivo and ex vivo FDA approved gene therapies for various conditions, only ex vivo cancer gene therapies have been approved so far [24]. RNA interference has become a popular approach recently, RNAi technology is applied for posttranscriptional gene silencing which triggered by siRNA [25, 26]. RNAi therapies have rapidly been advanced in clinical trials for the treatment of various human diseases, especially various human cancers, and the strategy of multiple gene-targeted siRNAs is considered to be a good way for controlling complex disease systems, such as HCC [27]. This study aims to evaluate *MDK* serum levels as a diagnostic biomarker for HCC, ascertain its sensitivity and specificity in predicting HCC, explore its role in HCC development and progression by analyzing its expression in tumor and adjacent non-tumor tissues, and consider *MDK* silencing through RNAi as a potential therapeutic avenue for HCC treatment.

Materials and Methods

Ethics statement

The study was approved by the Ethics Committee of National Hepatology and Tropical Medicine Research Institute (NHMRI) and Theodor Bilharz Research Institute (TBRI). Approval was granted by the Research Institute office for national hepatology and tropical medicine (NHTMRI-IRB) (Serial #36-23), and the Theodor Bilharz research ethics committee (TBRI-REC) (FWA00010609) (Serial #PT787). Written informed consents were signed by all participants to use their specimens for research purposes, in accordance with the institutional guidelines. All the experimental procedures in this study complies with the latest version of the Declaration of Helsinki and general guidelines for good clinical practice. Confidentiality and personal privacy were respected in all levels of the study, patients were informed to feel free to withdraw from the study at any time without any consequences; The study was carried out at the Department of Biochemistry and Molecular Biology, TBRI.

Study design and patients

This longitudinal study population comprised adult patients with HCC admitted to the medical unit of NHMRI, during the study period. One hundred and twenty adult patients above 18 years diagnosed on the basis of clinical features, laboratory investigations, and abdominal ultrasound findings suggestive of HCC. Patients with severe primary cardiopulmonary failure, intrinsic renal disease, and obstructive jaundice were excluded from the study.

Data Collection

A medical records abstractor trained in the diagnosis of HCC was in charge of the patients' enrollment. The data abstractor has been trained on 10 records prior to the study period. He collected the data, not knowing about the aim of the study. After thoroughly explaining the study to patients, individuals who gave informed consent were recruited and a questionnaire was administered to obtain sociodemographic data and clinical history. Relevant histories including alcohol use and clinical features of HCC (ascites, asterixis, hepatomegaly, splenomegaly, and abdominal vein collaterals) were obtained. Furthermore, an abdominal ultrasound scan was performed for all patients.

Sample collection

Five milliliters of venous blood were taken from all patients and 20 healthy volunteers served as controls for hematological, biochemical, and serological investigations. Samples were allowed to clot at room temperature, this usually takes 15-30 minutes then centrifuged at 2000 rpm for 10 min, serum was then aliquoted and stored at -80°C. All patients who underwent liver resection were sampled for tumour and adjacent non-tumour liver tissues, the surgery for liver resection was conducted within the department of surgery NHMRI Hospital, Egypt.

Laboratory Tests

Routine tests including alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, albumin (ALB) and alpha-fetoprotein (AFP) were performed for all participants.

Evaluation of serum MDK levels

Midkine levels in HCC patients and healthy volunteers' sera were measured by Enzyme-Linked Immunosorbent Assay (ELISA) method according to manufacturer's instructions using Human assay kit, (Sunlong Biotech Co., Ltd). Briefly, 100 µl of prepared standards and samples were added to appropriate wells of the ELISA plate and then assayed according to the manufacturer's instructions. The absorbance was measured at 450 nm in a microstrip plate spectrophotometer, (Abcamm CA, USA) and *MDK*

levels were quantified with a calibration curve. Each standard or sample was assayed in duplicate.

Molecular Analysis:
Cell Culture

The human primary liver cancer cells HepG2 from Egyptian Company for Production of Vaccines, Sera, and Drugs, (VacSera). Cells were cultured in RPMI medium supplemented with 10% inactivated FBS (Biowest), 100 U/ml penicillin, 100 µg/ml streptomycin (Thermo Fisher Scientific, Inc.), and 1% HEPES buffer (Invitrogen) and cell lines were allowed to grow in plastic tissue culture T-flasks 25 cm2 at 36oC and 5% CO2.

Cell plating

Cultured cells were checked for 85% confluency then trypsinized by 0.5 Trypsin /EDTA (Invitrogen). The cells were counted using hemocytometer by trypan blue dye exclusion assay. In 24 well tissue culture, 7x104 cells were plated in each well and incubated for overnight at 37°C.

In Vitro Knockdown of MDK using Small Interfering RNA Transfection

Midkine-specific small interfering RNA (siRNA-*MDK*), was purchased from commercial vendors (Flexi-Tube siRNA, Qiagen, Hilden, Germany) with stock concentration of siRNA (0.125µg /1µl). The lyophilized siRNA duplex powder was simply resuspended in RNase-free water to a final concentration of 20 µM. Transfection protocols require optimization for different cell types, the manufacturer's recommended procedures were used as a starting point for optimization. The HiperFect Transfection Reagent (Qiagen, Hilden, Germany) was used to transfect siRNA into HepG2 cells, while leaving some wells untransfected to act as control. The plate was incubated at 37°C and samples were withdrawn at 0 time, 6, 24, 48 and 72 hrs. post-transfection.

RNA extraction

The miRNeasy extraction kit was used to extract total RNA (Qiagen, Valencia, CA). The extraction procedures were conducted according to the manufacturer's instructions to extract RNA from serum samples, tumour, nontumor tissues and transfected and untransfected HepG2 cells. For serum 200 µl, and for tissue roughly 70 mg of tissue were homogenized with lysis solution prior to extraction. Samples were extracted in duplicates and quantitated using a Nanodrop ND-2000c (Thermo Scientific, Waltham, MA, USA), and stored at −80°C for further analyses.

Assessment of MDK Knockdown using Quantitative Real-Time Reverse-Transcription Assay (qRT-PCR)

For evaluation of *MDK* expression, 10 µl of the extracted RNA from serum, tissues and the HepG2 cells were reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). The PCR thermal cycler (BioRad-T 100, Singapore) program was as follows: 10 min at 25°C, 120 min at 37°C, and 5 min at 85°C. Five µl of the cDNA were used for the real time PCR amplification step using *MDK* and GAPDH specific primers (Table 1), and qRT-PCR assay was performed using (Maxima SYBR Green/ROX qPCR Master Mix 2X, Thermo-fisher Scientific, UK) in Applied Biosystems™ Step-One™ Real-Time PCR System. The program was initiated by 10 min at 95°C, followed by 15 sec at 95°C, then 60 sec at 60°C, and the last two steps were repeated 40 times. The ΔΔCT method was used for the relative quantification of miRNAs in all samples [28].

Proliferation assay

The transfected and untransfected HepG2 cells were seeded in triplicate in 24 well plates. After 6, 24, 48 and 72 hrs. of incubation, the cells were trypsinized, stained with trypan blue and counted using hematocytometer.

Statistical analysis

The data were analyzed using Microsoft Excel 2016 and statistical package for social science 'IBM SPSS Statistics for Windows, version 28 (IBM Corp., Armonk, N.Y., USA)'. Continuous normally distributed variables were represented as mean±SD. with 95% confidence interval and using the frequencies and percentage for categorical variables; a P value <0.05 was considered statistically significant. To compare the means of normally distributed variables between groups, the student's t test was performed. The diagnostic performance of the studied markers was assessed by receiver operating characteristic (ROC) curves. The area under the ROC (AUC) was calculated as an accuracy index for prognostic performance of selected tests. Effect modifications were evaluated by stratification, and statistical interaction was assessed by including main effect variables and their product terms in the logistic regression model.

Results

Patient characteristics

This study recruited 140 participants, including 120 HCC patients and 20 healthy volunteers with no history of liver disease or alcohol intake as a control group. Table 2 displays the clinical data of all subjects. The HCC group subjects were 50.099.73 years old on average, with 76 (63.3%) males and 44 (36.7%) females, whereas the healthy volunteers group subjects were 42.2411.78 years old on average, with 14 (70%) males and 6 (30%) females. In terms of demographic data, the mean age in the HCC group was substantially greater than in the control group (P<0.05), however there was no significant difference

Table 1. Sequence of Primers Used for qRT-PCR

Gene	Forward Primer Sequence (5'-3')	Reverse (5'-3')
MDK	AAAGAATTCGAGATGCAGCACCGAGG	AAACTCGAGCCAGGCTTGGCGTCTAGTC
GAPDH	ATTCCATGGCACCGTCAAGGCTGA	TTCTCCATGGTGGTGAAGACGCCA

in terms of sex between the HCC group and the control group (P = 0.3). A solitary hepatic mass was detected using computed tomography. A single hepatic mass was found in 59 individuals, two masses in 31 patients, three masses in 17 patients, and four masses in 13 patients. The tumor size is represented as Mean±SD= 4.2±2.3. Twenty-nine patients (24.2%) were classified as grade I, 59 (49.2%) were grade II and 32 (26.7%) patients were grade III. The histologic pattern of HCC patients was classified as 47 (39.2%) were acinar, 41 (34.2%) were solid and 32 (26.7%) were Acinar/Solid. Steatosis was represented as Mean±SD= 0.024±0.005. Seventy-six patients (63.3%) were with fibrotic liver, while 44 (36.7%) were staged cirrhotic liver. Regarding the hepatitis activity index (HAI), 22 (18.3%) were with grade I, the majority of patients 78 (65%) were II and 20 (16.7%) were III. Abdominal ultrasound detected hepatomegaly in 25 (20.8%), ascites in 39 (78%), OLL in 50 (41.7%) and splenomegaly in 38 (31.7%) patients of the HCC group.

Midkine expression levels in serum and tissues of HCC patients by qRT-PCR

The *MDK* expression levels were evaluated in serum of HCC patients, tumor tissue (T) and adjacent non-tumor tissues (NT) of human samples using qRT-PCR analysis for both HCC patients and healthy volunteers for serum. The results showed that the expression of *MDK* was significantly upregulated in the serum of HCC patients

Table 2. Demographic and Clinical Characteristics of HCC Patients and Healthy Volunteers, and Their Association with *MDK* Expression:

Parameters	Categories	HCC Patients	Healthy Volunteers	Association with MDK expression (OR/95% C.I)		P. value
		N=120	N=20	OR	95% C. I	
Age (years)		50.09±9.73	42.24±11.78			0.02*
Sex	Male	76 (63.3%)	14(70%)			0.3
	Female	44 (36.7%)	6(30%)			
ALT (U/L)		70.67± 5.36	8.1± 1.28			0.001**
AST(U/L)		80.08± 6.55	9.33± 2.01			0.001**
Bilirubin (mg/dl)		2.52 ± 1.03	0.51± 1.21			0.01*
Albumin (g/dl)		1.01± 0.89	3.50± 0.64			0.001**
AFP (ng/ml)		199.2±161.4	25.14 ± 0.45			0.001**
Number of masses	1	59 (49.2%)	-	0.904	0.751 - 1.089	0.289
	2	31 (25.8%)	-	2.347	1.621 - 3.398	<0.001**
	3	17 (14.2%)	-	1.254	0.954 - 1.648	0.105
	4	13 (10.8%)	-	0.904	0.751 - 1.089	0.289
Tumor size (cm)		4.2±2.3	-			
Tumor grade	I	29 (24.2%)	-			
	II	59 (49.2%)	-	1.12	0.93 - 1.348	0.233
	III	32 (26.7%)	-	1.755	1.361 - 2.263	<0.001**
Tumor pattern	Acinar	47 (39.2%)	-	-	-	-
	Solid	41 (34.2%)	-	1.203	1.018 - 1.422	0.03*
	Acinar/Solid	32 (26.7%)	-	1.17	0.981 - 1.396	0.08
Steatosis		0.024±0.005	-	-	-	-
Tumor stage	Fibrosis	76 (63.3%)	-	1.148	0.993 - 1.328	0.062
	Cirrhosis	44 (36.7%)	-			
HAI (I-III)	I	22 (18.3%)	-			
	II	78 (65%)	-	1.477	1.181 - 1.846	<0.001**
	III	20 (16.7%)	-	1.991	1.469 - 2.697	<0.001**
Hepatomegaly	Negative	95 (79.2%)	-	1.716	1.356 - 2.172	<0.001**
	Positive	25 (20.8%)	-			
Ascites	Negative	73 (60.8%)	-	1.067	0.929 - 1.226	0.357
	Positive	47 (39.2%)	-			
Oedema lower limb (OLL)	Negative	70 (58.3%)	-	0.944	0.824 - 1.082	0.41
	Positive	50 (41.7%)	-			
Splenomegaly	Negative	82 (68.3%)	-	1.038	0.899 - 1.2	0.608
	Positive	38 (31.7%)	-			

Age, ALT, AST, Bilirubin, Alb, AFP, tumor size and steatosis are represented as Mean±SD; the data were analyzed by t test. Sex, number of masses, tumor grade, tumor pattern, tumor stage, hepatitis activity index (HAI), hepatomegaly, ascites, OLL and splenomegally are represented as F(%); the data was analyzed by paired X test; for associations OR; Odd Ratio, C.I; Confidence Interval, P value calculated depend on log linear regression analysis. * P value <0.05 is significant, ** P value <0.001 is highly significant.

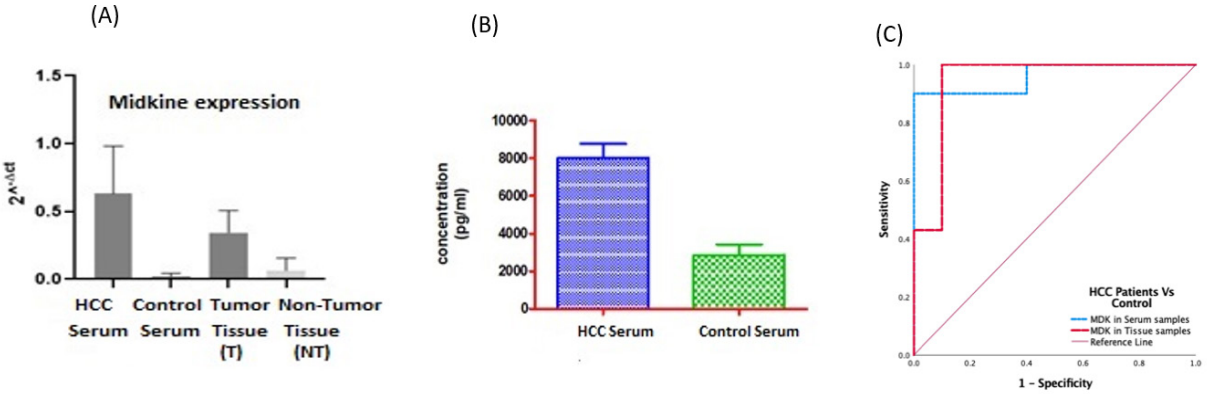

Figure 1. (A) Midkine expression levels in HCC serum, control serum, tumor tissue (T) and non-tumor tissue (NT). (B) Concentration of MDK in serum of HCC patients and controls using ELISA. (C) ROC Curve of MDK expression in serum and tissue samples.

Table 3. Diagnostic Performances of MDK in Serum and Tissue of HCC Patients

		Cutoff point	Sn. %	Sp. %	AUC	S. E	Asymptotic 95% C. I		P. value
							Lower Bound	Upper Bound	
MDK Expression	Serum	>0.113	90	100	0.96	0.017	0.928	0.992	<0.0001**
	Tissue	>0.1342	100	90	0.943	0.039	0.867	1	<0.0001**

Sn, Sensitivity; Sp, Specificity; AUC, Area under curve and C.I: 95% Confidence Interval; * P value <0.05 is significant; ** P value <0.01 is highly significant.

compared to control serum samples with P<0.001** and a highly significant upregulation expression levels of *MDK* in tumor tissues compared to non-tumor ones with P<0.001** (Figure 1A).

The associations between MDK expression levels and patient outcomes

The *MDK* expression level was significantly associated with many patient outcomes, from these parameters, an association with patients who have 3 tumor masses with an odd ratio (OR)=2.347 and 95% Confidence Interval (95% C.I) = 1.621 - 3.398 and P was calculated depend on log linear regression analysis <0.001, patients with tumor grade III with OR= 1.755, (95% C.I) = 1.361 - 2.263 and P <0.001, this association confirms the role of *MDK* in the disease progression. An association was observed with solid liver pattern with OR = 1.203, (95% C.I) = 1.018 - 1.422 and P = 0.03. A highly significant association was observed with stage II and III of hepatitis activity index (HAI) with OR, (95% C.I) = 1.477, 1.181 - 1.846 and 1.991, 1.469 - 2.697 respectively and P was <0.001 for both stages. Finally, *MDK* expression level was significantly associated with patients with negative hepatomegaly with OR = 1.716, (95% C.I) = 1.356 - 2.172 and P<0.001 (Table 2).

Table 4. Prognostic Performance of *MDK* in Serum and Tissue of HCC Patients

	OR	95% C. I	P. value
MDK in serum samples	2.05	0.051 - 6.34	0.043*
MDK in tissue samples	4.94	2.55 - 9.42	<0.001**

OR, Odd Ratio; C.I, Confidence Interval; P value calculated depend on log linear regression analysis. * P value <0.05 is significant; ** P value <0.001 is highly significant

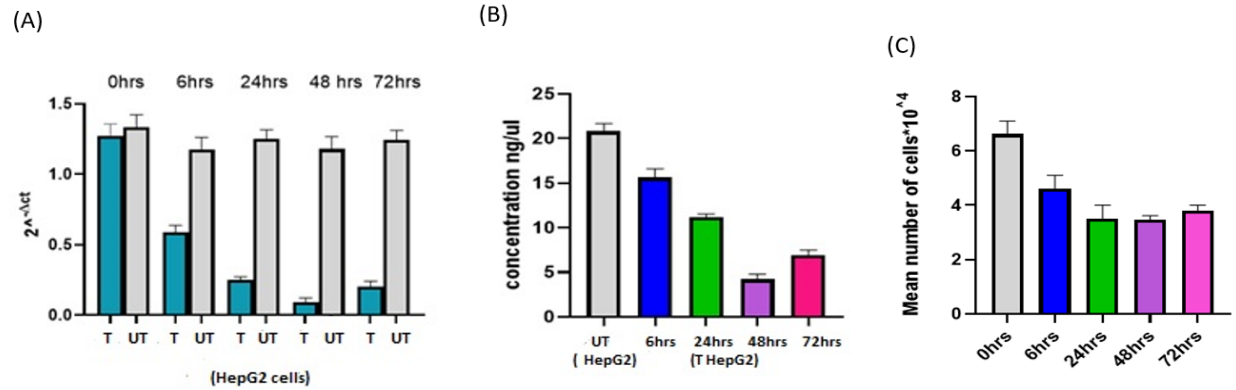

Figure 2. (A) Expression of MDK in transfected (T HepG2) and untransfected (UT HepG2) cells; (B) Concentration of MDK evaluated by ELISA in transfected (T HepG2) and untransfected (UT HepG2) cells during different time intervals post-transfection. (C) Growth rate of HepG2 cells during different time intervals.

Evaluation of MDK levels in serum of HCC by ELISA

Then, *MDK* concentration in the sera of both patients and healthy controls was determined using ELISA, which revealed an increased concentration of *MDK* in HCC serum samples to reach 8000 pg/ml compared to 3800 pg/ml for normal serum samples of the healthy volunteers as shown in Figure 1B.

Diagnostic performance of MDK in serum and tissue samples

Receiver Operating Characteristic (ROC) Curve was established to show the diagnostic performance of *MDK* regarding the studied groups. The results showed that *MDK* could be used as a powerful diagnostic biomarker and could differentiate between HCC serum and normal serum samples as the diagnostic performance was found at the cutoff >0.113, with sensitivity = 90% and specificity = 100%, an area under the curve (AUC)= 0.96 and (lower bound-upper bound, 95% confidence interval) 95% C.I (0.928-0.992) and P < 0.0001. While for tissue, the cut-off >0.1342, with sensitivity = 100% and specificity = 90%, an area under curve (AUC)= 0.943, 95% C.I (0.867-1) and P < 0.0001(Table 3, Figure 1C).

Prognostic performance of MDK in serum and tissue samples

Univariate logistic regression analysis was performed to characterize *MDK* as a predictor and/or prognostic parameter for HCC, the analysis showed that *MDK* expression in serum and tissue samples was statistically associated with HCC, and this gene may be used as a powerful predictor and/or prognostic parameter for HCC prospection, an increase in 1 degree of *MDK* in serum and tissue samples, increased the odds of being HCC by a factor of odd ratio and C.I; confidence interval OR (95% C.I) = 2.05 (0.051-6.34), P<0.05, 4.94 (2.55-9.42), P<0.001 respectively (Table 4).

In Vitro knockdown of MDK in HepG2 cell line & its effects

The statistical analysis of *MDK* expression level showed knockdown in the expression of the targeted gene. At zero time, the expression was (1.28±0.1, P N/S). After 6 hrs. of transfection, the inhibitory effect of the siRNA started to increase (0.58±0.09, P<0.05*) and reached its maximum inhibition (0.001±0.005, P<0.001**) at 48 hrs. post-transfection as the expression reaches maximum decrease, then at 72 hrs. the inhibitory effect of the siRNA decreases and the *MDK* expression level starts to increase (0.10±0.07, P<0.001**) compared to the expression level in the untransfected HepG2 cells (Figure 2A).

Examination of the functional effect of siRNA knockdown

Once knockdown of the siRNA-targeted gene is confirmed, assays can then be carried out to investigate the resulting functional effects. To confirm and determine the inhibitory effect of the siRNA, the concentration of *MDK* was measured by ELISA technique, the results revealed that, for untransfected HepG2 cells, the *MDK* concentration was (20.81±0.86) while decreasing dramatically to (15.63±0.97, 11.22±0.33 and 4.21±0.58) after 6, 24 and 48 hrs. respectively post-transfection, and released again to reach (6.94±0.56) after 72 hrs., these results confirm the inhibitory effect of siRNA and showed that the maximum effect appears after 48 hrs. post-transfection (Figure 2B).

The effect of midkine downregulation on HepG2 growth rate

The growth rate of the HepG2 was determined after *MDK*-siRNA transfection for 6, 24, 48 and 72 hrs., the results showed that the growth rate of the HepG2 began to decline after 6 hrs. and continued till 48 hrs. with the total cell number remained constant after 48 hrs., then the cells restore its growth ability after 72 hrs. with large number of dead cells and an abnormal cell morphology (Figure 2C).

Discussion

The rapid evolution of novel therapies based on RNA interference (RNAi) presents an exponential growth, offering promising avenues for treating genetic diseases and cancer by precisely targeting RNA sequences [29]. RNAi research traces back to the early 1990s when several scientists, studying plants and fungi, independently noted the ability of RNA molecules, under specific conditions, to impede gene expression at a post-transcriptional level [30]. Small interfering RNAs (siRNAs) emerged as therapeutic tools for various diseases, including cancer. These short double-stranded RNAs (20-24 nt) possess distinct structures featuring 5'-phosphate/3'-hydroxyl terminations and two 3'-overhang ribonucleotides on each duplex strand [31].

Upon cytoplasmic entry, siRNAs integrate into the "RNA-induced silencing complex" (RISC). Typically, only one strand, the guide strand, becomes part of the RISC, while the other strand, the passenger strand, is typically discarded and degraded. Once integrated into the RISC, the guide strand, exhibiting total complementarity with the target transcript, initiates endonucleolytic cleavage, leading to transcript degradation and inhibition of protein translation [26].

Midkine, initially discovered in embryonic carcinoma cells, plays a pivotal role in retinoic acid-induced differentiation's early stages [6, 14]. Functioning as a heparin-binding cytokine or growth factor, it orchestrates survival, growth, migration, gene expression, and other cellular activities. Its involvement in tumorigenesis and tumor invasion is notable, as it enhances the growth, survival, and angiogenic activity of tumor cells [10, 32]

Our study aims to assess *MDK* serum levels as a diagnostic biomarker for early hepatocellular carcinoma (HCC) detection, elucidating its sensitivity and specificity in predicting HCC, analyzing its expression in tumor and adjacent non-tumor tissues to explore its role in HCC development and progression, and investigating the potential of *MDK* silencing via RNAi as a therapeutic target for HCC [33, 34].

Utilizing qRT-PCR, our findings indicate a significant upregulation of *MDK* expression in the serum of HCC patients compared to control samples (P<0.05). ELISA

analysis of *MDK* levels in HCC serum samples revealed an elevated concentration of *MDK* (8000 pg/ml) compared to healthy individuals' serum (3800 pg/ml) (Figure 1). Our results align with Thokerunga et al, and Sheata et al, and others, demonstrating increased *MDK* levels in HCC serum samples compared to controls [10, 27, 35, 33]. In terms of association with patient features our findings showed that *MDK* was associated with Age, ALT, AST, AFP, number of masses, tumour grades, HAI, and hepatomegaly, which was the case for *MDK* abundance and its association with clinicopathological features as well as poor clinical outcomes in other studies involving prostate cancer and hepatocellular cholangiocarcinoma [36, 37]. Examining *MDK* expression in liver tissues exhibited highly significant upregulation in tumor tissues compared to non-tumor tissues ($P<0.001$). Previous studies by supported this finding, highlighting *MDK* expression predominantly in HCC tissues compared to surrounding tissues [1, 38–41].

Assessing *MDK*'s diagnostic performance revealed its potential as a robust biomarker distinguishing between HCC serum and healthy individuals' serum with a sensitivity of 90%, specificity of 100%, and an AUC of 0.96. These results, present some differences in sensitivity and specificity assessments but highlight similarities as well [10, 17, 33, 38, 39, 42, 43]. Regression analysis affirmed *MDK*'s prognostic significance in serum and tissue samples of HCC patients, consistent with Zheng et al, associating high *MDK* expression with tumor characteristics and patient prognosis [32, 44]. In light of delays in diagnosing asymptomatic HCC stages, leading to aggressive disease progression and limited treatment options, alternative strategies are imperative for targeting *MDK*, showed promise in our study where a decreased viability and growth rate of cancer cells were observed in vitro (Figure 2). SiRNA-*MDK* effectively silenced *MDK* in HepG2 cells in vitro, aligning with previous findings on other malignancies such as those by Zhong et al, and Ding, et al., Younis et al, and others indicating the potential for *MDK*-targeted therapy in HCC treatment [42, 45, 46, 42, 47, 48]. In conclusion, our study underscores *MDK*'s potential as a noninvasive diagnostic and prognostic biomarker for early HCC detection and progression. Targeting *MDK* presents a promising avenue for personalized medicine in HCC treatment. Further research into *MDK*'s role in carcinogenesis and therapeutic resistance is warranted. Additionally, this work suggests future progression on the possibility of using *MDK* silencing in animal models to further investigate its role, and what could be improved in this study is to increase the cohort for healthy controls, and this will be further considered in future work.

Author Contribution Statement

All authors contributed to equally to the research project. S.M. Conception and writing of manuscript, FK GH, MM practical work, and manuscript preparation, EH, data analysis, MS, study design, editing and reviewing the manuscript, AA samples, clinical data, and approval acquisition, and review of the manuscript which was done by all authors

Acknowledgements

Ethical approval and Consent to participate

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Research Institute office for national hepatology and tropical medicine (NHTMRI-IRB) (Serial #36-23), and the Theodor Bilharz research ethics committee (TBRI-REC) (FWA00010609) (Serial #PT787). Informed consent was obtained from all individual participants included in the study.

Availability of data

The datasets generated during and/or analyzed during the current study are available from the corresponding author or Samah Mamdouh on reasonable request.

Conflict of Interests

The authors have no relevant financial or non-financial interests to disclose.

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