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Original Research

Visfatin increases miR-21 to promote migration in HCC

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Abstract: Hepatocellular carcinoma (HCC) is a common human malignancy. In this study, we aimed to investigate the serum levels of visfatin and miR-21 in HCC patients, to analysis the relationship between the pathological features and the plasma level of visfatin or miR-21, and to explore the roles of visfatin and miR-21 in migration of HCC cells. Our results showed that the serum levels of visfatin and miR-21 were significant higher in HCC patients than healthy subjects. The diagnostic sensitivity of serum visfatin was 82.5% and the specificity was 65.0%. The serum visfatin was significantly associated with the histology and metastasis. Visfatin induced miR-21 expression and cell migration in HepG2 cells. Transfection of miR-21 inhibitor suppressed the visfatin-induced migration in HCC cells. These results suggested that visfatin induced HCC cell migration via upregulation of miR-21, which provides a novel basis for the diagnosis of HCC.

Key words: Visfatin; miR-21; HepG2; HCC; Metastasis.

Introduction

Hepatocellular carcinoma (HCC) is a common primary liver malignancy (1). The incidence of HCC is ranked fifth worldwide and its mortality rate ranks the third in malignant tumors of the digestive system, behind the gastric and esophageal cancers (2, 3). HCC is one of the worst prognostic malignancies (4). It had shown that about 1 million people die each year from HCC in the world corresponding to its high mortality rate and the increasing incidence of HCC (1, 2). There are greater than 1 million newly-added cases every year in the world, of which more than 80% cases occur in countries and regions in Africa and Asia (5, 6). There are approximately 500,000 newly-added cases each year in China where has become the country with the most HCC patients (1). The incidence of HCC is influenced by both environmental and genetic factors. The environmental factors mainly include hepatitis B virus and hepatitis C virus infection, alcoholic hepatitis, non-alcoholic fatty liver and cirrhosis. The majority of HCC in China are due to chronic hepatitis B virus infection (7, 8). Moreover, genetic factors play an important role in HCC, and whole genome association study (GWAS) illustrates the genetic basis of HCC susceptibility (9). HCC has become an important issue in the field of medical research due to the obvious symptoms of the early stage of HCC, the difficulty in early diagnosis, the high degree of malignancy, the difficulty in treatment and the high mortality rate. Therefore, it has attracted more and more attention and researches.

The relationship between the occurrence and obesity of tumors has gradually attracted people's attention in recent years, and many studies have shown that obesity is an independent risk factor for colon cancer, breast cancer, prostate cancer and esophageal cancer (10). Researches showed that adipose tissue is not only an energy-storage organ, but also an active endocrine organ, which can secrete various cytokines such as visfatin (NAMPT), resistin, IL-6 and TNF-α (11-13). Adiposity cytokines play an important role in proliferation, invasion and metastasis of tumor cells (14). It has demonstrated that visfatin can inhibit angiogenesis and is negatively correlated with the occurrence and development of a variety of malignant tumors including HCC (15, 16). Visfatin is highly expressed in many tumor cells such as small cell lung cancer cell, breast cancer cell, and gastric cancer cell (17-20). Visfatin increased gastric cancer cell proliferation and the telomerase gene expression (21). Visfatin inhibitor STF-118804 reduced the growth of pancreatic ductal adenocarcinoma cells (22). However, the relationship between visfatin and pathological features in HCC and the role of visfatin in migration of HCC cells are still unclear.

In this study, we explored the serum levels of visfatin in HCC patients and healthy subjects, and analyzed the clinical significance of serum visfatin and relationship between visfatin and pathological features of HCC patients. Moreover, we found that visfatin induced expression of microRNA (miR)-21. Then, we investigated the role of miR-21 in visfatin-induced migration of HCC cells.

Materials and Methods

Patients and clinical data

One hundred and fourteen patients who were diagnosed as hepatocellular carcinoma (HCC) from September 2014 to April 2016 were selected, including 93 males and 21 females, aged 25-87 years (mean 58.45 ±

Table 1. Diagnostic efficiency of serum visfatin in HCC.

Diagnostic efficiency	Values
Best cut-off value	>20.42 μg/L
Sensitivity (%)	82.5
Specificity (%)	65.0
Youden Index	0.475
AUC	0.872
95%CI	0.783-0.946

12.89) years old, 17 cases were ≤5 cm, 97 cases were >5 cm. There were 51 cases of metastasis including 19 cases of abdominal or other organ metastasis and 35 cases of inferior vena cava or portal vein tumor thrombus (based on CT data) (Table 1). All patients were confirmed by contrast-enhanced CT and serum AFP, have not received any radiotherapy, chemotherapy and surgical treatment. Patients with cardiopulmonary and vital organs dysfunction were excluded. Patients with severe endocrine disease and malignant tumor history were also excluded. Another 150 healthy volunteers in our hospital during the same period were selected as the control group, including 115 males and 35 females, aged 20-85 years, with an average of 57.32 ± 10.54 years. There were no significant differences in gender and age between the HCC patients and health subject groups (P>0.05). Venous bloods from HCC patients and healthy subjects were collected before treatment or physical examination on an empty stomach condition at room temperature. After centrifugation at 3000 r/min 30 min, the serum was collected and stored at -20 ℃. The study was approved by the hospital ethics committee, and all participants were signed the informed consent.

Detection of serum level of visfatin

The serum was warmed to 4℃, and then serum level of visfatin was detected using ELISA kit (K4907-100, Biovision, USA) according to the manufacture's instruction. Detection was performed by Mulytiskan MK3 ELISA instrument (Thermo, USA). Samples (100 μL) were incubated in the antibody-coated plate at 37 ℃ for 1 h. Detection antibody (100 μL) was added to each well and incubated at 37 ℃ for 1 h. Then, the plates were incubated with 1x HRP conjugated anti-rabbit IgG at 37 ℃ for 1 h and 100 μL TMB substrate for 10 min. The absorbance at 45o nm was read within 30 min.

Cell culture and transfection

HepG2 cells were obtained from ATCC, USA. Cells were maintained in DMEM containing 10% new born bovine serum. MiR-21 inhibitor and negative control (NC) were purchased from GenePharma, USA. Fifty μM miR-21 inhibitor or NC was transfected into HepG2 cells using Lipofectamine 2000 (Invitrogen, USA) according to the manufacture's instruction. Before treatment with different visfatin (Sigma, USA) concentrations at 0, 1, 5 μg/ml for 24 h, HepG2 cells were incubated with serum-free serum for 24 h. The transfection cells were treated with 5 μg/ml for 24 h.

qRT-PCR

Total RNAs from cells were extracted using TRIzol (Invitrogen, USA) according to the instructions provided by the manufacturer. cDNA was synthesized by reverse transcribing the total RNA using PrimeScript™ RT Master Mix (Takara, China). The expression level of miR-21 was detected by qRT-PCR using the Ultra SYBR Mixture with ROX (CWBio Co., Ltd.) and ABI7100 system (Applied Biosystems, Germany). U6 was used as internal control for miR-21. All qRT-PCR reactions were performed in triplicate. Relative quantification of gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. The primers used in this study were: miR-21-5p forward 5′- TAGCTTATCAGACTGATG-3′, reverse 5′-ACATCGAGTGTAGCATA -3′; U6 forward 5′-TGCGGGTGCTCGCTTCGGCAGC-3′ and reverse 5′-CCA GTGCAGGGTCCGAGGT-3′. PCR reaction was performed in condition: 94℃ 2min, 94℃ 20s, 58℃ 20 s, 72℃ 20s, 40 cycles.

Scratch wound migration assay

In order to evaluate the migratory response of miR-21 inhibitor transfected HepG2 cells following visfatin exposure, a scratch wound healing assay was performed. Cells were seeded at confluent status for 24 h and transfected with miR-21 inhibitor or NC prior to the addition of 0, 1, 5 μg/ml visfatin. A cell-free area (900-μm scratch wound) in each well was created using a 500-μl pipette tip. A total of 48 h subsequently, the wound area in the gap was counted using a light microscope at ×200 magnification. All of the data were obtained in six independent experiments.

Statistical analysis

All experimental data are presented as the mean ± SD and were analyzed using SPSS software (version 19.0; SPSS, USA). Statistical analysis was performed using one-way analysis of variance (ANVOA) and Student-Newman-Keuls (SNK) post hoc analysis. The ROC curve analysis was performed to evaluate the diagnostic value of serum visfatin for HCC. Relationships between clinical pathological features of HCC patients and serum visfatin or miR-21 were performed by χ^2 test. $P<0.05$ was considered as a statistically significant difference.

Results

Serum levels of visfatin and miR-21 in HCC patients

We firstly detected the serum level of visfatin and relative expression of miR-21 in HCC patients and normal healthy subjects (Figure 1). Results revealed that the serum level of visfatin was significant higher in HCC patients than in normal healthy subjects (Figure 1A). The serum level of miR-21 was also significant higher in HCC patients than in normal healthy subjects

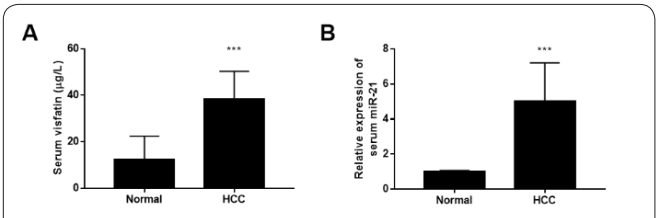

Figure 1. Differential serum level of visfatin and miR-21 between HCC patients and normal subjects. Levels of visfatin and relative expression of miR-21 in 114 HCC patients and 150 normal subjects were detected by ELISA and Qrt-PCR, respectively. ***p<0.001.

Table 2. The relationship between levels of visfatin and pathological features of HCC patients.

Pathological features		n	Visfatin positive rates n (%)	χ^2	*P*
Gender	Male	93	82 (88.17)	0.09	0.764
	Female	21	19 (90.48)		
Age	<55	42	36 (85.71)	0.547	0.460
	≥55	72	65 (90.28)		
Histology	≤5 cm	17	8 (47.06)	34.120	0.000
	>5 cm	97	93 (95.88)		
Metastasis	No	63	51 (80.95)	8.145	0.004
	Yes	51	50 (98.04)		

(Figure 1B).

Clinical significance of serum visfatin

To evaluate the diagnostic value of serum visfatin for HCC, we performed the ROC curve analysis (Table 1) and data showed that the AUC (area under ROC curve) of visfatin was 0.872 (95%CI was 0.783-0.946), with best cut-off value >20.42 μg/L. The diagnostic sensitivity was 82.5% and the specificity was 65.0%.

Relationship between clinical pathological features of HCC patients and serum levels of visfatin

We analyzed the relationship between serum levels of visfatin and clinical pathological features including gender, age, histology and metastasis of 114 HCC patients (Table 2). The serum visfatin was not associated with gender and age, but was significantly associated with the histology and metastasis.

Visfatin increases miR-21 in HepG2 cells

To explore the relationship between visfatin and miR-21, we treated HepG2 cells with different concentration of visfatin and detected the expression of miR-21 by qRT-PCR (Figure 2). Results showed that visfatin induced miR-21 expression in HepG2 cells. The increase in level of miR-21 was shown in a concentration dependent manner. After transfected with miR-21 inhibitors, the upregulation of miR-21 in HepG2 cells by vifatin was significantly inhibited.

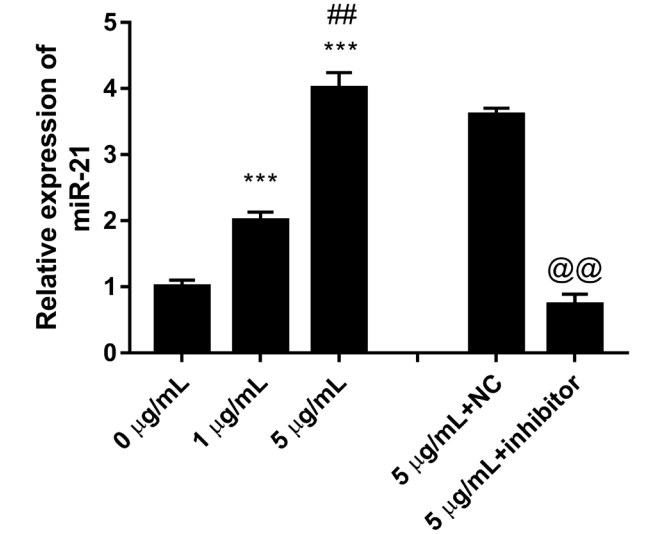

Figure 2. Expression of miR-21 in HepG2 cells with visfatin treatment. HepG2 cells were treated with 0, 1, and 5 μg/mL visfatin for 24 h, then the relative expression of miR-21 was detected by qRT-PCR. The level of miR-21 in cells after transfection of miR-21 inhibitors was also detected. ***P<0.001 vs. 0 μg/mL visfatin, ### P<0.001 vs. 1 μg/mL visfatin, ### P<0.001 vs. 1 μg/mL visfatin, @@ P<0.01 vs. 5 μg/mL visfatin+NC.

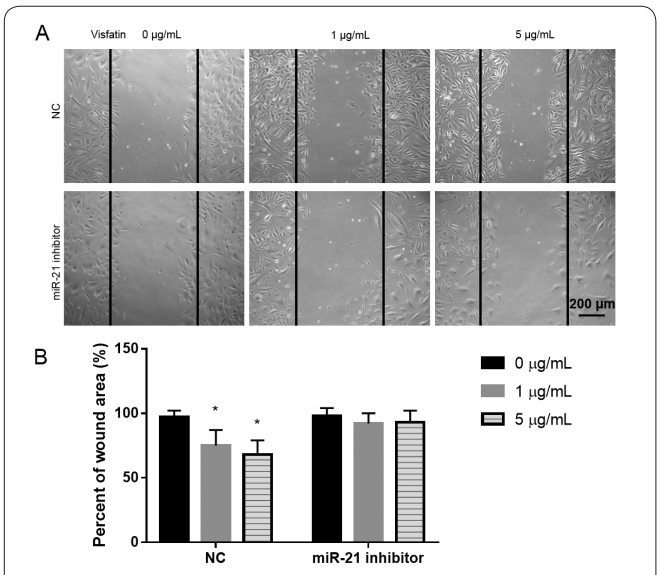

Figure 3. Role of vasfitin and miR-21 in cell migration of HepG2 cells. miR-21 inhibitor and NC transfected HepG2 cells were treated with 0, 1, and 5 μg/mL visfatin for 48 h, then the percent of scratch wound were detected by scratch wound migration assay. (A) Scratch wound. (B) percent of wound area. *P<0.05 vs. NC at 0 μg/mL.

miR-21 inhibitor suppresses visfatin induced migration of HCC cells

Roles of visfatin and its regulated miR-21 expression in migration of HCC cells were detected (Figure 3). Visfatin induced migration of HepG2 cells with concentration. After transfected with miR-21 inhibitor, the visfatin-induced migration of HepG2 cells was significantly inhibited. Thus, visfatin induced HCC cell migration via upregulation of miR-21.

Discussion

Visfatin is an adipocytokine, which has an insulin-like effect, can promote nicotinamide adenine dinucleotide synthesis, and is involved in mediating inflammation and immunity (23-25). In recent years, studies have shown that visfatin can stimulate tumorigenesis and metastasis by stimulating inflammatory mediators and proinflammatory cytokines that are involved in tumor development (26-28). In this study, we found that visfatin significantly associated with the histology and

metastasis, and induced HCC cell migration via upregulation of miR-21.

Studies have shown that the increase of abdominal adipose tissue is not only closely related to metabolic syndrome, IR and liver steatosis, but also directly related to liver tissue inflammation and fibrosis degree (23). In our previous studies, visfatin has been shown to promote the activation of rat hepatic stellate cells (HSCs) and to promote the development of hepatic fibrosis (23).

A number of studies have shown that visfatin is closely related to the occurrence and development of gastric cancer, colon cancer, pancreatic cancer, prostate cancer, and ovarian cancer (17-20, 25, 27). Visfatin is a bioactive factor secreted by visceral adipose tissue. It widely exists in tissues and organs such as the stomach, spleen and liver, and plays an important role in regulating the level of ATP in animals. It can be acted as an adipokine involved in diabetes, obesity and atherosclerosis and other lipid metabolism disorders (29, 30). Studies have shown that visfatin can participate in the development and progression of tumor by promoting angiogenesis, participating in insulin resistance and regulating cell cycle (29, 30). It has found that visfatin can promote the expression of oncogene STAT3 in ovarian cancer, and is involved in the development of breast cancer (31). Visfatin inhibitors such as STF-118804 inhibited the growth of cancer cells such as pancreatic ductal adenocarcinoma (22).

MiRNA is a kind of small non-coding 22-bp endogenous small RNA (32). It participates in the regulation of gene expression at the post-transcriptional level by binding to 3'UTR of target gene mRNA, and thus plays an important role in the regulation of gene expression (33). It was found that miRNA can participate in the regulation of more than one third of human gene expression, affecting a series of life processes, such as cell proliferation, differentiation, apoptosis, and various diseases including HCC development (34).

More and more studies have shown that miR-21 is closely related to the occurrence of a variety of tumors including HCC (35, 36). Studies have shown that upregulation of miR-21 in HCC can shorten the cell cycle, promote vascular growth, and reduce cell death (35, 36). In recent, metastatic mechanism in HCC is receiving more and more attention and researches (37, 38). In this study, we found that visfatin induced HCC cell migration via upregulation of miR-21. The same miRNA can regulate a number of different target genes, and different miRNAs can jointly regulate the same target gene affecting the target gene expression (39, 40). In HCC, PTEN is an important target gene of miR-21, miR-21 regulating cell proliferation, cell growth and tumor invasion through PTEN (41). It was demonstrated that overexpression of miR-21 increased cell survival in HCC via inhibiting the expression of PTEN to activate the PI3K-AKT pathway (42). In this study, our results implied serum visfatin level associates with tumor progression and metastasis, and the malignant degree of HCC. However, the details in the target genes and mechanism remain unclear, which should be explored by further experiments with visfatin inhibitors, nanotechnology, and other tools (43, 44).This study detected the serum levels of visfatin and miR-21 in HCC and healthy subjects, and analyzed their values in the diagnosis of HCC and its relationship with tumor differentiation, invasion and metastasis, which provides the basis for the diagnosis of HCC. However, the molecular mechanism is needed to be investigated in the future.

Acknowledgements

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Conflict of interest

The authors declared none of interest.

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