MicroRNA-550a Acts as a Pro-Metastatic Gene and Directly Targets Cytoplasmic Polyadenylation Element-Binding Protein 4 in Hepatocellular Carcinoma

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Abstract

MicroRNAs (miRNAs) are a class of small, non-coding RNA molecules that are often found at chromosomal breakpoints and play a vital role in human cancer. Our previous study found that miR-550a, a frequently amplified miRNA on 7p14.3, was upregulated in hepatocellular carcinoma (HCC). However, the possible functions and molecular mechanisms of miR-550a in HCC remain unknown. In this study, gain-of-function and loss-of-function assays revealed that miR-550a markedly promoted HCC cell migration and invasion. In addition, we discovered that cytoplasmic polyadenylation element binding protein 4 (CPEB4) was a potential target of miR-550a in HCC. Further analyses showed that knockdown of CPEB4 expression significantly facilitated HCC cell migration and invasion, which phenocopied the effects of miR-550a on HCC cells. Moreover, a decrease in CPEB4 expression mediated miR-550a-induced liver cancer cell migration and invasion. Interestingly, CPEB4 is frequently downregulated in HCC, and its expression levels correlate with the overall survival of HCC patients. Together, these results suggested that this newly identified miR-550a-CPEB4 axis may be involved in HCC cell metastasis. Moreover, the expression levels of CPEB4 could be used to predict outcomes in HCC patients. Our findings provide novel potential targets for HCC therapy and prognosis.

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Competing Interests: The authors have declared that no competing interests exist.

Abstract

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Introduction

MicroRNAs (miRNAs) are small, single-stranded, non-coding RNAs that are highly conserved between species [1]. They are key post-transcriptional regulators of gene expression that act mainly via binding to the 3′ untranslated regions (3′ UTRs) of target mRNAs and thus participate in various biological processes [2,3,4,5]. It has been predicted that miRNAs could regulate 60% of human genes, which makes miRNAs a powerful regulator in human physiology and pathology, including cancer [4,6,7,8]. In the last decade, emerging evidence has indicated that miRNAs are differentially expressed and play critical roles in many cancers [6,9].

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers, particularly among East Asian and Southeast Asian populations [10]. Half of HCC cases and deaths worldwide are estimated to occur in China [11]. Despite its high lethality, the molecular mechanism underlying HCC remains largely unknown. As one hallmark of cancer, genomic instability leads to the over- or under-expression of genes and enables cancer cells to acquire multiple mutations, which leads to the initiation and development of cancer phenotypes [6,12,13,14].

In the last decade, studies have revealed many chromosomal breakpoints related to genomic instability and identified numerous oncogenes/tumor suppressors that are involved in cancer pathogenesis. However, the non-coding RNAs located at chromosome breakpoints are largely unknown. Our laboratory has extensively analyzed the miRNAs in common recurrent chromosomal aberration regions and identified some miRNAs that were aberrantly expressed in HCC [12]. Among the miRNAs associated with the chromosomal breakpoints, miR-151 and miR-30d were found to be critical regulators of HCC invasion and metastasis. However, the roles of the remaining miRNAs identified are still unexplored in HCC. Therefore, we performed a preliminary functional screen of the remaining miRNAs and found that miR-550a could regulate HCC cell motility. miR-550a is located in 7p14.3, which is frequently amplified in many cancers, such as gastric carcinomas [15], malignant peripheral nerve sheath tumors (MPNSTs) [16,17], malignant mesotheliomas (MMs) [18], nasopharyngeal carcinoma (NPC) [19], esophageal squamous cell carcinoma (ESCC) [20], seminomas [21] and HCC [22]. Gene gains have also been reported at this site. For example, a gain of TCRG (TCR gene loci) was observed in T-cell lymphoma [23]. These findings suggested that genes located in this site may play
miR-550a is often upregulated in HCC and promotes HCC cell migration and invasion. (A, B) The relative expression of mature miR-550a in 48 pairs of HCC tissues and their corresponding noncancerous liver tissues were measured using TaqMan real-time PCR and normalized to U6 snRNA. (C, D) Transwell migration and invasion assays of Huh-7 and SK-Hep1 cells stably expressing miR-550a or mock control. (E, F) Transwell migration and invasion assays of MHCC-97L and SMMC-7721 cells transfected with a miR-550a inhibitor or negative control. Representative images are shown on the left, and quantification is shown on the right. The results are representative of at least three independent experiments, and the values shown are the mean ± SD.

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Materials and Methods

Human Liver Tumor Samples/Ethics Statement
HCC, the matched noncancerous liver tissues (3 cm from the tumour) and the cirrhosis liver tissues were obtained from the surgical specimen archives of the Qidong Liver Cancer Institute, Jiangsu Province, China. Ten normal liver tissue samples were obtained from people who died from accidents. Participants that these samples were obtained from provided their written informed consent to participate in the study, and the Ethical Review Committee of the WHO Collaborating Center for Research in...
Human Production authorized by the Shanghai Municipal Government approved this study as well as the consent procedure.

Cell Culture

HEK 293T, HepG2, Huh-7, SK-Hep-1, SMMC-7721, MHCC-LM3 and MHCC-97L cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum.
(FBS) and antibiotics. SNU-449 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and antibiotics.

RNA Extraction and Quantitative Real-time PCR
Total RNA was extracted with TRIzol reagent (Invitrogen). cDNA was synthesized with the PrimeScript RT reagent Kit (TaKaRa). Real-time PCR was performed with SYBR Premix Ex Taq (TaKaRa). Mature miRNAs were quantified with specific primers and probes using TaqMan microRNA Assays (Applied Biosystems). The primers used are listed in Table S1.

Vector Constructs
In the miR-550a lentivirus expression vector pWPXL-miR-550a, the primary miRNA sequence amplified from normal genomic DNA replaced the green fluorescent protein fragment of the pWPXL mock vector. In the luciferase reporter vector, the wild-type or mutant 3’UTR of CPEB4 was cloned downstream of the stop codon in the luciferase gene. Other potential target genes were cloned in a similar manner. The sequences of the primary miRNA and wild-type and mutant 3’UTR were confirmed by sequencing. The primers and the detail of miR-550a vector as well as 3’UTRs are separately listed in Table S2 and S3.

Lentivirus Production and Transduction
Virus particles were harvested from HEK 293T cells 48 h after pWPXL-miR-550a transfection with the envelope plasmid pMDG2 and the packaging plasmid psPAX2 using Lipofectamine 2000. Huh-7 and SK-HEP-1 cells were infected with recombinant lentivirus-transducing units and 6 µg/mL polybrene.

Oligonucleotide Transfection
The miRNA mimics and small interfering RNAs (siRNA) targeting CPEB4 were designed and synthesized by GenePharma. The miR-500a inhibitor was synthesized by Ribobio. Cells were transfected with mimic or inhibitor using Lipofectamine 2000, while siRNA transfection was performed with Lipofectamine RNAi MAX reagents according to the manufacturer’s instructions (Invitrogen). Commonly, 48 hours after transfection, cells were used in experiments. The sequences of siRNAs used are shown in Table S4.

Cell Proliferation Assays
Cell proliferation was assessed by the Cell Counting Kit-8 (CCK-8) assay kit (Dojindo Corp.). Approximately 10³ cells were seeded in each well of a 96-well plate, and 10 µL CCK-8 was added to 90 µL culture medium. After incubation at 37°C for 2 h, the absorbance was detected at 450 nm and the OD450 value is correlated with the number of live cells.

Migration and Invasion Assays
A 24-well plate containing 8 µm-pore size chamber inserts (BD Biosciences) was used to evaluate the migration and invasion of tumor cells. For the invasion assay, the membrane was coated with Matrigel to form a matrix barrier, and 10³ cells were placed in the upper chamber. For the migration assay, only 5 x 10⁴ cells were seeded in the upper chamber. In each lower chamber, 800 µL of Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) was added. After several hours of incubation at 37°C, cells that had migrated through the pore were fixed and stained with a mixture of 20% methanol and 0.1% crystal violet for 0.5 h. Then, the cells were photographed and counted under an IX71 inverted microscope.

Luciferase Reporter Assay
HEK 293T cells were cultured in 96-well plates and transfected with 50 ng puc3-3’UTR, 10 ng Renilla and 5 pmol miR-550a mimic or negative control. After 48 h of incubation, luciferase activity was detected with the dual-luciferase reporter assay system (Promega).

Western Blot Assays
Cell lysates were separated with 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with rabbit anti-CPEB4 polyclonal antibody (Abcam, Sigma), mouse anti-β-actin (Sigma) or mouse anti-GAPDH monoclonal antibody (Kangchen). The proteins were visualized using enhanced chemiluminescence reagents (Thermo Scientific).

Immunohistochemical Staining (IHC)
All of the samples were processed to analyze the expression of the CPEB4 protein in HCC tissues, matched noncancerous tissues, cirrhotic tissues and normal liver tissues. Paraffin-embedded tissues were cut into 5 µm thick sections and analyzed with IHC using a rabbit antibody against CPEB4 (Sigma). Scoring was based on the proportion of positively stained cells: <5% was scored as 0; 5–24% was scored as 1; 25–49% was scored as 2; 50–74% was scored as 3; and more than 74% was scored as 4.

Statistical Analysis
The data are shown as the mean ± standard deviation (SD). Statistical analyses were performed with a two-tailed Student t-test unless otherwise specified. Differences were considered statistically significant at p<0.05. Asterisks were used to represent statistical significance of p values in some figures, e.g. *p≤0.05, **p≤0.01, ***p≤0.001.

Results
miR-550a is Frequently Upregulated in HCC and Accelerates HCC Cell Migration and Invasion
To validate the expression of miR-550a in HCC, we detected mature miR-550a in 48 pairs of HCC and matched noncancerous liver tissues by real-time PCR. The results indicated that miR-550a expression was upregulated in 58% of HCC tissues compared with the noncancerous liver tissues (Figure 1A, B). In addition, the endogenous expression of miR-550a in various liver cancer cell lines was evaluated. miR-550a expression was relatively low in Huh-7 cells, whereas SMMC-7721 and MHCC-97L cells had a relatively high basal level of miR-550a (Figure S1).

To examine the biological function of miR-550a in HCC, a miR-550a mimic was transfected into two HCC cell lines to determine whether it could affect cell proliferation and motility. CCK-8 assays suggested that miR-550a did not influence HCC cell growth (Figure S2A, B), and transwell assays with or without Matrigel indicated that miR-550a markedly induced migration and invasion in HCC cell lines (Figure S3A, B). Next, a lentivirus vector expressing miR-550a was constructed and used to infect Huh-7 and SK-HEP1 cells to establish stable cell lines because these cell lines exhibited relatively low endogenous miR-550a levels. The expression of miR-550a in the two stable cell lines was measured by real-time PCR (Figure S4). In transwell assays, Huh-7 cells overexpressing miR-550a showed enhanced migratory and invasive abilities compared with vector overexpressing cells (Figure 1C, D). Similar results were obtained in SK-HEP1 cells (Figure 1C, D). To further confirm these findings, a miR-550a inhibitor was transfected into SMMC-7721 and MHCC-97L cells.
A

a Case 1 (HCC)  
b Case 1 (Noncancerous)  
c Case 2 (HCC)  
d Case 2 (Noncancerous)  
e Cirrhosis  
f Normal liver

B

The expression level of CPEB4 protein

| Scores | 0 | 1 | 2 | 3 | 4 |
|--------|---|---|---|---|---|
| Normal liver | 0 | 0 | 0 | 0 | 10 |
| Cirrhosis | 0 | 1 | 0 | 10 | 5 |
| Noncancerous | 0 | 3 | 10 | 76 | 147 |
| HCC | 21 | 40 | 47 | 54 | 74 |

p (HCC, Noncancerous) < 0.0001  
p (HCC, Normal liver) = 0.0004  
p (HCC, Cirrhosis) = 0.0070

C

Survival rate

Low
High

**p < 0.001**

time (day)

0.00  500.00  1000.00  1500.00  2000.00
miR-550a acts by repressing CPEB4 expression in HCC

To clarify the effects of CPEB4 in HCC cells, siRNAs against CPEB4 were designed and transfected into HEK 293T cells. The mRNA and protein levels of CPEB4 were markedly decreased, particularly in the si-CPEB4-1 group which was used in further experiments (Figure 4A, B). Next, SMMC-7721 cells were transfected with siRNAs and subjected to transwell assays. The results indicated that SMMC-7721 cells transfected with siRNA against CPEB4 exhibited enhanced migration and invasion potential (Figure 4C). These results mimicked the phenotype induced by miR-550a overexpression and further suggested that CPEB4 may be a functional target of miR-550 in HCC.

We next attempted to determine whether CPEB4 was involved in miR-550a-induced HCC cell migration and invasion. The miR-550a inhibitor and siRNAs targeting CPEB4 were co-transfected into SMMC-7721 cells in which miR-550a expression was relatively high. The subsequent transwell assays demonstrated that CPEB4 knockdown partly neutralized the suppressive effects of the miR-550a inhibitor on HCC cell migration and invasion (Figure 4C). These data provided further evidence that CPEB4 could inhibit miR-550a-induced HCC cell migration and invasion, suggesting that CPEB4 is a direct and functional target of miR-550 in HCC.

If miR-550a actually regulates the expression of CPEB4 in HCC, then the expression of these two factors should be inversely correlated in HCC. Therefore, we evaluated the expression of CPEB4 mRNA in various liver cancer cell lines (Figure 4D). The results showed that the mRNA level of CPEB4 was inversely correlated with the expression of miR-550a in these cell lines (Figure 4E). To extend our analysis to clinical cases, we assessed the mRNA level of CPEB4 in the previous 48 cases of HCC and the adjacent noncancerous liver tissues. CPEB4 mRNA was downregulated in HCC tissues compared with their respective noncancerous liver tissues (Figure 4F), consistent with the observed CPEB4 protein levels. Moreover, the downregulation of CPEB4 was correlated with the upregulation of miR-550a in these HCC samples (Figure 4G). These data suggest that CPEB4 mRNA expression is negatively correlated with miR-550a expression in HCC.

Discussion

In this study, we found that miR-550a is frequently upregulated in HCC and facilitates HCC cell migration and invasion. The direct, functional miR-550a target gene CPEB4 is commonly suppressed in HCC, and its expression is correlated with HCC patient outcome.

According to miRBase, hsa-miR-550 is located in chromosomal region 7p14, and two members of the hsa-miR-550 family have been identified: hsa-miR-550a and hsa-miR-550b. In a previous study...
MiR-550a Targets CPEB4 in HCC Metastasis

A. HEK 293T

Relative expression level of CPEB4 mRNA

B. SMMC-7721

Migration/invasive cells per field

C. nc miR-550a inhibitor si-CPEB4

anti-CPEB4

anti-β-actin

D. Relative expression level of CPEB4

E. miR-550a-U6

F. MHC-LM3

CPEB4/Actin

G. MHC-LM3

CPEB4/Actin

HCC Noncancerous
study from our group, miR-550a was identified in a screen and referred to as miR-550-2 [12]. We found that the miR-550a DNA copy number was distinctly amplified in HCC, and its expression was evidently upregulated. In this study, we verified the upregulation of miR-550a in an independent cohort of HCC samples, and the results were consistent with those of our previous report. Recent studies have demonstrated that miR-550 is differentially expressed in gastritis and gastric extranodal marginal zone lymphoma and may have a role in the transition from gastritis to monoclonal B-cell lymphoma [28]. In addition, miR-550 is differentially expressed in childhood acute lymphoblastic leukemia [29]. Together with our results, these findings indicated that the deregulation of miR-550a could be common to several cancers and may have a functional role. Additionally, miR-550 has been found to be upregulated in prolactinomas following bromocriptine treatment [30]. Furthermore, miR-550 is among the eight microRNAs that predict sensitivity to prednisone in childhood acute lymphoblastic leukemia [29]. These results suggest that miR-550 may act as an indicator of treatment responses in different cancers and is worthy of further investigation. To the best of our knowledge, although miR-550 was found to be deregulated in several tumors, there is little data about the function of miR-550a. In this study, for the first time, we found that miR-550a could promote the migration and invasion of HCC cells. Meanwhile, miR-550a expression was associated with the vascular invasion of HCC, which may be due to the invasion-promoting function of miR-550a in HCC.

This mechanistic insight into the effect of miR-550a on cell migration and invasion suggested that the target gene CPEB4 mediated the function of miR-550a in HCC. CPEB4 belongs to the cytoplasmic polyadenylation element-binding protein family, the members of which mainly regulate translation by controlling the polyadenylation of target genes. The CPEB family contains two subfamilies, CPEB1 and CPEB2. Although the biological function of CPEB1 has been studied extensively, the function of CPEB4, a member of the CPEB2 subfamily, remains largely unexplored. It has been reported that CPEB4 acts as a cell survival protein in neurons [31] and regulates meiotic cells [25]. In pancreatic ductal cancer and neuroblastoma, the expression of CPEB4 is upregulated, driving the growth and invasion of cancer cells [27]. In this study, we found that the mRNA and protein levels of CPEB4 were often downregulated in HCC. The tissue-specific feature and some other factors which are unexplored may attribute to this phenomenon in different kind of tumor tissues. Furthermore, we found that CPEB4 siRNA could promote the migration and invasion of HCC cells. Our results contradict those of a previous report. We assume that this contradiction might be due to the different downstream targets regulated by CPEB4 in different cells because CPEB4 can control the translation of many genes by binding to the CPE sequence in their 3′ UTR [32]. Intriguingly, relatively high levels of CPEB4 predicted a better outcome in HCC patients. These results indicated that CPEB4 could act as a prognostic factor in HCC. We also found that miR-550a and CPEB4 expression were inversely associated in HCC samples, which suggested that the downregulation of CPEB4 in HCC may be at least partially due to the upregulation of miR-550a. The regulation of CPEB4 by microRNAs has also been reported by Morgan et al. [32]. They found that members of the CPEB2 subfamily could be co-regulated by microRNAs through a conserved sequence in their 3′ UTR. Together with our results, these findings demonstrate that microRNA regulation is a common phenomenon in CPEB4 regulation.

In summary, our findings show that increased miR-550a expression due to DNA amplification increased the migratory and invasive abilities of HCC cells. Knockdown of the miR-550a target gene CPEB4 enhanced the migration and invasion of HCC cells. Importantly, CPEB4 expression is correlated with HCC patient outcome, and miR-550a/CPEB4 may represent a promising prognostic and therapeutic target in HCC.

Supporting Information

Figure S1 The relative expression of miR-550a in various liver cancer cells. The relative expression level of mature miR-550a was detected by TaqMan real-time PCR. The data were normalized to U6 snRNA. (TIF)

Figure S2 miR-550a has no significant effects on HCC cell growth in vitro. (A) CCK-8 assays of Huh-7 and HepG2 cells were performed every other day after transfection with a miR-550a mimic or negative control (nc). (B) Transwell invasion assays of SMMC-7721 cells transfected with a miR-550a inhibitor, CPEB4 siRNA or a negative control (nc). The protein level of CPEB4 was detected through western blot assays. There is statistical significance between group 1 and group 2 as well as between group 2 and group 4. The results are presented as the mean ± SD. (D) The relative expression of CPEB4 at the mRNA level was analyzed through real-time PCR, normalized to β-actin. (E) The correlation between CPEB4 expression and mature miR-550a in various liver cancer cell lines was analyzed by linear regression. (F) The miRNA level of CPEB4 was determined in 48 pairs of HCC tissues and matched noncancerous liver tissues by real-time PCR. (G) The correlation between CPEB4 expression and mature miR-550a was analyzed in the same HCC samples by linear regression. The expression data were normalized to β-actin and U6 snRNA, respectively. doi:10.1371/journal.pone.0048958.g004

Figure 4. CPEB4 suppresses miR-550a-induced migration&invasion and its expression is inversely correlated with miR-550a in HCC. (A, B) Real-time PCR and western blot of CPEB4 expression in HEK 293T cells transfected with siRNAs targeting CPEB4 or a negative control (nc). In (A), there is statistical significance between the nc group and either the si-RNA group. And the data are shown as the mean ± SD. (C) Transwell migration and invasion assays of SMMC-7721 cells were performed after transfection with a miR-550a inhibitor, CPEB4 siRNA or a negative control (nc). The protein level of CPEB4 was detected through western blot assays. There is statistical significance between group 1 and group 2 as well as between group 2 and group 4. The results are presented as the mean ± SD. (D) The relative expression of CPEB4 at the mRNA level was analyzed through real-time PCR, normalized to β-actin. (E) The correlation between CPEB4 expression and mature miR-550a in various liver cancer cell lines was analyzed by linear regression. (F) The miRNA level of CPEB4 was determined in 48 pairs of HCC tissues and matched noncancerous liver tissues by real-time PCR. (G) The correlation between CPEB4 expression and mature miR-550a was analyzed in the same HCC samples by linear regression. The expression data were normalized to β-actin and U6 snRNA, respectively. doi:10.1371/journal.pone.0048958.g004

Figure S3 A miR-550a mimic facilitates HCC cell migration and invasion in vitro. (A) Transwell migration assays of Huh-7 and SMMC-7721 cells transfected with the miR-550a mimic or vector control, CPEB4 siRNA or a negative control (nc). In (A), the correlation between CPEB4 expression and mature miR-550a in various liver cancer cell lines was analyzed by linear regression. The expression data were normalized to β-actin and U6 snRNA, respectively. doi:10.1371/journal.pone.0048958.g004

Figure S4 The expression of miR-550a in stable cell lines. The relative expression level of mature miR-550a was detected by TaqMan real-time PCR. The data were normalized to U6 snRNA. (TIF)

Figure S5 The identification of potential miR-550a target genes. (A) The mRNA expression levels of the predicted genes in Huh-7 and SK-Hep1 cells expressing miR-550a or vector were evaluated by real-time PCR. (B) Dual-luciferase activity assays were used to determine the binding potential between miR-550a and the 3′UTR of these candidate genes. Renilla luciferase activity was detected as an internal control. (C) Western blot assays of the TRAK2 and CPEB4 protein levels in Huh-7, SMMC-7721 and HEK 293T cells.
MiR-550a Targets CPEB4 in HCC Metastasis

Table S1 The primer sequences for real-time PCR.

Table S2 The nested PCR primer sequences for miR-550a or 3′UTR of potential target genes.

Table S3 The information of miR-550a and its 3′UTR vectors.

Table S4 The sequences of siRNAs against CPEB4.

Table S5 The possible target genes for miR-550a in HCC cells.

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Author Contributions
Conceived and designed the experiments: QT XH. Performed the experiments: QT LJ JD RZ HS QW SH WG CG JL. Analyzed the data: QT JD XH. Contributed reagents/materials/analysis tools: TC. Wrote the paper: QT LJ XH.