Original Paper

Tumor-Suppressing Effect of MiR-4458 on Human Hepatocellular Carcinoma

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Key Words

Hepatocellular carcinoma • Apoptosis • Micro-RNAs

Abstract

Background: Besides multiple genetic and epigenetic changes of protein coding genes in hepatocellular carcinoma (HCC), growing evidence indicate that deregulation of miRNAs contribute to HCC development by influencing cell growth, apoptosis, migration, or invasion. *IKBKE* is amplified and over-expressed in a large percentage of human breast tumors and identified as an oncogene of human breast tumor. Microarray analysis showed that miR-4458 was down-regulated in HCC tissues. **Methods:** The level of miR-4458 was up-regulated by miR-4458 mimics transfection, or down-regulated by miR-4458 ASO transfection. Cell proliferation was assayed by MTT analysis. MiRNAs and mRNA expression were assayed by qRT-PCR. These potential targeted genes of miR-4458 were predicted by bioinformatic algorithms. Dual luciferase reporter assay system was used to analyze the interaction between miR-4458 and *IKBKE*. *IKBKE* protein level was assayed by Western blot. The role of miR-4458 or *IKBKE* in the survival of HCC patients were revealed by Kaplan-Meier plot of overall survival. **Results:** Lower miR-4458 expression level or higher *IKBKE* level in HCC tissues correlated with worse prognosis of HCC patients. Overexpression of miR-4458 inhibited the HCC cells growth and vice versa. MiR-4458 played its role via targeting 3'UTR of *IKBKE*. **Conclusions:** MiR-4458 or *IKBKE* may be potential predictors of HCC prognosis. Restoration of miR-4458 or inhibition of *IKBKE* could be a prospective therapeutic approach for HCC.

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Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes for cancer-related death [1]. Although HCC is the sixth most common neoplasm worldwide, poor prognosis makes HCC the third leading cause of cancer-related mortality, responsible for 600,000 deaths annually [2]. In some countries, especially in China, HCC accounted for 70%-85% of primary liver cancer cases [3], with the burden of disease expected to increase in coming years [4]. Besides multiple genetic and epigenetic changes of protein coding genes in HCC [5], growing evidence indicates that deregulation of miRNAs can also contribute to HCC development by influencing cell growth, apoptosis, migration, or invasion [6-22]. Thus, more extensive investigations are needed to identify miRNAs which can be employed as prognosis predictor or therapeutic target for HCC.

Previous study had proved that the human cells can be transformed by the cooperation of activated ERK and signaling pathway of PI3K (phosphatidylinositol 3-kinase) [23]. PI3K signaling can be replaced by various kinases, which can render cell tumorigenic. *IKBKE* (Inhibitor of nuclear factor kappa-B kinase subunit epsilon also known as I-kappa-B kinase epsilon) was found to be amplified and over expressed in breast carcinoma cell lines and patient-derived tumors [23].

Algorithms predicted that miR-4458 could bind the 3' UTR of *IKBKE*, thus we guessed that miR-4458 may play a role in the pathogenesis of HCC. Here, the functions of miR-4458 and its underlying mechanisms in HCC development were investigated. We found that HCC patients with worse prognosis correlated with the lower level of miR-4458 in HCC tissues. Furthermore, the cell growth of HCC cell lines would be suppressed if we restored miR-4458 expression. *IKBKE* was confirmed as the target of miR-4458. Therefore, our finding indicated that miR-4458 is a new prognosis predictor for HCC patients and a new potential therapeutic target for HCC.

Materials and Methods

Human Tissue Specimens

Liver tissue samples were obtained from patients undergoing resection from Eastern Hepatobiliary Surgery Hospital (Shanghai, China) [24]. Tissue samples were frozen immediately in liquid nitrogen until analysis. Normal human liver tissues were obtained from distal normal liver tissue of liver hemangioma. Tissue samples and matched control were achieved from HCC patients. The study was performed according to the Declaration of Helsinki. All patients provided written informed consent. And the study was approved by the Ethics Committee of Second Military Medical University, Shanghai, China. All patients clinical data were recorded by electronic file and we followed the patients status more than 3 years.

Cell Lines and reagent

Huh7 cell line and Normal human hepatic cell line L-02 were maintained in RPMI1640 with 10% FBS (PAA Laboratories, Pasching, Australia). Human HCC cell lines HepG2, Hep3B, SMMC-7721, Huh7 and Normal human hepatic cell line Chang were maintained in DMEM with 10% FBS (PAA Laboratories, Pasching, Australia). All cell lines were obtained from the Cell Bank of Chinese Academy of Science (Shanghai, China). 5-fluorouracil (5-FU) was purchased from Shenggong company (Shanghai, China). The concentration of 5-FU used here is 0.5 µg/ml.

RNA quantification

Expression of miR-4458 was detected with miRCURY LNA Universal RT microRNA PCR kit (Exiqon, Boston) based on the manufacturer's instructions. MicroRNAs quantification was normalized to U6 small nuclear RNA.

MicroRNA target prediction [25-28]

MiRNAs targets were predicted using the algorithms TargetScan (<https://www.targetscan.org>) [29], miRand (<http://www.cbio.mskcc.org/mirnaviewer>) [25], PicTar (<http://pictar.mdc-berlin.de>) [30], miRGen

[31, 32] and miRBase (<http://www.mirbase.org>) [33]. The algorithm produces hundreds of target genes for miRNAs by searching for the presence of conserved 8-mer and 7-mer sites matching the seed region of miRNAs.

MiRNAs mimics, MiRNAs antisense oligonucleotides and overexpression plasmid

MiR-4458 mimics and miR-4458 antisense oligonucleotides (ASO) were obtained from GenePharma (GenePharma, China). Following the manufacturer's instructions, miRNAs mimics, negative control (NC) were transfected into cells at 50 nM concentration with Lipofectamine® 2000 transfection reagent. 48 h, or 72 h later cells were used for further experiments. The overexpression plasmid (pBabe-Neo-Flag-*IKBKE*) and the knock siNRA (si-*IKBKE*) were constructed and confirmed by Shengong Company (Shanghai) according to previous paper [23, 34].

MTT assay

For MTT assay, we cultured 5×10^3 cells per well in a triplicate using a 96 well plate with complete growth medium. We counted the cells over 5 days with the MTT assay (Promega, Fitchburg, WI, USA) as described previously [35, 36].

Apoptosis assay

After different treatments, cells were marked with Annexin V-FITC and PI (propidium iodide) using an apoptosis detecting kit (Invitrogen, Canada) as described previously [37]. Samples were determined by FACS assays and the results were analyzed using CellQuest software (Becton Dickinson, San Jose, CA) [38].

Luciferase Reporter Assay

The 3' UTR fragments of *IKBKE* containing putative binding sites for miR-4458 were cloned into pMIR-Report construct (Ambion, Austin, TX). The primers were synthesized by Shengong company (Shanghai, China) according to the method as detailed elsewhere [39, 40]. And the detail information were showed in previous paper [40]. The complementary site of miR-4458 carried a mutated sequence in the seed region, mutant 3' UTR of *IKBKE*, were generated using fusion PCR. As described previously, luciferase reporter assay was performed in Huh-7 cells [24].

Soft Agar Assay for Colony Formation

Growth of cells in soft agar was determined by 5×10^4 cells in triplicate in 0.4% (HEK) or 0.3% (HMEC) Noble agar. After 8 weeks of plating, colonies greater than 100 μm in diameter were counted by microscope. Colonies greater than 200 nm in diameter were counted macroscopically 8 weeks after planting [23].

Survival-based tissue microarray of HCC

Survival-based tissue microarray of HCC were provided by Jianhua Yin [41]. Tissue microarray were processed and analyzed by Xinchao company (Shanghai, China).

Statistical analysis

Data is presented as the mean $\pm$ SD from at least three independent experiments. The difference between groups was analyzed using two-tailed Student's *t* test when only two groups were compared. The difference between groups was analyzed using ANOVA when three or more than three groups were compared. The Wilcoxon matched-pairs signed rank test was used to determine if there was a statistically significant difference in the expression of miR-4458 or *IKBKE* between matched pairs. Correlation analysis was performed by two-tailed Person's correlation coefficient analysis. Overall survival of patients was estimated by the Kaplan-Meier method. Statistical analysis was performed using SPSS software (version 16.0). $P < 0.05$ was considered significantly different.

Results

Lower miR-4458 expression level in HCC tissues correlated with worse prognosis of HCC patients

Expression of miR-4458 in HCC cell lines were assayed by qRT-PCR. As comparing with normal liver, miR-4458 expression in HepG2, Hep3B, SMMC-7721 Huh-7 and Chang is lower. (Fig. 1A). 30 human hepatocellular carcinoma tissues were collected. Expression

Fig. 1. MiR-4458 expression in HCC and HCC patient survival analysis according to miR-4458. A, miR-4458 expression in normal liver, L-02, HepG2, Chang, Hep3B, SMMC-7721 and Huh-7 were assayed by qRT-PCR. MiR-4458 quantification were normalized to U6 small nuclear RNA. The miR-4458 expression in normal liver was arbitrary defined as 100%. Data was the mean $\pm$ SD of three independent test. B, 30 human hepatocellular carcinoma tissues and their paired normal tissues were assayed for miR-4458 expression by qRT-PCR. miR-4458 quantification were normalized to U6 small nuclear RNA. The miR-4458 expression in normal liver was arbitrary defined as 100%. Data was the mean $\pm$ SD of 30 samples. C, miR-4458 expression in 30 human hepatocellular carcinoma tissues and in their matched normal tissues were compared by qRT-PCR. miR-4458 quantification were normalized to U6 small nuclear RNA. Data was the mean $\pm$ SD of three independent test for each sample. D, Kaplan-Meier plot of overall survival in HCC patient post-operation according to the expression of miR-4458. MiR-4458 median value of all 30 cases was

chosen as the cutoff point for separating miR-4458 high expression tumors ($n=15$) from miR-4458 low expression cases ($n=15$). *, $P<0.05$.

of miR-4458 in 30 human hepatocellular carcinoma tissues were assayed. We found that the average expression of miR-4458 in HCC tissues was lower than paired normal human liver tissues (Fig. 1B). In the 30 HCC specimens, there were 23 specimens, in which the miR-4458 expression is lower than matched normal tissues (Fig. 1C). To evaluate the clinical significance of miR-4458 in HCC, we investigated whether the level of miR-4458 expression was associated with overall survival in HCC patients. 30 HCC patients have been followed-up for 30 months. During follow-up period, 11 out of 30 (35%) patients died as a result of disease progression. Survival curve showed that patient with high miR-4458 level (15 case) had a significantly longer overall survival than those with low miR-4458 level (15 case) (Fig. 1D).

MiR-4458 inhibited the HCC cells viability in vitro

For studying the role of miR-4458 in HCC, miR-4458 in SMMC-7721 and Huh-7 were over-expressed by miR-4458 mimics, and confirmed by qRT-PCR 48 h or 72 h after mimics transfection (Fig. 2A). To investigate the role of miR-4458 in cell proliferation, MTT assay

Fig. 2. MiR-4458 mimics inhibited SMMC-7721 and Huh-7 cells proliferation. A, SMMC-7721 and Huh-7 cells (5×10^5 cells/well) were transfected with miR-4458 mimics or miR-NC (NC) separately. 48h or 72h later, the miR-4458 expression was assayed by qRT-PCR. Data were normalized to U6 small nuclear RNA. The miR-4458 expression in normal liver tissue were arbitrarily defined as 100%. Data are mean $\pm$ SD of three separate independent sample. B, After the miR-4458 mimics transfection, the cell proliferation were assayed by MTT at the indicted time points (from 1 to 4 hours after transfection). Data are mean $\pm$ SD of three independent sample assays. C, After 48 h miR-4458 mimics transfection, the cell apoptosis rate of SMMC-7721 and Huh-7 cells was assayed by FACS. D, Following miR-4458 mimics transfection, SMMC-7721 and Huh-7 cells were treated 5-Fu (0.5 μ g/ml). 48 h later, the cell apoptosis rate of SMMC-7721 and Huh-7 cells was assayed by FACS analysis. Data are mean $\pm$ SD of three separate assays*, $P < 0.05$.

was performed, and results showed that up-regulation of miR-4458 in SMMC-7721 and Huh-7 inhibited cells proliferation (Fig. 2B). Cell apoptosis assay showed that miR-4458 induced more cell apoptosis in SMMC-7721 and Huh-7 (Fig. 2C). Besides, SMMC-7721 and Huh-7 were pretreated with 5-Fu, then the cell apoptosis was analyzed, we found that, still, miR-4458 induced more cell apoptosis under the treatment of 5-Fu (Fig. 2D).

Down-regulation of miR-4458 promoted cells proliferation

As miR-4458 expression in L-02 and HepG2 cell lines were higher than other cell lines, we down-regulated the miR-4458 expression by transfection of miR-4458 ASO. We found that miR-4458 ASO transfection down-regulated the miR-4458 level to 40% level of control (Fig. 3A). Then, cell proliferation were assayed by MTT, we found that miR-4458 ASO promoted cell proliferation as expected (Fig. 3B). Next, Cell apoptosis assay showed that miR-4458 ASO inhibited cell apoptosis in L-02 and HepG2 cell lines than control (Fig. 3C). Similarly, L-02 and HepG2 cell were pretreated with 5-Fu, then the cell apoptosis was analyzed, we found that, still, miR-4458 ASO inhibited cell apoptosis under the pretreatment of 5-Fu (Fig. 2D).

MiR-4458 targeted *IKBKE*

To identify which genes miR-4458 targets, bioinformatic algorithms were applied. We found that miR-4458 could bind 11 genes (Fig. 4A). Of these predicated genes, *IKBKE* was identified as a breast cancer oncogene [23]. For the importance of *IKBKE* and our interests, *IKBKE* was chosen for further study.

To confirm whether miR-4458 could target *IKBKE*, the putative miR-4458 binding site in 3' UTR *IKBKE* was mutated (Fig. 4B). A dual luciferase reporter gene assay was performed

Fig. 3. MiR-4458 ASO promoted L-02 and HepG2 cells proliferation. A, L-02 and HepG2 cells (5×10^5 cells/well) were transfected with miR-4458 ASO or control separately. 48h or 72h later, the miR-4458 expression were assayed by qRT-PCR. Data were normalized to U6 small nuclear RNA. The miR-4458 expression in normal liver tissue were arbitrary defined as 100%. Data are mean $\pm$ SD of three separate independent sample. B, 48 h after the miR-4458 ASO transfection, the cell proliferation were assayed by MTT at the indicted time points (from 1 to 4 hours after transfection). Data are mean $\pm$ SD of three independent sample assays. C, 48 h after miR-4458 ASO transfection, the cell apoptosis rate of L-02 and HepG2 cells was assayed. D, Following miR-4458 ASO transfection, L-02 and HepG2 cells were treated 5-Fu (0.5 μ g/ml). 48 h later, the cell apoptosis rate of L-02 and HepG2 cells was assayed by FACS analysis. Data are mean $\pm$ SD of three separate assays*, $P < 0.05$. *, $P < 0.05$.

to test whether miR-4458 could repress the expression of IKBKE. We constructed luciferase reporter plasmids containing wild-type or mutated 3' UTR of IKBKE. We found that miR-4458 mimics significantly reduced the luciferase activity of IKBKE reporter with wild-type 3' UTR but had no dramatic effect on the luciferase activity of the IKBKE reporter with mutated 3' UTR (Fig. 4C). Next, we transfected Huh-7 cells with miR-4458 mimics, 48h later, the IKBKE protein expression was inhibited (Fig. 4D). Person's correlation coefficient analysis showed that there was an inverse correlation between miR-4458 and IKBKE mRNA in the HCC cell lines and normal lung tissue (Fig. 4E). Thus, our data indicated that miR-4458 inhibited IKBKE expression.

High expression of IKBKE correlated with short overall survival

To investigate the role of IKBKE expression in HCC, HCC tissue microarray of 222 patients' samples was analyzed. We found that IKBKE protein expression was higher in HCC tissue than in normal liver tissue (Fig. 5A, B). In the figure 5B, we found that IKBKE was significantly increased in 200 of 222 HCC tissues compared with the matched adjacent normal tissues, suggesting that IKBKE may be an important protein associated with the development of HCC. To evaluate the clinical significance of IKBKE overexpression in HCC, we investigated whether the levels of IKBKE expression were associated with overall survival in HCC. 222 HCC patients had been followed-up for 3 years. Kaplan-Meier curves indicated that patients with higher IKBKE expression had a significantly shorter overall survival ($p < 0.05$) than those with lower IKBKE expression (Fig. 5C). To confirm the effect of IKBKE, we transfected IKBKE overexpression plasmid into L-02 and HepG2, and found the colony number of tumor cell was increased (Fig. 5D). Then, we over-expressed the IKBKE after the miR-4458 transfection, we found that IKBKE over-expression increased the cell numbers, and partly reduced the inhibitory effect of miR-4458 in SMMC-7721 and Huh-7 (Fig. 5E). Next we down-regulated

Fig. 4. MiR-4458 targeted *IKBKE*. A. 11 putative miRNA were predicted by bioinformatic analysis. B. The mutation of *IKBKE* 3' UTR (miR-4458 binding site). C. Analysis of luciferase activity. Huh7 cells were co-transfected with indicated RNA duplex, pRL-TK, and pMIR-Reporter firefly luciferase reporter plasmid containing wild-type or mutant 3' UTR of putative target gene. Firefly luciferase activity was measured and normalized by Renilla luciferase activity 24h after transfection. Data are mean $\pm$ SD of separate transfection (n=6) The normalized luciferase activity of NC transfectant in each experiment was set as relative luciferase activity 1. D. the *IKBKE* protein in Huh7 cells were assayed by Western blot 48h after miR-4458 transfection. E. Inverse correlation between miR-4458 and *IKBKE* mRNA in the HCC cell lines and normal lung tissue. Data are mean $\pm$ SD of three independent sample assays. *, P<0.05.

the *IKBKE* expression by si-*IKBKE* transfection, we found that down-regulation of *IKBKE* and miR-4458 mimics transfection both decreased the cell numbers (Fig. 5F).

Discussion

A number of miRNAs has been suggested to play important roles in HCC development [3-22]. Thus, exploring and understanding the more aberrantly expressed miRNAs may help to better reveal the mechanisms underlying HCC carcinogenesis and progression [24]. Here, we identified the role of miR-4458 in HCC, and proved that miR-4458 is a new prognosis predictor for HCC patients and a new potential therapeutic target for HCC. It is very important to identify the molecular markers correlating with the survival of cancer patients. Deregulated expression of both coding genes and miRNAs has been suggested to have considerable potential predicting the prognosis of HCC patients [16]. Previous reports showed that deregulated miR-26 miR-29, miR-199a/b-3p and miR-99a correlated with the survival of HCC [16, 24, 42, 43]. Detecting the expression level of these miRNAs, in combination with miR-4458 and other coding genes, may be valuable to predict the prognosis of HCC patients more accurately.

With informatics prediction and sequential experimental demonstration. *IKBKE* were identified as target of miR-4458 in our study. *IKBKE* has been identified as a breast cancer oncogene [23]. Our clinical data showed that *IKBKE* was significantly increased in 200 of 222 HCC tissues compared with the matched adjacent normal tissues, suggesting the importance of *IKBKE* in the development of HCC. We also demonstrate that higher expression of *IKBKE* in

Fig. 5. Higher IKBKE expression level in HCC tissue correlates with worse prognosis of HCC patients. **A.** Immunohistochemical assay of IKBKE in HCC tissues and matched adjacent normal tissues (Peri-HCC). 6 representative samples were shown. **B.** IKBKE protein expressed level in tumor were compared with IKBKE protein expressed level in matched adjacent normal tissues ($n=222$). **C.** Kaplan-Meier plot of overall survival in HCC patients post-operation according to the immunostaining results of IKBKE. IKBKE median value of all 222 cases was chosen as the cutoff point for separating IKBKE high expression tumors ($n=111$) from IKBKE low expression cases ($n=111$). **D.** 24h after IKBKE overexpression plasmid transfection, the colony formations of L-02 and HepG2 were counted. **E.** SMMC-7792 and Huh-7 (6×10^5 cells/well) were transfected with miR-4458 or miR-NC (NC) separately. 12h later, these cell were transfected with empty plasmid, or IKBKE overexpression plasmid. Then the MTT assay were performed. The OD value from MTT assay were presented as the relative cell number. The relative cell number in miR-NC+Vector (negative control) was defined as 100%. NS, not significantly, Data are mean $\pm$ SD of three independent sample assays. **F.** SMMC-7792 and Huh-7 (6×10^5 cells/well) were transfected with miR-4458 or miR-NC (NC) separately. 12h later, these cell were transfected with si-NC, or si- IKBKE Then the MTT assay were performed. The OD value from MTT assay were presented as the relative cell number. The relative cell number in miR-NC+ si-NC, (negative control) was defined as 100%. NS, not significantly, Data are mean $\pm$ SD of three independent sample assays. *, $P < 0.05$.

HCC tissues significantly correlated with shorter survival of HCC patients. Our data indicated that *IKBKE* protein may be a predictor of prognosis of HCC. Similarly, we proved that high expression of *IKBKE* led the colony number increased in HCC cell lines as in breast cancer cells [23], indicating that *IKBKE* may play the similar roles in HCC.

Informatics prediction showed that *NRAS* and *EZH2* are the potential targeted genes of miR-4458. Previous study showed that *NRAS* are key cancer-related gene in the RAS/RAF signaling pathways which play central roles in carcinogenesis of HCC [44]. *EZH2* in HCC could be targeted by miR-26a which inhibits HCC proliferation [45]. Whether miR-4458 plays its role via *NRAS* and *EZH2* needed further investigation.

HCC is the most fatal cancer around the world, especially in China. HCC confers a poor prognosis when diagnosed at advanced stages. The diminished treatment efficacy may be due to multidrug resistance transporter protein and hyper-activated drug-metabolizing pathways. Thus, alternative approaches are urgently needed. Tumor inhibition effect of miR-4458 showed in our data indicated the therapy drug possibility of miR-4458. Also, viral and nonviral vectors are being developed by delivery of synthetic RNA to liver [46-48]. Hence, restoration of miR-4458 should have a considerable potential for HCC molecular therapy.

In conclusion, we proved the role of miR-4458 in HCC, and miR-4458 inhibited HCC cell viability via targeting *IKBKE*. We also highlight *IKBKE* as a HCC prognosis predictor.

Disclosure Statement

The authors have declared that no competing interests exist.

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