Hsa-miR-331-3p inhibits VHL expression by directly targeting its mRNA 3'-UTR in HCC cell lines

Yiyi Cao, Jinnan Zhang, Dongmei Xiong, Dan Wang, Ting Wu, Ailong Huang and Hua Tang

Key Laboratory of Molecular Biology on Infectious Diseases, Ministry of Education, Chongqing Medical University, Chongqing 400016, China

INTRODUCTION

MicroRNAs (miRNAs) are small single-stranded non-coding RNA molecules composed of 21–22 nucleotides and account for about 1% of the entire genome. miRNAs widely exist in eukaryotic cells and regulate gene expression by interacting preferentially with the 3’ untranslated regions (3’-UTRs) of target mRNAs, which may cause either inhibition of translation or degradation of the targeted mRNA (Bartel, 2004; Calin & Croce, 2006). miRNAs and their target mRNAs form complex regulatory networks, involved in cells proliferation, apoptosis, cell differentiation, stress response and other complex regulatory networks (Gaal & Olah, 2012; Lin et al., 2013). A large number of researches indicates that deregulation of miRNAs is common in human tumors. miRNAs can inhibit target mRNAs which are involved in the occurrence, development and progression of cancer as either oncogenes or tumor suppressors (Zhang et al., 2007). miRNAs expression disorder maybe a common cause of human tumor (Croce, 2009).

HCC is one of the most common and typical malignancies, and most cases are attributable to persistent hepatitis B virus (HBV) infections. It has also been reported that virus infection can interfere with cellular mRNA expression (Lin & Flemington et al., 2011). The Von Hippel-Lindau (VHL) syndrome is a dominantly inherited familial cancer syndrome predisposing to a variety of malignant and benign tumors. The basis of familial inheritance of VHL syndrome is a germline mutation of the VHL gene (Maher & Kaelin, 1997). This gene encodes a component of a protein complex including elongin B, elongin C, and cullin-2, and it possesses ubiquitin ligase E3 activity. VHL protein is associated with the ubiquitination and degradation of hypoxia inducible factor (Cockman & Masson, 2000).

Here, we detected the expressions of miR-331-3p in different HCC cell lines and confirmed VHL was the target gene of miR-331-3p. These data will be helpful for investigating the potential association between miR-331-3p and HBV.

MATERIALS AND METHODS

Cell culture and transfection. HepG2 and HepG2.2.15 were cultured in a minimum essential medium (HyClone, China) with 10% fetal bovine serum

Abbreviations: miRNAs, microRNAs; HCC, hepatocellular carcinoma; qRT-PCR, quantitative real time polymerase chain reaction; PCR, polymerase chain reaction; 3’-UTRs, 3’ untranslated regions; HBV, hepatitis B virus; VHL, Von Hippel-Lindau; S.D., mean standard deviation; Ad-HBV, recombinant adenoviruses expressing HBV; Ad-GFP, recombinant adenoviruses expressing GFP; NC, negative control
(Gibco, USA), 100 units/ml penicillin and 100 μg/ml streptomycin (Hyclone, China) and 1.2% sodium pyruvate (Hyclone, China). SMMC7721 was cultured in RPMI 1640 medium (Hyclone, China) with 10% fetal bovine serum (Gibco, USA) and 100 units/ml penicillin and 100 μg/ml streptomycin (Hyclone, China). HepG2, HepG2.2.15 and SMMC7721 were both maintained in a humidified incubator at 37°C with 5% CO₂. HepG2 and HepG2.2.15 were seeded in a 6-well plate at a density of 60% and SMMC7721 at a density of 45%, and transfected with the target vectors respectively by using Lipo-fectamine™2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

Plasmid and adenovirus construction. For constructing pTARGET-miR-331-3p, the following primers were used. Forward primer: 5'-CCGCCTCCGGCCCTCCACCTC-3', reverse primer: 5'-AGGATCCCATCCTGTTAGTGTG-3'. The primers for VHL were forward primer: 5'-ACATCGTCAGTGTTGCTCTAC-3' and reverse primer: 5'-ATCCCTACCTCGTGTG-3'. β-actin was used as an endogenous control, forward primer: 5'-GTGAGATCCAAGCCAGAGGT-3' and reverse primer: 5'-TGTGTGGACTTGGAGAGGA-3'. The qRT-PCR reactions were performed in triplicate and included no-template controls. Relative changes in gene expression were calculated using the 2-ΔΔCT method (Livak et al., 2001).

Western blot. SMMC7721 cells were seeded in a 60 mm plate at a density of 45%, transfected with pTARGET (5 μg), pTARGET-miR-331-3p (5 μg), NC (100 pmol) or miR-331-3p inhibitor (100 pmol) respectively. Cells were harvested 48 h after transfection and lysed with RIPA buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA, leupeptin) with PMSF (Beyotime, China). The supernatants were collected, and protein concentrations were measured using the Enhanced BCA Protein Assay Kit (Beyotime, China). Western blot analysis was performed as described in previous research (Zou et al., 2014) with primary antibodies: rabbit anti-human VHL (Bioword, USA), rabbit anti-human β-actin (Bioword, USA) and goat anti-rabbit secondary antibody (Bioword, USA).

Luciferase reporter assay. For the luciferase reporter assay, SMMC7721 cells were seeded in a 24 well plate at a density of 45% and co-transfected with 250 ng pTARGET-miR-331-3p or pTARGET vector, 150 ng of pGL3-Control-VHL-WT or pGL3-Control-VHL-MUT constructs and 25 ng pRL-TK plasmid expressing renilla luciferase (Promega, Madison, WI). HepG2.2.15 cells were seeded in 24 well plates at a density of 60% and co-transfected with 70 pmol miR-331-3p inhibitor or inhibitor NC, 150 ng of pGL3-Control-VHL-WT and 50 ng pRL-TK. Cells were collected 48 h after transfection and analyzed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Relative luciferase activity was normalized to renilla luciferase activity. Transfections were done in triplicate and repeated at least 3 times in independent experiments.

Statistical analysis. Data are expressed as mean standard deviation (S.D.). Statistical analysis was performed by using the independent t-test. P value of less than 0.05 was considered statistically significant.

RESULTS

MiR-331-3p was up-regulated in HBV expressing HCC cell lines

Previous studies have shown that there are large numbers of different expression miRNAs in HepG2 and HepG2.2.15 cell lines (Zhang et al., 2011). By using qRT-PCR analysis, we found that miR-331-3p expressions were markedly higher in HepG2.2.15 cells (5.76±2.29) (P=0.035) compared to HepG2 (1.02±0.27) (Fig. 1a), which were consistent with previous miRNA microarray data. To further confirm this data, HepG2 cells were infected with recombinant adenoviruses expressing HBV (Ad-HBV) or GFP (Ad-GFP). We found that miR-331-3p was also up-regulated in HepG2 cells transiently expressing HBV (3.62±0.86) (P=0.006) compared to its control (1.00±0.05) (Fig. 1b). SMMC7721 cells were transfected with pCH9 or pCH9/3091 plasmids, and miR-331-3p expression was detected. The same
Hsa-miR-331-3p inhibits VHL expression by targeting its mRNA 3'-UTR in HCC

Changes were observed, miR-331-3p was up-regulated in SMMC7721 cells transiently expressing HBV (4.08±0.62) (P=0.001) compared to its control group (1.00±0.16) (Fig. 1c). These results indicated HBV could promote miR-331-3p expression in HCC cell lines.

MiR-331-3p inhibited VHL expression

To further investigate the function of miR-331-3p in HCC, we used bioinformatics website miRBase (http://mirbase.org/index.shtml) to search for the downstream effect genes of miR-331-3p, and found that VHL might be one of its target mRNAs (http://mirdb.org/cgi-bin/search.cgi?searchType=miRNA&full=mirbase&searchBox=MIMAT0000760).

In order to evaluate if VHL is a target gene of miR-331-3p, we first constructed miR-331-3p over-expression plasmid pTARGET-miR-331-3p, and verified the over-expression efficiency by qRT-PCR. MiR-331-3p expression was markedly increased in pTARGET-miR-331-3p expressing SMMC7721 (50.65±19.52) than pTARGET control group (1.00±0.12) (P=0.01) (Fig. 2a). Later, qRT-PCR and Western blot analysis were used to determine the effects of pTARGET-miR-331-3p on the expression of VHL in HCC cells. qRT-PCR result revealed that VHL expression was decreased in pTARGET-miR-331-3p expressing SMMC7721 cells (0.30±0.17) than pTAR-
GET expressing SMMC7721 cells (1.00±0.05) (P=0.002) (Fig. 2b); the same conclusion was achieved with the western blot result (Fig. 2c). Finally, miR-331-3p inhibitor was used to silence miR-331-3p expression, which lead to a remarkable decrease of miR-331-3p expression (0.10±0.03) in comparison to the NC control group (1.01±0.20) (P=0.001) (Fig. 2d) and resulted in an increase of VHL expression (2.37±0.37) compared with the control (1.03±0.28) (P=0.007) (Fig. 2e and 2f) in SMMC7721 cells. Therefore, we concluded that VHL expression could be inhibited by miR-331-3p in HCC cell lines.

**MiR-331-3p inhibited VHL expression by directly targeting its 3′-UTR**

As we know, miRNAs regulate gene expression by interacting preferentially with the 3′-untranslated regions (3′-UTRs) of their target mRNAs. We wondered whether miR-331-3p inhibit VHL expression in the same way. To confirm the mechanism of action, pGL3-Control-VHL-WT vector which contained 3′-UTR binding site (CCAG GGG) for miR-331-3p, and pGL3-Control-VHL-MUT vector which contained a mutation site (AATC CCC) were constructed respectively (Fig. 3a). The 3′-UTR binding site was predicted by miRDB (http://mirdb.org/cgi-bin/target_detail.cgi?targetID=284590). Luciferase reporter gene assay indicated that the luciferase activity was lower in SMMC7721 cells which were co-transfected with pTARGET-miR-331-3p (6.69±1.22) and pGL3-Control-VHL-WT than that in SMMC7721 cells which were co-transfected with pTARGET-miR-331-3p and pGL3-Control (11.29±2.24) (P=0.035), and the luciferase activity was recovered when SMMC7721 cells were co-transfected with pTARGET-miR-331-3p

Figure 3. MiR-331-3p down-regulated the expression of VHL through its 3′-UTR binding sites.

(a) Schematic diagram of wild-type 3′-UTR binding site or mutated type of miR-331-3p. (b) Luciferase reporter assays for SMMC7721 cells which were co-transfected with different plasmid DNAs as indicated. (c) Luciferase reporter assays for HepG2.2.15 cells which were co-transfected with different plasmid DNAs and RNAs as indicated. *P<0.05, **P<0.01.

Figure 4. VHL expression was decreased in HepG2.2.15 cells compared to HepG2 cells.

(a) Relative expression of VHL in HepG2 and HepG2.2.15 cells was analyzed by qRT-PCR. β-actin was used as an internal quantitative control. *P<0.05, **P<0.01. (b) VHL protein expression levels in HepG2 and HepG2.2.15 cells were analyzed by western blot. (c) VHL protein expressions were analyzed by western blot in HepG2.2.15 cells transfected with miR-331-3p inhibitor or inhibitor NC.
and pGL3-Control-VHL-MUT vector (11.20±1.38) (Fig. 3b). The luciferase activities were decreased in miR-331-3p up-regulated HepG2.2.15 cells (32.46±7.77) and increased when co-transfected with miR-331-3p inhibitor (92.73±10.4) (Fig. 3c). These data suggested that miR-331-3p inhibited VHL expression by directly targeting its 3'-UTR.

VHL expression was decreased in HepG2.2.15 cells compared to HepG2 cells

Since miR-331-3p was up-regulated in HepG2.2.15 cells, could VHL have been influenced by miR-331-3p in HepG2.2.15 cells? VHL expression was checked with qRT-PCR analysis and western blot analysis. The results showed that VHL was down-regulated in HepG2.2.15 cells (0.07±0.001) in comparison with HepG2 (1.01±0.20) (P<0.001) (Fig. 4a, b) both in mRNA and protein levels, and increased in HepG2.2.15 cells transfected with miR-331-3p inhibitor or inhibitor NC (Fig. 4c).

DISCUSSION

In recent years, miRNAs have been reported frequently to undergo different expression patterns in many biological events, especially tumor genesis (Ambros, 2004; Griffiths-Jones et al., 2008). miRNA expression profiling studies have shown that HBV infection resulted in alterations of many miRNA expression (Gao et al., 2011; Yip et al., 2011). As HBV is the main cause of HCC, maybe HBV plays an important role in HCC occurrence and development in miRNA-related manners. According to our studies, miR-331-3p was up-regulated by HBV, but whether miR-331-3p was involved in the HBV-miRNA-HCC mode of action needs a further research.

It has been reported that VHL mutation is associated with several types of tumors like pheochromocytoma, clear-cell renal cancer, central nervous system and retinal angiomas, which suggests that VHL gene may play a role as a tumor suppressor in those cancers (Latif et al., 1993; Kim & Kaelin, 2004). According to miRNA bioinformatics analysis, VHL was predicted to be a target gene of miR-331-3p. We detected VHL expression after overexpression or inhibition of miR-331-3p in SMMC7721 cells by qRT-PCR and western blot. As we expected, VHL was inhibited by miR-331-3p. To further investigate its mechanism, SMMC7721 cells were co-transfected with the expression plasmid of miR-331-3p and VHL 3'-UTR wild type or 3'-UTR mutated type to detect their luciferase activities. Luciferase reporter gene assay results show that miR-331-3p can decrease the luciferase activity of pGGL3-Control-VHL-WT and has little influence on pGGL3-Control-VHL-MUT. Luciferase activities of HepG2.2.15 cells transfected with miR-331-3p inhibitor were higher than inhibitor NC. These indicated the inhibitory effect of miR-331-3p on VHL depended on its 3'-UTR, so we confirmed that VHL is one of the target genes of miR-331-3p.

MiR-331-3p were reported to be down-regulated in several types of tumors like glioblastoma multiforme, prostate cancer and gastric cancer and plays a role as a tumor suppressor (Guo et al., 2010; Epis et al., 2011; Epis et al., 2012; Epis et al., 2014). But interestingly, our studies showed that miR-331-3p was up-regulated in HepG2.2.15 cells in comparison to HepG2 cells, and inhibited the expression of tumor suppressor gene VHL. It means miR-331-3p might act as a promotion factor in HCC cells. This may be due to its different roles in different cancers. More detailed mechanisms and functions of miR-331-3p need to be further elucidated. As VHL is a target gene of miR-331-3p which can be regulated by HBV in HCC cell lines and we also observed that VHL was down-regulated in HepG2.2.15 cells and recovered in HepG2.2.15 cells transfected with miR-331-3p inhibitor, but we did not observe the same variation tendency in HBV transient transfection HCC cell lines, so whether VHL is involved in HBV-related HCC still needs further study.

Acknowledgements

This work was supported by The Major National S&T program (2013ZX10002002, ALH), Major project of Chongqing Science & Technology Commission (cstc2013jcyjC1002, ALH), and Natural Science Foundation Project of CQ CSTC (2010BB3539).

REFERENCES


